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Immunological Mechanisms Controlling Chronic Inflammatory Diseases

By

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ABSTRACT

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Autoimmune diseases (AID) are chronic inflammatory diseases (CID) mediated by self-reactive T and B cells and are generally the results of the breakdown of T cell tolerance to self-antigen and failure of peripheral regulatory mechanisms. In this thesis I studied different mechanisms controlling the development of CIDs. I investigated the initial events involved in the activation of self-reactive $CD4^+$ T cells which mediate the destruction of the thyroid in a mouse model of spontaneous thyroiditis. TAZ10 transgenic mice express a human T cell receptor (TCR) specific for a cryptic epitope of thyroid peroxidise (TPO) generated upon endogenous processing by thyroid epithelial cells (TEC), and a naturally occurring antagonistic epitope presented by dendritic cells (DC) upon exogenous processing of TPO.

I have characterized the function of myeloid derived suppressor cells (MDSCs) in TAZ10 mice. MDSCs accumulate in lymphoid and non-lymphoid organs of TAZ10 mice during acute phases of inflammation and their number decrease as inflammation is fading. Despite their strong inhibitory function on T cell function and proliferation, MDSCs fail to prevent the activation of self-reactive T cells. I showed that the manipulation of MDSCs generated DCs that efficiently promoted the activation of T cells from TAZ10 mice. By contrast, peripheral T cells from patients with rheumatoid arthritis (RA) and lupus had a high proliferative activity compared to controls. Further analysis revealed that RA patients had reduced amounts of inhibitory MDSCs in peripheral blood.

I showed that in TAZ10 mice TEC upregulate MHC class II molecules and present the cryptic epitope to TAZ10 T cells inducing their activation. I have demonstrated that DCs are responsible for the spreading of the TPO cryptic epitope from the thyroid to draining lymph-nodes (DLN) resulting in the strong activation of transgenic T cells from TAZ10 mice. By adoptive transfer experiments, I showed that the activation of naive TAZ10 T cells occurs within days both in the thyroid and draining lymph-nodes (DLN) and resulted in the destruction of the thyroid. Altogether, this work shows for the first time that in a model devoid of any environmental insults, the normal turnover of TEC is sufficient to induce the activation of self-reactive T cells and the development of AID.

In this thesis, I have highlighted the potential role of tissue transglutaminse 2 (TG2) in the treatment of CIDs. TG2 contributes to the pathogenesis of celiac disease and I have showed that TG2 activity promotes inflammation in patients with cystic fibrosis (CF). Mutation of the cystic fibrosis transmembrane regulator gene (CFTR) in CF patients is associated with increased TG2 expression and activity. In CF, TG2 promoted the crosslinking of the anti-inflammatory peroxisome proliferator-activated receptor γ (PPARγ) into perinuclear agresomes. The functional sequestration of PPARγ was leading to increased inflammation. The finding of this function of TG2 in CF was relevant in TAZ10 mice as *in-vivo* inhibition of TG2 downregulated common markers of inflammation.

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DECLARATION OF AUTORSHIP

I, Olivier Cexus declare that the thesis entitled

Immunological Mechanisms Controlling Chronic Inflammatory Diseases

and the work presented are my own. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this university;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- Parts of this work have been published as:

    - "Tissue Transglutaminase Activation Modulates Inflammation in Cystic Fibrosis via PPARγ Down-Regulation" Luigi Maiuri, Alessandro Luciani, Ida Giardino, Valeria Raia, Valeria R. Villella, Maria D'Apolito, Massimo Pettoello-Mantovani, Stefano Guido, Carolina Ciacci, Mariano Cimmino, Olivier N. Cexus, Marco Londei, and Sonia Quaratino (2008). Journal of Immunology (2008) 180(11): 7697-7705.

    - "Lysosomal accumulation of p31-43 induces inflammation via ROS-TG2 axis in intestinal epithelial cells and celiac mucosa" Alessandro Luciani, Valeria R. Villella, Angela Vasaturo, Ida Giardino, Massimo Pettoello-Mantovani, Stefano Guido, Olivier N. Cexus, Nick Peake, Marco Londei, Sonia Quaratino, Luigi Maiuri. *Paper Submitted*.

    - "Role and fate of self-antigen specific regulatory T cells" Ester Badami, Olivier N. Cexus, Marco Londei, Sonia Quaratino. *Paper submitted*.

- "Myeloid Derived Suppressor Cells in a mouse model of autoimmune thyroiditis" Olivier N. Cexus, Delphine Labrousse, Marco Londei and Sonia Quaratino. *Paper in preparation*.

Signed: ……

Date: ……

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This Thesis is dedicated to my Mum,
Who showed me how and where to find the strength to Fight…

This Thesis is also dedicated to my Dad,

I could never tell you enough…
How much I Love you

ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AICD	Antigen-Induced-Cell-Death
AID	Autoimmune Diseases
AIRE	Auto-Immune REgulator
AnV	Annexin V
ARG1	Arginase 1
APC	Antigen Presenting Cells
APL	Altered Peptide Ligand
ATRA	All-Trans Retinoic Acid
BM-DCs	Bone-Marrow derived Dendritic Cells
Ca^{2+}	Calcium
CD	Cluster of Differentiation
CDis	Celiac Disease
CFA	Complete Freund's Adjuvant
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CIA	Collagen Induced Arthitis
CIITA	Class II Transactivator gene
CLIP	Class-II Invariant chain Peptide
CRP	C-Reactive Protein
cTEC	cortical Thymic Epithelial Cells
CTL	Cytotoxic T-Lymphocyte
CXC	Chemokine Receptor
DAS	Disease Activity Score
DCs	Dendritic Cells
DLN	Draining Lymph-Nodes
DN	Double Negative
DP	Double Positive
DRiPs	Defective Ribosomal Products
dsDNA	double stranded DNA
EAE	Experimental model of Autoimmune Encephalitis

EAU	Experimental model of Autoimmune Uveitis
EBV	Epstein-Barr Virus
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FSC	Forward Scatter
G-CSF	Granulocyte Colony Stimulating Factor
G-EAT	Granulomatous Experimental Autoimmune Thyroiditis
GM-CSF	Granulocyte-Macrophages Colony Stimulating Factor
HDAC6	Histone Deacetylase 6
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
Hsp	Heat Shock Proteins
ICAM-1	Inter-Cellular Adhesion Molecule 1
iDCs	immature Dendritic Cells
IFNγ	Interferon gamma
Ii	Invariant chain
imm	immature
iNOS	inducible Nitric Oxide Synthase
IP	ImmunoPrecipitation
IPOD	Insoluble Protein Deposit
IRBP	Intra-Retinal Binding Protein
JUNQ	Juxta-nuclear Quality Compartment
kDa	kilo Dalton
LCMV	Lymphocytic ChorioMeningitis Virus
Lin	Lineage
LN	Lymph-Nodes
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinases
mat	mature
mDCs	Mature Dendritic Cells
MDSC	Myeloid Derived Suppressor Cells

MFG-E8	Milk Fat Globule EGF Factor 8
mg	milligrams
MHC	Major Histocompatibility Complex
MLR	Mixed Leucocyte Reaction
MSC	Mesenchymal Stem Cells
mTEC	medullary Thymic Epithelial Cells
MTOC	Microtubule Associated Center
NDLN	Non Draining Lymph-Nodes
ng	nanograms
NK	Natural Killer cells
NO	Nitric Oxide
NOD	Non-Obese Diabetic
NOS	Nitric Oxide Synthase
PBS	Phosphate Buffered Saline
PBMC	Peripheral Blood Mononuclear Cell
PCD	Programmed Cell Death
PI	Propidium Iodine
PLC	Peptide Loading Complex
PPARγ	Peroxisome proliferator-activated receptors γ
RA	Rheumatoid Arthritis
RAG/Rag	Recombination Activating Gene
RNOS	Reactive Nitrogen Oxygen Species
ROS	Reactive Oxygen Species
SC	Schwann Cells
siRNA	small interfering RNA
SP	Single Psotive
SSC	Side Scatter
SED	*Staphylococcus aureus* Enterotoxin D
SLE	Systemic Lupus Erythematosus
TAP	Transport associated with Antigen Processing
TEC/TECs	Thyroid Epithelial Cells
TGN	Trans Golgi Network
T_{CM}	Central Memory T cells
TCR	T-Cell Receptor

Teff - T_E	effector T cells
T_{EM}	Effector Memory T cells
TG2	Tissue Transglutaminase / Transglutaminase 2
Tg	Transgenic
TGFβ	Tumor Growth Factor β
TGN	Trans-Goldi Network
Th	helper T cell
T_N	Naïve T Cells
TPO	Thyroid Peroxidase
Treg	regulatory T cells
TSA	Tissue Specific Antigens
TILs	Tumor Infiltrating Lymphocytes
TLR	Toll-Like Receptor
TNFα	Tumor Necrosis Factor alpha
TPO	Thyroid Peroxidase
TUNEL	Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling
UPR	Unfolded Protein Response
WB	Western-Blot
WCC	White Cell Count
WT	Wild-Type
YFP	Yellow Fluorescent Protein
μg	microgram

1/ Introduction

1-1/ The Immune Response: the combination of Innate and Adaptive Immune systems

Evolving in an environment full of infectious agents, living creatures represent a rich habitat for the replication of bacteria, parasites and viruses. In order to keep these pathogens at bay, immune responses have been developed to provide a line of defense against infections and any events altering cellular or tissue homeostasis. These systems have been shaped throughout evolution when pathogens have developed complicated ways to evade these systems (Hartl et al. 2007; Finlay et al. 2006), and are composed of two major components that are characterized by their specificity to the infectious agents. Present in nearly all forms of life, the innate immune response displays a rapid first line of defence regardless of the infectious agent. In vertebrates, the immune system has evolved and an adaptive immune system has been developed that is not present in amphibians. The adaptive immune system is made by specialized cells, is pathogen-specific and carries immunological memory.

1-1-1/ The innate immune response

First lines of defense, epithelia are considered as physiological barriers preventing the intrusion of pathogens into adjacent tissues. Although the epithelium of the skin is the major mechanical barrier, epithelium of the trachea or the gut also constitute an important line of defence against pathogens (Elias. 2007; Holt et al. 2008). The second arm of this systems is constituted by the cells of the immune systems whose response are based on the recognition of pathogen associated molecular patterns (PAMPs) that are not expressed by the host. The innate immune system therefore has no memory and each breach is treated individually. Mechanisms have however been put in place so as to distinguish infectious parasites from parasites that have co-evolved with the host such as the flora of the digestive system (Lee et al. 2006; Paragraph 1-2-1-1/). Defects in cells of the innate systems have been linked with an inability to mount an effective immune response against common pathogens leading to severe chronic inflammation such as Crohn's disease in the guts.

Immunity developed by vertebrates has long been shown to be highly effective when considering infection by pathogens. If first physical barriers are broken by the pathogen, the

innate immune system will be the next line of defence. This system is crucial in promoting and coordinating the activity of the acquired or adaptive immune system that has long been described as the "optimal solution" despite some evolutionary weaknesses (Hedrick. 2004).

1-1-2/ The adaptive immune response

The importance of the adaptive immune system is well established and has been largely demonstrated. The acquired immune response feeds back by amplifying the innate immune response; arming it with specific receptors and functions so as to efficiently promote the destruction of foreign agents. It also feeds back on itself through cytokines and other mediators. Numerous studies of bacterial infections have demonstrated that adaptive immunity is crucial in resolving infections and in establishing memory to the individual so that protection will be provided in case of a further infection. Moreover, individuals with an acquired immunodeficiency are more vulnerable to common parasitic or bacterial infections (Fischer. 2004). In this respect, re-establishment of the immune system in immunodeficient children (X-linked severe combined immunodeficiency / SCID-X1) by gene therapy has been highly successful in restoring the immune system and curing the affected children (Cavazzana-Calvo et al. 2000; Gaspar et al. 2004).

In order to efficiently mount a specific immune response, the adaptive immune system must recognised non-self antigens from pathogens whereas the recognition of self antigens by the host must be avoided so as to avoid any autoimmune diseases. Although this system is highly efficient, to avoid autoimmunity or "horror autotoxicus" (Himmelweit F. Collected Papers of Paul Ehrlich, London: Pergamon; 1956–1960), where the immune system is prone to attack and destroy its host, it must made a clear distinction between what is self and non-self.

Following this concept of "horror autotoxicus", Jerne proposed the "natural selection theory" (Jerne. 1955) where he suggested that there was a pre-existing repertoire of antibodies from which antigen selects. In this respect, he suggested that antigen-antibody complexes are taken into cells where the antibody is then replicated. Burnet proposed a totally different approach; "the clonal selection theory" (Burnet. 1961; Burnet. 1976) that was based on the differences between self and non-self. The immune system only mounts an immune response against non-self whereas avoiding any attack against self. His work was based on the analysis of experiments on skin-graft where an individual will reject a graft from

another donor whereas accepting his own graft. However, this theory could not explain the emergence of autoimmune diseases; the same “horror autoxicus” where the immune system would not only recognise the non-self but also the self. To address this question, Matzinger proposed the “danger model” (Matzinger. 2001; Matzinger. 2002). In this theory, the immune system triggers an immune response only when confronted with sources of “Danger” regardless of self / non-self discrimination. Thus an immune response could be mounted against either self or non-self as long as a danger signals are present.

The nature of the danger modulates dendritic cell function to polarise different T cell responses triggering the adaptive immune response (Germain et al. 2004).

1-2/ Dendritic cells at the interface between innate and adaptive immune systems

Dendritic cells (DCs) have been described as playing a major role in immune responses as they are at the interface between innate and adaptive immunity. This is due to their pleiotropic activities as sensors of danger as well as their efficiency in promoting both B and T cell responses. In the resting state, dendritic cells are immature and patrol the tissues until activated by danger signals. These signals have different origins and can result from the recognition of pathogens by cells of the innate immune system such as epithelial cells, dendritic cells or macrophages. In this context, immature DCs capture and process antigens shed in the microenvironment (Banchereau et al. 2000). Maturing and migrating to the nearest proximal lymphoid organs, mature DCs present antigens to lymphocytes that are then stimulated and able to mount an effective adaptive immune response (Rossi et al. 2005; Colonna et al. 2006).

It is therefore clear that in the "danger model", DCs play a crucial role between the quick innate immune response and the antigen specific adaptive immune system.

1-2-1/ Phenotype and maturation of dendritic cells

In an inflammatory context, professional antigen presenting cells such as DCs, uptake extracellular components (Figure 1-1). Proteins are then processed following either endogenous or exogenous pathways and resulting epitopes are presented in the context of Major Histocompatibility Molecule Complex (MHC) Class I or II molecules to be recognised by $CD8^+$ and $CD4^+$ T cells respectively; this is known as the "first activation signal" (Banchereau et al. 2000). The efficient activation of T cells will however depend on the ability of dendritic cells to provide costimulatory signals to T cells via costimulatory molecules such as proteins of the B7 family constitutively expressed by DCs and CD28 expressed by T cells (Greenfield et al. 1998). This first costimulatory signal (or "second activation signal") between DCs and T cells triggers the release of DCs derived cytokines and the engagement of other costimulatory molecules newly expressed by T cells and mDCs. This

provides the "third activation signals" necessary to fully activate T cells (Anderton et al. 2002).

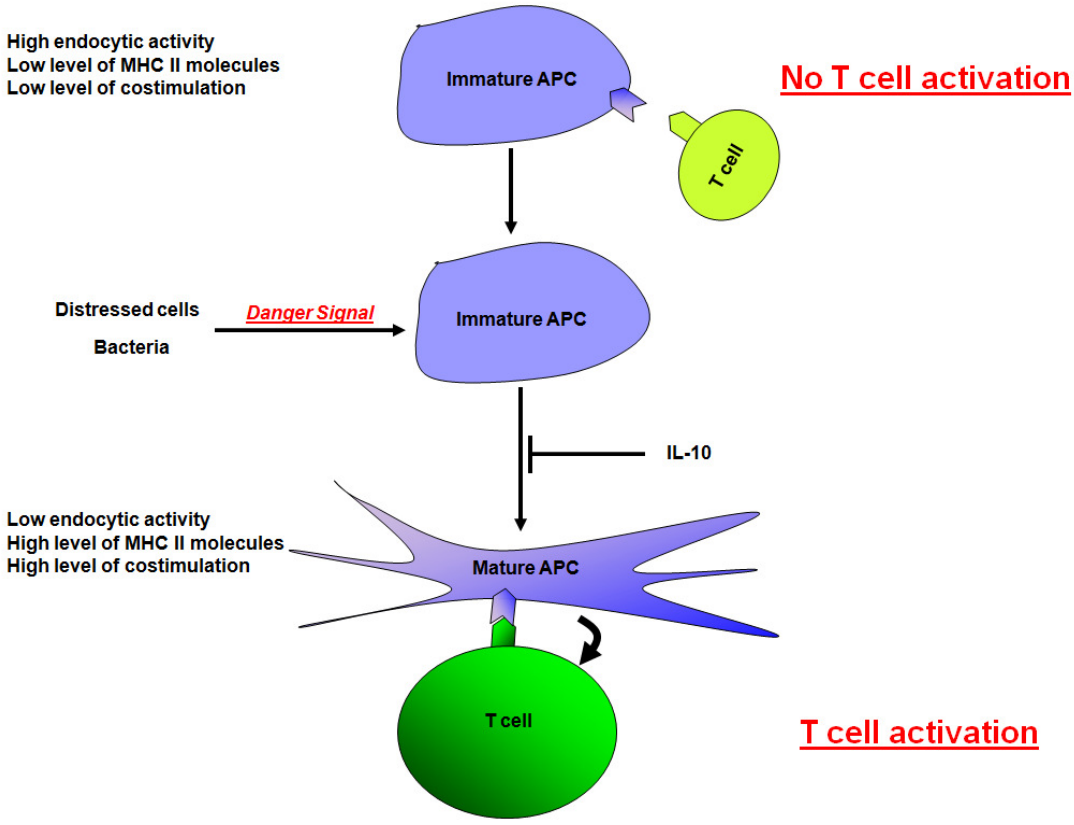


Figure 1-1: The "Danger model" and activation of dendritic cells
Danger or stress signals released by bacteria or cells undergoing necrosis activate immature antigen presenting cells (APC). Immature APC highly efficient in uptaking extracellular elements but showing a low expression of both MHC-II and costimulatory molecules differentiate into mature APCs. Mature APCs upregulate the expression of MHC-II and costimulatory molecules enabling an efficient T cell activation. The maturation of APCs is characterized by a reduced endocytic activity.

Danger signals are of various natures. These signals are differently recognised by immature DCs (iDCs) bearing a wide range of specific receptors, thus allowing the immune response to be finely tuned.

1-2-1-1/ Nature of the "Danger signals"

Danger signals are represented by molecules from the pathogen or molecules shed by dying cells from the host. These molecules characterized by a specific molecular structure or PAMP are recognized by antigen presenting cells by pattern recognition receptors (PRR). Among these PRRs, Toll-like receptors (TLR) are the most studied as they induce different immunological responses depending on the nature of the PAMP detected (Beutler. 2004). The interaction between a specific TLR and its ligand triggers a signalling cascade involving the pro-inflammatory protein NF-κB and mitogen–activated protein kinases (MAPK). This first signalling is often followed by a late pro-inflammatory response involving proteins such as interferon-regulatory factors (IRF) (Akira et al. 2004; Trinchieri et al. 2007). This results in the production of pro-inflammatory proteins and cytokines that are critical for the generation of the appropriate immune response against invading pathogens.

To date, up to 13 TLR have been identified and can be present at the cell-surface or inside the cells (Table 1-1). TLR5 recognizes flagellin, TLR3 binds double stranded RNA while TLR7 recognizes single strand RNA and TLR9 binds viral and bacterial DNA that are rich in unmethylated CpG motifs. TLR ligands also include lipopolysaccharides (LPS), the main lipid constituting bacterial walls. LPS has been shown to induce the maturation of DCs via the ligation to Toll-like receptor 4 (Spörri et al. 2005).

Receptors	Ligands
TLR1 / TLR2	Lipoproteins: e.g. OspA Triacylated Lipopeptides
TLR2	Bacterial lipoproteins Glycosylphosphatidyl-inositol Glycolipids Porins
TLR3	Double-stranded RNA Poly I:C mRNA/tRNA
TLR4	Lipopolysaccharides Monophosphoryl lipid A Flavolipin Glycoinositolphospholipids
TLR6 / TLR2	Mycoplasmal Macrophage-activating lipopeptide Diacetylated Lipopeptide Soluble Tuberculosis Factor
TLR5	Flagellin
TLR11	Profilin
TLR7	Single-stranded RNA Polyuridylic acid
TLR8	Single-stranded RNA Polyuridylic acid
TLR9	Unmethylated CpG DNA

Table 1-1: Members of the Toll-Like Receptor (TLR) family bind a wide range of ligands
Description of the ligands recognized by the different members of the toll-like receptor family. Adapted from www.nature.com/nri/focus.tlr and (Akira et al. 2004).

It is now clear from numerous studies that the nature of the LPS allows DCs to finely tune the immune response (Netea et al. 2002). As a matter of fact, LPS molecules from different bacteria have been shown to bind TLR molecules with different affinity according to the shape of the Lipid A (from cylindrical to conical), a major component of LPS molecules. Indeed, LPS from *Rhodobacter Sphaeroides* (cylindrical Lipid A) can engage both TLR4 and TLR2, LPS from *Porphytomonas Gingivalis* (conical Lipid A with a small base) binds TLR2 while *Neisseria Meningitidis* LPS (conical Lipid A with a large base) binds either TLR4 or TLR2 / TLR1 (Figure 1-2).

The response to danger signals must however be controlled to provide immune tolerance to commensal bacteria in the airways or the guts. Indeed, it is estimated that up to $1x10^4$ bacteria families are present in the human colon. A defective or inappropriate immune

response to commensal bacteria of the gut is involved in numerous pathologies such as Crohn's disease or ulcerative colitis (McGukin et al. 2009). A study (Lee et al. 2006) has recently shown how the immune response to commensal bacteria through TLR9 depends on the polarity of intestinal epithelial cells. Activation of TLR9 expressed at the basolateral surface of intestinal epithelial cells leads to the activation of the NF-κB pro-inflammatory pathway. By contrast, stimulation of TLR9 present at the apical membrane leads to the ubiquitination of Iκ-Bα that prevents NF-κB from being activated. Interestingly, the stimulation of TLR9 at the apical membrane prevented any subsequent TLR stimulation at the basolateral membrane of gut epithelial cells. Therefore, the configuration of the mucosa (and ultimately of epithelial cells) modulate TLR responses to control tolerance and inflammation to commensal and non-commensal pathogens.

Recognition by TLRs must also allow some degree of flexibility to adapt to strategies developed by pathogens avoiding recognition by the immune system. We previously exposed that the shape of Lipid A from different LPS influences its ability to bind different TLRs (Figure 1-2). In a strategy to adapt to the environment, environmental changes and to escape the innate immune system, bacteria can acquire the capacity to modulate the structure of LPS by promoting a different degree of acetylation of the Lipid A recognized by TLR molecules. Indeed, Lipid A composing LPS from *Pseudomonas Aeruginosa* is hexa-acetylated in cystic fibrosis and penta-acetylated in chronic lung bronchiectasis or conventional bacterial culture. Interestingly, penta-acetylated LPS binding TLR4 does not lead to the generation of a strong and robust inflammatory response which is observed with hexa-acetylated LPS from patients with cystic fibrosis (Hajjar et al. 2002). This difference of responses to LPS from *Pseudomonas Aeruginosa* in cystic fibrosis and lung bronchiectasis depends on an 82 amino-acid region of human TLR4; murine TLR4 molecules being unable to distinguish between the two different forms of LPS. Thus, human TLR4 is capable of recognizing specifically the degree of bacterial adaptation in the microenvironment and to adapt the strength of the immune response to bacterial infections.

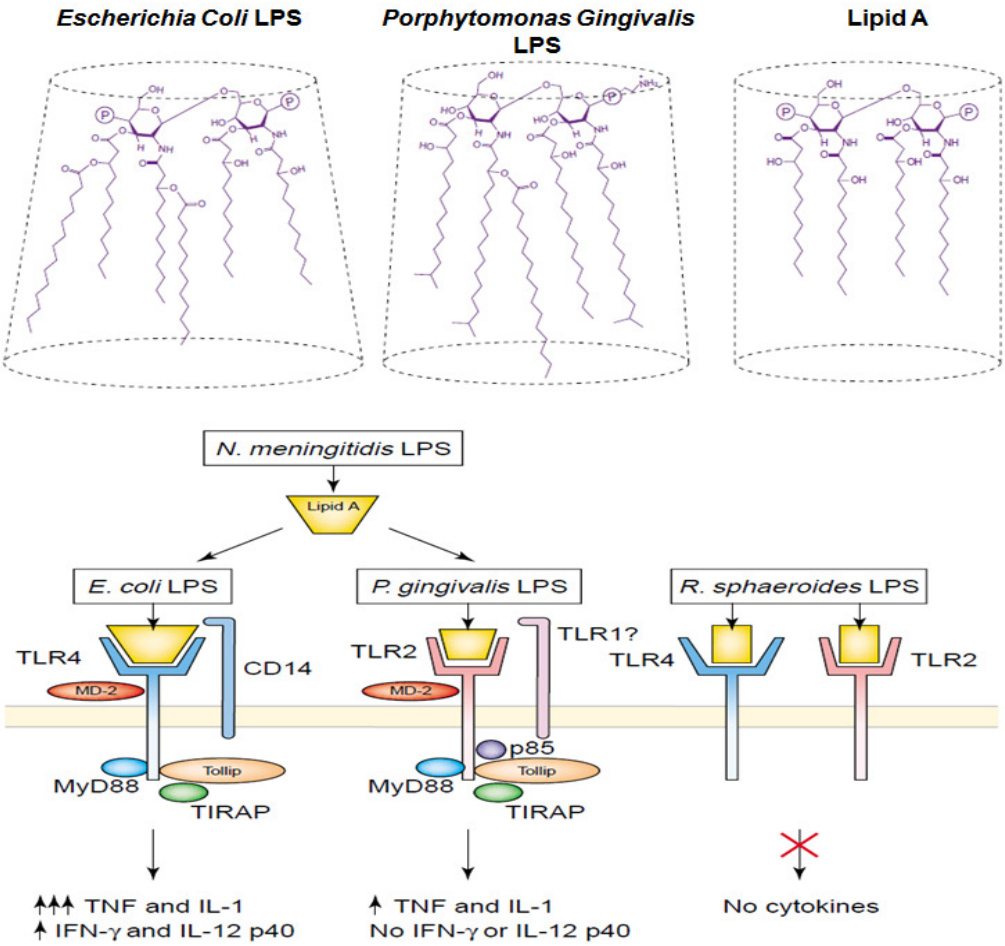


Figure 1-2: Binding activities of LPS from different bacterial strains induces different inflammatory responses
The nature and morphology of LPS and more specifically the shape of the Lipid A dictates its ability to bind different TLRs, thus dictating the nature of the immune response generated. LPS from *Neisseria Meningitidis* (conical Lipid A) binds TLR1/TLR2 and TLR4 molecules while LPS from *Escherichia Coli* (conical Lipid A) binds only TLR4. LPS from *Porphytomonas Gingivalis* is recognized by TLR2 / TLR1. LPS from *Rhodobacter Sphaeroides* has a cylindrical Lipid A and can therefore bind either TLR4 or TLR2. Figure is adapted from (Netea et al. 2002).

In the "Danger Model" proposed by Matzinger (Matzinger. 2001; Matzinger. 2002), the nature of the danger is sensed more than the self or non-self nature of the antigenic peptide. Indeed, although components of foreign infectious pathogens such as LPS or flagellin can trigger an immune response, cells or tissues from the host can be stressed and release "danger signals". By contrast to apoptosis, necrosis leads to the release of endogenous Hsp70 (Vabulas et al. 2002) and Hsp60 (Vabulas et al. 2001) into the microenvironment. Hsp60 and Hsp70 trigger an immune response through TLR4 and can therefore be considered as danger signals.

1-2-1-2/ Activation of the adaptive immune response by dendritic cells

In presence of "danger", iDCs undergo multiple functional and morphological changes and start to go through maturation steps to become mature DCs (mDCs). mDCs upregulate the expression of MHC-II as well as costimulatory molecules necessary for the full subsequent activation of T cells. Once activated, mDCs migrate to lymphoid tissues such as the lymph-nodes draining the tissue affected by inflammation, where they encounter T and B lymphocytes (Banchereau et al. 2000) (Figure 1-3).

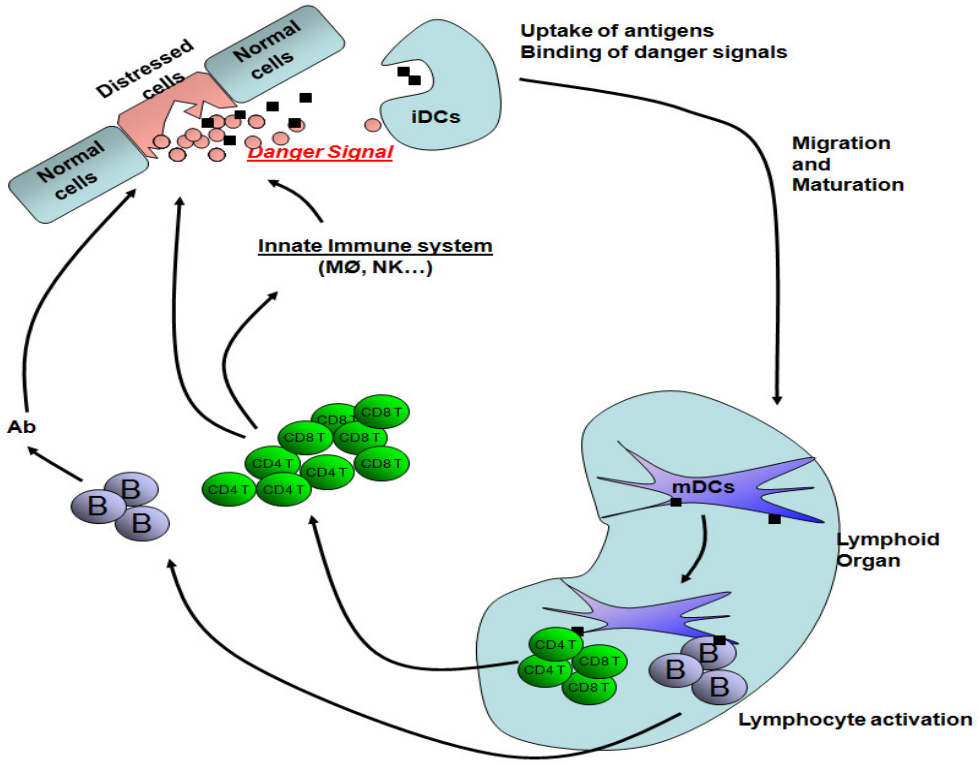


Figure 1-3: Cooperation of the Innate and Adaptive immune systems in response to danger signals
Upon stimulation by danger signals, iDCs uptake antigens present in the microenvironment. Maturing iDCs migrate to the nearest lymphoid organ. In the lymph-nodes, fully competent mature DCs (mDCs) present antigens to B cells, $CD4^+$ and $CD8^+$ T cells. Activated lymphocytes then migrate to the site of inflammation where they coordinate with cells of the innate immune system to mount an effective immune response. Figure is adapted from (Banchereau et al. 2000). **NK**: Natural killer cells; **MØ**: macrophages.

Naïve T cells circulating in the blood and lymph-nodes make contact with mDCs within lymphoid organs. As soon as they recognise the cognate peptide presented by MHC molecules, naive T cells will differentiate into specific T cell subsets depending on the nature of the peptide and the cytokine environment.

T cell activation by mDCs requires different signals. Activation signal 1 is represented by the interaction between the T cell receptor (TCR) and the peptide-MHC molecule complex (Anderton et al. 2002). Alone, this signal leads to T cell anergy unless full T cell activation is achieved via "Activation signals 2 and 3". These latest signals are the production of DC- and T cell-derived cytokines (IL-6 secreted by mDCs, and IL-2 secreted by T cells etc.) and the engagement of costimulatory molecules (CD80/86 on mDCs interacting with CD28 on T cells) between T cells and mDCs (Anderton et al. 2002). Resulting T cell activation has been largely described and depends on the length and frequency of contact between mDCs and naive T cells. Stable and prolonged interactions between mDCs and T cells lead to T cell activation and immunity. However, in tolerogenic conditions and despite early activation and proliferation, naïve T cells will remain motile, thus establishing multiple short contacts with mDCs (Hugues et al. 2004).

It is now established that not all mature DCs are able to efficiently promote a full T cell response involving proliferation and acquisition of effector functions. The production of IL-10 by many tumours has been shown to have a detrimental effect on the ability of mDCs to activate T cells (Morel et al. 1997). Indeed, IL-10 can for example lead to the downregulation of costimulatory molecules by mDCs that leads to T cell anergy upon mDC/T cell encounter (Steinbrink et al. 2002). iDCs matured with bystander inflammatory molecules (such as TNFα or IFNα) resulting from an infection are not fully licenced to promote the differentiation and acquisition of effector functions of $CD4^+$ T cells. However, iDCs directly stimulated with pathogen products via TLR allows mDCs to induce the proliferation and differentiation of $CD4^+$ T cells that acquire the effector functions required to mount an efficient immune response (Spörri et al. 2005; Heath et al, 2005).

Activated T and B cells migrate towards the site of inflammation where they cooperate with cells of the innate system (natural killer cells, macrophages…) and dendritic cells to get rid of the source of danger (Banchereau et al. 2000). Once this goal is achieved, most of the T cells undergo apoptosis whereas a small proportion becomes memory T cells. Memory cells present within the blood-stream and lymphoid organs will trigger an immediate immune response once in the presence of the cognate peptide (Lanzavecchia et al. 2005).

1-2-2/ Antigen processing and presentation

The efficient activation of antigen-specific $CD8^+$ and $CD4^+$ T cells requires first the recognition by T cells of the appropriate peptide presented in the context of MHC-I and MHC-II molecules respectively.

1-2-2-1/ Endogenous pathway of antigen processing and presentation onto MHC-I molecules

All nucleated cells present their pool of proteins or foreign proteins (from viral or bacterial origin) on MHC-I molecules. Proteins are ubiquitinylated by three different enzymes (U1; U2 and U3 ubiquitin ligases) to be recognized by the proteasome for degradation. 9-mer peptides resulting from the degradation are transported into the endoplasmic reticulum (ER) by chaperone membrane proteins known as Transporter Associated Peptide 1 and 2 (TAP1 and TAP2). In order to bind MHC-I molecules, these peptides are further trimmed down to 8 amino-acids in the ER by different enzymes such as ERAP (ER aminopeptidase) or Bip (Saveanu et al. 2002; Elliott et al. 2005).

MHC-I molecules are composed of a heavy-chain formed of 3 subunits (α1, α2 and α3), and the β2-microglubulin subunit. The binding of a specific antigen into the groove of MHC-I molecules (formed by the α1 and α2 subunits of the heavy-chain) is controlled and occurs in different stages. Once synthesized, the heavy-chain is associated with chaperones such as calnexin and ERp57 (a thiol oxido-reductase) and the β2-microglobulin (β2m) subunit binds the heavy-chain. At this stage, the calnexin-ERp57 complex is replaced by calreticulin-ERp57 until a peptide is loaded onto MHC-I molecule (Saveanu et al. 2002; Elliott et al. 2005). The association of TAP proteins (Transporter associated with Antigen Processing and has two subunits: TAP1 and TAP2), the different chaperones and the empty MHC-I molecule forms the Peptide Loading Complex (PLC) that allows the binding of peptides with the correct affinity onto MHC-I molecules. MHC-I molecules stabilised by the bound peptide are then freed from the different protein chaperones and migrate to the cell-surface to prime MHC-I restricted $CD8^+$ T cells (Figure 1-4).

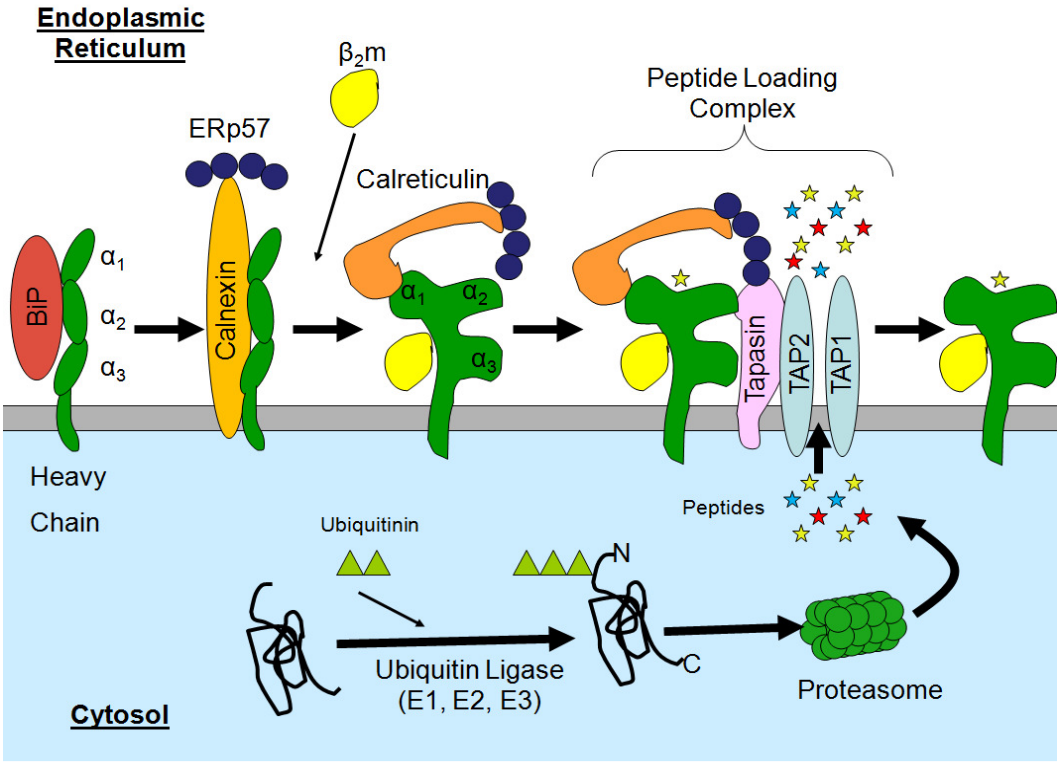


Figure 1-4: Processing of endogenous proteins and presentation in the context of MHC-I molecules
Nascent α chain of the MHC molecule bound to the β2-microglobulin subunit (β2m) associates with different chaperones to form the Peptide Loading Complex (PLC). These chaperones include ERp57, calreticulin and the Transporter Associated with Antigen Processing (TAP1 and TAP2). Misfolded proteins or proteins at the end of their "life" are ubiquitinated and degraded by the proteasome into peptides. These peptides are transporter into the endoplamic reticulum via the TAP proteins and loaded onto MHC-I molecules. The stabilized MHC:peptide complex then migrates to the cell-surface to be recognized by $CD8^+$ T cells.

During synthesis, nascent proteins are associated with chaperones and undergo complex folding to acquire the tridimensional structure required to exert their function(s). Protein folding is an active process that requires chaperones such Hsp70, Hsp40 and Hsp90 (Hartl et al. 2002). While this system has long been considered to be efficient, recent studies have highlighted that this is not the case. Cells are opting for high speed protein synthesis thus increasing the probability to produce large amounts of misfolded proteins. These defective ribosomal products (DRiPs) are then ubiquitinated and degraded by the proteasome to avoid the accumulation of misfolded proteins that could potentially be toxic to the cells. Indeed, the accumulation of misfolded proteins in the ER leads to cellular stress and unfolded protein response (UPR). UPR is associated with numerous diseases such as prion or Alzheimer's disease (Kim et al. 2009). A common feature to several of these diseases and mainly neurodegenerative diseases is the accumulation of misfolded proteins into inclusions bodies such as aggresomes (Kopito. 2000; Garcia-Mata et al. 2002; Kawaguchi et al 2003; Kaganovitch et al. 2008).

After folding and quality control has been performed by chaperone proteins, it has been estimated that for one protein, about 30% are DRiPs that are targeted to the ubiquitin-proteasome degradation pathway along with fully folded proteins at the end of their life-span (Schubert et al. 2000). These DRiPs have got important immunological functions. Indeed, peptides generated from the degradation of DRiPs by the proteasome are the major source of peptides for TAP and MHC-I binding. This mechanism allows the quick presentation of peptides from pathogens and mainly viruses to patrolling $CD8^+$ T cells for rapid recognition and clearance of infected cells (Schubert et al. 2000; Reits et al. 2000). This system however is not perfect and viruses have developed strategies to escape the immune system by altering the presentation of antigens in the context of MHC-I and MHC-II molecules (Ploegh. 1998; Hewitt et al. 2003).

1-2-2-2/ Exogenous pathway of antigen processing and presentation onto MHC-II molecules

Although the MHC-I processing pathway is ubiquitous in nucleated cells, professional APCs such as dendritic cells, macrophages and B cells constitutively express MHC-II molecules. However, the expression of MHC-II is not restricted to professional APCs as other cells such as epithelial cells can be induced to express these molecules under pro-inflammatory conditions such as exposure to IFNγ. These non-professional APCs can present self-antigens to self-reactive T cells that have not been eliminated during the shaping of the TCR repertoire in the thymus: central tolerance (Kingston et al. 1989; Suda et al. 1995; Quaratino et al. 1996; Chan et al. 2006). Central tolerance will be discussed Paragraph 1-4-1/.

Exogenous proteins are taken-up by endocytosis and digested in the lysosomes by acid-dependent proteases such as cathepsins (Watts. 2004; Robinson et al. 2002). As for MHC-I molecules, MHC-II molecules are synthesised in the ER and are dimeric consisting of α and β chains. Transported into the lysosomes and MHC-II rich compartments, MHC-II molecules are associated with the invariant chain (Ii) (Stumptner et al. 1997). The Ii chain is digested in the lysosomal compartment by the pH sensitive cathepsin-S protease. A small fragment called CLIP (Class-II Invariant chain Peptide) remains in the groove of MHC-II molecules. The CLIP peptide is then removed from the peptide-binding groove by the MHC-II like molecule HLA-DM (H2-M in mice) and replaced with high affinity peptides (Gautam et al. 1995; Thayer et al. 1999). The MHC:peptide complexes formed are then stable, dissociate

from HLA-DM molecules and are transported to the cell surface for the priming of $CD4^+$ T cells (Watts. 2004; Robinson et al. 2002) (Figure 1-5).

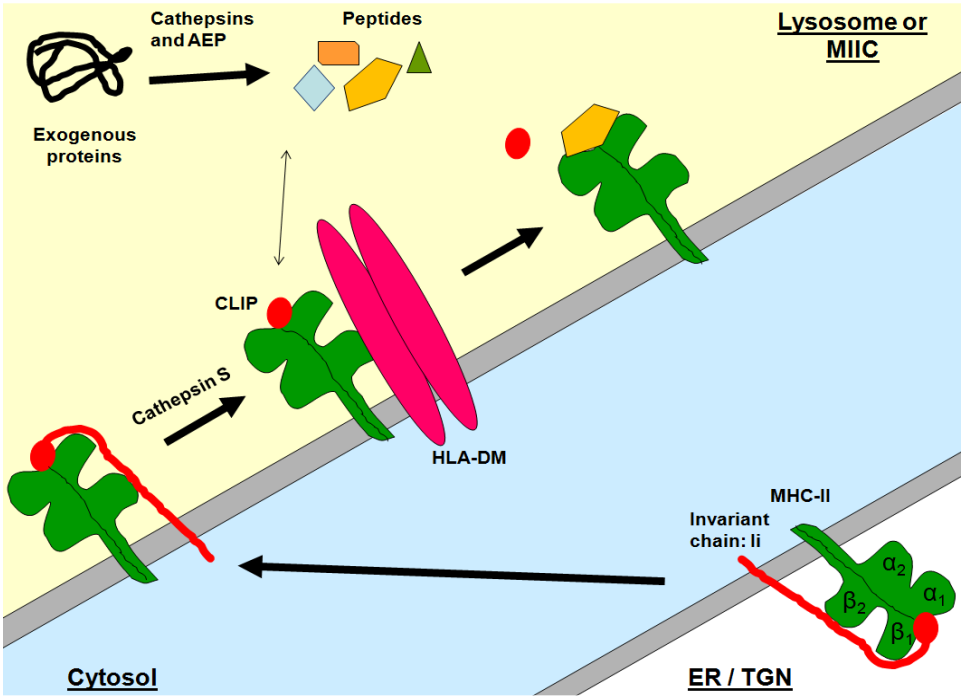


Figure 1-5: Processing of exogenous proteins and presentation in the context of MHC-II molecules
MHC-II molecules are dimeric and formed of two chains (α and β chains) synthesized in the endoplasmic reticulum (ER). Folded MHC-II molecules are transported to the trans-golgi network (TGN) and then to the lysosomes and MHC-II rich compartments (MIIC). The invariant chain (Ii) is digested and a CLIP peptide remains in the peptide-binding groove of MHC-II molecules (cleft formed by the α1 and α2 subunits). Competition with higher affinity peptides for the groove of MHC-II molecules leads to the binding of high affinity peptides. The MHC-II:peptide complex is then transported to the plasma membrane for the priming of $CD4^+$ T cells.

By binding MHC-II molecules, Ii has the ability to regulate and optimize the loading of peptides through the interaction between CLIP and the peptide-binding groove of MHC-II molecules. Some MHC-II alleles have a low affinity for CLIP (I-A^k, I-A^d, I-E^k in mice) and therefore are less dependent on H-2M for the generation of the MHC-II:peptide repertoire. By contrast, some MHC-II alleles have a high affinity for the CLIP peptide (I-A^b in mice) and rely on HLA-DM to exchange CLIP with MHC-II peptides (Gautam et al. 1995). It has been suggested that a decreased affinity for CLIP facilitates the loading of MHC-II peptide thus escaping the editing process mediated by H2-M (Nanda et al. 2000). This would lead to the generation of a larger repertoire of MHC-II peptide presented to T cells and therefore would increase the probability to develop autoimmune diseases (Hitzel et al. 1996; Nanda et al. 2000; Honey et al. 2004).

Ii also plays a critical role in the transport of newly synthesized MHC-II molecules from the ER to lysosomes / MHC-II rich compartments (MIIC). Indeed, the inhibition of Ii's phosphorylation reduces the trafficking of MHC-II molecules to antigen-processing compartments (Anderson et al. 1999). Interestingly, a recent study has highlighted the important role of Ii in the regulation of the motility of mDCs. While the movements of mDCs alternate between episodes of high and low motility, Ii deficient DCs have fast and uniform movements (Faure-Andre et al. 2008).

The role of Ii in the presentation of peptides in the context of MHC-II molecules is therefore crucial as it is involved in the generation of the peptide repertoire presented to $CD4^+$ T cells. Ii also regulates the trafficking of newly synthesized MHC-II molecules from the ER to the antigen-processing compartments. Through its actions, Ii couples antigen processing with cell motility thus enhancing DC's functions during inflammation.

1-2-2-3/ Cross-presentation of endogenous antigens onto MHC-II molecules and of exogenous antigens onto MHC-I molecules

Common pathways of antigen-presentation consist in the presentation of antigens from exogenous origin onto MHC-II molecules and antigens from endogenous origin onto MHC-I molecules.

Some professional APCs are able to perform the phenomenon of cross-presentation, in which an exogenous antigen is loaded onto MHC-I molecules (Kurts et al. 1996). Endocytosed antigens can be directly loaded onto recycling MHC-I molecules. Endocytosed proteins can also be degraded in the lysosomes and exported into the cytoplasm via some transporters such as Sec61 (Lilley et al. 2004; Di Pucchio et al. 2008). These antigens can be further degraded by the proteasome or chaperones within the ER. Eventual binding of these epitopes onto MHC-I molecules allows the presentation of peptides from exogenous origin to $CD8^+$ T cells (Kurts et al. 1996; Burgdorf et al. 2008). DCs bearing the mannose receptor DEC-205 or CD8α have been shown to be efficient at cross-priming (Dudziak et al. 2007).

Although endogenous proteins are presented in the context of MHC-I molecules, cross-presentation onto MHC-II molecules can naturally occur. Cytosolic proteins are degraded in the cytosol before being translocated into the lysosome for loading onto MHC-II molecules

(Dani et al. 2004). The cross-presentation of endogenous has an important immunological role. Indeed, viral proteins can be presented onto MHC-II molecules thus promoting an efficient recognition by the immune system and the rapid clearance of infected cells (Wan et al. 2005).

A study has recently highlighted the ability of DCs to cross-present exogenous antigens to T cells several days after receptor-mediated uptake of the Ag. DCs were storing the antigen in early endosomes for up to 3 days thus allowing its presentation to $CD4^+$ and $CD8^+$ T cells (van Montfoort et al. 2009).

1-3/ Lineage decision of T cells: a phenomenon orchestrated by dendritic cells

The generation of an appropriate T cell response relies on the ability of a TCR to recognize a specific antigen presented by APCs in the context of MHC molecules. This first signal alone leads to T cell anergy and costimulatory signals are necessary to initiate an efficient T cell response.

1-3-1/ T cell priming: TCR recognizes MHC:peptide Complex

T cells recognize the cognate peptide in the peptide-binding groove of MHC molecule through the TCR. This interaction known as activation signal 1 also dictates the fate of thymocytes during the selection occurring in the thymus: central tolerance (mechanisms of selection in central tolerance will be discussed Paragraph 1-4-1/). Indeed thymocyte progenitors arriving at the thymus from the bone-marrow are selected on their ability to recognise self-MHC molecules but not self-antigens. This prevents the survival of self-reactive T cells and the occurence of autoimmune diseases.

The interaction of a TCR with a MHC:peptide complex depends on the overall avidity of the complex MHC:peptide/TCR. This avidity relies on the affinity of the MHC molecule for the specific antigen, the affinity of the TCR for a specific MHC:peptide complex but also on the concentration of the antigen (Anderton et al. 2002). By definition, the avidity of the TCR

for a specific peptide/MHC complex is the concentration of peptide leading to the activation of 50% of the pool of antigen-specific T cells. This in turn defines a threshold of activation (van den Boorn et al. 2006). The strength of the signalling resulting from this interaction dictates the fate of T cells towards anergy or activation. The level of expression of MHC:peptide and TCR by APCs and T cells respectively is therefore a key element in inducing a T cell response (Figure 1-6).

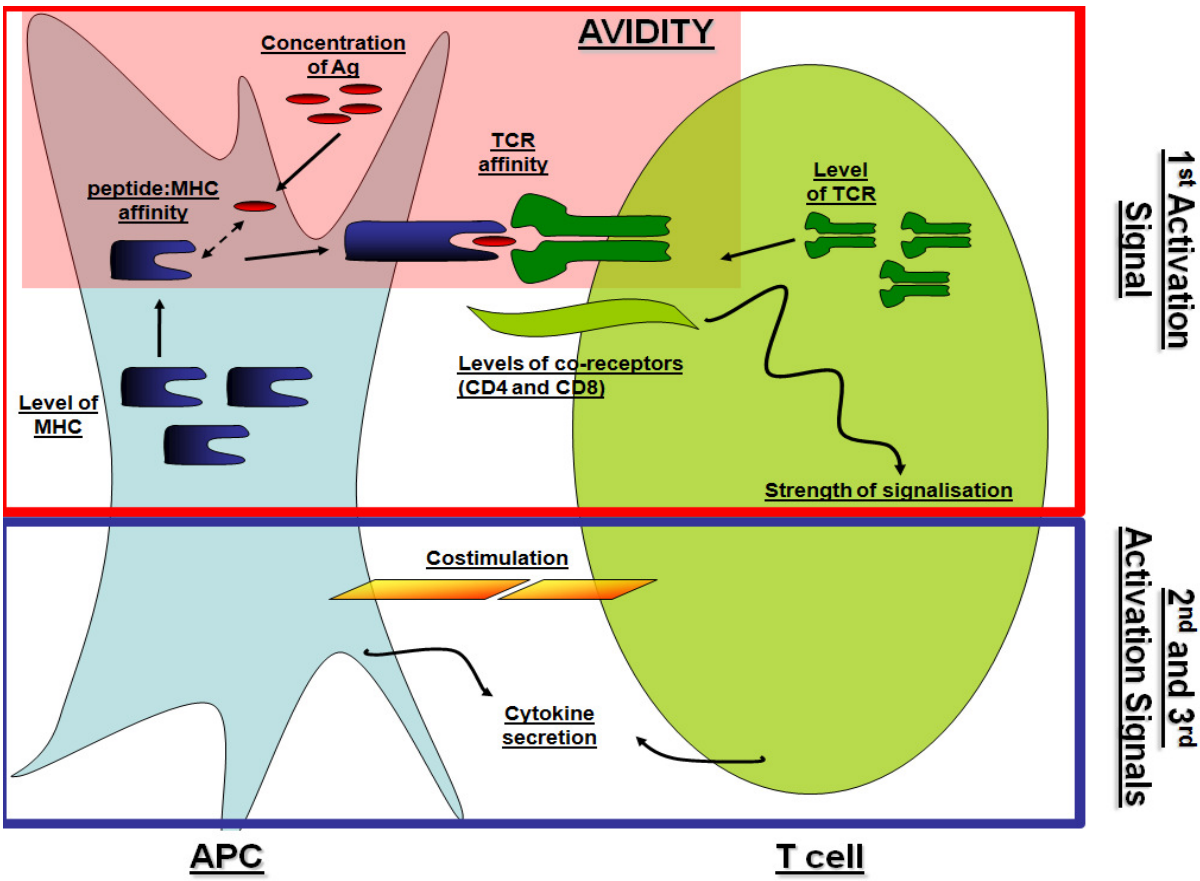


Figure 1-6: Elements dictating T cell avidity and activation
The interaction between a T cell receptor (TCR) and a peptide bound to an MHC molecule presented by antigen presenting cells (APC) depends on numerous factors. The avidity of the complex MHC:peptide/TCR is defined by the affinity between a peptide and the MHC molecule, the affinity of the TCR for the MHC:peptide complex and the concentration of antigen (Ag) (*Red-filled area*). The first activation signal based on the recognition of the MHC:peptide complex by the TCR depends on the avidity of the MHC:peptide/TCR complex, the level of expression of MHC molecules, TCR and co-receptor (CD4 and CD8α) but also the strength of the signal after T cell engagement (*red rectangle*). A successful T cell activation will require the engagement of costimulatory molecules and the presence of cytokines secreted by DCs and T cells (*blue rectangle*).

Peptides bind MHC through anchoring points in the peptide-binding grooves formed by the α1 and α2 subunits of MHC-I molecules and α1 and β1 subunits of MHC-II molecules. The association constant (Ka) or off-rate binding of the MHC-peptide complex depends on the strength and affinity of this interaction. Low affinity peptides have a low Ka or fast off-

rate binding while high affinity peptides interact more strongly with MHC molecules and have a high Ka or low off-rate binding. The definition of anchor amino-acid is at the origin of predilection techniques assessing the ability of a peptide to bind a specific MHC molecule (Young et al. 1995).

Several studies have also highlighted the importance of manipulating peptides via the modification of some key anchor amino-acids to promote a T cell response. This technique has been developed to improve the affinity of self- and tumour specific- peptides for MHC molecules and therefore their immunogenicity in therapeutic strategies in cancer (Vertuani et al. 2004; Borbulevych et al. 2005). The generation of altered-peptide ligands (APL) that naturally occurs *in-vivo* has been shown to be important in the generation of immune responses both in cancer and autoimmune diseases. Indeed, peptides presenting post-translational modifications such as phosphorylation (Mohammed et al. 2008) or glycosylation (Wood et al. 1998) can present increased affinity to MHC molecules and contribute to the peptide repertoire binding a specific MHC molecule (Engelhard et al. 2006). This will be discussed Paragraph 1-5-2/.

A single TCR has therefore the ability to bind different epitopes with different strength (T cell degeneracy / cross-reactivity) (Kersh et al. 1996; Anderson et al. 2000). This highlight that the nature of the processed peptide, rather than the type of APC, determines the outcome of T cell activation: anergy or proliferation.

1-3-2/ Different pathways for the generation of T cell response

According to the nature of the pathogen or inflammation, DCs have the ability to polarize naïve $CD4^+$ T cells into different $CD4^+$ T effector cells. This phenomenon depends on the cytokines produced by DCs in the context of inflammation and induced after TCR engagement.

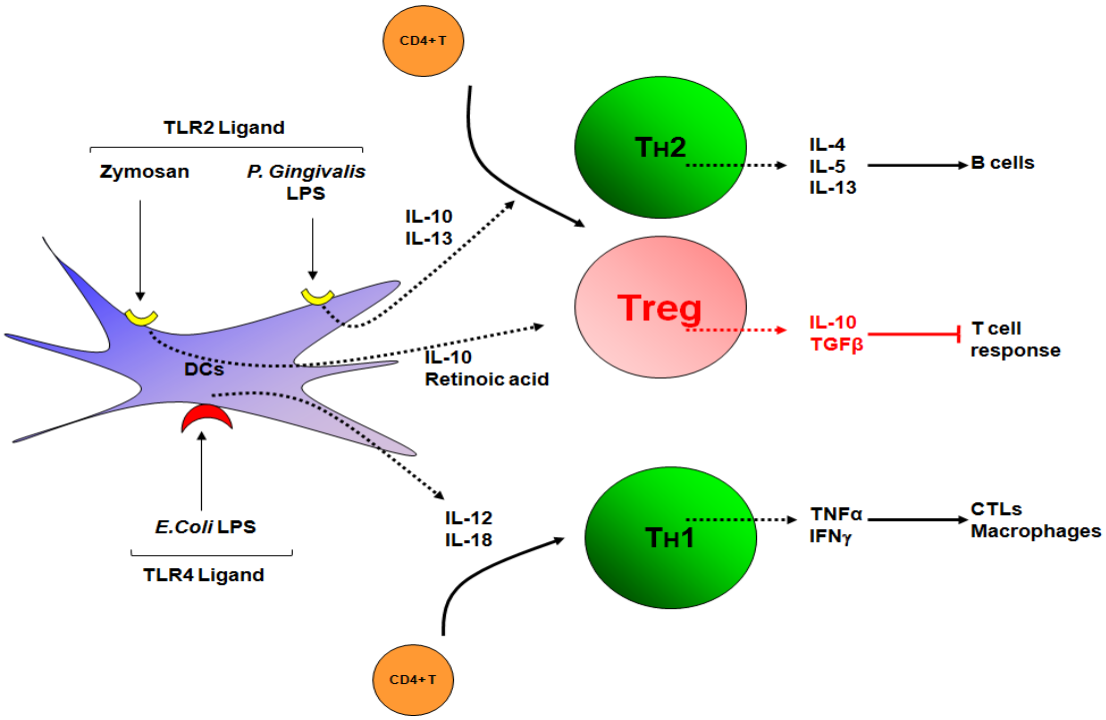


Figure 1-7: DCs polarize the response of CD4$^+$ T cell in response to different TLR ligands
The binding of different TLR ligands by dendritic cells shape the CD4$^+$ T cell response.
* DCs exposed to TLR4 ligands (such as LPS from *Escherichia Coli*) release IL-12 and IL-18 that induce the generation of a Th1 response. Th1 cells release of TNFα and IFNγ.
* DCs activated through TLR2 by LPS from *Porphytomonas gingivalis* produce IL-10 and IL-13 that favor the generation of a Th2 response and the subsequent release of IL-4 and IL-5 and IL-13.
* Stimulation of DCs with the TLR2 ligand zymosan leads to the secretion of IL-10 and retinoic-acid that support the generation of regulatory T cells (Treg). Treg inhibit T cell response via the release of IL-10 and TGFβ.

Different LPS bind different TLR on APCs (Paragraph 1-2-1-1/). One principal aspect of these receptors is the ability to polarize the CD4$^+$ T cell response into a Th1-, a Th2- or a Treg-like response (Netea et al. 2002; Germain. 2004). Exposure of DCs to LPS from *Escherichia Coli* results in the binding of TLR4 and the subsequent release of IL-12 and IL-18 by DCs (Banchereau et al. 2000). This enhances an IFNγ-like Th1 response that will ultimately leads to the stimulation of cytotoxic T lymphocytes (CTLs) and macrophages. LPS from *Porphytomonas Gingivalis* will bind mainly to TLR2 and induces the release of IL-10 and IL-13 by DCs. This promotes a Th2-like response that will favour the action of B cells (Agrawal et al. 2003; Dillon et al. 2004). By contrast, stimulation of DCs with the TLR2 ligand Zymosan leads to the production of IL-10 and retinoic-acid that promote the generation of regulatory T cells (Treg). Treg inhibit T cell response through the secretion of IL-10 and TGFβ (Manicassamy et al. 2009) (Figure 1-7).

1-3-2-1/ The different subsets of helper T cells – Plasticity of the T cell response

The hallmark of adpative immunity is the existence of lymphocytes that induce and regulate immune responses. Activated in a specific cytokine environment in which APCs such as DCs have a crucial role, naïve $CD4^+$ T cells proliferate and differentiate into different subsets such as Th1, Th2, regulatory T cells or the newly described Th17 cells (Figure 1-8):

- **Th1** cells are induced by exposure to cytokines such as IL-2, IL-12, IFNγ, IL-1β and TNFα. In this cytokine environment, naïve $CD4^+$ T cells express the transcription-factors STAT-1 and T-BET (Szabo et al. 2000; Afkarian et al. 2002). Further stimulation with DC-derived IL-12 and IL-18 induces the expression of the transcription-factor STAT4. At this point, $CD4^+$ T cells are mature Th1 cells. Th1 cells secrete different cytokines such as IL-2, very low amounts of IL-10, members of the TNF family and high amounts of IFNγ. Th1 cells are generally generated in response to intracellular infections such as mycobacteria (Murphy et al. 2002).

- In an environment rich in IL-4, IL-10, IL-5, IL-13 and GM-CSF, naive $CD4^+$ T cells upregulate the expression of STAT6 and GATA3 transcription-factors skewing their phenotype toward **Th2** (Farrar et al. 2001; Zhu et al. 2001). Th2 cells secrete diverse cytokines such as IL-4, IL-5, IL-13 and IL-10 (low amounts) and stimulate the production of IgE by B cells to promote the clearance of extracellular pathogens. Th2 cells have a preponderant role in mucosal barriers (trachea, gut) and promote the functions of mast-cells and eosinophils (Murphy et al. 2002).

- Recently a newly recognised subset of $CD4^+$ T cells has been described. These cells secrete IL-17A and have a major role in autoimmune diseases. Upon stimulation with IL-6 (or IL-21) and TGFβ, naïve $CD4^+$ T cells express the transcription-factors STAT-1 and RORγT (Manel et al. 2008). This first phase allows for the secretion of IL-21 by maturing **Th17** cells and the full expression of the IL-21 receptor (Korn et al. 2007; Zhou et al. 2007). IL-21 is required for the amplification of the Th17 response in an autocrine/paracrine manner. This amplification leads to the expression of a functional IL-23 receptor on Th17 T

cells. IL-23 secreted by APCs promotes the full maturation and stabilization of Th17 cells that secrete IL-17A, IL-17F, IL-22 and IL-10 (Volpe et al. 2008). The generation of Th17 is altered by IL-25, IL-27, IL-4 and IFNγ (Kleinschek et al. 2007).

- Similar to Th17 cells, inducible $Foxp3^+$ **Treg** require TGFβ to differentiate while IL-6 inhibits their generation (Betelli et al. 2006). IL-6 plays an important role in a TGFβ rich environment as it promotes the generation of pathogenic Th17 cells and inhibits the development of Treg. $CD4^+$ $CD25^+$ $Foxp3^+$ Treg are at the forefront of the mechanisms of peripheral tolerance and a deficiency of Tregs has been associated with the development of AID (Sakaguchi et al. 1995; Asano et al. 1996; Suri-Payer et al. 1998). Tregs inhibit T cell proliferation and function via different mechanisms (Brandenburg et al. 2008). They exert their function via the neutralization of cytokines such as IL-2 (Pandiyan et al. 2007) and the release of IL-10 and TGFβ. By aggregating on DCs, Treg also alter the function of DCs by reducing the expression of costimulatory molecules and cytokines (Onishi et al. 2008). While Treg are involved in cancer and AID, they play an important role in other pathologies such as the recovery process following a stroke (Liesz et al. 2009).

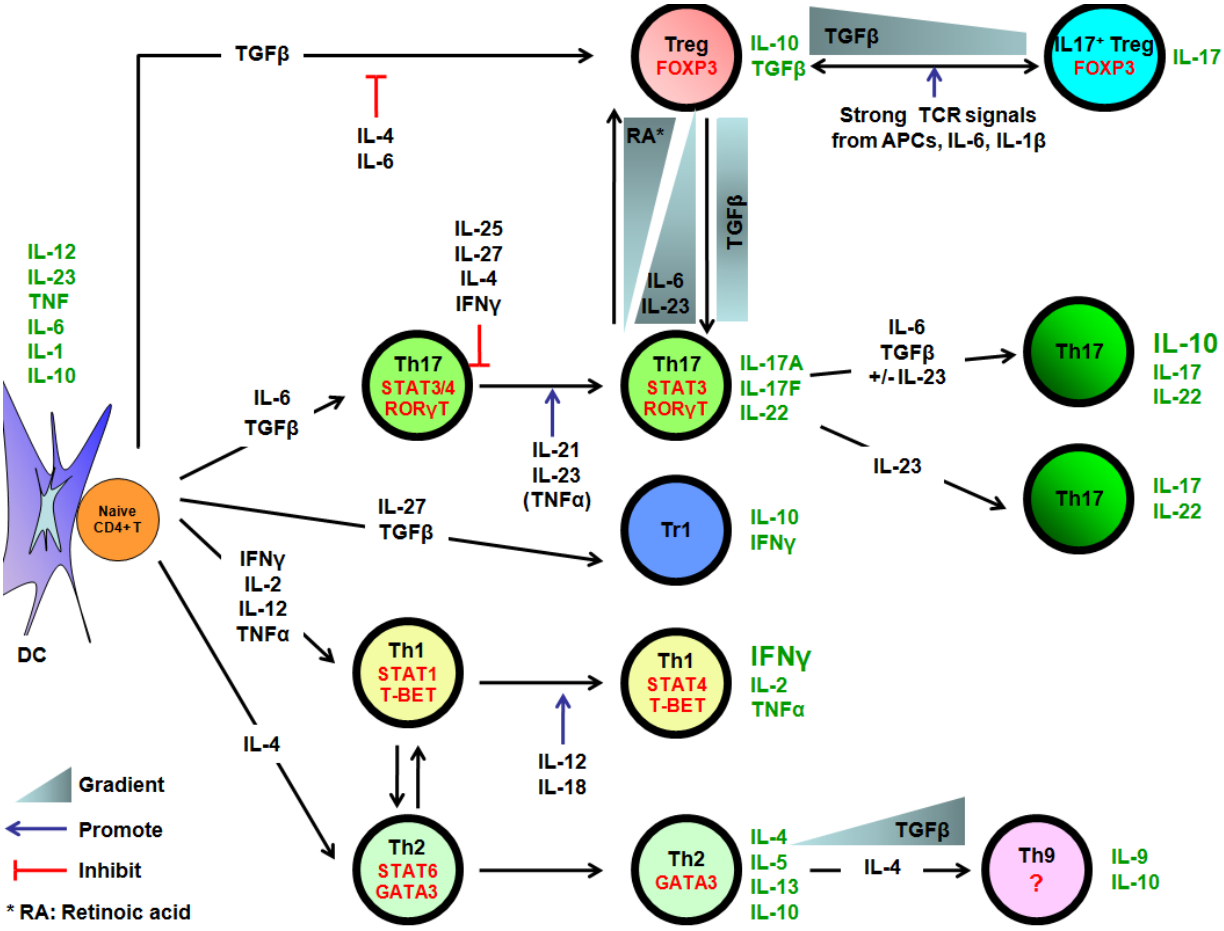


Figure 1-8: Plasticity of the T cell response mediated by dendritic cells and the cytokine environment
Dendritic cells (DC) induce the development of various $CD4^+$ T cell subsets such as Th1, Th2, Th17 or Tregs. TGFβ favors the generation of $Foxp3^+$ **Tregs** that inhibit T cell response via the release of IL-10 and TGFβ. Il-6 blocks the generation of Tregs. Acting in synergy with TGFβ, IL-6 promotes the generation of IL-17A/F and IL-22 secreting $ROR\gamma T^+$ **Th17** T cells. While IL-27 inhibits the generation of Th17 T cells, its association with TGFβ leads to the generation of suppressive **Tr1** T cells characterized by the production of IL-10 and IFNγ. In an environment rich in IL-2, IL-12 and IFNγ, naïve T cells express the transcription factors STAT1 and T-BET and differentiate into **Th1** T cells secreting IFNγ, IL-2 and TNFα. Upon IL-4, naïve T cells express STAT6 and GATA3 and acquire a **Th2** phenotype. Th2 T cells release different cytokines such as IL-4, IL-5, IL-10 and IL-13.

Upon stimulation with TGFβ and IL-4, Th2 T cells skew their phenotype and secrete IL-9 and IL-10 (**Th9** T cells). Th17 T cells adjust their phenotype according to the inflammatory nature of the microenvironment. If Th17 cells are still in an environment containing IL-6 and TGFβ, Th17 cells release less IL-10 and become more and more **pathogenic**. On the other hand, absence of TGFβ and IL-6 will skew the phenotype of Th17 cells into Th17 cells producing more IL-10, thus presenting a more **regulatory** and a less pathogenic phenotype. In the presence of IL-6, IL-1β, and strong TRC signals delivered by APCs, TGFβ secreting Tregs express the Th17 cytokine IL-17. **$Foxp3^+$ $IL17^+$ Tregs** express IL-17 in this cytokine environment and contribute to the inflammatory response.

In **green** are represented the cytokines secreted by the specific Th subsets. In **red** are represented the transcription factors and in **black** the cytokines promoting or preventing the generation / differentiation of the different T cell subsets.

The ability of DCs to promote the differentiation of $CD4^+$ T cells into different T cell subsets must allow a certain degree of plasticity to finely tune the T cell response to the inflammation over time (Figure 1-8).

- The first evidence on the plasticity of the adaptive immune response is the ability for Th1 and Th2 cells to interconvert. This phenomenon is antigen-specific and depends on the expression of costimulatory molecules, ICAM-1 and the level of IL-12, IFNγ and IL-12 present in the microenvironment. However, this reversibility is lost in fully differentiated Th1 and Th2 cells (Murphy et al. 1996; Radhakrishnan et al. 2007).

- The role of Th17 in AID and host-defence against pathogens has been largely described (Bettelli et al. 2008). The combinations of TGFβ / IL-6 and TGFβ / IL-21 promote the generation of $ROR\gamma T^+$ Th17 T cells. In a situation of continuous inflammation, a prolonged exposure of Th17 to TGFβ and IL-6 (associated or not with IL-23) abrogates their pathogenic functions while increasing the amounts of IL-10 they secrete. IL-10 producing Th17 T cells are less pathogenic and display a more regulatory phenotype associated with the dampening of the immune response. In the absence of IL-6 and TGFβ, stimulation of Th17 with IL-23 preserves the secretion of IL-17. IL-6 and TGFβ have therefore a dual role as they promote the differentiation of Th17 while restraining their pathogenic functions (McGeachy et al. 2007).

- After stimulation with IL-4 and in the presence of TGFβ, Th2 T cells upregulate the expression of Foxp3. Foxp3 directly interacts with GATA3 resulting in the downregulation of Th2 specific genes. $Foxp3^+$ $GATA3^+$ Th2 cells do not secrete common Th2 cytokines (IL-4, IL-5 and IL-13) but produce IL-9 and IL-10 to become "**Th9**" T cells. Although Foxp3 and GATA3 are required throughout this differentiation process, terminally differentiated Th9 T cells do not express any of these transcription factors, and no specific transcription factors have so far been identified (Tato et al. 2008). Interestingly, Th9 cells provide protection in response to infections by the helminth *Trichuris muris* probably via the recruitment of mast cells (Veldhoen et al. 2008). Th9 cells do not inhibit the proliferation and functions of effector T cells in a mouse model of autoimmune colitis. Yet, adoptive-transfer of Th9 cells worsens the disease and mice presented a more severe inflammation of the guts (Dardalhon et al. 2008). Thus, Th9 lacking suppressive function on T cells are

beneficial in host-pathogen infections and detrimental in T cell driven autoimmune diseases.

- TGFβ has an important role in the generation of Th17, Tregs and Th9 cells. Studies in mice have recently indicated that Tregs secreting TGFβ and IL-10 can produce IL-17 upon stimulation with IL-6 (Xu et al. 2007; Radhakrishnan et al. 2008). Only a specific subset of Tregs ($CD4^+$ $CD25^{hi}$ $CCR6^+$ HLA-DR^- $Foxp3^+$) in humans could express IL-17. This subset retained its suppressive functions in response to weak TCR stimulation after binding APCs. However, upon high TCR stimulation and the secretion of IL-6 and IL-1β by APCs, these Tregs transiently lose their suppressive activity and secrete IL-17. Inflammation can therefore drive HLA-DR^- Tregs to acquire an effector Th17-like phenotype. This mechanism allows the rapid adaptation of T cell response to suppress or activate pro-inflammatory responses (Beriou et al. 2009).

1-3-2-2/ The importance of Th17 and Th1 in the development of autoimmune diseases - The new Th1 / Th2 / Th17 paradigm?

For many years, Th1 and Th2 T cells have been at the centre of immunological-classification of different chronic inflammatory diseases. Th2 T cells were associated with allergy and asthma whereas Th1 T cells were linked with the development of autoimmune diseases. This Th1/Th2 classification or paradigm could not explain various diseases and results obtained. Indeed, treatment of EAE models developing multiple sclerosis with IFNγ does not worsen but reverses disease (Voorthuis et al. 1990). In contrast, blocking IFNγ is detrimental as disease worsens (Billiau et al. 1988). This highlights that IFNγ has different roles according to the stage of disease.

The recent identification of Th17 T cells has helped to clarify some of the discrepancies resulting from the Th1/Th2 paradigm (Korn et al. 2007; Zhou et al. 2007; Manel et al. 2008). However new findings have highlighted a new dilemma when considering the action of Th1 and Th17 cells in the pathogenesis of AID such as experimental autoimmune uveitis (EAU mice) resembling experimental autoimmune encephalomyelitis (EAE mice) in the pathogenesis of the disease (Steinman et al. 2008; Kroenke et al. 2008; Luger et al. 2008).

In EAU mice, injection of the cognate antigen (IRBP: intra-retinal binding protein) in CFA induces disease. Interestingly, addition of blocking αIL-17A antibodies (Ab) dampened clinical signs in ongoing disease while it altered the onset of the disease. On the other hand, blocking of IL-23 with αIL-23 Ab was only beneficial if added early during priming phases of the disease. This was further confirmed by the use of specific knock-out mice. Mice lacking subunits for IL-23 and IL-12/IL-23 ($p19^{-/-}$ and $p40^{-/-}$ mice respectively) were protected against disease when immunized with IRBP and CFA. Lack of IL-12 in $p35^{-/-}$ mice resulted in increased disease activity upon immunization. These results confirmed that IL-23 is necessary for the priming of the disease and Th17 cells but not for their effector functions mediated by IL-17, IL-17F and IL-22 (Luger et al. 2008) (Figure 1-9/A).

Interestingly, Luger *et al.* demonstrated that adoptive transfer of IRBP-specific Th1 and Th17 cells were able to induce EAU in WT mice. WT mice were immunized with IRBP and CFA, draining lymph-nodes harvested and T cells polarized to Th1 (IRBP peptide, IL-12 and αIL-4 antibody) or Th17 (IRBP peptide, IL-23, TGFβ, IL-6 and anti-IL-4 Ab) T cells. Adoptive transfer of Th1 polarized T cells induced EAU in WT mice treated or not with αIL-17 Ab. However, blocking of IFNγ with an αIFNγ Ab prevented the occurrence of EAU. Interestingly, WT and $IFN\gamma^{-/-}$ mice develop EAU upon immunization with IRBP-specific Th17 T cells (Figure 1-9/B) (Luger et al. 2008).

Analysing cellular infiltrates in the central nervous system (CNS) of WT mice immunized with pathogenic Th1 and Th17 T cells from EAE mice, Kroenke *et al* demonstrated that EAE mediated by Th1 and Th17 T cells was associated with macrophage and neutrophil infiltration respectively (Kroenke et al. 2008). This confirmed another study demonstrating that Th17/Th1 ratio dictated the severity of EAE disease. Indeed, mice with high Th17/Th1 ratio develop severe EAE mainly in the CNS. Mice with low Th17/Th1 presented a mild disease activity that localized in the spinal cord (Stromnes et al. 2008)

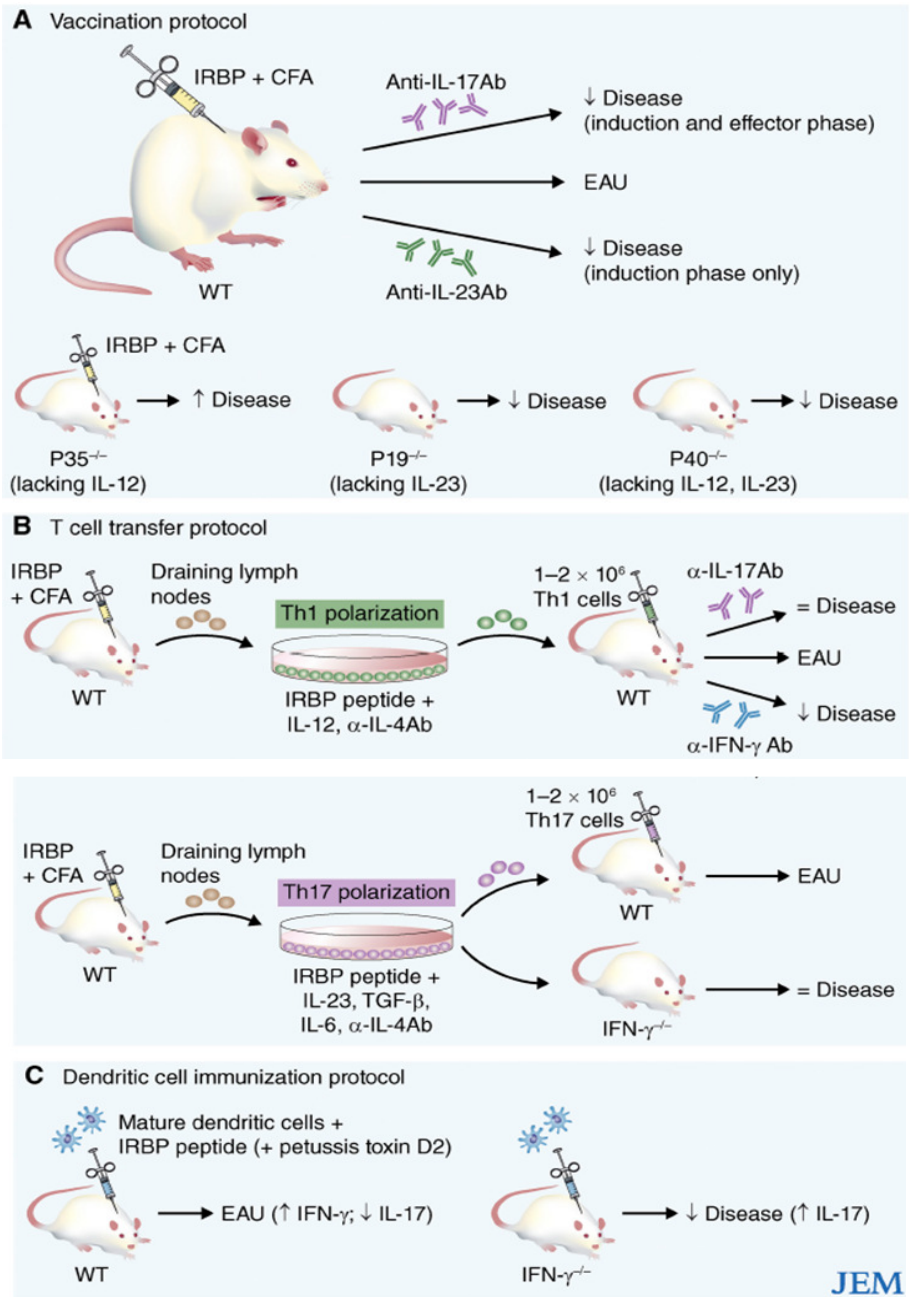


Figure 1-9: Influence of Th1 and Th17 cells on the pathogenesis of experimental autoimmune uveitis (EAU) – Place of Th17 in the Th1/Th2 paradigm

(A) EAU is induced in WT mice by immunization with IRBP and CFA. Blockade of IL-17 using αIL-17 antibodies (Ab) reduced disease activity at the onset, after induction and in ongoing disease activity. Blockade of IL-23 with αIL-23 Ab was only effective during disease induction. A reduction of EAU in $p19^{-/-}$ and p40**-/-** mice lacking IL-23 and IL-12/13 respectively was observed, whereas increased disease activity was observed in $p35^{-/-}$ mice lacking IL-12. **(B)** Adoptive transfer of *in-vitro* polarized Th1 and Th17 cells induced EAU in WT mice. The severity of the disease was not affected when $IFN\gamma^{-/-}$ mice where immunized with polarized Th17 cells. However, blockade of IFNγ in WT mice immunized with Th1 polarized cells reduced the severity of the disease; blocking of IL-17 did not have any effects. **(C)** IRBP pulsed matured DCs were injected into mice subsequently inoculated with pertussis toxin after 2 days. Immunized WT mice developed EAU associated with high production of IFNγ and low production of IL-17. EAU was decreased in $IFN\gamma^{-/-}$ mice despite a strong IL-17 production. Figure is adapted from (Steinman. 2008)

Immunization of WT mice with IRBP pulsed-DCs promoted EAU associated with high amounts of IFNγ and reduced amounts of IL-17. IFNγ$^{-/-}$ mice however seemed to be protected against EAU upon immunization with IRBP pulsed DCs despite a robust IL-17 production (Figure 1-9/C). These results are different from results exposed figure 1-9/B and 1-9/C. This can be explained by the use of CFA adjuvant in the immunization of WT with IRBP peptide. Indeed, CFA contains killed mycobacteria that are able to skew T cell phenotypes. Indeed CFA is a strong IL-6 inducer while IFA fails to promote the induction of IL-6 and contributes to the conversion of Foxp3^{-} cells to Foxp3^{+} cells *in-vivo* (Dr. Thomas Korn, British Society for Rheumatology Annual Meeting 2008, Liverpool, UK; Korn et al. 2008).

These data clearly demonstrated that Th1 and Th17 can act independently of each other to induce autoimmune diseases. We cannot conclude that Th1 are more pathogenic and able to induce AID than Th17 and *vice versa*. This question was at the centre of a recent review on the role of Th17 in immunity and for many reasons such as those highlighted in this paragraph, the conclusion was that both Th1 and Th17 are able to induce AID (Bettelli et al. 2007, Steinman, 2007; Steinman, 2008). Thus, rushing into a new Th1/Th2/Th17 paradigm seems inadequate and one must consider that the nature of inflammation, cellular interactions and cellular signalling pathways dictates the outcome and the different subsets involved in AID. Finally, precautions must be taken when considering results from studies requiring the use of adjuvants and *in-vitro* polarized T cell subsets as they may not reflect reality.

The ability of the adaptive immune response to be shaped or formed in relation to the nature of inflammation is essential to mount an effective immune response. This however requires that the immune system is strictly recognizing self-MHC molecules whilst not reacting against self antigens so as to avoid the emergence of AID. The shaping of the T cell repertoire is therefore essential to prevent the immune system to recognise self antigens.

1-4/ Shaping of the T cell repertoire – Role of central and peripheral tolerance

The shaping of the T cell repertoire is a prerequisite for the efficient activity of the immune system. Two systems exist to prevent the generation and activation of self-reactive T cells: central and peripheral tolerance. In this paragraph will be exposed the different mechanisms governing these two systems.

1-4-1/ Central Tolerance

Central tolerance is a mechanism developed by mammals throughout evolution by which developing T cells are selected on their ability not to recognize self antigens. By contrast to peripheral tolerance, central tolerance occurs while T cell precursors mature in the thymus before being released into the peripheral blood and the periphery.

Thymocyte progenitors from the bone-marrow enter the thymus through the cortex-medulla junction. At this stage, they do not express either the CD4 or CD8α co-receptor chains but a pre-TCRβ chain (von Boehmer et al. 2003). Only those Double Negative cells (DN) that have correctly rearranged their TCRβ chain with an endogenous α chain, upregulate both co-receptors to become $CD4^+$ $CD8\alpha^+$ double positive (DP) thymocytes. DP thymocytes then go through a very stringent selection process within the thymus resulting in the death of about 95% of thymocyte precursors (Figure 1-10). The selection is based on the overall avidity of the peptide-MHC:TCR complex (Paragraph 1-3-1/).

During **positive selection** DP thymocytes are selected through the binding of their TCR to self-MHC molecules expressed by cortical thymic epithelial cells (cTEC). DP that bind self-MHC molecules receive survival signals and migrate to the medulla. Those unable to bind self-MHC molecules undergo apoptosis by neglect. Among the high and low affinity DP thymocytes positively selected, some can recognise “self” and therefore could promote AID (Baldwin et al. 2004).

To eliminate these self-reactive T cells, a second selection occurs in the medulla of the thymus: the **negative selection** (Reviewed in Palmer. 2003). This crucial mechanism leads to the clonal deletion of thymocytes recognising self-MHC:self-antigen complexes. TCRs with a very high affinity for self-MHC:self-antigen complexes die by apoptosis. Thymocytes with

intermediate affinity will become Treg whereas those with low affinity TCR will be selected and become mature naive T cells that leave the thymus into the blood stream (Zuniga-Pflucker et al. 2004**;** Hogquist et al. 2005; Ladi et al. 2006).

Negative selection is possible as peripheral antigens or tissue specific antigens (TSA) are expressed in the thymus (Palmer. 2003). The expression of high number of genes is specific of thymic epithelial cells from the medulla (mTEC). It allows the presentation of a broad range of self-peptide representing the self-repertoire under the influence of a transcription factor: the autoimmune regulator AIRE (Derbinski et al. 2001; Gillard et al, 2005). This transcription factor controls the expression of a cluster of genes that encode for TSA. The importance of this transcription factor has been shown in AIRE knock-out mice and patients with a deficiency in AIRE (Autoimmune PolyEndocrynopathy Candidiasis Ectodermal Dystrophy or APECED) expression, as they develop multiple AID (Anderson et al. 2002). Moreover, it has been shown recently that the induction of antigen specific tolerance and therefore the prevention of autoimmune diseases could be performed by inducing the thymic expression of a single self-antigen. The self antigens chosen are not constitutively presented by mTECs, and their expression is induced intra-thymically using lentiviruses (DeVoss et al. 2006; Marodon et al. 2006).

Cortical thymic epithelial cells do not express AIRE and therefore do not mediate negative selection. On the contrary immature medullary thymic epithelial cells (Imm mTEC) express this transcription factor at a low level. As they mature, they upregulate the expression of AIRE after engagement of lymphotoxin-β receptor (LTβR) and have therefore an increased ability to express TSAs and mediate the negative selection (Kyewski et al. 2004). The importance of dendritic cells has also been shown in mediating both negative selection and the generation of naturally arising regulatory T cells (Treg).

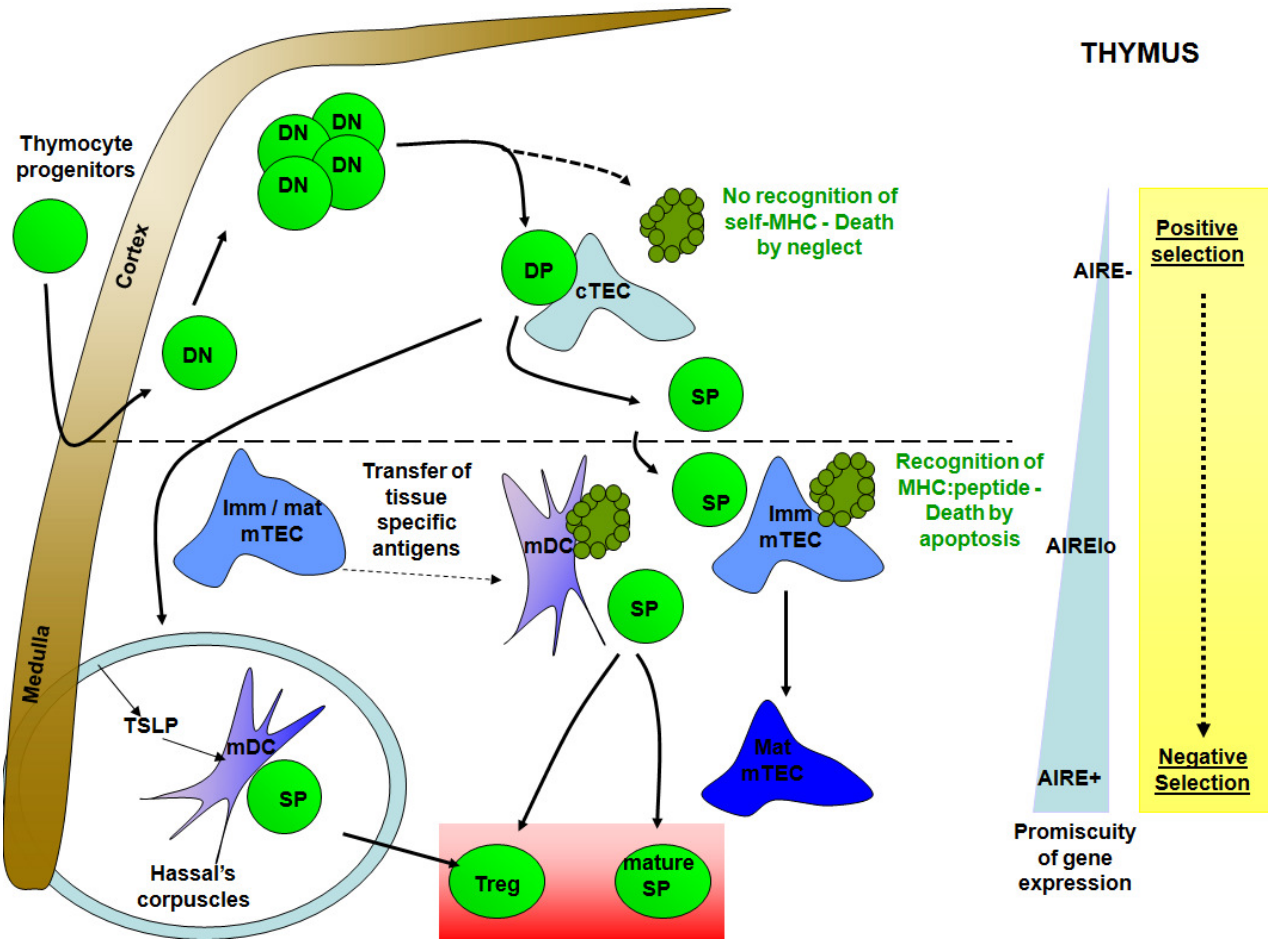


Figure 1-10: Central tolerance in the thymus: positive and negative selections
Thymocyte progenitors enter the thymus through the cortex medulla junction where they proliferate and differentiate in double-negative (DN) cells. Double positive (DP) thymocytes bind cortical thymic epithelial cells (cTEC) and are going through positive selection. Thymocytes positively selected migrate toward the medulla and are going through negative selection via the binding of their TCR to self tissue associated antigens/ MHC molecule complexes. According to their affinity to the complex they are either depleted by negative selection or positively selected to become mature single positive (mature SP) effector T cells or regulatory T cells. Immature medullary thymic epithelial cells (Imm mTEC) express low level of AIRE. As they mature (mat mTEC), they upregulate the expression of AIRE via the contact of LTβR present at the surface of thymocytes. Tissue specific antigens from Imm or mat mTEC can be transferred to mature DCs (mDCs) thus enhancing negative selection. Hassall's corpuscles secreting TSLP lead to the maturation of DCs that subsequently favor the generation of regulatory T cells (Treg). Figure is adapted from (Zuniga-Pflucker et al. 2004) and (Anderson et al. 2001).

Thymic medullary DCs have an important role in central tolerance by cross-presentation of antigens from dying immature and mature thymic epithelial cells (Hogquist et al. 2005). Dendritic cells in Hassall's corpuscle in the thymus can be activated by thymic stromal lymphopoietin TSLP released by cells from the corpuscles (Liu et al. 2006; Watanabe et al. 2005). Resulting mature DCs are then able to induce both proliferation and differentiation of naturally occurring $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs. This function has been described to depend on the expression of costimulatory molecules like CD80 and CD86 as well as the secretion of IL-2 (Watanabe et al. 2005).

Central thymic selection is very efficient and depends on the avidity displayed by a TCR for a specific TSA/MHC complex (Figure 1-11). The majority of thymocytes with a low TCR avidity undergo death by neglect. Thymocytes with intermediate TCR avidity mature and become either regulatory or effector T cells. Finally, thymocytes with high avidity TCRs undergo negative selection and death by apoptosis.

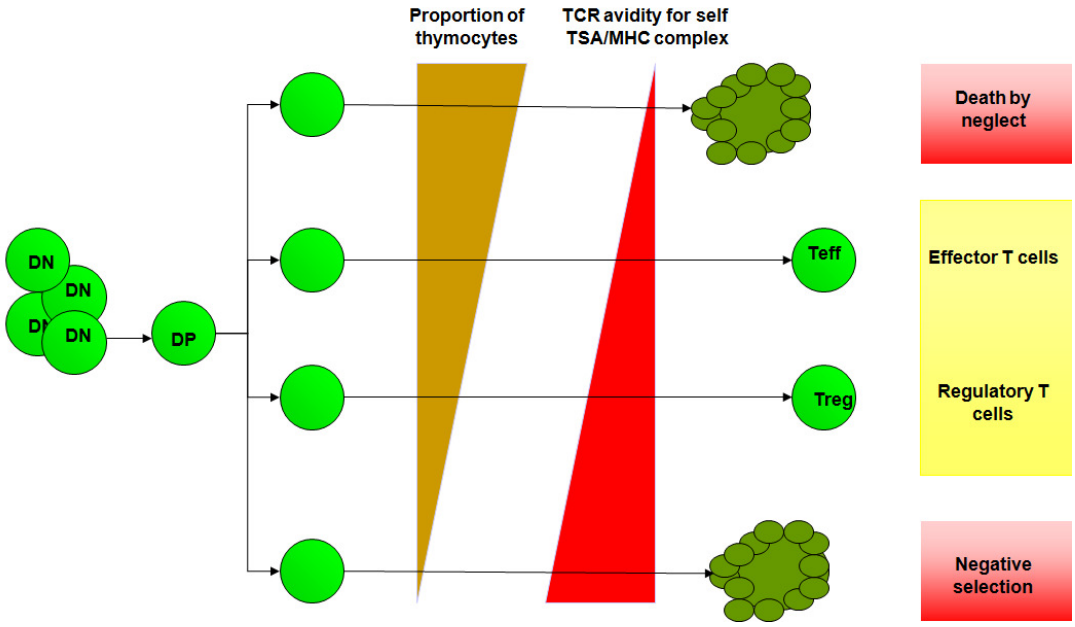


Figure 1-11: TCR avidity of a T cell for a MHC:self-TSA complex defines its fate
Selection of T cells depends on the T cell avidity for self-Tissue specific antigen (TSA):MHC complex. The majority of the cells exhibits a low affinity for the MHC molecules and die by neglect (Positive selection). On the other hand, TCR presenting a high avidity for the TSA/MHC complex are eliminated by negative selection. T cells with intermediate avidity are positively selected and give rise to effector or regulatory T cells. Figure is adapted from (Hogquist et al. 2005).

Thymocytes or naïve effector T cells that have been positively selected leave the thymus and circulate between the blood and lymphoid organs until they recognise the cognate peptide in a context of "danger".

This system is however not perfect and some self-reactive T cells are not eliminated. This is the case if the TCR has a high affinity for a TSA whose expression in the thymus is not dependent on AIRE, or if its expression occurs later in life when the T cell repertoire has already been created. These T cells are in a state of anergy in the periphery. With a very high activation threshold, these cells are very unwilling to proliferate and need a high concentration of antigen to be activated (Jordan et al. 2001). Other mechanisms are in place

in order to keep these self-reactive T cells at bay; this is the phenomenon of peripheral tolerance.

1-4-2/ Peripheral Tolerance

Peripheral tolerance regroups different components to provide immunological tolerance after mature naive T cells leave the thymus to the periphery. Two different systems are in place; intrinsic (depending on the antigen and the costimulation) and extrinsic (relying on inhibitory cells of the immune system) (Walker et al. 2002).

1-4-2-1/ Mechanisms of intrinsic peripheral tolerance

Mechanisms of intrinsic T cell peripheral tolerance depend on the level of MHC:Ag complexes as well as on the nature and amount of costimulation. Four different cases can be considered:

- Self-reactive T cells in the periphery might never encounter the self-peptide. This occurs if the peptide is not accessible to T cells or if the amount of antigen present is not able to trigger T cell activation despite their intermediate TCR avidity (Walker et al. 2002). These T cells are therefore **ignorant**.

- The recognition of a peptide presented by APCs can lead to T cell **anergy** (van Duivenvoorde et al. 2006). At the beginning it was considered that the lack of costimulation was responsible for T cell anergy. It is now acknowledged that the involvement of specific receptors such as PD-1 (Programmed cell Death-1) or CTLA-4 (Cytotoxic T Lymphocyte Associated antigen-4) on T cells and PDL1/2 or CD80/86 on APCs leads to the functional inactivation of T cells. Evidence support the participation of these specific receptors, as mice lacking either of these receptors develop autoimmune and lymphoproliferative disorders (Eagar et al. 2002).

- T cell activation results from the recognition of an antigen presented by APCs in a context of “danger”. In this respect, engagement of the TCR leads to strong signals. However, variants of the antigenic peptides or **Altered Peptide Ligands** have been

shown to act either as TCR agonists or TCR antagonists producing weak signals resulting in T cell anergy (Quaratino et al. 2000). These peptides have been shown to be both MHC Class I and II restricted (Jameson et al. 1993; De Magistris et al. 1992).

- Self-reactive T cells encountering a high concentration of self-antigen or being in a highly inflammatory microenvironment are deleted via apoptosis. This **activation induced cell-death** (AICD) is linked to the upregulation of Fas-L on T cells (Zhang et al. 2004; Ryan et al. 2005).

1-4-2-2/ Mechanisms of extrinsic peripheral tolerance

Extrinsic mechanisms of peripheral T-cell tolerance involve different cell subsets of the innate and adaptive immune systems.

- **$CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells** have been largely described to play an important role in peripheral tolerance (Kim et al. 2007; von Herrath et al. 2003). Two subsets of Tregs exist: thymic derived and peripheral antigen-induced regulatory T cells. These cells have been shown to inhibit T cells via cell-cell contact and the release of IL-10 and TGFβ respectively. Tregs also induce the apoptosis of effector T cells by depleting the micro-environment of cytokines such as IL-2 (Pandiyan et al. 2007).

- Other regulatory T cells have been described such as IL-10 secreting **Tr1** and TGFβ secreting **Th3** cells. Recent work has also focussed on the role of **double negative** T cells in the inhibition of T cell responses (Zhang et al. 2000; Fischer et al. 2005).

- Early precursor cells of the myeloid lineage, **Myeloid Derived Suppressor Cells** (MDSCs) have been shown to strongly inhibit T cell proliferation (Bronte et al. 2000) (This is discussed Paragraph 1-4-3/).

- Although extremely powerful at inducing autoimmune diseases, **dendritic cells** have been shown to play a major role in inhibiting T cell response (partially

described Figure 1-10) (Rutella et al. 2006). The intrinsic mechanisms previously described can be applied to DCs inducing tolerance. It is noteworthy that the same type of tolerance can be induced by non professional APCs such as epithelial cells. We will here focus on the cellular side of peripheral tolerance that depends on DCs (Figure 1-12).

Uptake of antigens via the DEC-205 mannose receptor or uptake of apoptotic bodies does not induce maturation of DCs. iDCs presenting antigens to T cells can lead to T cell death or T cell anergy. This is highlighted in recent studies, targeting of the DEC-205 receptor using an α-DEC-205 antibody conjugated to an Ag (Bonifaz et al. 2002; Bonifaz et al. 2004). Sole injection of the Ab:Ag complex leads to tolerance to the Ag whereas co-injection with αCD40 results in the stimulation of $CD4^+$ T cells as well as $CD8^+$ T cells by Ag cross-presentation (Bonifaz et al. 2002; Bonifaz et al. 2004) (Figure 1-12/A).

De-novo induction of regulatory T cells (Treg) by both immature and mature DCs has been largely documented. This phenomenon has been shown to be mediated by TGFβ but also other molecules such as integrin receptor CD11b on DCs (von Herrath et al. 2003) (Figure 1-12/B).

In the presence of the cognate peptide presented in the context of MHC-II molecules, thymic derived Tregs are able to expand. Their inhibitory activity on T cells is well documented and deficiency in Tregs has been linked with an increased frequency of autoimmune disorders (Kim et al. 2007) (Figure 1-12/C). mDCs presenting the Ag to T cells can lead either to T cell anergy (involvement of CTLA-4 and PD-1 on APCs) or T cell death (involvement of Fas and Fas-L) (Figure 1-12/D). In certain conditions, iDCs and mDCs can release immunoregulatory cytokines that have been shown to inhibit DCs and T cell functions (Rutella et al. 2006) (Figure 1-12/E). Under stimulation by various factors such as IFNγ, PGE2, mDCs upregulate the expression of indoleamine 2,3-dioxygenase (IDO). IDO degrades tryptophan into kynurenines. This leads to the inhibition of T cells, the alteration of DC functions and the generation of Tregs (Puccetti et al. 2007) (Figure 1-12/F).

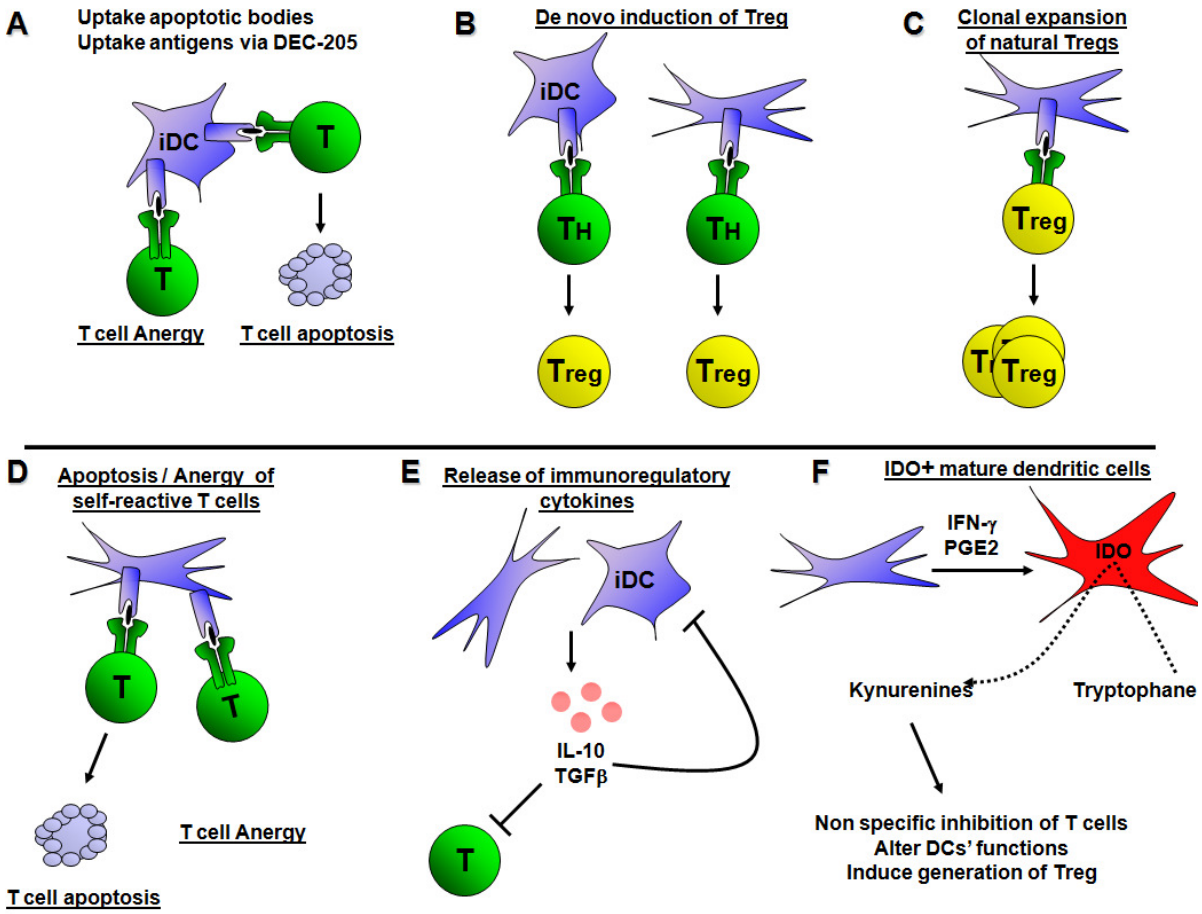


Figure 1-12: Examples of peripheral tolerance mediated by dendritic cells
(**A**) Uptake of antigens via the DEC-205 mannose receptor or uptake of apoptotic bodies does not induce maturation of dendritic cells (DCs). iDCs presenting antigens to T cells can lead to T cell death or T cell anergy. (**B**) The de-novo induction of regulatory T cells (Treg) by both immature and mature DCs has been largely documented. (**C**) In the presence of the cognate peptide presented in the context of MHC-II molecules, thymic derived Tregs are able to expand. (**D**) As described in the extrinsic mechanisms of peripheral tolerance, mDCs presenting the Ag to T cells can lead to either T cell anergy (involvement of CTLA-4 and PD-1) or T cell death (involvement of Fas and Fas-L). (**E**) In certain conditions, iDCs and mDCs can release immunoregulatory cytokines that inhibit DCs and T cells. (**F**) Under stimulation by various factors such as IFNγ, PGE2, mDCs upregulate the expression of IDO. IDO degrades tryptophan into kynurenines leading to the inhibition of T cells, the alteration of DCs' functions and the generation of Tregs.

DCs play therefore a dual role in promoting an efficient immune response while they are also key players in peripheral tolerance.

1-4-3/ Myeloid Derived Suppressor Cells (MDSCs)

Myeloid derived suppressor cells (MDSCs) express the myelomonocytic markers Gr1 and CD11b. MDSCs constitute a heterogeneous population of myeloid cells originating from Common Myeloid Precursor cells and MDSCs differentiate into macrophages, granulocytes or DCs depending on the nature of inflammation (Bronte et al. 1998; Bronte et al. 2000).

Although they are present in the spleen and bone-marrow of healthy mice, they accumulate in different lymphoid and non-lymphoid tissues both in tumour bearing mice and humans with cancer (Bronte et al. 2000; Almand et al. 2000) (Figure 1-13). A significant decrease in the number of myeloid DCs is associated with an increased number of MDSCs in the blood of patients with head and neck squamous-cell carcinomas and breast cancers (Almand et al. 2000).

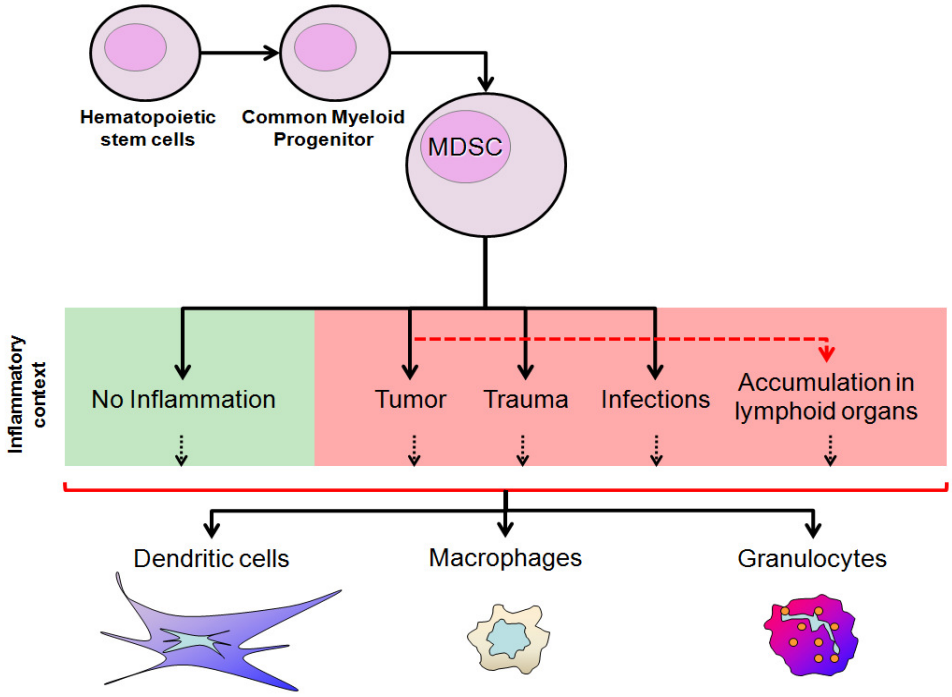


Figure 1-13: MDSCs and inflammation
Myeloid Derived Suppressor Cells (MDSCs) are precursor cells of the myeloid lineage. Upon inflammation such as cancer, infection or trauma, MDSCs accumulate in lymphoid organs and the tissue affected by the inflammatory process. MDSCs differentiate in mature myeloid cells (dendritic cells or macrophages or granulocytes) according to the nature of inflammation.

Attracted to the site of inflammation via the release of inflammatory cytokines such as GM-CSF, MDSCs play an important role in anti-tumor immunity by inhibiting $CD8^+$ T cell proliferation and functions in response not only to α-CD3/CD28 antibodies or superantigens but also to antigens in part via the modulation of L-arginine metabolism (Pak et al. 1995; Bronte et al. 2000; Bronte et al. 2005). Different mechanisms by which MDSCs inhibit T cell function and activity have been described including the inducible nitric oxide synthase (iNOS) and / or Arginase I (ARG1). The activity of these two enzymes depends on the levels of Th1 and Th2 cytokines respectively. Bacterial products (such as LPS) and Th1 cytokines (such as IFNγ or TNFα) lead to an increased production of nitric oxide (NO) via the activation of iNOS and inhibition of ARG1 (Mazzoni et al. 2002) (Figure 1-14/A). Release of

NO alters the IL-2R signalling pathway thus rendering T cells non-responsive to IL-2. By contrast, Th2-like cytokines (such as IL-4, IL-10 and IL-13) strongly induce ARG1 synthesis and inhibit iNOS activity (Figure 1-14/B). ARG1 activation is associated with the increased expression of the cationic amino-acid transporter 2B (CAT2B) (Yeramian et al. 2006; Bronte et al. 2005) and with the degradation of L-arginine into urea and polyamines. This leads to the depletion of the extracellular environment in L-arginine and impaired T cell function resulting from a default in the synthesis of CD3ζ chains. Products of degradation of L-arginine also alter T cell functions. Urea alters the translation of various mRNA species while polyamines have an anti-inflammatory role by inhibiting the release of pro-inflammatory cytokines by monocytes (Rodriguez et al 2004). Finally, both ARG1 and iNOS can act in synergy to generate superoxides, thus promoting the production of reactive nitrogen oxide species (RNOS) and reactive oxygen species (ROS) (Bronte et al. 2005). Together, this production has a strong inhibitory effect on T cells that can potentially lead to T cell apoptosis.

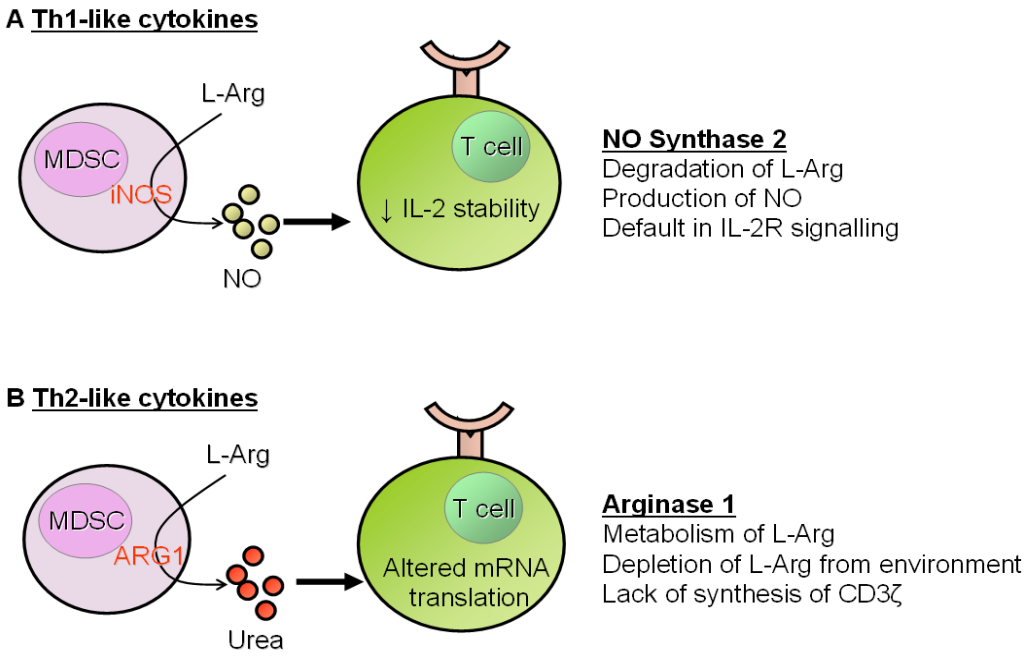


Figure 1-14: Mechanisms of action of Myeloid Derived Suppressor Cells (MDSCs)
(A) Upon stimulation by Th1-like cytokines, MDSCs express the inducible nitric oxide synthase (iNOS). iNOS degrades L-arginine (L-Arg) into citrulline and nitric oxide (NO). This leads to T cells having a default in the IL-2 receptor (IL-2R) signaling pathway. **(B)** MDSCs stimulated by Th2-like cytokines such as IL-4, 10 and 13 upregulate the expression of arginase 1 (ARG1). Degradation of L-arginine into urea and polyamines leads to a decrease in CD3ζ chain expression in T cells.

While very efficient, some self-reactive T cells can escape negative and positive selection occurring in the thymus during central tolerance. Peripheral tolerance is indeed a

supplementary mechanism that allows the inhibition of self-reactive T cells in the periphery and therefore the emergence of AID. In some situations however, a breakdown of tolerance can occur that will trigger AID.

1-5/ Breakdown of tolerance and emergence of autoimmune diseases

Breakdown of tolerance has been linked to genetic susceptibility. The expression of specific HLA molecules is associated to increased risk in developing some AID. Indeed, some self-peptides are more prone to binding some specific MHC molecules than others. Indeed people bearing HLA-DR3, HLA-DR4 and HLA-DQ8 are at risk to develop type 1 diabetes while HLA-DR2 is protective (Aly et al. 2006). Genetic influence on certain specific genes such as FAS or CTLA-4 has also been linked to some AID (Ueda et al. 2003).

Beyond this genetic susceptibility, some factors lead to the activation of self-reactive T cells that have not been eliminated during central tolerance. This means that the different arms involved in peripheral tolerance have failed to keep self-reactive T cells at bay, thus triggering AID. The activation of self-reactive T cells means that somehow the TCR has been able to recognize some epitopes in the context of MHC-molecules that are hidden or do not exist in physiological conditions.

1-5-1/ Molecular mimicry, epitope spreading and bystander activation

External factors able to induce AID are factors that can cause tissue damage and therefore the dissemination of auto-antigens that can lead to the activation of self-reactive T cells. The following theories have considered the involvement of persistent microbial infections leading to a breakdown of tolerance via different mechanisms (Vanderlugt et al. 2002).

- Following tissue destruction during an infection, self-antigens released by dying cells can mimic foreign antigens from the pathogens thus amplifying the activation of pathogen-specific or self-reactive T cells (Benoist et al. 2001; Christen et al. 2004). This **mimicry** is possible if self-peptides share a similar antigenic surface

with bacterial (Elli et al. 2005) or viral peptides (Christen et al. 2004) (Figure 1-15/A).

Shape mimicry is an alternative situation when peptides are shifting in the MHC groove (Bankovich et al. 2004). Two peptides from thyroid peroxidase (TPO) antigen (TPO$_{536-547}$: N-LDPLIRGLLARPAKLQ-C) could induce the proliferation of the same T cell clone. The two peptides (TPO$_{535-55}$: N-DPLIRGLLARPA-C) and TPO$_{539-550}$: N-IRGLLARPAKLQ-C) had different anchor residues in the HLA molecule but shared a similar antigenic surface (Quaratino et al. 1995).

- Inflammatory and pathogen specific T cells attracted to the site of inflammation can generate a powerful environment of pro-inflammatory cytokines (Figure 1-15/B). This cytokine milieu promotes the **bystander activation** or non-specific activation of self-reactive T cells that in turn increases tissue damage or trigger the development of autoimmune diseases in tissues other than the one infected (Bangs et al. 2006).

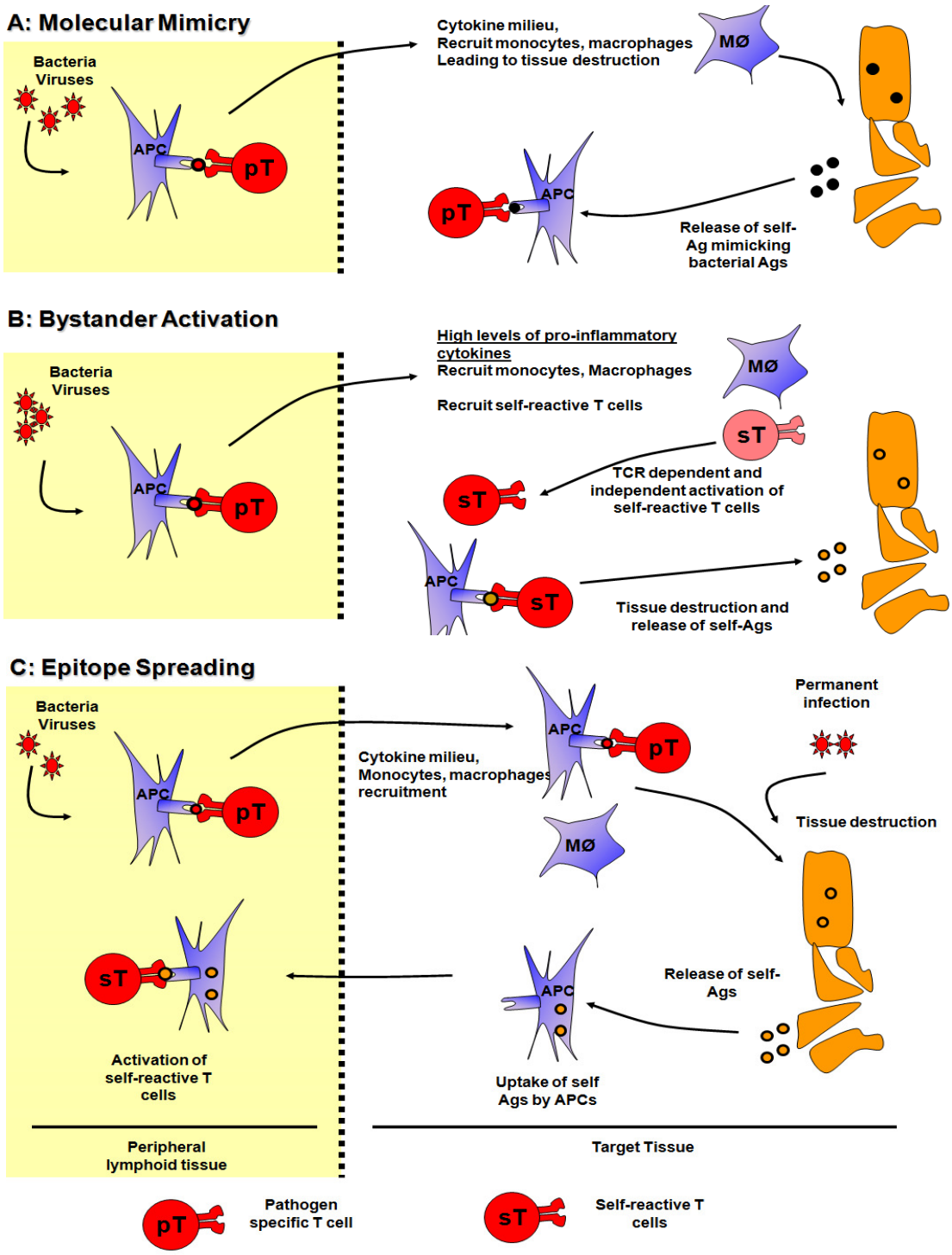


Figure 1-15: Breach of tolerance can induce autoimmune diseases
This is based on the consequences of a primary bacterial or viral infection. **(A)** Molecular mimicry occurs when self antigens released are similar in antigenic surface to bacterial antigens. **(B)** Epitope spreading: foreign antigens presented by APCs stimulate T cells that migrate to the target tissue along with inflammatory cells. This promotes tissue damage and the release of self-antigens that in turn activate self-reactive T cells. **(C)** The pro-inflammatory cytokine milieu caused by persistent infections leads to the non specific bystander activation of self-reactive T cells. **pT**: pathogenic specific T cells; **sT**: self-reactive T cells, **Ags**: antigens; **APCs**: antigen presenting cells; **MØ**: macrophages.

- In the situation of a persistant infection / inflammation where T cells are continuously activated, tissue damage can occur resulting in the release of self antigens. These self-antigens are loaded by APCs that activate self-reactive T cells. This **intermolecular epitope spreading** can also result in more self-antigens being released by the damaged tissue, which promotes the stimulation of a wider range of self-reactive T cells (Figure 1-15/C) (McMahon et al. 2005). As the inflammation persists, more self-antigens are shed and multiple epitopes can be generated by APCs from the same antigen, thereby amplifying the extent of the inflammatory process (**intramolecular epitope spreading**) (Sercarz et al. 2000; Vandergult et al. 2002).

1-5-2/ Generation of cryptic epitopes

During thymic selection, some self-reactive T cells are not depleted as the epitope for which they are specific may not be available or is inefficiently processed and presented in the thymus. Therefore, if these epitopes are then displayed in the periphery, self-reactive T cells can be activated and become pathogenic. AID are therefore the result of the unexpected pathogenic presentation of normally hidden **cryptic epitopes** (Lanzavecchia et al. 1995). Cryptic epitopes can be unmasked in different conditions such as:

- In physiological conditions, the cryptic epitope might not be produced in sufficient quantities to be efficiently presented or recognize by T cells. **Increased concentration of Ag** as a result of tissue destruction leads to an increased production of cryptic epitopes that are then presented to T cells (Figure 1-16/A).
  Processing of antigens can be boosted by receptor down-regulation. gp120 protein from HIV promotes the down-regulation of cell-surface CD4 marker. Increased degradation of CD4 leads to the generation of a previously-invisible cryptic epitope that elicits the activation of self-reactive T cells (Salemi et al. 1995; Lanzavecchia et al. 1995).

- An **Ab binding to the Ag** can modulate Ag uptake and processing by APCs and the generation of different epitopes is either increased or suppressed. The presentation of a cryptic epitope to T cells can occur if the generation of an immunodominant

epitope is downregulated as a result of altered processing (Simistek et al. 1995; Lanzavecchia et al. 1995; Quaratino et al. 2005) (Figure 1-16/B).

- During inflammation, high levels of **pro-inflammatory cytokines** can affect the level of expression of MHC-I and MHC-II molecules as well as the protease content of the cells. In this respect, inflammation mimicked by IFNγ leads to the upregulation of MHC-I and MHC-II molecules, affects the content of proteases and promotes new pathways of endocytosis (Delamarre et al. 2005). Upon these changes, APCs can generate or unmask cryptic epitopes capable of activating self-reactive T cells (*left panel*, Figure 1-16/D).
  The differential processing of an antigen by the proteasome (in iDCs) and immunoproteasome (mDCs and stimulation with IFNγ) is largely admitted. Upon stimulation with IFNγ, three subunits of the proteasome are replaced by the inducible subunits LMP2, LMP7 (low-molecular mass polypeptide 2 and 7) and MECL-1 (multicatalytic endopeptidase complex-like 1) conferring new catalytic activities to immunoproteasomes (Goldberg et al. 2002). Processing of ubiquitinated proteins by the immunoproteasome has been associated with the generation of cryptic epitopes from HIV and LCMV viruses (Sewell et al. 1999; Schwarz et al. 2000). Proteasome activity is also involved in the shaping of the T cell repertoire (Osterloh et al. 2006) (*right panel*, Figure 1-16/D).

- Cryptic epitopes can be naturally generated by **epithelial cells** but the absence of MHC-II molecules is not allowing their presentation. Upon inflammation astrocytes can present self-antigens to self-reactive T cells in a mouse model of experimental autoimmune encephalomyelitis (EAE). These effects can be mimicked *in-vitro* upon stimulation with IFNγ (Soos et al. 1998; Stüve et al. 2002) and have been described in other epithelial cells such as hepatocytes (Herkel et al. 2003) (Figure 1-16/C).

- Cryptic epitopes may arise from **alternative translational products** of a single mRNA if its translation has occured from a different open reading frame. This mechanism is not restricted to tumors or virally infected cells, and can occur in normal cells (Review by Ho et al. 2006). This mechanism has been shown to provide immune protection against retroviruses by eliciting a strong $CD8^+$ T cell

response (Ho et al. 2006) while providing additional source of peptides to generate T cell tolerance (Schwab et al. 2003).

- **Post-translational modifications** of certain proteins such as by phosphorylation (Mohammed et al. 2008), glycosylation (Wood et al. 1998) or oxidation of certain amino-acids (Kurien et al. 2008) can increase the affinity of a peptide for MHC binding (Engelhard et al. 2006).

- The **disruption of protein structure** during oxidative stress such as ROS can result in alternative processing and production of cryptic epitopes. This phenomenon has been demonstrated in AID of the kidneys and lungs (Goodpasture Syndrome) characterised by the generation of an adaptive immune response to collagen.

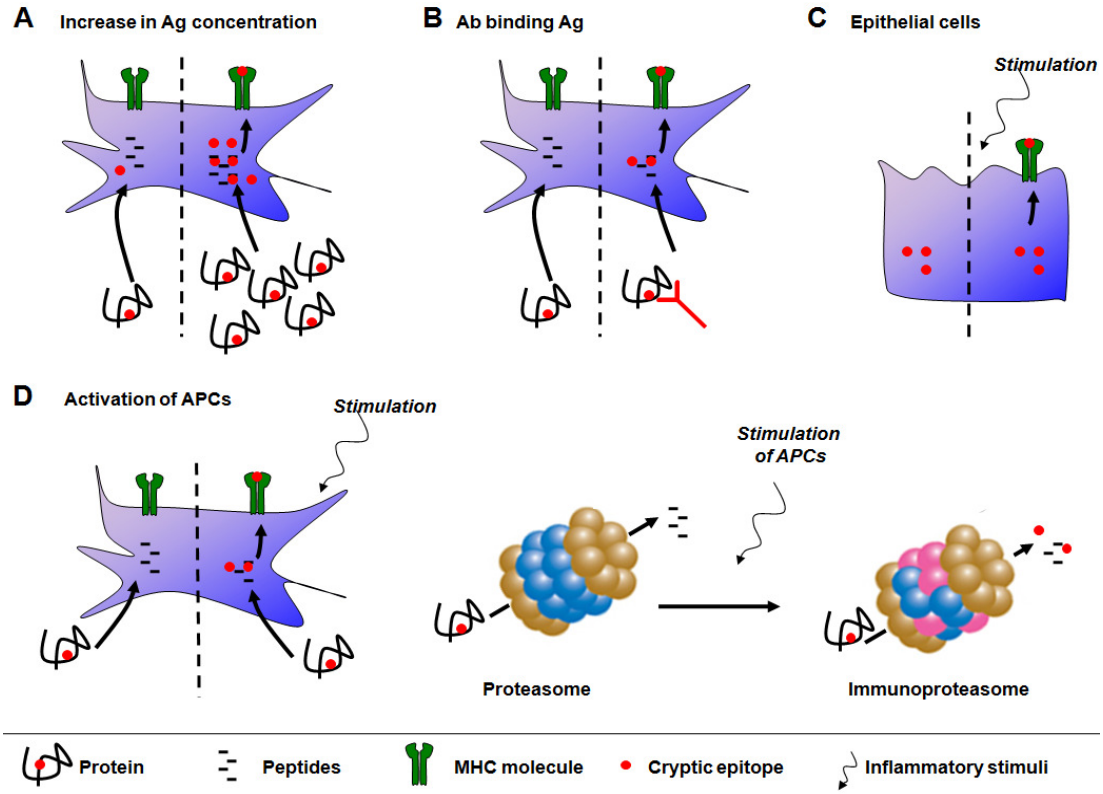


Figure 1-16: Generation of cryptic epitopes
Cryptic epitopes are revealed through different processes. **(A)** Increased antigen (Ag) concentration processed by antigen presenting cells (APCs) leads to more cryptic epitope being produced and presented to T cells. **(B)** Auto-antibodies (Ab) binding the Ag can modulate the route of uptake and processing of Ag by APCs, thus favoring the generation of cryptic epitopes. **(C)** Epithelial cells naturally generate the cryptic epitope but lack the appropriate MHC molecule. Upon inflammation, epithelial cells upregulate the expression of MHC molecules allowing the presentation of cryptic epitopes to T cells. **(D)** Upon stimulation, APCs change the pool of proteases necessary to process Ag to generate the cryptic epitope (*left panel*). Upon maturation, APCs synthesize new catalytic subunit of the proteasome to form the immunoproteasome. Degradation of ubiquitinated Ag by the immunoproteasome generates or destroys cryptic epitopes (*right panel*).

These inflammatory mechanisms are of no or little impact when considering mice models developing spontaneous autoimmune diseases in specific-pathogen-free conditions where the actions of external factors such as bacterial or virus infections are excluded.

In a recent study by Sakaguchi's group (Hirota et al. 2007), this question was addressed in a mouse model of autoimmune arthritis. In these mice self-reactive T cells are activated in the periphery by APCs presenting the cognate peptide. This activation resulted in the release of IL-6 by DCs that was driving the differentiation of naïve self-reactive T cells into pathogenic Th17 cells. Deficiency of either IL-6 or IL-17 prevented the development of the disease (Hirota et al. 2007; Bailey et al. 2007). This breakdown of tolerance was also observed in a mouse model of multiple sclerosis (EAE mice - Experimental mouse model of Autoimmune Encephalitis). The activation of self-reactive T cells in these mice are the result of epitope-spreading into the central nervous system (CNS) mediated by DCs (McMahon et

al. 2005). In these two studies, it was clear that the genetically determined self-reactive TCR induced the formation of a cytokine milieu favouring the development of AID. This was possible by the spreading of the cognate epitope by DCs in the CNS (EAE model) and peripheral lymphoid organs (autoimmune arthritis) (McMahon et al. 2005; Hirota et al. 2007 respectively)

1-6/ The TAZ10 transgenic mouse model of autoimmune thyroiditis

In this thesis, we will study some mechanisms influencing the pathogenesis of some chronic inflammatory diseases such as autoimmune thyroiditis.

TAZ10 transgenic mice express the human TCR (Vβ1/Vα15) isolated from the thyroid infiltrating T cell clone 37 from a patient with Hashimoto's thyroid disease. In the thyroid of this patient nearly half of the T cell-clones generated were specific for a main thyroid auto-antigen: thyroid peroxidase (TPO). Among them, 18% were specific for the immunodominant TPO epitope $TPO_{535-551}$ (N-LDPLIRGLLARPAKLQ-C) (Dayan et al. 1991; Quaratino et al. 1996).

The immuno-dominant T cell clone 37 is specific for $TPO_{536-547}$ (N-DPLIRGLLARPA-C), identified as a cryptic epitope as it was presented only by thyroid epithelial cells (TEC) and not by DCs (Quaratino et al. 1996). On the contrary, DCs pulsed with exogenous TPO presented a natural antagonist epitope ($TPO_{537-548}$: N-PLIRGLLARPAK-C) that induced T cell anergy in T clone 37. $TPO_{537-548}$ and $TPO_{536-547}$ induced a similar down-regulation of the TCR but $TPO_{537-548}$ did not lead to T cell proliferation and IL-2 secretion. $TPO_{537-548}$ is therefore an altered peptide ligand (antagonist peptide of $TPO_{536-547}$) that is recognized by the same TCR but inducing T cell anergy (Quaratino et al. 2000).

Interestingly, two peptides from thyroid peroxidase (TPO) antigen ($TPO_{535-551}$: N-LDPLIRGLLARPAKLQ-C) could induce the proliferation of T cell clone 37. The two peptides ($TPO_{536-547}$: N-DPLIRGLLARPA-C) and $TPO_{539-550}$: N-IRGLLARPAKLQ-C) had different anchor residues in the HLA-DQ6 molecule but shared a similar antigenic surface. This highlighted that rather than identical sequences, the shape of the antigenic surface characterized epitope mimicry (Quaratino et al. 1995).

From these studies, it appeared that T cell clone 37 was interesting and further in-vivo studies were needed to ascertain its major role in autoimmune thyroiditis, thus the generation of the TAZ10 model. The extra cellular domain of human TCR Vβ1/Vα15 was ligated to the intracellular domain of the mouse TCR to allow normal TCR signalling in mice (Quaratino et al. 2000). The chimeric TCR α and β chains were then cloned in the expression cassette VahCD2 under the hCD2 promoter and injected in fertilized eggs from C57BL/6xCBA F2 offspring pregnant females. Extensive backcrossing led to the generation of TAZ10 mice (Quaratino et al. 2004).

Interestingly, both $CD4^+$ and $CD8^+$ T cells from TAZ10 mice could recognize the TPO cryptic epitope when presented by APCs in the context of human HLA DQB1*0602-DQA1*0102 (DQ6) and murine H2-A^b and H2-A^k MHC-II molecules. Transgenic T cells from TAZ10 mice recognize the human TPO cryptic epitope ($TPO_{536-547}$: N-DPLIRGLLARPA-C) and the murine equivalent ($TPO_{524-535}$: N-DPIVRGLLARPA-C) when presented in the context of H2-A^k murine MHC-II molecules. Molecular modelling of human $TPO_{536-547}$:HLA-DQ6 and human $TPO_{536-547}$:H2-A^k compared with molecular modelling of human $TPO_{536-547}$:H2-A^k and mouse $TPO_{524-535}$:H2-A^k revealed that H2-A^k and HLA-DQ6 molecules display a close similar antigenic surface of $TPO_{536-547}$ recognized by the same TCR Vβ1/Vα15. It also showed that the murine $TPO_{524-535}$ and human $TPO_{536-547}$ cryptic epitope presented a similar antigenic surface despite the residue differences (amino-acids at position 3 and 11).

TAZ10 transgenic mice spontaneously developed autoimmune thyroiditis sharing the same clinical signs (gain of weight), hormonal changes (increased amounts of TSH and decreased levels of T4) and histological modifications (destruction of the thyroid associated with mononuclear cellular infiltrates) as patients with Hashimoto's thyroiditis (Quaratino et al. 2004). Interestingly, $CD4^+$ and $CD8^+$ T cells from $Rag^{-/-}$ TAZ10 mice presented an activated phenotype as they did not express CD62L while expressing high levels of CD44. At 4 months of age, almost 100% of TAZ10 mice had developed severe autoimmune thyroiditis highlighted by extensive destruction of the thyroid (Quaratino et al. 2004).

Although the study on TAZ10 mice provided evidence of the role of cryptic epitopes in the pathogenesis of human autoimmune diseases, it also highlighted the role played by

unconventionally MHC-II selected $CD8^+$ T cells in the development of the disease. Early experiments have addressed the ability of thyroid epithelial cells (TECs) to upregulate MHC-II molecules in Hashimoto's thyroiditis. These cells were able to promote the activation of thyroid specific T cells, suggesting that thyroid epithelial cells can present antigens to T cells (Londei et al. 1984; Londei et al 1985). However the role of TEC and the origin of the cryptic epitope leading to the activation of T cells from TAZ10 mice in the induction of the disease are still elusive.

1-7/ The role of Tissue transglutaminase 2 in the pathogenesis of Celiac Disease

It has been described that patients with autoimmune thyroiditis (Hashimoto's thyroiditis) have an increased chance of developing celiac disease (CDis) and *vice-versa* (Naiyer et al. 2008; Duntas, 2009). CDis is a common pathology caused by an inappropriate immune response to wheat and gluten and is characterised by local (small intestine) and systemic manifestations (Schuppan et al. 2000). Important histological features of untreated patients with CDis include the activation of gliadin specific T cells, the infiltration of intraepithelial lymphocytes and the flattening of the mucosa of the small intestine (Schuppan et al. 2000).

CDis is triggered by a non-immunodominant gliadin peptide which stimulates the innate immune response and induces epithelial damage and death by apoptosis (Maiuri et al. 2003). This non-immunodominant peptide induces the upregulation of tissue transglutaminase 2 (TG2) whose inhibition controls the activation of epithelial changes and damages and the subsequent activation of pathogenic gliadin-specific T cells (Maiuri et al. 2005 and Submitted). While the non-immunodominant gliadin peptide p31-43 induced the death of epithelial cells of the gut via Fas/Fas-L engagement (Maiuri et al, 2001; Maiuri et al. 2003), these effect are abrogated by blocking TG2 expressed at the cell-surface with a monoclonal antibody (Maiuri et al. 2005).

In celiac disease (CDis), TG2 plays a fundamental role in the pathogenesis of the disease by operating a crucial deamidation of glutamine to glutamic acid of immunodominant gliadin epitopes that drive the adaptive immune response (van de Wal et al. 1998; Arentz-Hansen et al. 2000; Molberg et al 2008). However, a recent study has highlighted a new unexpected role

of TG2 in celiac disease where the inhibition of TG2 activity controlled the activation of gliadin-specific T cells. Patients with CDis present high titres of autoantibodies to TG2 that has therefore been described as the main autoantigen in CDis (Dieterich et al. 1997; Marsh, 1997).

However, the enzymatic activity of TG2 is not its only function as it displays other multiple biological activities (Akimov et al. 2000 and 2001) and is upregulated upon inflammation (Lorand et al. 2003; Ientile et al. 2007). TG2 is a calcium-dependent enzyme with a transamidating activity that also results in the cross-linking of proteins via ε(γ-glutamyl) lysine bonds (Fesus et al. 2002). More recent studies have highlighted the important role played by TG2 in the pathogenesis of numerous neurodegenerative diseases. Indeed, in Parkinson's disease, TG2 mediates the cross-linking and aggregation of α-synuclein in Lewy inclusion bodies (Junn et al. 2003).

AIMS:

The aims of my project are to characterize different mechanisms controlling the development and inflammation of different chronic inflammatory diseases (CIDs). In this thesis I have investigated the early events leading to the presentation of the TPO cryptic epitope in the TAZ10 mouse model of autoimmune thyroiditis whereas also addressing the impact of a new regulatory network formed by MDSCs. I also described how the manipulation of MDSCs *in-vitro* affected the proliferation of self-reactive T cells from TAZ10 mice. I then assessed whether these findings on MDSCs from TAZ10 mice could be transposed to human CIDs such as rheumatoid arthritis and systemic lupus erythematosus.

Finally, I evaluated the impact of tissue transglutaminase 2 (TG2) in the generation of the inflammatory response in patients with cystic fibrosis. These findings were transposed into TAZ10 mice to address whether TG2 could control the ongoing inflammation that leads to the destruction of the thyroid.

2/ Materials and Methods

2-1/ Material and Methods Part 1: Mouse work

2-1-1/ Mice and cell-lines

Wild-type C57Bl/6 and CBA, TAZ10 and $Rag2^{-/-}$ trasngenic mice were housed and bred at the Tenovus animal facility in accordance with home-office guidelines for animal welfare. All mice used were aged from 3 weeks to 6 months of age. TAZ10 Rag-/- transgenic mice were maintained under specific pathogen-free conditions. B16 melanoma and CT26-GM colon carcinoma cell-lines was grown in complete RPMI-1640 medium (Cambrex, UK) supplemented with 10% Foetal Calf Serum (Globepharm, UK), 0.3 mg/mL L-glutamine (Cambrex, UK), 100 U/mL Penicillin/streptomycin (Cambrex, UK), 1mM non essential amino-acids (Cambrex, UK) and 1 mM sodium pyruvate (Cambrex, UK) at 37°C with 5% CO_2.

2-1-2/ Flow-Cytometry and antibodies

Cells were collected and washed in cold FACS Buffer (1X PBS supplemented with 0.1% FCS and 0.05% Sodium Azide). Antibodies used were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allophycocyanin (APC). Tandem dyes were also used: cyanin conjugated to phycoerythrin (PE-Cy5 and PE-Cy7), allophycocyanin (APC-Cy7) and peridinin chlorophyll protein (PerCP-Cy5). Cells were stained with monoclonal antibodies against MHC class-II (**I-A^b**, Rat IgG2b, clone M5/114.15.2; BD Pharmingen), **CD4** (rat IgG2b, clone L3T4; BD Pharmingen), **CD8α** (rat IgG2a, clone Ly-2; BD Pharmingen), **CD11b** (rat IgG2b, clone M1/70; eBiosciences), **CD11c** (N418, Hamster IgG; BD Pharmingen), **CD19** (rat IgG2a, clone 1D3; BD Pharmingen), **CD25** (rat IgM, clone 7D4; BD Pharmingen), **CD31** (rat IgG2a, clone 390; BD Pharmingen), **CD62L** (mouse IgG1, clone DREG-56; eBiosciences), **CD80** (hamster IgG, clone 16-10A1; BD Pharmingen), **CD86** (rat IgG2a, clone GL1; eBiosciences), **CD95/Fas** (mouse IgG1, clone 15A7; eBiosciences), **CD95-L/Fas-L** (Hamster IgG, clone MFL3; eBiosciences), **CD127** (Rat IgG2a, clone A7R34; eBiosciences),**CD205/DEC-205** (rat IgG2a, clone NLDC-145; made in house), **Gr1** (rat IgG2b, clone RB6-8C5; eBiosciences), **F4/80** (rat IgG2a,κ, clone BM8; eBiosciences) and **human TCR Vβ1** (rat IgG1, clone BL37.2; Beckman Coulter). Samples were acquired with FACSCanto and analysed using the FACS Diva Software (BD Biosciences).

2-1-3/ ELISA

Production of cytokines was assessed by ELISA. Supernatants were tested for cytokine content such as IL-10 and GM-CSF (All purchased from R&D Systems, UK). Flat-bottom 96-well maxisorp plates (Nunc, UK) were coated overnight at room temperature with capture antibody diluted in PBS (100µL per well). Capture antibody was discarded and plate was washed three times with washing-buffer (0.05% Tween-20 in PBS, pH 7.2-7.4) by inverting the plate onto clean paper towels. Plate was blocked for a minimum of 1 hour with 300µL of blocking-buffer (1% BSA in PBS, pH 7.2-7.4) per well at room temperature. Plate was washed three times as previously described and 100µL of standard or sample diluted in blocking-buffer was added per well and the plate incubated at room temperature for 2 hours. Plate was washed as previously described and biotinylated detecting antibody diluted in blocking-buffer was plated out (100µL per well) and incubated for 2 hours at room temperature.

Wells were washed 3 times with washing-buffer and 100µL/well of a 1:1000 dilution of avidin alkaline-phosphatase (Sigma, UK) in blocking-buffer and the plate incubated for 40 minutes at room temperature. After 3 washes, 100µL Sigmafast pNPP substrate (Sigma, UK) was added per well and watched for color development. Absorbance was read on Biorad model 680 plate reader using Microplate manager software (BioRad, UK) at 405nm. Results were analysed and graphs plotted using Prism 4 (GraphPad).

2-1-4/ Purification of T cells and $Gr1^+$ cells from wild-type and TAZ10 mice

Where indicated, T lymphocytes were enriched from spleens or lymph-nodes using the MACS magnetic technology (Myltenyi Biotech, Germany). Briefly, splenocytes were obtained by passing the spleen through a 70µm cell strainer. Red blood cells were lysed by incubating the cell suspension in ACK red blood cell lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA). Splenocytes were resuspended in MACS buffer (2 mM EDTA and 0.5% BSA in PBS) and incubated with micro-beads conjugated to mAb anti-CD4 (clone L3T4) and anti-CD8 (clone Ly-2) for 15 minutes at 4°C. Excess of free micro-beads was washed away with MACS buffer and labelled cells passed through a separating MS column on a magnetic field. In all experiments, the purity was assessed by flow cytometry and ranged from 80 to 95%.

For the isolation of $Gr1^+$ cells, splenocytes were incubated with micro-beads conjugated to mAb anti-Gr1 (BD Biosciences, San Diego, CA) for 15 minutes at 4°C before being run through an MS column (Miltenyi Biotec, Germany).

All experiments were performed using RPMI-1640 medium (Cambrex, UK) supplemented with 10% Foetal Calf Serum (Globepharm, UK), 0.3 mg/mL L-glutamine (Cambrex, UK), 100 U/mL Penicillin/streptomycin (Cambrex, UK), 1mM non essential amino-acids (Cambrex, UK) and 1 mM sodium pyruvate (Cambrex, UK) at 37°C with 5% CO_2.

2-1-5/ Generation of bone-marrow derived dendritic-cells

4 to 12 week old mice were used. Bone-marrow from femurs and tibiae was flushed-out and red blood cells lysed in ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4). DCs were generated by culture in RPMI-1640 medium (Cambrex, UK) supplemented with 10% low-endotoxin FCS (Autogen Bioclear, UK), 0.3 mg/mL L-glutamine (Cambrex, UK), 100 U/mL Penicillin/streptomycin (Cambrex, UK), 1mM non essential amino-acids (Cambrex, UK) and 1 mM sodium pyruvate (Cambrex, UK) at 37°C with 5% CO_2. CT26-GM supernatant was used as a source of GM-CSF and IL-4 was from Peprotech, UK.

Immature and mature DCs were used for functional assays and their phenotype analysed by FACS.

"Inaba's method" (Inaba et al. 1992)

$2x10^6$ bone-marrow cells were seeded at 1mL of medium per well of 24 well-plates. Every two days, the plate was gently swirled and 75% of the medium replaced with fresh medium. iDCs obtained 7 days later were replated overnight before LPS (1µg/mL) was added overnight for complete maturation.

"Son's method" (Son et al. 2002)

$1x10^6$ bone-marrow progenitors were seeded in 5mL of medium per well of 6 well-plates. At day 4 and 7, the medium was only supplemented with GM-CSF until DCs were considered to be immature at day 8. Non-adherent iDCs were replated overnight and full maturation achieved by overnight stimulation with LPS (1µg/mL).

"Lutz's method" (Lutz et al. 1999)

$2x10^6$ cells were seeded in 100 mm petri-dishes in medium supplemented with 25% of GM-CSF from CT26-GM cell-conditioned-medium. The concentration of GM-CSF assessed by ELISA (Biosource, UK) ranged from 200 to 400ng/mL. On Day 3, 10 mL of fresh medium supplemented with GM-CSF was added to each culture dish. On day 6 and 8, 10 mL of the culture medium was collected, centrifuged, the cell pellet resuspended in 10mL of fresh GM-CSF containing medium and seeded back into the original petri-dishes. Immature DCs obtained at day 10 were harvested and seeded at $2x10^6$ cells per well of a 6 well-plate.

For complete maturation, 1 µg/mL of LPS E.Coli E26:B6 (Sigma, UK) was added per well on day 11 and the cells cultured for 18 hours.

2-1-6/ Primary culture of thyroid epithelial cells

Primary cultures of thyrocytes were prepared as previously described (Jeker et al. 1999). Thyroid lobes were aseptically removed from the trachea of wild-type (C57Bl/6) or TAZ-10 transgenic mice, placed in a petri dish in Eagle`s Minimum Essential Medium (EMEM) (Autogen Bioclear, UK) and cut into pieces using a scalpel. Disrupted thyroid lobes were digested in 1 mL of EMEM containing 5ug/mL type I collagenase (Sigma, UK) and 2,5mg/mL Dispase I (Roche, UK) for 45 minutes at 37 ° C. The tube was gently swirled every 10 minutes to allow a homogeneous digestion. The digestion allowed the release of single thyroid follicles with few single thyrocytes. After digestion, the mix was resuspended in 10mL of Hams F-12 medium (Autogen Bioclear, UK) and spun down for 3 minutes at 1200rpm. Supernatant containing mainly single thyrocytes is discarded and the pellet containing partially digested thyroid follicles resuspended in complete thyroid medium. The culture medium was: Nu-Serum IV (BD Biosciences, San Diego, CA) diluted 2.5 times in Hams F-12 medium and supplemented with 10ng/mL somatostatin (Sigma, UK) and 2ng/ml glycyl-L-histidyl-L-lysine acetate (Sigma, UK). Medium was changed one day after the start of the culture and replaced every 2 days. IFNγ (Peprotech, UK) was added when stated at 200 units/mL; but only one day after the beginning of the culture. Thyrocytes were seeded either in 96 well-plates for proliferation assay using thymidine incorporation or 6 well-plates for expansion or fluorescence microscopy.

2-1-7/ T cell proliferation assay by (^{3}H) thymidine incorporation

T cell proliferation was assessed by thymidine incorporation. Briefly, freshly isolated splenocytes were cultured in triplicate in 96 well plates at 2.5 x10^5 cells per well in culture medium containing soluble or plate-bound α-CD3 (clone RC5-4C11, CRUK) at 10µg/mL and/or soluble α-CD28 (clone 37.51, made in house). Where indicated mature bone-marrow derived dendritic cells seeded at 1x10^5 cells per well were pulsed with $TPO_{536-547}$ (N-DPLIRGLLARPA-C) peptide at 0.1µg/mL for 3 days. T cells where then added at DCs: T cell ratio of 1:30 and myeloid derived suppressor cells (MDSCs) were eventually added at a T:MDSC ratio of 1:1 or 1:5.

When thyroid epithelial cells were used, 25µL of thyroid digestion mix (Paragraph 2-1-6/) was cultured in 96 well-plates in 200µL for 3 days prior to the addition of T cells.

Cells were co-cultured for 72 hours at 37°C in 5% CO_2. Each well was then pulsed with 1 µCi ^{3}H thymidine for the last 12 hours of culture. Plates were harvested onto filtered plates (Perkin Elmer) and ^{3}H thymidine incorporation measured in a microplate scintillation counter (Packard). Data are expressed in cpm (count per minute) and were plotted as mean of the triplicates.

2-1-8/ CFSE labeling of T cells for in-vitro proliferation assays

In some experiments, proliferation was assessed by CFSE labelling. Purified lymphocytes from either WT or TAZ10 mice were resuspended at 2 to 4 x10^7 cells per mL in FCS free medium. 5µM CFSE (Carboxyfluorescein succinimidyl ester, Invitrogen, UK) was added to the cells for 10 minutes at 37°C. Cells were washed three times in FCS supplemented medium to remove any excess of CFSE. Cells were plated at the appropriate concentrations with thyroid epithelial cells with or without SED (staphylococcal enterotoxin D) at 10ng/mL or αCD3 (made in house). After 3 to 5 days, cells were harvested and stained with mouse monoclonal antibodies for T cell markers. Proliferation was assessed by flow cytometry as CFSE dilution.

2-1-9/ Adoptive transfer of CFSE labelled T cells for in-vivo tracking and proliferation

Purified lymphocytes were washed twice in PBS and resuspended in PBS at 1x10^7cells/mL. CFSE was added at 5µM and cells incubated at 37°C in the dark for 15

minutes. Cells were washed three times in PBS. Cells were resuspended in PBS at the appropriate concentration and $2x10^6$ cells were injected to $Rag2^{-/-}$ mice intravenously (IV) into the lateral vein at the base of the tail. After 7 days, cardiac blood samples were collected with from mice under anesthesia following conditions imposed by the Home Office. Blood was collected in eppendrof tubes supplemented with heparin. Mice were culled and spleen, blood, cervical and mesenteric lymph-nodes, and thyroids removed. Cells were stained for specific markers and analysed by flow cytometry.

2-1-10/ MTT assay to assess cell-viability

Thyroid epithelial cells were seeded in wells of 96 well plates (flat-bottom) and incubated at 37°C. At the end of the assay, 20µL of a 5mg/mL solution of MTT (3-(4,5-Dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide, Sigma, UK), in PBS. The plate was incubated at 37°C for 4 hours. Supernatant was discarded from the well and cells washed in PBS. 200µL of DMSO was added to each well and mixed thoroughly. Plate was incubated for 5 minutes at 37°C to help for the removal of air bubbles. Absorbance was read at 550nm.

2-1-11/ Single cell lysis assay

Lysis assay was performed as described in Schwab et al (Schwab et al. 2005)

- Staining of Target cells

10^7 Purified lymphocytes from WT and TAZ10 mice were resuspended in 0.5ML Diluent C. 0.5mL of a 4µM PKH26 dye solution (Sigma, UK) diluted in Diluent C was added to the cells and incubated for 3 to 5 minutes at room temperature with periodic shaking. 1mL of Fetal Calf Serum was added to the cells and incubated for 1 minute at room temperature. 2mL of medium was then added to the cells before being centrifuged for 5 minutes at 300g. Supernatant was poured-off and cells resuspended in 10mL of PBS. Cells were centrifuged for 10 minutes at 200g to clean as much as possible the cells from cell debris and pellet resuspended in medium to final concentration.

- Cell Lysis assay

10^5 target cells were used per well and assays were done in triplicate. Incubations were kept as short as possible so that the dead cells have not completely disintegrated and are thus

visible by flow cytometry analysis at the end of the assay. In our case, TECs and T cells were co-cultured for 6 hours.

- Staining for dead cells using YO-PRO1 / Cell type specific antibody staining

At the end of the assay, YO-PRO1 (Molecular Probes, Invitrogen, UK) was added to a final concentration of 1µM and cells were stained with anti-CD4 antibodies to target purified $CD4^+$T cells. Cells were incubated on ice for 30 minutes before being centrifuged at 300g for 3 minutes. Cells from the wells in triplicate were pooled together were resuspended in medium before being analysed by flow cytometry.

2-1-12/ Determination of nitric-oxide (NO) production using the Griess method

Cells were seeded at $2x10^5$ in 200µL in 96 well-plates in the presence of anti-CD3 alone or in combination with anti-CD28 antibody. After 72 hours, the amount of NO released in the medium was measured using the Griess reagent system (Promega, UK). Briefly, 50µL of culture supernatant was incubated in the dark with 50µL sulfanilamide solution (1% sulfanilamide in 5 % phosphoric acid) for 10 minutes at room temperature in a 96 well flat bottom plate. The same volume of NED solution (0.1% N-1-napthylethylenediamine dihydrochloride diluted in water) was added to the reaction. After 10 minutes of incubation in the dark, absorbance at 550nm was read using a microplate reader (BioRad). Measurements were performed in duplicate and NO concentrations were determined by comparing the absorbance values obtained for the sample to the standard curve generated by the serial dilution of a 0.1M nitrite sodium solution. Medium used to assess the amount of NO was phenol-red free (Sigma, UK).

2-1-13/ Measurement of intracellular NO by flow cytometry

Intracellular NO concentration was measured using the DAF-FM diacetate probe (Molecular Probes, UK). Briefly, 10^6 splenocytes were incubated for 1 hour at 37°C in phenol-red free RPMI medium (Sigma) containing 5µM DAF-FM diacetate. Cells were washed in medium to remove excess probe and incubated for an additional 20 minutes to allow complete de-esterification of the intracellular diacetates. Cells were then washed in cold PBS and stained for MSCs' markers. Treated samples were analysed by flow cytometry.

2-1-14/ Fluorescence microscopy

When fluorescent microscopy was done on DCs and TECs, coverslip were pre-coated with poly-L-lysine (Sigma, UK) for 10 minutes at room temperature and washed three times with 1X PBS.

Cells were washed 3 times with 1X PBS, and 4% paraformaldehyde (PFA) in PBS added to the cells for 7 minutes at room-temperature (RT). PFA was washed off by rinsing the cells 3 times with PBS. 200 µL of 0.1% Triton X-100 in PBS was added to the cells for 5 minutes at RT and cells were subsequently washed 3 times with PBS. First antibody was added at the appropriate concentration in PBS containing 1% BSA (Sigma, UK) to the cells and incubated for 20 minutes at RT in the dark. Coverslips were washed 3 times with PBS and the secondary antibody added in PBS with 1% BSA for a further 20 minutes at RT in the dark. Cells were washed three times and DAPI (Molecular Probe, UK) nuclear counterstain added at 1 µg/mL in PBS with 1% BSA for 10 minutes at RT in the dark. Coverslips were washed 4 times in PBS and mounted on a slide with some mounting medium (VectroLab, UK). Slides were left overnight in the dark at 4° C to allow the mounting medium to harden. Slides were read on a fluorescent microscope (Leica, UK) at different magnifications (x40 and x100) and meaning of fluorescence analysed using OpenLab3.1.5 software (OpenLab, USA).

For cytokeratin detection, mouse mAb to human cytokeratin 5, 6, 8, 17 and probably 19 (mouse IgG1, clone MNF116; Dako-Denmark) and cross reactive with mouse cytokeratin was used. Secondary antibody was a FITC-labelled rabbit antibody to mouse F(ab)'2 (DakoCytomation). Mouse MHC II molecule was detected using a pan mouse MHC II molecules (rat IgG2b, clone M5/114.15.2; Santa-Cruz) and the secondary was a FITC labelled goat anti-rat IgG. Tissue transglutaminase 2 was detected using a rabbit polyclonal antibody H-237 clone (rabbit IgG, Santa Cruz).

2-1-15/ Detection of TG2 activity

TG2 enzyme activity was detected by fluorescence microscopy. Thyroid epithelial cells were gorwn in medium or stimulated with a combination of IFNγ (Peprotech, UK; 200 units /mL), TNFα (Peprotech, UK; 200 units /mL) and IL-1β (Peprotech, UK; 200 units /mL). At the end of the assay, cells were incubated in TG2 assay buffer (965µL of 100mM Tris/HCl (pH 7.4), 25µL of 200mM $CaCl_2$) for 15 minutes. 10µL of a 10mM solution of biotinylated monodansyl-cadaverine (Cambridge Biosciences, UK) for 1 hour at room temperature. The reaction was stopped with 25mM EDTA for 5 minutes at room temperature. The cells were

gently washed once with PBS abd fixed with 4% paraformaldehyde for 10 minutes at room temperature. The detection for the incorporation of the cadaverine substrate, cells were incubated which was detected using streptavidin-FITC (Sigma, UK, 1:75).

Coverslips were washed 4 times in PBS and mounted on a slide with some mounting medium (VectroLab, UK). Slides were left overnight in the dark at 4° C to allow the mounting medium to harden. Slides were read on a fluorescent microscope (Leica, UK) at different magnifications (x40 and x100) and data analysed using OpenLab3.1.5 software (OpenLab, USA).

2-1-16/ Treatment of TAZ10 mice with Cystamine

As described in a transgenic mouse model of Huntington diseases (Karpuj et al, 2002; Dedeoglu et al 2002), TAZ10 mice were treated with 100uL of 0.01M daily injection of cystamine dihydrochloride (Sigma, UK) dissolved in PBS. Treatment lasted for 7 days.

Cardiac blood samples were collected from mice under anesthesia following conditions imposed by the Home Office. Blood was collected in eppendorf tubes supplemented with heparin. Mice were culled and spleen, cervical and mesenteric lymph-nodes isolated. Cells were stained for specific markers and analysed by flow cytometry.

On the other hand thyroids were frozen down in LAMB OCT embedding medium (Raymond A Lamb, UK) for further histology.

2-1-17/ Statistical Analysis

Statistical differences between groups were determined with the unpaired Student's t test using Prism software (GraphPad, USA). Values of probability were considered to be significant when $p < 0.05$.

2-2/ Material and Methods Part 2: Human Work

2-2-1/ Cell-lines

IB3-1 (human bronchial cell-line bearing the common CF mutation ΔF508 of the CFTR gene) and C38 (Isogenic CFTR rescued IB3-1 cell-line) cell lines were grown in petri-dishes pre-coated in LHC-Basal medium (Invitrogen, UK) containing 0.5mg/mL Fraction V BSA (Calbiochem, UK), 10μg/mL fibronectin (BD Biosciences, San Diego, CA) and 35μg/mL collagen II (BD Biosciences, San Diego, CA) for a minimum of 2 hours at 37°C. Cells were grown in LHC-8 medium (Invitrogen, UK) supplemented with 5% of low-endotoxin FCS (Autogen Bioclear, UK) and 100 U/mL Penicillin/streptomycin (Cambrex, UK).

Where specified, were stimulated for 6 h with R283 (250 μM) or KCC009 (250 μM), ionomycin (1 μM; Calbiochem), BAPTA-AM (5 μM, Calbiochem), EUK 134 (50 μg/ml), rosiglitazone (10 μM), NAC (10 mM), proteasome inhibitor MG132 (50 μM for 6 h; Calbiochem), or R283 for 24 h, followed by 6-h rosiglitazone. Normal human bronchial epithelial 16HBE cells were cultured in the same way.

2-2-2/ Patients

All patients signed consent form prior to the study. Study was performed according to the protocol approved by the National Research Ethics Service, Southampton and south west Hampshire Research Ethics Committee (Reference Number 07/H0502/117): "Understanding the role played by myeloid derived suppressor cells (MDSCs) in patients with rheumatoid arthritis (RA) and the effect of anti-TNFα therapy".

2-2-3/ Flow cytometry and antibodies

PBMCs were collected and washed in cold FACS Buffer (1X PBS supplemented with 0.1% FCS and 0.05% Sodium Azide). Antibodies used were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or peridinin chlorophyll protein (PerCP) or allophycocyanin (APC). Tandem dyes were also used: cyanin conjugated to phycoerythrin (PE-Cy5 and PE-Cy7) or allophycocyanin (APC-Cy7) or peridinin chlorophyll protein (PerCP-Cy5). Samples were acquired with FACSCanto and analysed using the FACSDiva Software (BD Biosciences). Cells were stained for **CD11b** (mouse IgG1, clone CBRM1/5; eBiosciences), **CD11c** (mouse IgG1, clone 3.9; eBiosciences), **CD14** (mouse IgG1, clone

61D3; eBiosciences), **CD15** (mouse IgM, clone HI98; eBiosciences), **CD33** (mouse IgG1, clone WM53; eBiosciences), **CD34** (mouse IgG1, clone 563; BD Pharmingen) and **HLA-DR** (mouse IgG2b, clone LN3; eBiosciences).

Samples were acquired with FACSCanto and analysed using the FACS Diva Software (BD Biosciences).

2-2-4/ ELISA

Production of cytokines was assessed by ELISA. Supernatants were tested for cytokine content such as IL-1β, IL-6, IL-8 and TNFα (All purchased from R&D Systems, UK). Flat-bottom 96-well maxisorp plates (Nunc, UK) were coated overnight at room temperature with capture antibody diluted in PBS (100µL per well). Capture antibody was discarded and plate was washed three times with washing-buffer (0.05% Tween-20 in PBS, pH 7.2-7.4) by inverting the plate onto clean paper towels. Plate was blocked for a minimum of 1 hour with 300µL of blocking-buffer (1% BSA in PBS, pH 7.2-7.4) per well at room temperature. Plate was washed three times as previously described and 100µL of standard or sample diluted in blocking-buffer was added per well and the plate incubated at room temperature for 2 hours. Plate was washed as previously described and biotinylated detecting antibody diluted in blocking-buffer was plated out (100µL per well) and incubated for 2 hours at room temperature.

Wells were washed 3 times with washing-buffer and 100µL/well of a 1:1000 dilution of avidin alkaline-phosphatase (Sigma, UK) in blocking-buffer and the plate incubated for 40 minutes at room temperature. After 3 washes, 100µL Sigmafast pNPP substrate (Sigma, UK) was added per well and watched for color development. Absorbance was read on an Biorad model 680 plate reader using Microplate manager software (BioRad, UK) at 405nm. Results were analysed and graphs plotted using Prism 4 program.

2-2-5/ Isolation of PBMCs from the blood of control individuals, patients with Rheumatoid Arthritis and Systemic Lupus Erythematosus

50mLs of blood from patients was harvested and diluted 1:1 with sterile PBS, 20mLs of diluted blood was carefully layered onto 20mLs of FicoLite-H at a density of 1.077 g/mL (LINARIS Biologische Produkte GmbH, Germany) and spun down for 20 minutes at 600g at

21°C. The interphasse containing PBMCs was carefully harvested using a Pasteur pipette and washed twice in PBS. Cells were counted and used in subsequent assays.

2-2-6/ Purification of $CD34^+$ cells from PBMCs

$CD34^+$ cells were isolated using micro-beads conjugated CD34 microbeads (Myltenyi Biotech, Germany) as previously described.

Briefly, purified PBMCs (Paragraph 2-2-5/) were resuspended in MACS buffer (2 mM EDTA and 0.5% BSA in PBS) and incubated with micro-beads conjugated with anti-CD34 mAb (clone L3T4) for 15 minutes at 4°C. Excess of free micro-beads was washed away with MACS buffer and labelled cells passed through a separating MS column on a magnetic field.

All experiments were performed using DMEM medium (Cambrex, UK) supplemented with 10% Foetal Calf Serum (Globepharm), 0.3 mg/mL L-glutamine (Cambrex, UK), 100 U/mL Penicillin/streptomycin (Cambrex, UK), 1mM non essential amino-acids (Cambrex, UK) and 1 mM sodium pyruvate (Cambrex, UK) at 37°C with 5% CO_2.

2-2-7/ T cell proliferation by (^3H) thymidine incorporation

T cell proliferation was assessed by measurement of tritiated thymidine incorporation. Briefly, freshly isolated PBMCs were cultured in triplicate in 96 well plates at $5x10^4$ cells per well in culture medium containing soluble α-CD3 (clone OKT-3, made in house) at 10µg/mL. Purified $CD34^+$ cells were eventually added at a PBMC:$CD34^+$ cells ratio of 1:1 or 1:5.

Cells were co-cultured for 72 hours at 37°C in 5% CO_2. Each well was then pulsed with 1 µCi ^3H Thymidine for the last 12 hours of culture. Plates were harvested onto filtered plates (Perkin Elmer) and ^3H thymidine incorporation measured in a microplate scintillation counter (Packard). Data are expressed as cpm (count per minute) and were plotted as mean average of the triplicates.

2-2-8/ Fluorescence microscopy

C38 and IB3-1 cell-lines: coverslips were pre-coated for a minimum of 2 hours in LHC-Basal medium (Invitrogen, UK) containing 0.5mg/mL Fraction V BSA (Calbiochem, UK),

10μg/mL fibronectin (BD Biosciences, San Diego, CA) and 35μg/mL collagen II (BD Biosciences, San Diego, CA).

Cells were washed 3 times with 1X PBS, and 4% paraformaldehyde (PFA) in PBS added to the cells for 7 minutes at room-temperature (RT). PFA was washed off by rinsing the cells 3 times with PBS. 200 μL of 0.1% Triton X-100 in PBS was added to the cells for 5 minutes at RT and cells were subsequently washed 3 times with PBS. The first antibody at the appropriate concentration in PBS containing 1% BSA (Sigma, UK) was added to the cells and incubated for 20 minutes at RT in the dark. Coverslips were washed 3 times with PBS and the secondary antibody added in PBS with 1% BSA for a further 20 minutes at RT in the dark. Cells were washed three times and DAPI (Molecular Probe, UK) nuclear counterstain added at 1 μg/mL in PBS with 1% BSA for 10 minutes at RT in the dark. Coverslips were washed 4 times in PBS and mounted on a slide with some mounting medium (VectroLab, UK). Slides were left overnight in the dark at 4° C to allow the mounting medium to harden. Slides were read on a fluorescence microscope (Leica, UK) at different magnifications (x40 and x100) and data analysed using OpenLab3.1.5 software (OpenLab, USA).

For primary antibodies, anti-**phosphotyrosine** PY99 mAb (mouse IgG2b, Santa Cruz), anti-**PPARγ** (clone E8, mouse IgG1, Santa Cruz) and anti **TG2** mAb (clone CUB1402, NeoMarkers) were used. FITC conjugated rabbit anti mouse IgG (Sigma) was used as a secondary antibody.

2-2-9/ Confocal microscopy

Cell lines were fixed in methanol and permeabilized with 0.5% Triton X-100 prior incubation with primary Abs. Frozen tissue sections were fixed in acetone for 10 min. Anti-phospho-p42/p44 (1:500; Cell Signaling Technology), anti-phosphotyrosine PY99 mAb (1:80, mouse IgG2b), anti- PPARγ (clone E8, sc-7273, 1:100, mouse IgG1; clone H100, sc-7196, 1:100, rabbit polyclonal IgG; Santa Cruz Biotechnology), anti-ubiquitin (1:100 clone FL-76, rabbit polyclonal IgG), anti-histone deacetylase 6 (HDAC6; 1:100 clone H300, rabbit polyclonal IgG), anti-p65 NF-κB (F-6; 1:100), and anti-ICAM-1 (15.2; 1:200) (Santa Cruz Biotechnology) Abs were used for first Ab. The Ag expression and distribution were visualized by indirect immunofluorescence at confocal microscope (Leica).

2-2-10/ Detection of TG2 activity

TG2 enzyme activity was detected by fluorescence microscopy. Thyroide epithelial cells were gorwn in medium or stimulated with a combination of IFNγ (Peprotech, UK; 200 units /mL), TNFα (Peprotech, UK; 200 units /mL) and IL-1β (Peprotech, UK; 200 units /mL). At the end of the assay, cells were incubated in TG2 assay buffer (965µL of 100mM Tris/HCl (pH 7.4), 25µL of 200mM $CaCl_2$) for 15 minutes. 10µL of a 10mM solution of biotinylated monodansyl-cadaverine (Cambridge Biosciences, UK) was added for 1 hour at room temperature. The reaction was stopped with 25mM EDTA for 5 minutes at room temperature. The cells were gently washed once with PBS abd fixed with 4% paraformaldehyde for 10 minutes at room temperature. The detection for the incorporation of the cadaverine substrate, cells were incubated which was detected using streptavidin-FITC (Sigma, UK, 1:75).

Coverslips were washed 4 times in PBS and mounted on a slide with some mounting medium (VectroLab, UK). Slides were left overnight in the dark at 4° C to allow the mounting medium to harden. Slides were read on a fluorescent microscope (Leica, UK) at different magnifications (x40 and x100) and data analysed using OpenLab3.1.5 software (OpenLab, USA).

2-2-11/ Quantitative and semi-quantitative RT-PCR

Quantitative RT-PCR was performed using iCycler iQ Multicolour Real-Time PCR Detector (Bio-Rad) with iQ TM SYBR Green supermix (Bio-Rad). A relative quantitative method was applied for TG2 mRNA (Qiagen; catalog QT00081277), normalized by the control GAPDH mRNA.

Semi-quatitative PCR was performed to assess the relative expression of **TNFα** (forward primer: 5'-ggcccaggcagtcagatcatcttctcg-3'; reverse primer 5'-ggatgttcgtcctcctcacagggcaatg-3'), **IL-1β** (forward primer: 5'-ccctaaacagatgaagtgctccttccagg-3'; reverse primer: 5'-gggatctacactctccagctgtagag-3') and **TG2** (forward primer: 5'-ccgttttccactaagagatgc-3'; reverse primers: 5'-aacataccttggaattttggg-3') and **β-actin** (forward primer: 5'-gatgacccagatcatgtttgagacc-3'; reverse primer: 5'-ggagcaatgatcttgatcttcattg-3').

2-2-12/ RNA interference

IB3-1 cells were transfected with 50 nM human TG2, human PPARγ, and scramble small interfering RNAs (siRNAs) duplex using Hiperfect Transfection Reagent (Qiagen) at 37°C

for 72 h. The target sequence of TG2 siRNA was 5'-CCGCGTCGTGACCAACTACAA-3', and the whole-cell lysate was then analyzed for Western blot. The PPARγ siRNA is a pool of three sequences, as follows: CCAAGUAACUCUCCUCAAAtt, GAAUGUGAAGCCCAUUGAAtt, and CUACUGCAGGUGAUCAAGAtt. Transfected cells were then analyzed by confocal microscopy for expression using anti-PPARγ mAb clone E8

2-2-13/ Western-Blot analysis

First Abs anti-phospho-p42/p44 MAPKs (Cell Signaling Technology), PPARγ (clone E8 sc-7273; Santa Cruz Biotechnology), TG2 (clone CUB7402; DakoCytomation), Nε(γ-L-glutamyl)-L-lysine isopeptide (clone 81DIC2; Covalab), and ubiquitin (1:100 clone FL-76, rabbit polyclonal IgG) were counterstained by a HRP-conjugated anti-IgG Ab (Amersham, General Healthcare). The amounts of proteins were determined by a Bio-Rad protein assay to ensure equal protein loading before Western blot analysis. Fifty micrograms of cell lysate were loaded in each lane.

2-2-14/ Statistical Analysis

Statistical differences between groups were determined with the unpaired Student's *t* test using Prism software. Values of probability were considered as significant when < 0.05.

3/ Characterization of different subsets of myeloid cells: myeloid Dendritic Cells (DCs) and Myeloid Derived Suppressor Cells (MDSCs)

3-1/ Introduction

Dendritic cells (DCs) can act as powerful inducers of an adaptive immune response. Because of their properties, DCs have been and are still used as tools in immunotherapeutic strategies to boost the immune system in cancer (Banchereau et al. 2005; Cerundolo et al, 2004; Figdor et al. 2004). Tumor induced defects in DCs have been extensively studied. Patients with early-stage head and neck squamous-cell carcinoma have low levels of DCs (decrease of approximately 2 fold) in their peripheral blood with even fewer cells at later stages (4 fold decrease) (Pak et al. 1995; Garrity et al. 1997; Almand et al. 2000). This decrease in the amount of immunocompetent DCs was associated with an increased number of immature DCs and immature myeloid cells. The accumulation of myeloid precursors at the tumor site and other lymphoid and non-lymphoid organs has been the focus of numerous studies in the last decade to explain the failed anti-tumor immune response (Reviewed in Bronte et al. 2005; Kim et al. 2006; Frey. 2006; Gabrilovitch. 2004).

Myeloid Derived Suppressor Cells (MDSCs) express mainly the myelomonocytic markers Gr1 and CD11b (Bronte et al. 1998 and 2000) and are recruited at the site of inflammation by numerous cytokines such as G-CSF or GM-CSF that are often actively secreted by tumor cells (Kim et al. 2006; Serafini et al. 2004). MDSCs have been shown to strongly inhibit T cell proliferation both in mouse and human cancer (Bronte et al. 2000; Pak et al. 1995). Indeed, therapies aiming at reducing the pool of MDSCs (Suzuki et al. 2005) or inhibiting their actions (Bronte et al. 2005) have been shown to strongly improve T cell proliferation and therefore anti-tumor immunity.

While the role of MDSCs in tumor expansion has been extensively documented (Gabrilovitch et al. 2004), it is however unclear if MDSCs have any regulatory role in inflammation in general and in autoimmune diseases both mouse and human. It is now apparent that there is a strict correlation between anti-tumor responses and autoimmune diseases; in patients with melanoma for example, a good prognostic factor is the development of an autoimmune disease of the skin, vitiligo (Yee et al. 2000; Gogas et al. 2006).

Indeed, MDSCs might prevent autoimmunity by inhibiting pathogenic self-reactive T cells in a similar fashion as operated by the classic $CD4^+$ $CD25^+$ regulatory T cells (Tang et al. 2004) whilst suppressing anti-tumor responses. To address the role of MDSCs in autoimmunity, we are using a humanized transgenic mouse model of autoimmune thyroiditis

(TAZ10 model; Quaratino et al. 2004). In the TAZ10 model T cells express a human TCR isolated from T cells infiltrating the thyroid of a patient with thyroiditis (Quaratino et al. 2004). The TAZ10 transgenic mice spontaneously develop autoimmune thyroiditis sharing the same clinical and histological changes as patients with Hashimoto's thyroiditis.

In this chapter I will discuss how using different methods to generate DCs from the bone-marrow, and therefore the manipulation of MDSCs, can influence both the yield and quality of the DC population obtained. I will then describe the role and functions of MDSCs in the TAZ10 model of autoimmune disease. Finally, with the results obtained in mice, we will discuss how a defect of MDSCs influences the function of $CD34^+$ cells in patients with Rheumatoid Arthritis and Systemic Lupus Erythematosus. Amid confusions on the nature and phenotype of MDSCs in human, I will clarify their phenotype to reflect their function and myeloid origins.

3-2/ Results Part 1: Generation of potent dendritic cells: Different methods, different outcomes

Strategies to boost the immune system against cancer have been extensively studied and some are now being tested in various clinical trials. Although the results are encouraging, the effectiveness by which tumor cells create a powerful immuno-suppressive environment is detrimental to anti-tumor strategies (Khong et al. 2002; Dunn et al. 2002; Zitvogel et al. 2006).

DCs are at the forefront of an effective immune response as they bridge innate and adaptive immune responses. Numerous studies have exploited the ability of DCs to migrate to lymphoid tissues and efficiently induce the activation and proliferation of T lymphocytes. One example used DCs engineered to present specific tumor antigens to induce the activation of antigen-specific T cells (Pardoll et al. 2002).

Before we embarked on such a project, it was important to optimize the technique to obtain a reproducibly high number of DCs with the highest presenting capacity. Here we describe how different techniques used to generate DCs from bone-marrow precursors can have an impact on the quality and yield of DCs obtained

3-2-1/ Introduction – The spleen as a limited source of dendritic cells

Different subsets of dendritic cells have been described in mouse tissues such as the spleen, lymph-nodes or thymus (Vremec et al. 1992 and 2000; Kamath et al 2000; Henri et al. 2001 and Hochrein et al 2001). In the spleen, three major populations of DCs expressing CD11c can be distinguished according to their expression of CD4 and CD8α. $CD11c^+$ DCs not expressing CD4 or CD8α favour the generation of regulatory responses by inducing the development of IL-10 secreting $CD4^+$ T regulatory 1 cells via the release of TGFβ (Zhang et al. 2005). Both $CD4^+$ $CD8\alpha^-$ and $CD4^-$ $CD8\alpha^+$ $CD11c^+$-DCs have been shown to promote strong T cell responses with $CD8\alpha^+$ DCs being more efficient as they also express the DEC-205 marker involved in the cross-presentation of exogenous antigens onto MHC-Class I molecules (Paragraph 1-2-2/; Dudziak et al. 2007; Bonifaz et al 2002 and 2004).

We first explored the mouse spleen as a potential source of DCs. Flow cytometric analysis was performed on splenic cells using antibodies directed against the DC-specific marker CD11c, MHC Class-II molecules ($I\text{-}A^b$) and activation marker CD86. Both CD86 and $I\text{-}A^b$ markers are expressed at low levels by immature DCs (iDCs) and are upregulated upon

inflammation (Banchereau et al. 2000). Finally, expression of CD8α by DCs has been associated with an increased ability of dendritic cells to cross-present antigens to MHC Class-I molecules, especially those from apoptotic cells (Dudziak et al. 2007).

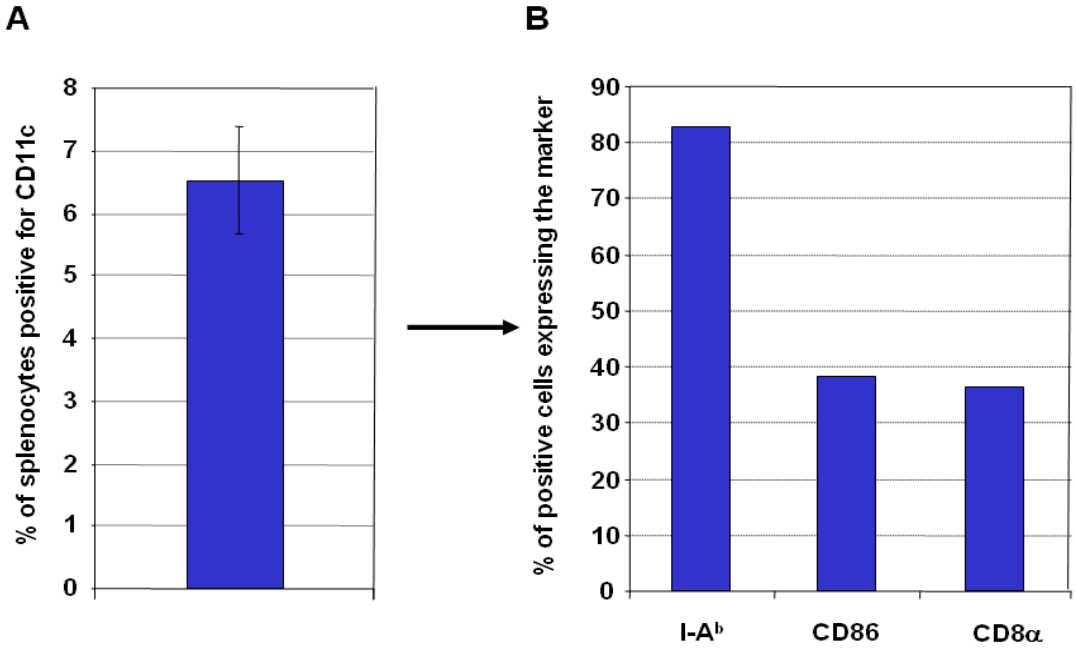


Figure 3-1: The spleen is a limited source of dendritic cells
Splenocytes from wild-type mice were stained for the expression of the CD11c marker **(A)**. $CD11c^+$ cells were then assessed for their expression of MHC Class-II molecules $I\text{-}A^b$, CD86 and CD8α **(B)**. Samples were analyzed by flow cytometry. Data shown is representative of 2 experiments.

6 to 7% of the total splenic population expressed the myeloid marker CD11c (Figure 3-1/A). These cells express a high level of $I\text{-}A^b$ MHC class-II molecules (80%) and CD86 (~ 40 %). Also, more than 30 % of the $CD11c^+$ cells expressed the CD8α marker (Figure 3-1/B).

Despite a phenotype associated with potent immunogenic DCs, their numbers were too low to perform experiments with freshly isolated DCs or to produce the high quality DCs required for the development of DC based vaccines. Thus, other sources had to be considered to generate high numbers of professional DCs.

3-2-2/ Three different methods to generate bone-marrow derived dendritic cells

Different methods have been published describing the generation of DCs from bone-marrow progenitors capable of generating a potent T cell response (Inaba et al. 1992; Lutz et al. 1999; Son et al. 2002). All these methods have in common the use of GM-CSF at 20ng/mL throughout the generation and LPS used to induce maturation. GM-CSF is a cytokine that promotes haematopoietic stem-cells to differentiate into granulocytes and monocytes (Burgess et al. 1980), justifying its use for the generation of dendritic cells from bone-marrow precursors. Methods are described in Figure 3-2.

The supernatant from the culture of CT26GM, the CT26 colon carcinoma cell-line, stably transfected with a retrovirus containing the gene for murine GM-CSF (Dranoff et al. 1993) was used as a source of GM-CSF for the generation of BM-DCs using the three methods. Content of GM-CSF was assessed by ELISA and used at a final concentration of 20ng/mL. In many tumors, the production of IL-10 has been shown to alter the ability of mature dendritic cells to efficiently promote antigen-specific T cell proliferation and the maturation of immature DCs through the decreased expression of costimulatory molecules (Reviewed in Gabrilovitch et al. 2004). By ELISA, I confirmed that CT26GM culture supernatant did not contain IL-10. Qualitatively and quantitavely, the use of CT26GM culture medium produced better BM-DCs than commercial GM-CSF. This could be due to other factors secreted by the tumor cell-line that could enhance the differentiation and maturation of BM-DCs (Gabrilovitch et al. 2004).

One method known as "Inaba's method" (Inaba et al. 1992) (Figure 3-2/A) involved the seeding of bone-marrow cells at $2x10^6$ cells per mL in 24 well-plates. Medium is removed every two days and replaced with 1mL of fresh medium supplemented with GM-CSF. iDCs obtained 7 days later are replated before LPS is added the following day and incubated overnight for complete maturation.

A second method or "Son's method" (Son et al. 2002) (Figure 3-2/B), seeds $1x10^6$ bone-marrow progenitors in 5mL of GM-CSF containing medium per well of 6 well-plates. At day 4 and 7 additional GM-CSF is added until DCs were considered to be immature 8 days later. Non-adherent iDCs were replated overnight and full maturation achieved by overnight stimulation with LPS.

The third method or "Lutz's method" (Lutz et al. 1999) (Figure 3-2/C) seeds bone-marrow progenitors in 100mm petri-dishes at $2x10^6$ cells in 10mL of medium supplemented with GM-CSF. At Day 3 of the culture, 10mL of medium supplemented with GM-CSF is added to the petri-dishes. At day 6 and 8, plates are swirled to homogeneously resuspend the cells and 10mL of medium was harvested, centrifuged and the pellet resuspended in 10mL of fresh medium with GM-CSF. The resuspended cells are then seeded back to the same petri-dishes. At day 10, non-adherent cells considered as iDCs were harvested and replated overnight at $2x10^6$ cells per well on 6 well-plates with 5 mL of medium. DCs obtained at this stage were considered as being semi-mature before being matured by an overnight stimulation with LPS.

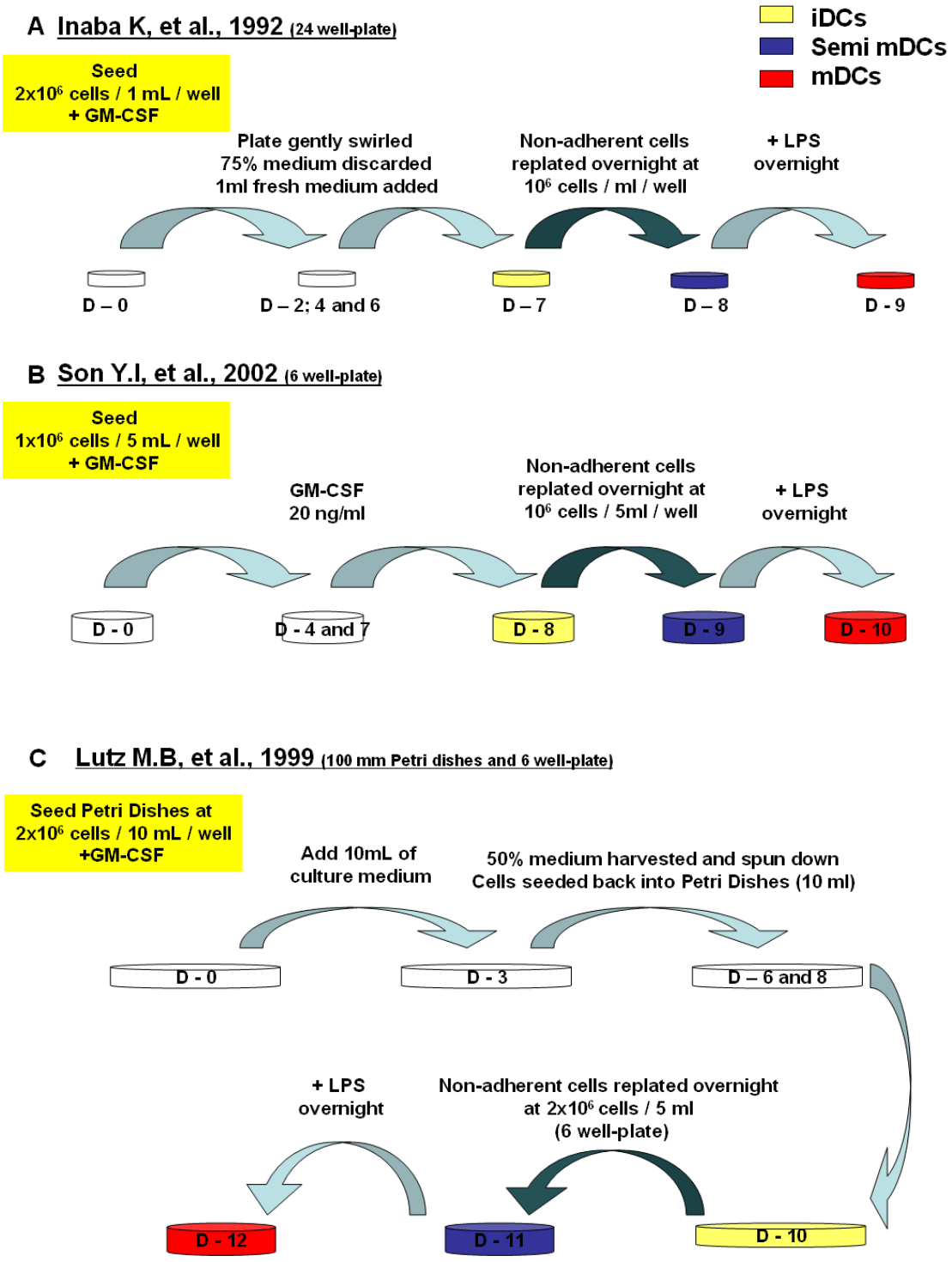


Figure 3-2: Description of the three different methods to generate bone-marrow derived dendritic cells
(A) Method described by (Inaba et al. 1992). **(B)** Method described by (Son et al. 2002). **(C)** Method described by (Lutz et al. 1999). In all the different methods, GM-CSF was used at 20 ng/mL.

3-2-3/ Differential phenotype of BM-DCs following different methods of generation

To compare their efficiencies, bone-marrow derived dendritic cells (BM-DCs) were generated using these different protocols and their ability to stimulate T cells evaluated.

During the generation of DCs following Inaba's method (Inaba's DCs), 75% of the medium was discarded after the plate had been gently swirled before being replaced with 1mL of fresh GM-CSF containing medium. In contrast, cells harvested during Lutz's method were not discarded but resuspended into fresh medium supplemented with GM-CSF before being seeded back to the same petri-dishes. I counted and analysed these cells by flow cytometry to assess whether any cells of the myeloid lineage were removed during the washing steps (Figure 3-3).

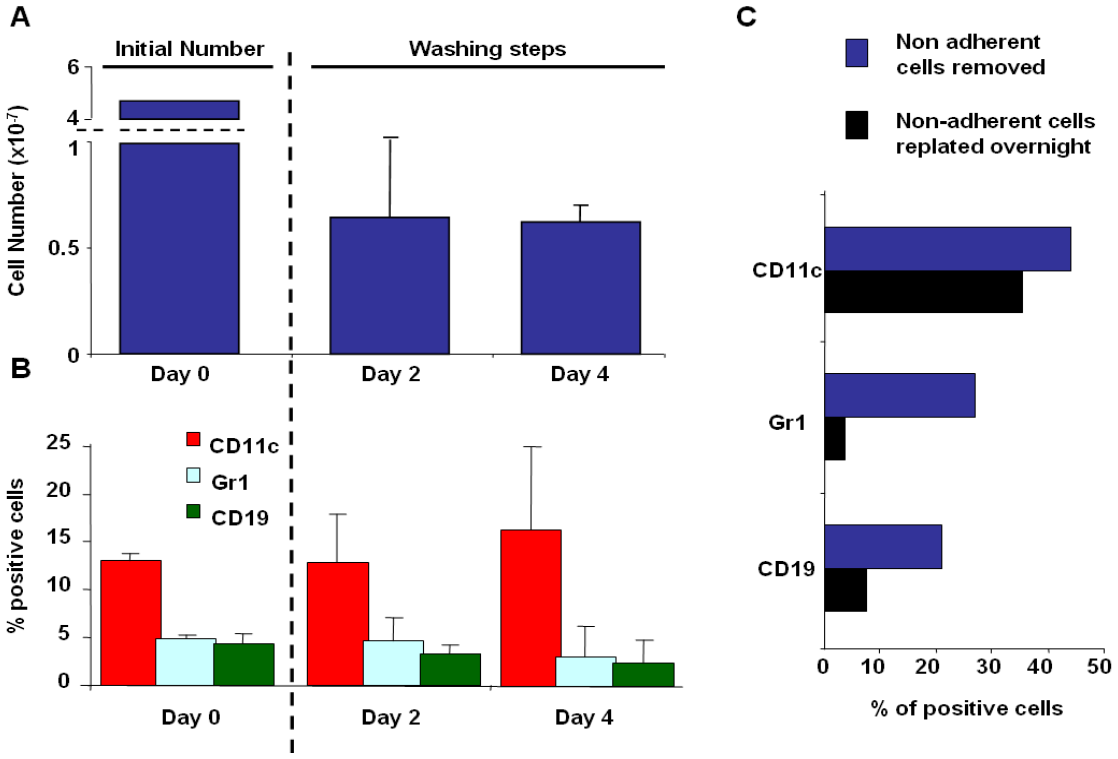


Figure 3-3: Removal of non-adherent cells during the generation of BM-DCs removes myeloid cells and precursors
Cells removed in Inaba's method at day 2 and 4 were counted **(A)** and their phenotype analyzed by flow cytometry using the CD11c, Gr1 and CD19 markers **(B)**. Cells harvested at day-8 during the generation of BM-DCs following Lutz's method were replated overnight and the expression of CD11c, Gr1 and CD19 assessed by FACs **(C)**. Data are representative of two experiments.

About $5x10^7$ cells were seeded at the start of the culture whereas about $5x10^6$ cells were removed in the washes performed at day 2 and 4 (Figure 3-3/A). Cells removed during each

wash were then analysed for their expression of the myeloid marker CD11c, the B cell marker CD19 and Gr1 as a marker for cells of the granulocyte lineage (Figure 3-3/B). Although $CD19^+$ cells were discarded after each step, high levels of $CD11c^+$ myeloid cells were also removed. Furthermore, at day-2 and 4, about 2 to 5 % of the cells removed were expressing the Gr1 marker. Because Gr1 has been described as a marker for cells of the granulocyte lineage, I could conclude that the washing steps removed this contamining population. However, the Gr1 marker is also primarily expressed in the bone-marrow by Myeloid Derived Suppressor Cells (MDSCs) (Refer to Chapter 3-2). MDSCs expressing the Gr1 and CD11b markers have been shown to be the progenitors of cells of the myeloid lineage such as dendritic cells, macrophages and granulocytes (Bronte et al. 2000). Therefore, the removal of the $Gr1^+$ cells during Inaba's method was detrimental as it meant the removal of myeloid precursors. In fact, the generation of BM-DCs following Inaba's method led to a reduced yield compared to the two other methods (Figure 3-4/A).

To further assess the effect of replating cells removed during washing in both Inaba's and Lutz's methods, non-adherent cells harvested at day 8 of the Lutz's protocol were seeded back into fresh wells overnight and their phenotype analysed (Figure 3-3/C). Replating induced a decrease in expression of Gr1 (30% to 5%) and CD19 (20% to 8%). Decreased expression of Gr1 by MDSCs is associated with their differentiation, the replating steps would further promote differentiation and maturation of myeloid precursors. Indeed, Lutz's method whereby cells are re-seeded in the dishes after washing should enhance both the quality and yield of the final DC preparation.

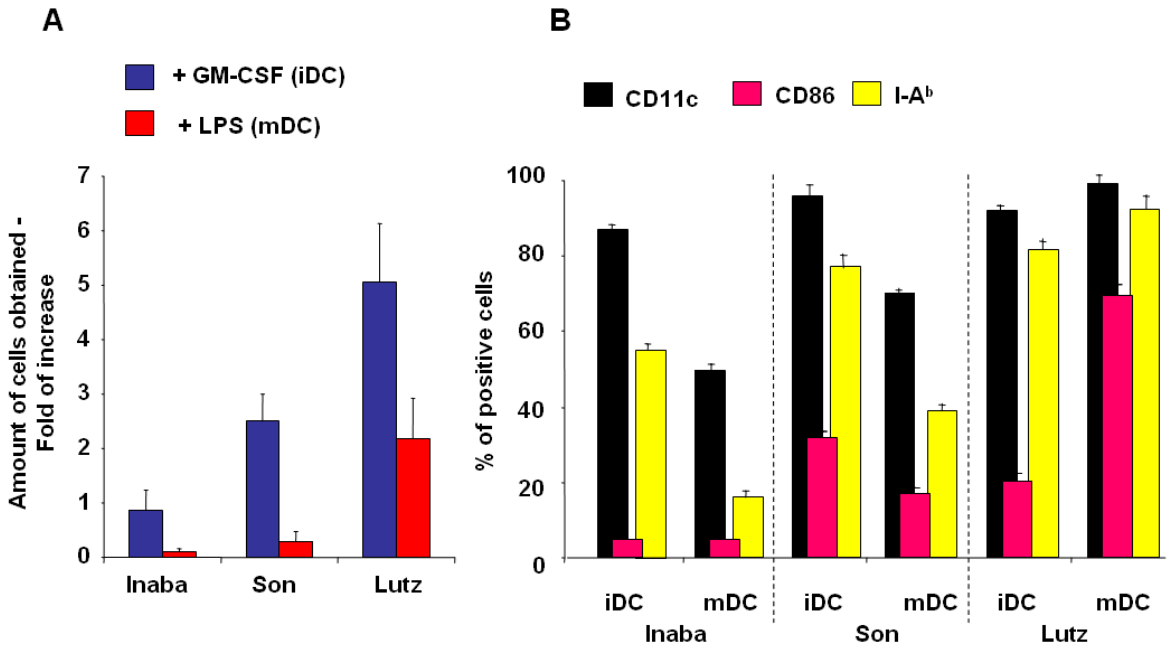


Figure 3-4: Yield and quality of immature and mature DCs (iDCs and mDCs respectively) obtained varies with the method used

(A) Amount of iDCs and mDCs was evaluated for each method. Number of cells obtained was evaluated as a fold of increase compared to the number of cells seeded at day-0. Results are a pool of 5 different experiments. **(B)** Cells obtained were analyzed by flow cytometry for their expression of CD11c, CD86 and I-A^b MHC Class-II molecules. Data is representative of 5 different experiments.

The quantity of iDCs obtained using Son's method was 2.5 fold higher compared to Inaba's method; whereas the amounts of mDCs obtained using both Inaba's and Son's methods were low. The yields of iDCs and mDCs obtained when generating DCs using Lutz's method were considerably higher (5 and 2 ½ fold increase respectively) compared to the other methods (Figure 3-4/A). Flow cytometric analysis was conducted on both iDCs and mDCs obtained with each of the methods. Although the CD11c marker for myeloid DCs was highly expressed by all iDCs (85 to 95% expression), its level was lower after maturation with LPS for Inaba's and Son's mDCs (50% and 70% respectively) whereas it remained unchanged when considering mDCs generated according to Lutz's method (Figure 3-4/B).

iDCs generated according to Inaba's method expressed low amounts of CD86 (less than 5%) with 50% of the cells expressing I-A^b molecules. However, further maturation with LPS lead to an abrupt drop in the expression levels of all the markers. Similarly, upon maturation, the percentage of DCs expressing CD86 and I-A^b MHC Class-II molecules decreases (15% and 40% respectively) with the second method.

iDCs obtained following Lutz's method showed a high percentage of cells expressing CD11c (90%) and MHC-II (85%) whereas 18 % of the cells expressed the maturation marker CD86. iDCs matured overnight with LPS increased their expression of CD86 and I-Ab molecules (2.5 and 4 fold respectively) with more than 90% of cells still expressing the CD11c myeloid marker (Figure 3-4/B).

Since efficient stimulation of antigen-specific $CD4^+$T cells requires two signals provided by mDCs (Anderton et al. 2002; Chapter 1), the generation of BM-DCs following Lutz's method generated high numbers of DCs expressing high amounts of MHC-II and costimulatory molecules.

The removal of 75% of the supernatant in Inaba's method leads to the removal of myeloid and precursor cells. The small surfaces of culture of Inaba's and Son's method are detrimental as it does not allow for the proper expansion and differentiation of myeloid cells into dendritic cells.

3-2-4/ Addition of IL-4 increases the yield but reduces the quality of the BM-DCs obtained

Generation of DCs from human PBMCs requires both GM-CSF and IL-4. Indeed, omission of IL-4 drastically reduces the yield and the DCs generated present an immature phenotype, with poor stimulation and antigen presentation capacity (Sallusto et al. 1994). To try to further increase the number of cells obtained and their phenotype, IL-4 was added to the culture (Figure 3-5). IL-4 enhanced the yield by 350 fold (Figure 3-5/A) with an identical expression of CD11c, $I\text{-}A^b$ and CD86 (Figure 3-5/B). As previously mentioned, the expression of both DEC-205 and CD8α is associated with potent DCs, capable of efficient uptake and cross-presentation to generate potent $CD4^+$ and $CD8^+$ T cell immune response. The addition of IL-4 lead to a reduction of CD8α expression (versus 32% for GM-CSF alone) whereas only 35% (versus 95% for GM-CSF) of the cells generated expressed DEC-205 (Figure 3-5/C). Interestingly, all BM-DCs expressing CD8α also expressed DEC-205.

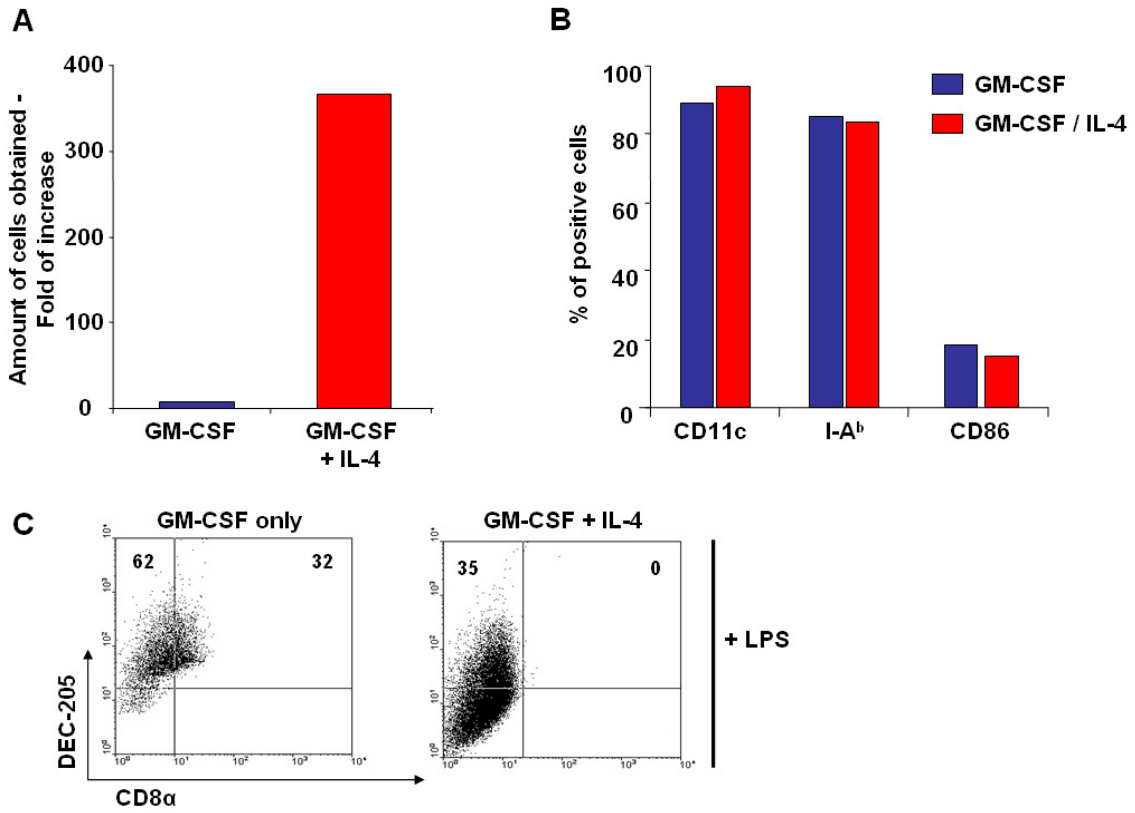


Figure 3-5: Effect of IL-4 on the generation of BM-DCs
(A) BM-DCs were generated either with GM-CSF alone or GM-CSF + IL-4 and the yields determined as fold increase compared to the original number of cells seeded. **(B)** BM-DCs generated according to Son's method with or without the addition of IL-4 were stained for CD11c, I-A^b and CD86 and analysed by flow cytometry. **(C)** mBM-DCs were also analysed by flow cytometry for their expression of CD8α and DEC-205. Data is representative of two different experiments.

It seemed therefore that the increase in yield obtained with GM-CSF and IL-4 was made at the expense of the quality of the cells obtained. This phenomenon has previously been observed as DCs generated using GM-CSF and IL-4 showed a significant reduction in the expression of MHC-II molecules (Masurier et al. 1999). I therefore decided not to supplement the culture medium with IL-4 when generating BM-DCs.

3-2-5/ Full maturation of BM-DCs with LPS limits their half-life

DCs are present in two major forms; immature (iDCs) and mature (mDCs). To further analyse the different processes leading to the full maturation of iDCs obtained at day 10, we assessed the effect of replating (day 11) and addition of maturing agent (day 12) on the phenotype of cells obtained following Lutz's method.

Replating of iDCs induced further maturation and upregulation of CD86 and MHC-II molecules. 96 % of these semi-mature DCs still expressed the myeloid CD11c marker. At this

point, semi-mature dendritic cells did not show any major differences in their morphology; their size (Forward Scatter or FSC) and granularity (Side Scatter or SSC) remained unchanged (Figure 3-6).

Multiple agents have been used to induce the full maturation of dendritic cells in culture. However, it is now established that although indirect inflammatory signals such as TNFα or IFNα cause the maturation of DCs, these DCs do not trigger the differentiation of T cells and their effector functions; these mature but unlicensed DCs promote the proliferation but not the full differentiation of $CD4^+$ T cells. On the other hand, direct contact with pathogen products via TLR receptors ensures that mature DCs are fully licenced to generate, not only the proliferation of $CD4^+$ T cells, but also to promote their differentiation and effector functions (Spörri et al. 2005; Heath et al, 2005). Taking this into consideration, I focussed our interest on the use of LPS to further mature DCs. I used LPS from *Escherichia Coli (E.Coli)* as it binds both CD14 and TLR4 and is potent at inducing the production of key pro-inflammatory cytokines TNFα, IL1-β, IL-12 and IFNγ (Netea et al. 2002).

Throughout culture CD11c expression remained constant at 96 % (Figure 3-6) whereas the size (FSC) and granularity (SSC) of the cells increased with the maturation process, reflecting the morphological changes affecting DCs as previously reported (Burns et al. 2004). Similarly, throughout the maturation, the expression of CD86 increases by two fold after replating (18% to 36 %) to reach 69% after addition of LPS.

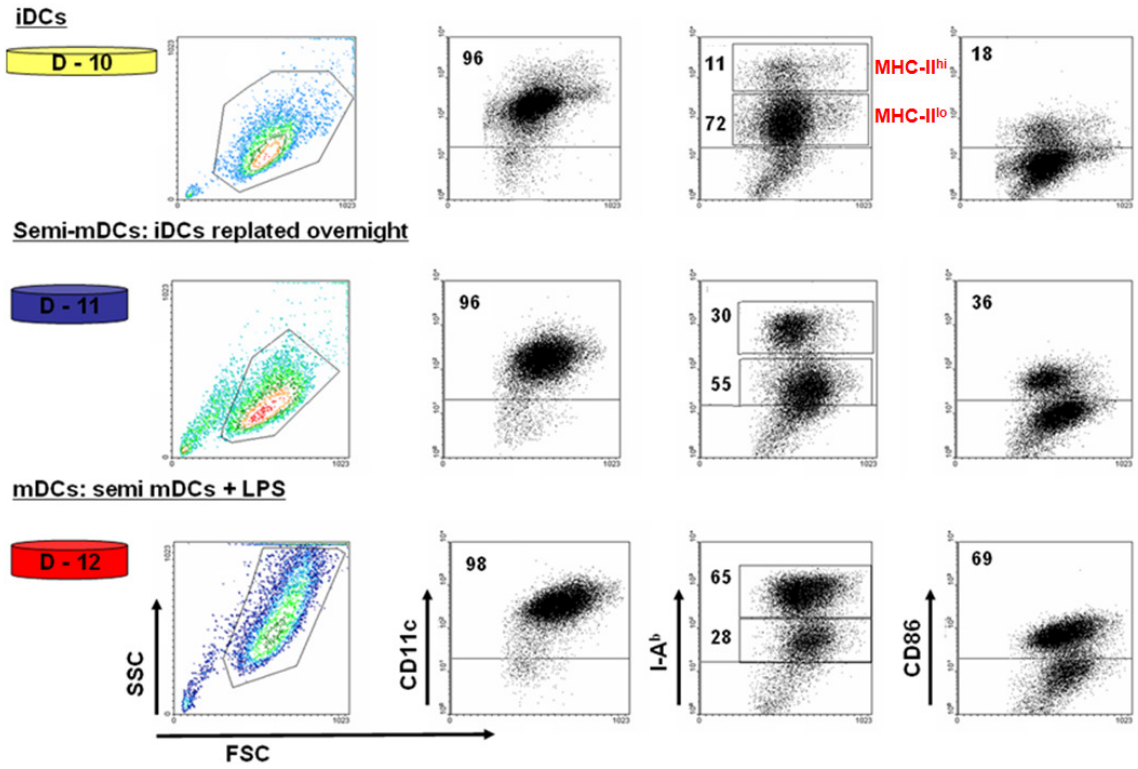


Figure 3-6: Replating produce an incomplete maturation of BM-DCs that is only achieved by the addition of LPS
Lutz's iDCs obtained at day 10 were first replated overnight before LPS was added or complete maturation. Cells obtained at the different stages were assessed for the expression of CD11c, I-A^b and CD86 molecules by flow cytometry. Keys for the culture recipient are described in the legend of Figure 3-2. Data is representative of more than 3 different experiments.

Interestingly, the expression of I-A^b could be detected as two distinct populations: high (MHC-II^{hi} DCs) and low expressors (MHC-II^{lo} DCs). Although the total amounts of MHC-II^+ DCs remained stable during the maturation, the proportion of high expressers increased after replating (11% to 30%). Further maturation with LPS stimulation led to 65% of BM-DCs expressing high levels of MHC Class-II molecules (increase from 30% DCs). Thus, replating of iDCs in Lutz's method promoted maturation but complete maturation was only achieved after the addition of the TLR4 ligand LPS.

The differential expression of MHC Class-II molecules was associated with the expression of the maturation marker CD86. Indeed, MHC-II^{hi} DCs also expressed the highest levels of CD86 compared to MHC-II^{lo} DCs. "Lutz's method" led to the generation of 65% MHC-II^{hi} DCs. These have previously been shown to promote a greater T cell proliferation in allogeneic Mixed Leucocyte Reaction (MLR). In addition, MHC-II^{hi} DCs pulsed with tumor-antigen are able to provide protection after vaccinating mice with the tumor (Masurier et al. 1999).

To assess the lifespan of mDCs, their phenotype was analysed after they were matured with LPS from *Escherichia Coli* (Figure 3-7).

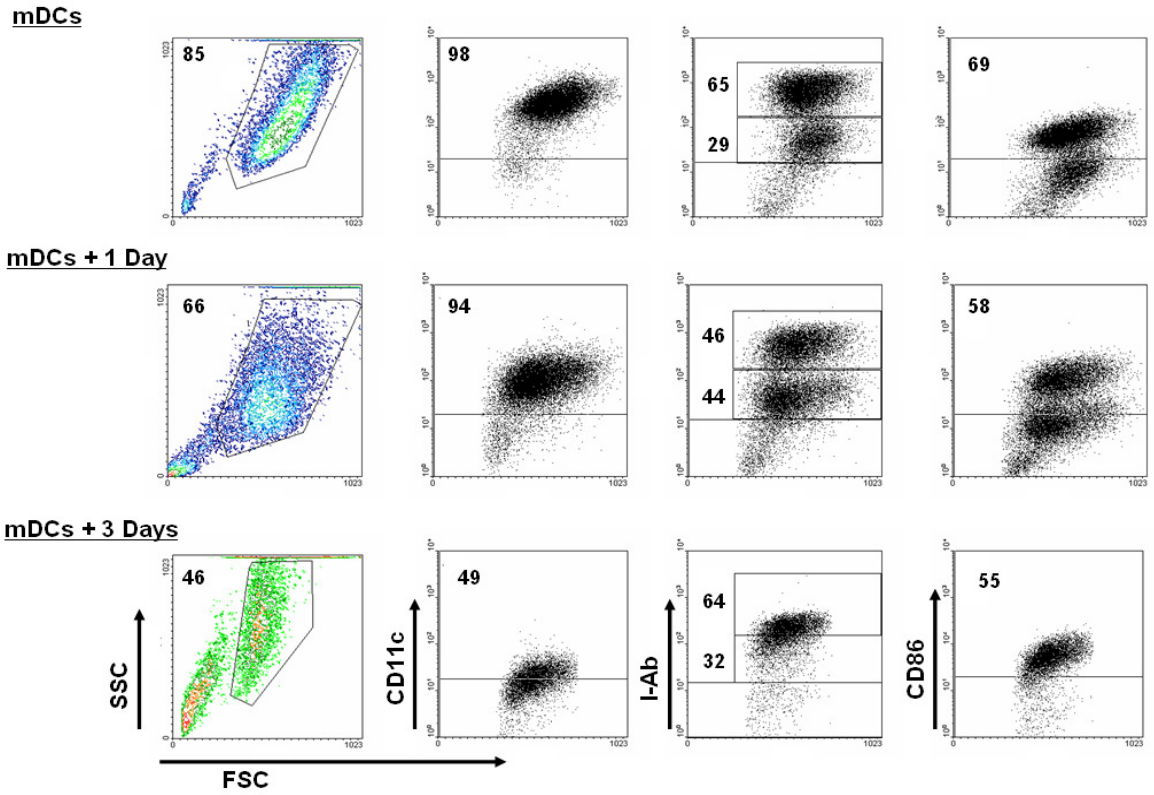


Figure 3-7: Lifespan of mDCs generated according to Lutz's method
mDCs obtained after LPS stimulation at day 12 when Lutz's method was used were left in culture for up to 3 days. Amount of viable cells as judged by the FSC-SSC profile was evaluated along with the expression levels of CD11c, I-A^b and CD86. Data is representative of more than three different experiments.

Upon maturation, 69% of the cells express the co-stimulatory marker CD86 and 65% of the cells expressed high amounts of I-A^b MHC Class-II molecules (Figure 3-7). 1 day after maturation, although 94% of the cells were still expressing CD11c, the level of expression was much lower (4 fold lower) with only 66% of the cells being viable compared to 85 % after LPS stimulation. 10% of the cells lost their expression of CD86 whereas about 20% of cells reduced their expression of I-A^b (44%).

After 3 days, only 46% of cells were viable and the expression of CD11c had dramatically decreased (46% of the cells had extremely low level of expression). 55 % of cells still expressed CD86 although at lower levels. This confirmed previous studies showing that BM-DCs have a short lifespan when they reach their mature status. This short lifespan prevents an unwanted over amplification of the immune response that would be detrimental. Indeed, a recent study has found that in certain conditions, mature dendritic cells can switch

phenotype to become regulatory and therefore dampen the immune response (Svensson et al. 2004; Zhang et al. 2004).

3-2-6/ Reduction of the endocytic activity of dendritic cells upon maturation with a TLR ligand

In figure 3-5, I described that mature mDCs were either DEC-205$^+$ CD8α$^-$ (62%) or DEC-205$^+$ CD8α$^+$ (32%); highlighting their probable efficiency in antigen processing and presentation but also their ability to cross-present extracellular antigens on MHC-I molecules. The differences in size (FSC) and granularity (SSC) of the cells obtained at day 10 and 12 (Figure 3-6) prompted me to analyse their morphology by light microscopy. iDCs obtained at day 10 displayed some dendritic processes increasing the surface of the cells for potential uptake of antigens from the surrounding environment. In contrast, adherent cells presented with the typical morphology of macrophages (Figure 3-8).

Upon maturation with LPS, mDCs displayed numerous dendritic processes and irregularity in their morphology compared to day 10- adherent cells, thus reflecting the high granularity of the cells previously observed by flow cytometry (Figure 3-6). This change in morphology observed for mDCs is associated with the formation of podosomes generated from reorganisation of the actin cytoskeleton (Burns et al. 2004). The formation of podosomes is crucial for the migratory capacity of mDCs via cell motility and degradation of the extracellular matrix (West et al. 2008; Svensson et al. 2008).

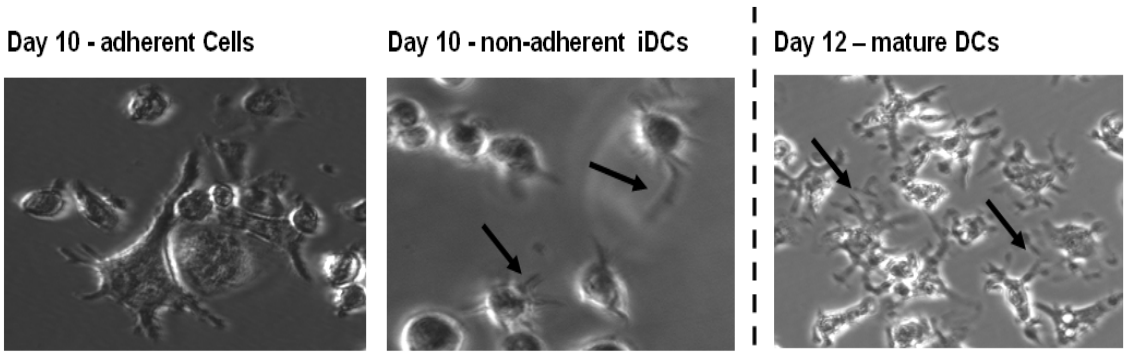


Figure 3-8: Maturation using TLR ligand induces a change in morphology of mDCs
Adherent and non-adherent iDCs obtained at day 10 as well as day 12 mDCs were observed using a light microscope (x40 magnification) for their morphology. Arrows highlight the presence of podosomes on iDCs and mDCs. Cells were then observed through a fluorescent microscope (magnification: x100).

To associate the morphology of the DCs obtained with their ability to uptake exogenous materials, day 10 adherent cells, iDCs and mDCs generated following Lutz's method were fed with latex beads for 30 minutes and observed with a fluorescence microscope after being stained for the myeloid markers CD11c and MHC Class-II molecules (Figure 3-9).

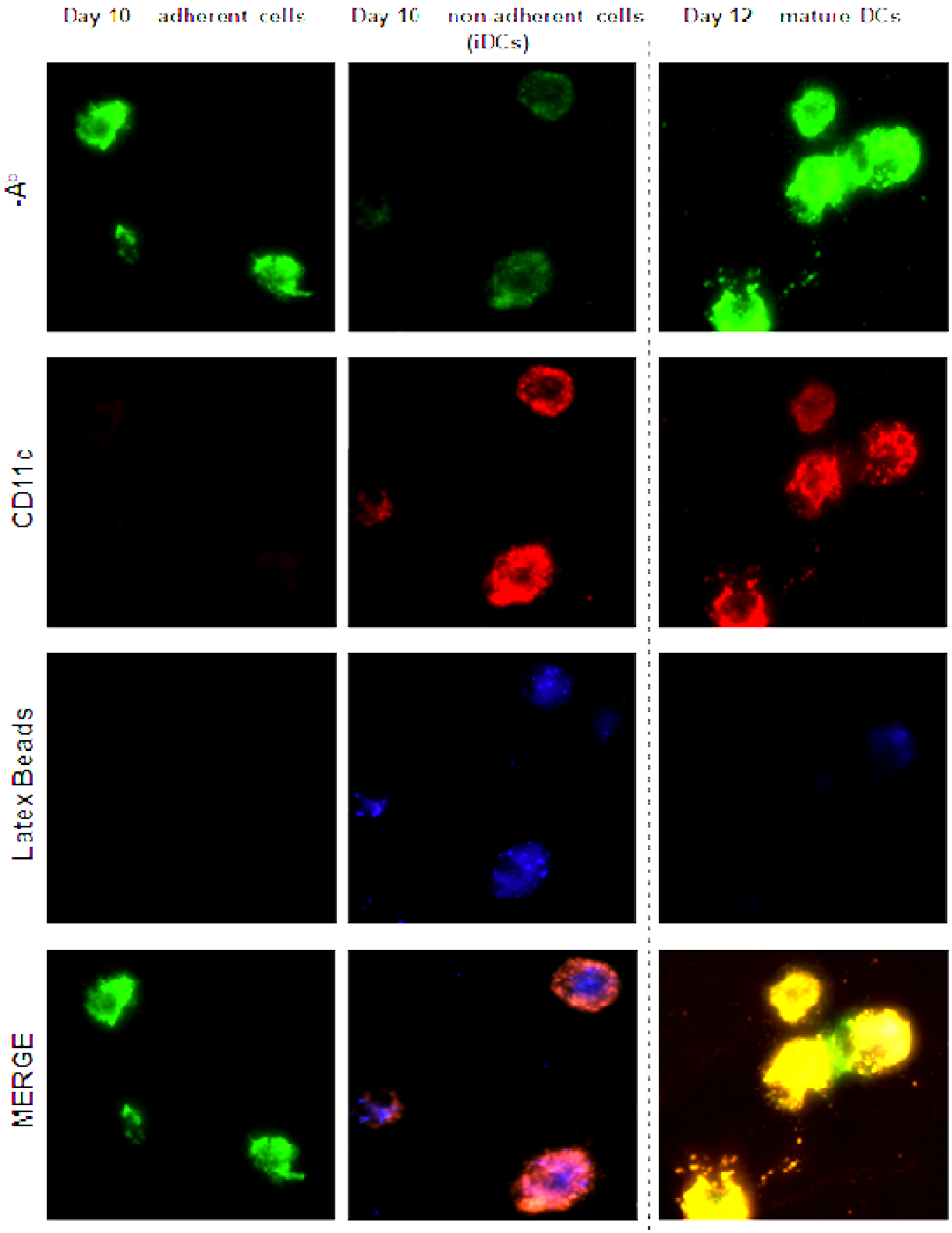


Figure 3-9: Maturation reduces the capacities of mDCs to uptake latex beads
The different cell populations obtained at day 10 (non-adherent iDCs and adherent cells) and 12 (mature DCs) were incubated with latex beads (blue) for 30 minutes and stained for CD11c (red fluorescence) and I-A^b (green fluorescence). Cells were then observed through a fluorescent microscope (magnification: x100).

Adherent cells obtained at day 10 showed a high expression of MHC-II molecules that was not associated with the expression of the myeloid marker CD11c or the uptake of latex beads, thus confirming their macrophage-like phenotype. iDCs generated at day 10 expressed high amounts of CD11c and were efficient in uptaking latex beads while surface expression of MHC-II molecules was low (Figure 3-9). Upon maturation with LPS, mDCs displayed numerous dendritic processes and expressed high levels of I-A^b at the cell-surface while their ability to take-up latex beads was nearly abrogated (Figure 3-9).

3-2-7/ Bone-marrow derived dendritic cells generated following Lutz's method are potent at activating antigen specific T cells

Activation of T cell proliferation is dependent on three signals. The first signal is the recognition by the TCR of the MHC:peptide complex present on antigen presenting cells. However, this signal needs to be associated with the second costimulatory signals and the third signals (from DC- and T- derived cytokines such as IL-2 and IL-12) that allow full activation of T cells (Anderton et al. 2002). Failure to provide efficient costimulation leads to T cell anergy and a deficient T cell response. I therefore used an assay to evaluate the ability of mDCs to deliver strong costimulatory signals to T cells when using an agonistic αCD3 antibody that mimicked the TCR engagement (Figure 3-10).

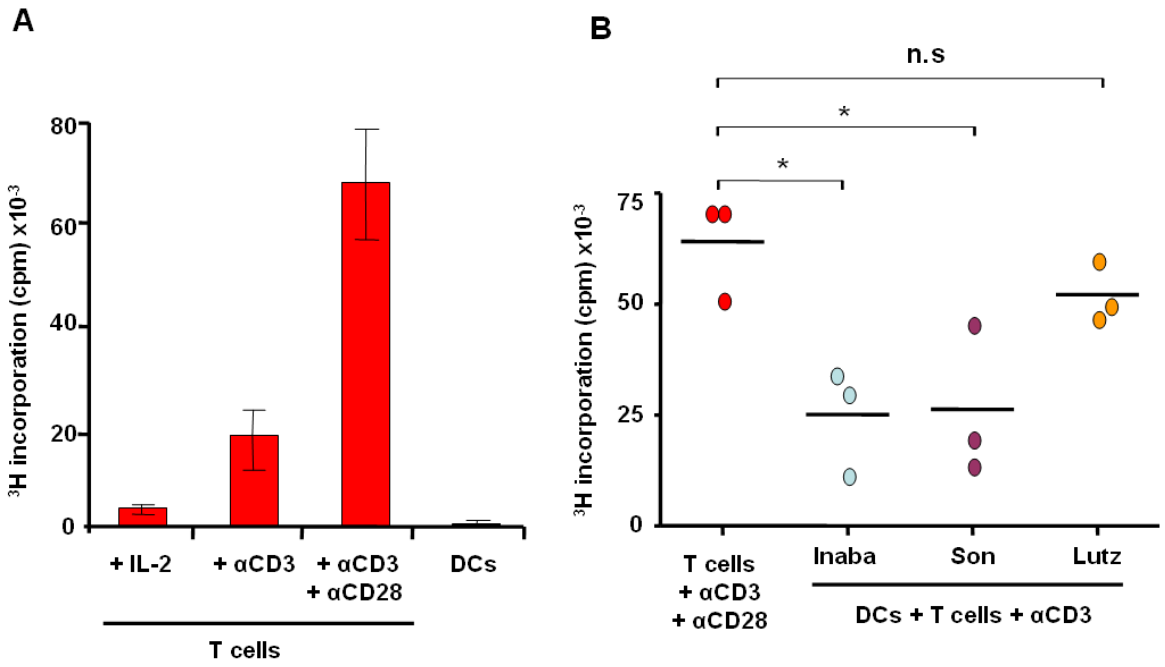


Figure 3-10: mDCs provide strong costimulatory signals to T cells
Proliferative activity of T cells was assessed by the measurement of (^{3}H) thymidine incorporation. **(A)** Stimulation of T cells with IL-2, αCD3 and αCD3/28. **(B)** The costimulatory activity of mDCs was assessed by co-cultivating T cells with mDCs generated following the different methods and αCD3. Data are representative of more than three different experiments.

The addition of IL-2 to T cells induced a low proliferation. The addition of αCD3 antibody increased proliferation. Subsequent addition of αCD28 antibodies provided strong costimulatory activity to T cells resulting in a 3.5 fold increase in proliferation (Figure 3-10/A). To address whether the mDCs generated can provide such a powerful costimulation and can therefore be substituted for αCD28 antibodies, we compared the proliferative activity of T cells with αCD3 and mDCs obtained using the three different methods. mDCs generated following Inaba's and Son's methods could provide weak costimulatory signals to T cells compared to αCD3/28 (p=*). On the other hand, high costimulatory activity was provided by mDCs generated according to Lutz's method; confirming the mature phenotype previously observed (Figure 3-4 and 3-6).

I then assessed the ability of mDCs to stimulate antigen specific T cell proliferation (Figure 3-11). To answer this question, we used the TAZ10 mouse model which spontaneously develops destructive thyroiditis and is characterized by the infiltration of lymphocytes into the thyroid. These transgenic mice express a human TCR specifically recognizing $TPO_{536-547}$, a highly stimulatory agonistic cryptic antigen of thyroid peroxidase

(TPO). mDCs were co-cultured with T cells from TAZ10 mice at a DC:T cell ratio of 1:30, with increasing concentrations of the$TPO_{536-547}$ antigen (from 0.01 to 10 µg/mL).

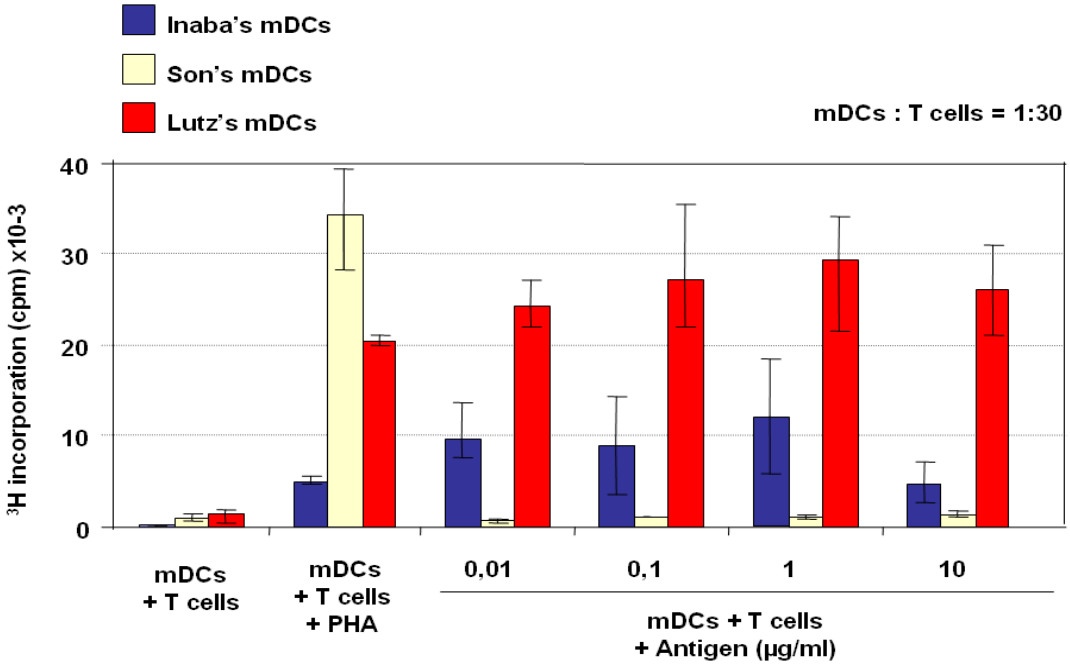


Figure 3-11: mDCs generated following Lutz's method are efficiently activating antigen specific T cells from TAZ10 mice
mDCs were assessed their ability to induce antigen specific T cell proliferation. DCs:T cell ratio was 1:30 and the concentration of the antigen ($TPO_{536-547}$) ranged from 0.01 to 10 µg/mL. T cell proliferation was measured by (^{3}H) thymidine incorporation. Data shown is representative of two different experiments.

Son's mDCs induced a very low level of T cell proliferation, Inaba's DCs could induce a relatively strong T cell response. Lutz's mDCs were highly immunogenic as they could promote an important T cell proliferation.

3-2-8/ Conclusions

IL-10 is an important cytokine produced by tumor cells, which alters the function of dendritic cells and their ability to promote efficient T cell response. IL-10 alters DC functions through different mechanisms such as the downregulation of costimulatory molecules (Gabrilovitch et al. 2004; Steinbrink et al. 2002) or the blockade of DC maturation (Corinti et al. 2001, Morel et al. 1997).

Beyond this alteration of the maturation of DCs by different soluble factors such as IL-10, it is now apparent that the choice of inflammatory signal used to mature DCs is critical to ensure an efficient and full T cell response. Indeed, only pathogen products (LPS, CpG etc.) signaling through TLR are capable of promoting the generation of mature licensed DCs that are capable of inducing not only T cell proliferation, but also T cell differentiation and effector functions. Maturation of iDCs with the soluble factors released by bystander cells upon inflammation, such as TNFα, led to the generation of mature unlicensed DCs that were only capable of inducing T cell proliferation (Spörri et al. 2005; Heath et al. 2005).

This study addressed whether the quality of the DCs generated from murine bone-marrow was affected by the methodology used. In this respect, I used CT26GM culture supernatant as the unique source of GM-CSF while the plastics (petri-dishes and plates) used were identically treated and from the same company for all the different methods.

Both the size of the plastic dish/plate used and the washing steps used to add fresh cytokines had an important impact on the quality and yield of iDCs and mDCs obtained. The use of petri-dishes in Lutz's method allowed the efficient expansion of myeloid progenitors that would otherwise not be able to expand up to 5-6 fold compared to 2 fold in Son's method or no change for Inaba's method (Figure 3-4). The washing steps performed in "Inaba's method" resulted in the loss of an important amount of myeloid progenitors that differentiated into myeloid DCs when replated in Lutz's method (Figure 3-3).

Phenotypic (Figure 3-4 and 3-6) and functional studies (Figure 3-9, 3-10 and 3-11) revealed that Lutz's method is the most efficient technique to generate high numbers of potent iDCs and highly immunogenic mDCs.

3-3/ Results Part 2: Characterization of Myeloid Derived Suppressor cells (MDSCs) in TAZ10 mice with spontaneous autoimmune thyroiditis

Autoimmune diseases (AID) are chronic-inflammatory diseases. AID arise from a breakdown of immunological tolerance to self-antigens and the subsequent activation and expansion of self-reactive T cells (Reviewed in Vanderlugt et al. 2002 and Paragraph 1-4/). This activation of self-reactive T cells together with the generation of self-reactive B cells, the release of soluble factors (pro-inflammatory cytokines such as IL-1β, IL-6 or TNFα or nitric-oxide) and the activation of common signaling pathways of inflammation (NF-κB and p38 MAPK) are responsible for the destruction of the target tissue.

Although the initial stimulus leading to these events are unknown, it is now clear that mechanisms of peripheral tolerance fail to prevent the activation of self-reactive T cells. $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (Tregs) have been shown to be at the forefront of the mechanisms of peripheral tolerance as a deficiency of Tregs has been linked with the development of AID (Sakaguchi et al. 1995; Asano et al. 1996; Suri-Payer et al. 1998).

In the last decade another subset of regulatory cells has been extensively studied. Myeloid Derived Suppressor Cells (MDSCs) are cells of the myelomonocytic lineage that have been shown to differentiate into cells of the myeloid lineage such as granulocytes, macrophages or DCs. Numerous studies have described MDSCs as expressing both the myelomonocytic CD11b and Gr1 markers (Figure 3-12/A and 3-14/A), other markers have also been described, such as CD31 or lineage specific markers such as F4/80 (macrophage) and CD11c (myeloid DCs) (Bronte et al. 2000).

MDSCs have been shown to strongly inhibit T cell proliferation (Kusmartsev et al. 2000). We assessed the ability of MDSCs from WT mice to inhibit T cell proliferation. The removal of MDSCs ($Gr1^+$ cells) from WT splenocytes increased the proliferation in response to the agonistic αCD3 antibody (Figure 3-12/B).

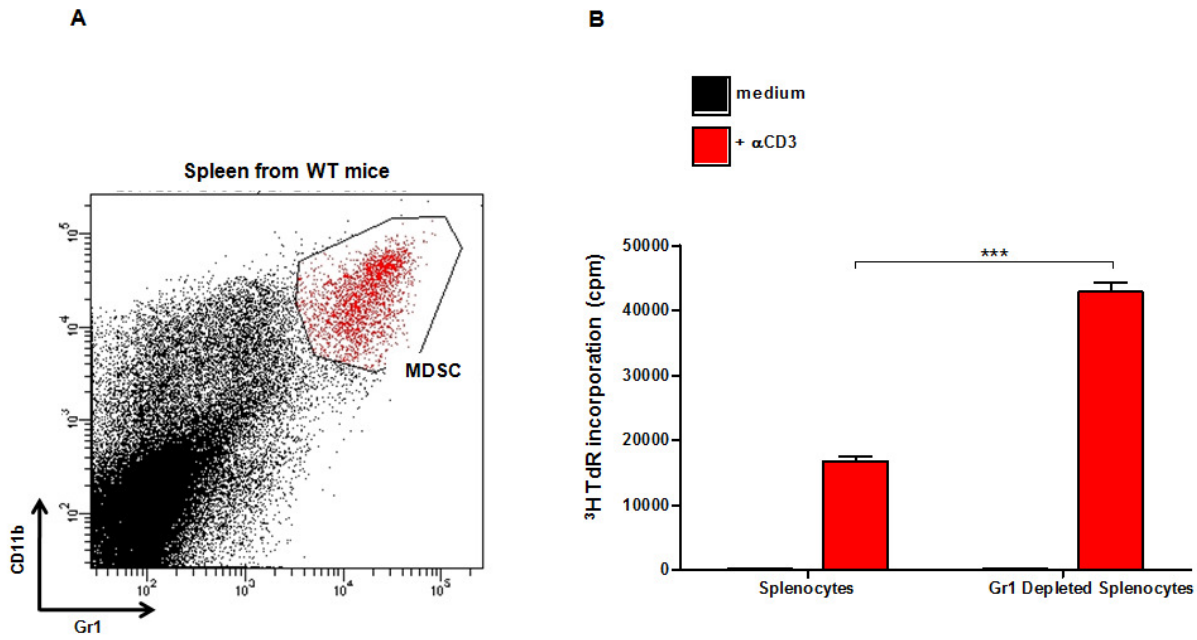


Figure 3-12: MDSCs from WT mice inhibit T cell proliferation in response to αCD3
(A) Staining of splenocytes from wild-type mice with Gr1 and CD11b reveals the presence of MDSCs. **(B)** Proliferative activity of splenocytes from WT mice was assessed by (^{3}H) thymidine incorporation with or without the depletion of Gr1$^+$ MDSCs. Data is representative of more than three different experiments.

The inability of the immune system to mount an effective anti-tumor response is a crucial immunological problem limiting the effects of therapeutic strategies in cancer. Although tumor specific T cells can be found in the blood and tumor (Tumor Infiltrating Lymphocytes or TILs) in melanoma, for example, they are rendered unable to effectively mount an anti-tumor response. Two studies based on the vaccination of melanoma patients using melanoma tumor-antigens suggest that this unresponsiveness can be abrogated by the activation and expansion of T cells specific for tumor antigens not present in the vaccine (Germeau et al. 2005; Lurquin et al. 2005). Activation of the few vaccine-specific T cells present leads to a change in the local microenvironment of the tumor which allows existing TILs, specific for non-vaccine tumor antigens, to be reactivated and new T cells and monocytic cells to be recruited.

Thus, inefficacies of active immune strategies against cancer are, in part, due to mechanisms of evasion of the immune system by tumor cells. A recent study on patients with prostate cancer described how the activity of two enzymes degrading L-arginine was involved in rendering TILs inactive at the tumor site (Bronte et al. 2005). Indeed, inhibition of nitric-oxide synthase (NOS) and arginase (ARG) was able to abrogate the unresponsive status of the TILs.

MDSCs have been shown to mediate their action on T cells via the products of degradation of L-arginine by inducible NOS and ARG (Reviewed in Bronte et al. 2005). Studies in tumor-mouse models (Song et al. 2005; Bunt et al. 2006) and human cancers (Almand et al. 2001) have highlighted an accumulation of MDSCs in tumors and lymphoid organs. Indeed, wild-type mice (C57BL/6) had an increase in the amount of MDSCs present in the bone-marrow (Figure 3-13/A) and lymphoid tissues (Figure 3-13/B) 7 and 15 days after challenge with the melanoma cell-line B16. The presence of MDSCs in the LN not draining the original tumor could be explained as B16 tumor quickly metastasizes in the lungs.

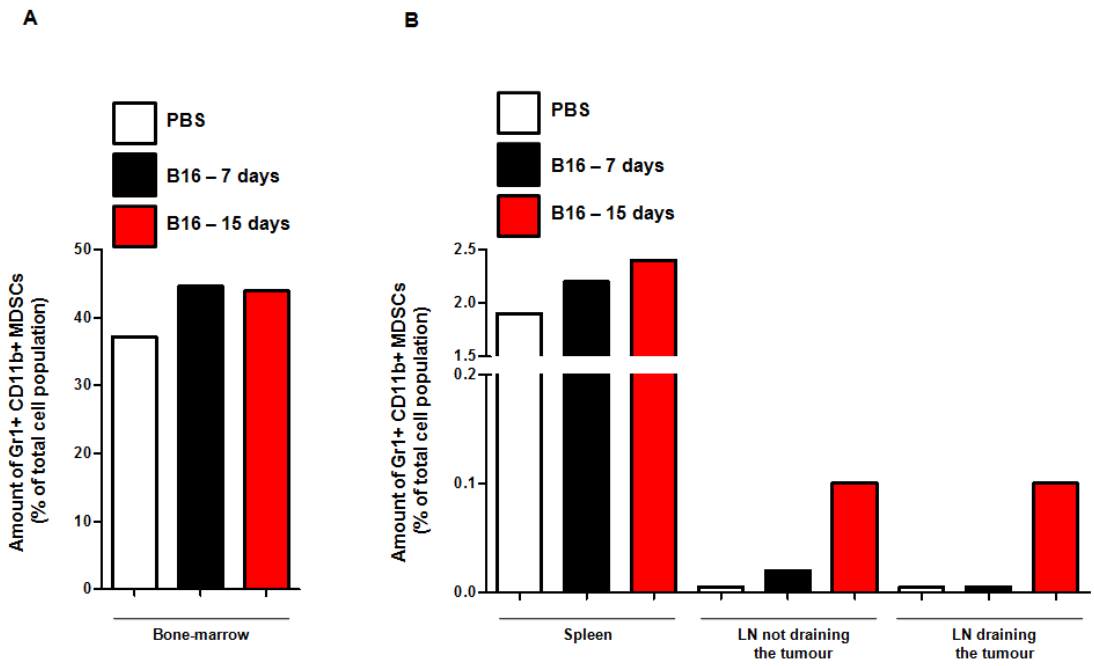


Figure 3-13: MDSCs accumulate in the spleen of mice injected with the B16 melanoma cell-line
Accumulation of Gr1+ CD11b+ MDSCs in the bone-marrow **(A)** and different lymphoid tissues **(B)** draining and not draining the tumor in mice challenged with the melanoma B16 tumor cell-line. Data shown is representative of one experiment involving 4 mice (1 control mouse injected with PBS and 3 challenged with B16) out of two different experiments.

MDSCs accumulating in tumor mice models and cancer patients inhibit T cell function and proliferation and therefore promote tumor growth. These results imply an important role of MDSCs in cancer, I wanted to explore their function in another model of chronic inflammatory disease such as AID.

TAZ10 transgenic mice spontaneously develop autoimmune thyroiditis characterized by the activation of self reactive T cells that infiltrate the thyroid triggering its destruction. To further address the presence of any other regulatory network in autoimmune disease, we

assessed whether MDSCs were present and had any role in the TAZ10 transgenic mouse model of autoimmune thyroiditis.

3-3-1/ Myeloid Derived Suppressor Cells accumulate in mice affected by an autoimmune pathology

From studies in tumor mouse models, it seemed likely that MDSCs would accumulate in TAZ10 mice as a result of ongoing inflammation. As they seemed to be unable to prevent the activation of self-reactive T cells leading to the destruction of the thyroid, we hypothesized that MDSCs were not present in autoimmune disease or had their function altered in these mice.

By three-colour FACS analysis, I investigated whether MDSCs were present in TAZ10 transgenic mice. In the spleen of TAZ10 mice, a cell population expressing very high levels of both Gr1 and CD11b markers was found (Figure 3-14/A). This cell population represented 3 to 8 % of the total splenic population in TAZ10 mice and was heterogeneous as the CD31 marker is not equally expressed (Fig 3-14/A). The heterogeneity of these cells was not only restricted to the markers expressed but also to their morphology. Indeed, MDSCs present with various sizes (FSC) and granularity (SSC) (Fig 3-14/B).

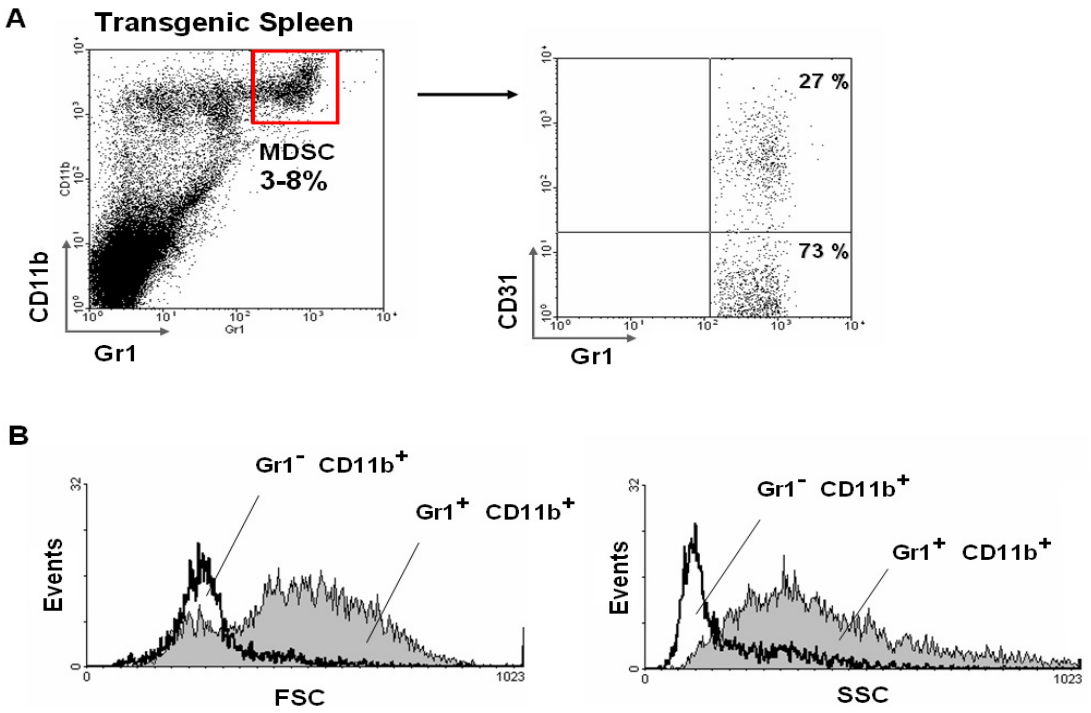


Figure 3-14: MDSCs are present in TAZ10 mice and express high levels of Gr1 and CD11b markers **(A)** Splenocytes from TAZ10 transgenic mice were analyzed by three-color FACS analysis for the expression of Gr1 and CD11b markers. The gated $Gr1^+$ $CD11b^+$ population was further analyzed for the expression of the CD31 marker. **(B)** Analysis of the size (FSC) and granularity (SSC) of $Gr1^+$ $CD11b^+$ cells. Data is representative of more than five different experiments.

Along with Ly6-C, CD31 has been described as a differentiation marker for the myeloid lineage and therefore as being a marker characteristic of mature granulo-monocyte precursors (Bailey et al. 2006; Zhu et al. 2007). Interestingly I detected a different expression of CD31 as 27% of MDSCs expressed this marker (Figure 3-14/A). This result confirms the notion that MDSCs are composed of precursor cells at different stages of differentiation toward granulocytes, macrophages or DCs. From this result, I was confident I could use the Gr1 and CD11b markers to describe the population of MDSCs in TAZ10 mice.

I then assessed whether TAZ10 mice developing AID were deficient in MDSCs compared to WT mice, which could explain the development of the disease. Using Gr1 and CD11b markers, I investigated the distribution of MDSCs in WT and TAZ10 transgenic mice. At the age of 4 months, TAZ10 mice spontaneously develop histological, hormonal and clinical changes comparable with human Hashimoto's autoimmune thyroiditis (Quaratino et al. 2004). From 1 to 4 months of age, the destruction of the thyroid by self-reactive T cells is such that inflammation would be at its maximum and would therefore be beneficial for the function and accumulation of MDSCs. I assessed the proportion of these cells in different

tissues by flow cytometry both in WT and TAZ10 mice (1 month of age) affected by autoimmune thyroiditis.

As shown Figure 3-15/A, MDSCs preferentially accumulated in the spleen of diseased mice. The number of MDSCs within the spleen showed a 3 fold increase. They also accumulated in the lymph-nodes (LN) draining the thyroid affected by the disease of TAZ10 mice whereas negligible amounts (0.18%) of MDSCs were detected in the LN of WT mice. Analysis of these cells in other tissues (Figure 3-15/B) confirmed this trend as they also accumulated in LNs not draining the thyroid.

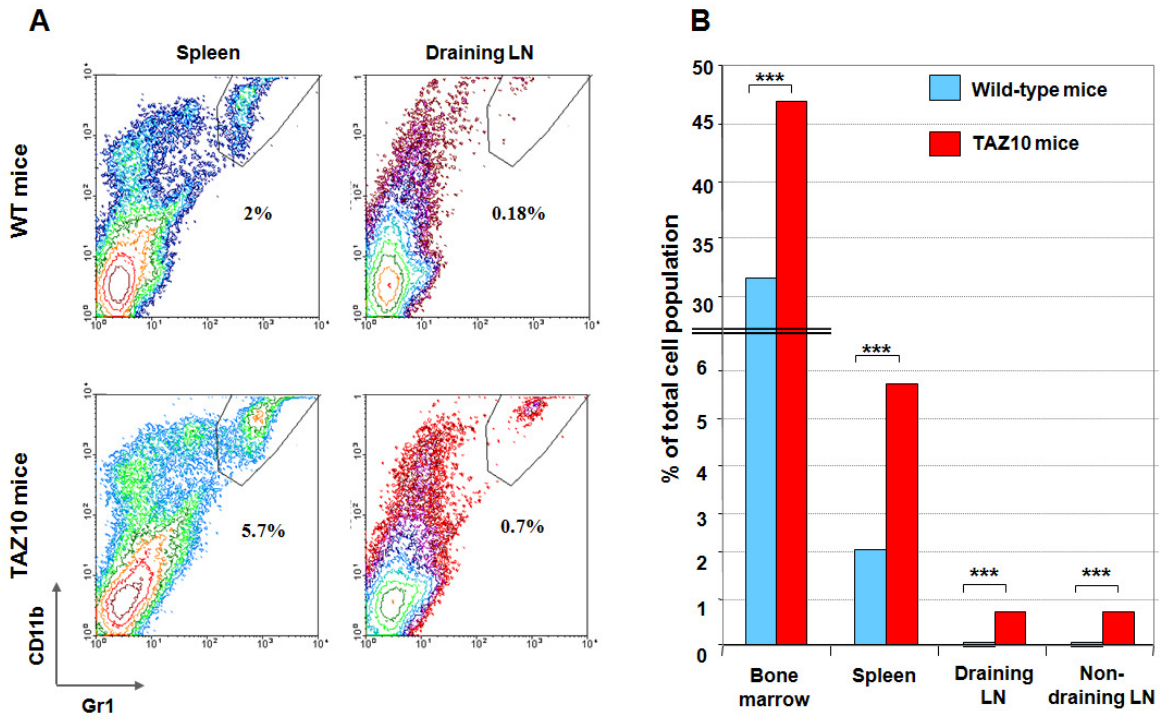


Figure 3-15: Myeloid Derived Suppressor Cells accumulate in different lymphoid and non-lymphoid organs in TAZ10 transgenic mice
(A) Two-color flow cytometry analysis was performed to assess the percentage of cells expressing Gr1 and CD11b markers in the spleen and draining lymph-node of both wild-type and TAZ10 mice. Result is representative of more than 20 mice. **(B)** Percentage of $Gr1^+$ $CD11b^+$ myeloid suppressor in different organs from both wild-type and TAZ10 mice. Data is representative of more than 20 mice.

MDSCs are generated from common myeloid progenitor within the bone-marrow and normally account for about 30% of the bone-marrow cell population (Bronte et al. 2000). In the TAZ10 model, MDSCs accounted for nearly half of the total cell population in the bone-marrow highlighting an important change in haematopoiesis in these mice (Figure 3-15/B).

This finding is in contradiction with the study of another subset of regulatory cells in TAZ10 mice. In the last decade, the classic $CD4^+$ $CD25^{high}$ $CD62L^{high}$ $Foxp3^+$ regulatory T cells (Tregs) have been extensively studied both in autoimmune diseases and in cancer. Tregs have been shown to play an important role in the prevention of autoimmune diseases and in the suppression of anti-tumor responses (Tang et al. 2004). It appeared that there was an important reduction of the number of Tregs in the spleen of TAZ10 (2.5%) compared to WT (8%) mice (Badami et al. 2005 and Badami E, Cexus O, Londei M and Quaratino S. Manuscript in preparation). Interestingly, the proportion of regulatory T cells in TAZ10 mice decreased with time and 6 month-old mice (1.3%) had a reduced amount of Tregs compared to younger mice.

3-3-2/ Distribution of $Gr1^+$ $CD11b^+$ Myeloid Derived Suppressor Cells in the spleen during the course of autoimmunity

In the light of these findings on Tregs, I wanted to evaluate any link between the numbers of MDSCs and the evolution of the disease in TAZ10 mice. We therefore assessed the proportion of these cells in the spleen of diseased and WT mice over time.

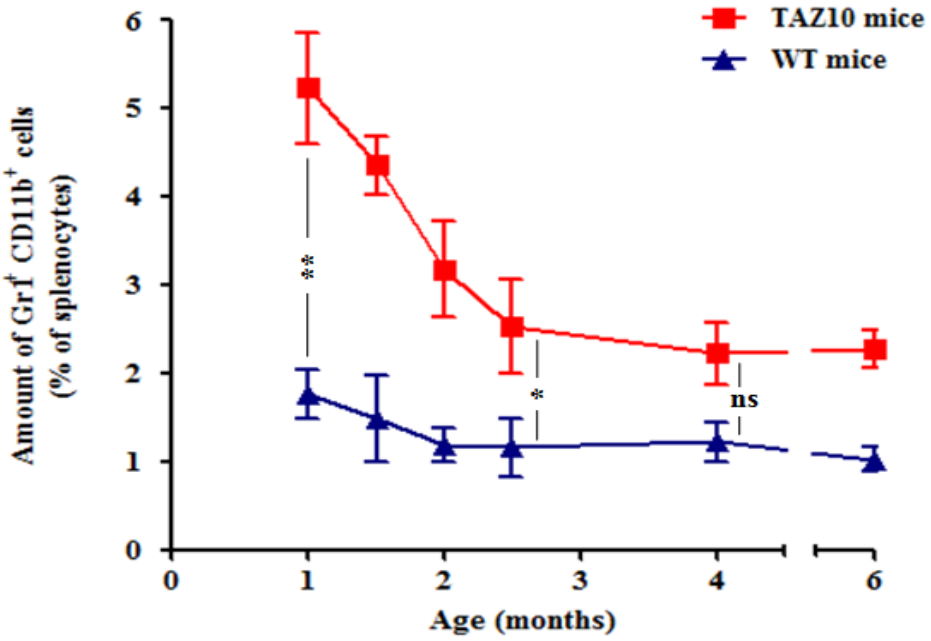


Figure 3-16: In TAZ10 mice, the percentage of MDSCs is higher than WT mice and decrease over time
FACS analysis was performed on splenocytes using Gr1 and CD11b markers in WT and TAZ10 mice for up to 6 months of age. Pooled results of more than 20 mice.

Although $Gr1^+$ $CD11b^+$ MDSCs are present in the spleen of WT mice, their distribution suggests that they accumulate in the spleen of TAZ10 mice during the acute phase of inflammation (average of 5% at 1 month of age versus 2% in WT) while they decrease as the inflammatory process fades, to remain just above the numbers present in WT mice (2.5% from 2 ½ month to 6 months of age) (Figure 3-16).

Mice below the age of 1 month could not be used to quantify the numbers of MDSCs as they were still not weaned. This would raise a question in relation to the amount of MDSCs at the onset of the disease. To address this question, current experiments aimed at performing adoptive transfer of naïve TAZ10 T cells (TAZ10-T_N) (TCR-$V\beta1^+$ $CD4^+$ $CD44^{lo}$ $CD25^-$ $CD62L^{hi}$) into Rag-2 knock-out transgenic mice ($Rag2^{-/-}$ mice) to mimic the development of the disease are underway. From the first results recently obtained, we can hypothesize that the onset of the disease in TAZ10 mice is early and occurs in the first few days of life as TAZ10-T_N proliferate in the thyroid and draining LNs of $Rag2^{-/-}$ mice within a week after the adoptive transfer occurred (Paragraph 4-3-3/).

We have observed a similar time course in another model of autoimmune disease (arthritis). In the collagen-induced arthritis model (CCIA model), the transgenic mice which express the TCR-$V\beta_{12}$ transgene, develop chronic arthritis which resembles rheumatoid arthritis (RA) after immunization with collagen-type II and Complete Freund's Adjuvant (CFA) (Mauri et al. 1997). Immunized mice were culled 40 days after immunization with a mean time of onset of disease of 20 days post-immunization. MDSCs peaked at the acute phase of inflammation whilst they decreased to the level present in WT mice as the disease became chronic (Tan LA, Cexus O et al. Manuscript in preparation). This result, in keeping with the present results, suggests a correlation between the numbers of MDSCs and the stage of the disease.

In TAZ10 transgenic mice the inflammatory process leads to a progressive destruction of the thyroid (Quaratino et al. 2004). At the time of onset and during the early stages of thyroiditis, $Gr1^+$ $CD11b^+$ MDSCs accumulate in the lymphoid organs of TAZ10 mice.

These results confirmed the presence of MDSCs in a mouse model of autoimmune disease. It is now known that the accumulation of MDSCs is detrimental both in mouse and human cancers as they promote a strong inhibition of anti-tumor responses (Almand et al. 2001; Bunt et al. 2006). However, the accumulation of MDSCs in TAZ10 mice was not able to prevent the initiation and development of the destructive thyroid disease by inhibiting the

activity of self-reactive T cells. Therefore, I wondered whether the increased numbers of MDSCs in TAZ10 mice have the same regulatory functions on T cells as it has been described in cancer.

3-3-3/ Myeloid Derived Suppressor Cells display a strong inhibitory function on T cells *in-vitro*.

The functional activity of MDSCs was investigated by measuring their effect on the proliferative activity of TAZ10 T cells. Splenocytes were depleted of MDSCs with anti-mouse Gr1 microbeads (BD Biosciences, UK). Purity was assessed by flow cytometry using anti-mouse Gr1-FITC and anti-mouse CD11b-PE. Splenocytes were then stimulated with either αCD3 or αCD3/CD28 agonistic antibodies and their proliferation measured by quantification of (^{3}H) thymidine uptake.

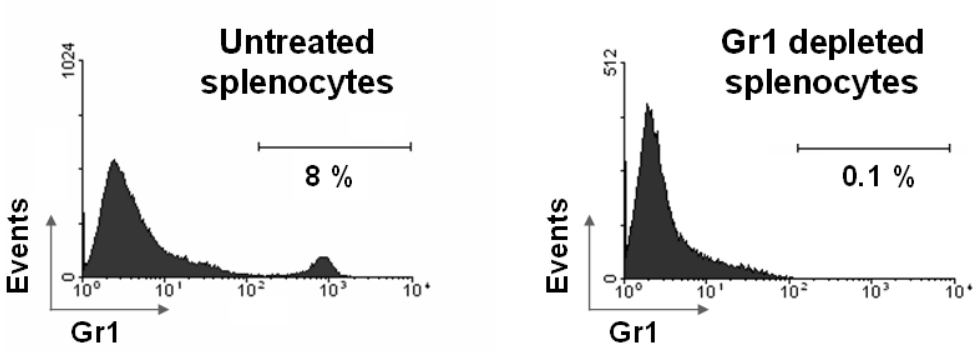


Figure 3-17: Depletion of Gr1 positive cells remove the population of myeloid derived suppressor cells
Spleens from wild-type or TAZ10 mice were stained with a FITC labeled anti-Gr1 antibody and Gr1-microbeads for further depletion. Efficiency of depletion was assessed by flow cytometry. Data is representative of more than 20 different experiments.

The efficiency of the in-vitro depletion of Gr1 positive cells was assessed by flow cytometry after depletion using an antibody targeting the Gr1 marker (Clone RB8-6C5) (Figure 3-17). As previously reported and observed in WT mice (Figure 3-12/B), T cell proliferation in response to the agonistic αCD3 antibody was drastically enhanced after depletion of $Gr1^+$ cells. Interestingly, the strong stimulation provided by the αCD28 antibody was not able to abrogate the strong inhibition on T cells displayed by MDSCs, suggesting a suppressor role for these MDSCs (Figure 3-18/A).

$CD4^+$ $CD25^+$ $Foxp3^+$ Tregs have been shown to dampen the immune response by inhibiting the proliferation of effector T cells. Upon depletion of Tregs I could observe a

threefold increase in T cell proliferation in response to αCD3 stimulation. However, Tregs were not able to abrogate the strong stimulation provided by both αCD3 and αCD28 antibodies as previously reported (Thornton et al. 1998). This suggested that the suppressive functions mediated by Tregs from TAZ10 mice can be broken by a strong stimulation provided by αCD3/CD28 (Figure 3-18/B) (Work performed by E. Badami).

In order to assess the ability of MDSCs to suppress T cell suppression, I performed the same assays as performed with Tregs. Removal of MDSCs led to an increased T cell proliferation in response to αCD3 stimulation. However, the suppressive function of MDSCs was bypassed by the addition of αCD28. As both Tregs and MDSCs from TAZ10 mice inhibited T cell proliferation in response to αCD3, only MDSCs abrogated the strong stimulation provided by αCD3 and αCD28 antibodies. This suggested that MDSCs had an important suppressive function on T cells (Figure 3-18/A).

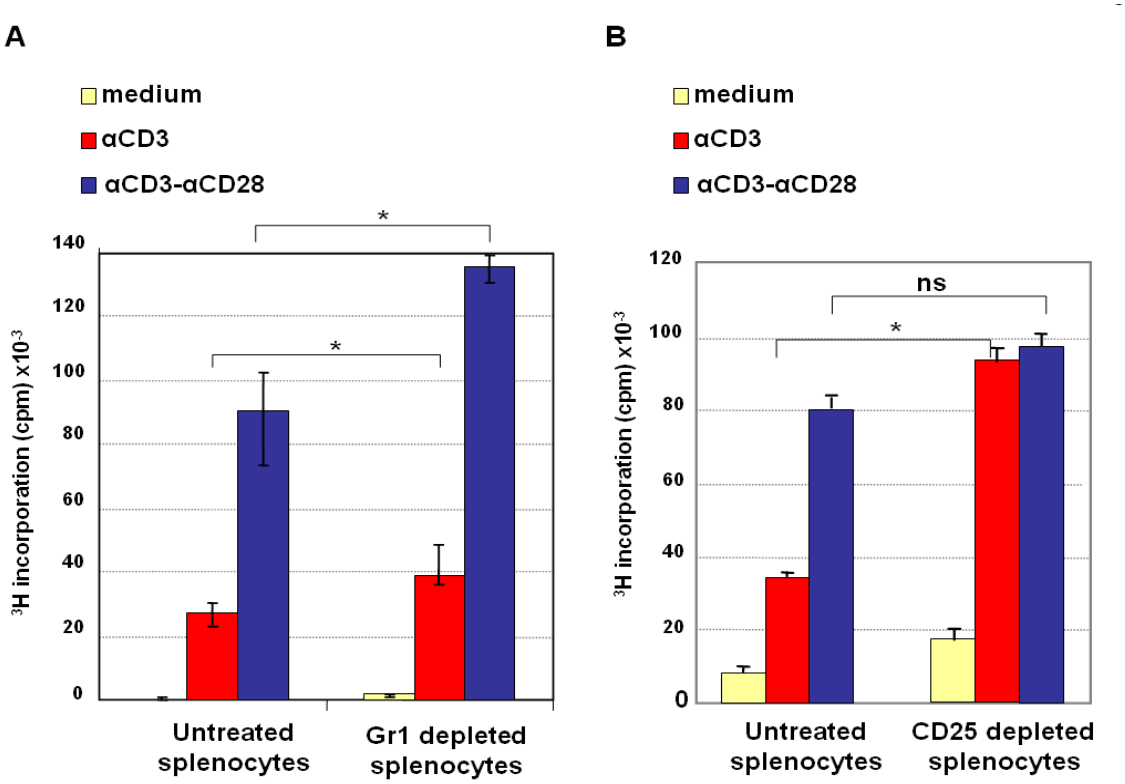


Figure 3-18: MDSCs display a stronger inhibitory function on T cell proliferation in response to both αCD3 and αCD28 antibodies than CD4$^+$ CD25$^+$ regulatory T cells
Proliferative activity of TAZ10 T cells with or without depletion of MDSCs **(A)** or regulatory T cells **(B)** was assessed by (^{3}H) thymidine incorporation assay. Untreated or depleted splenocytes were stimulated with αCD3 or αCD3-αCD28 antibodies. Data on the effect of the depletion of CD25 cells were generated by Ester Badami **(B)**. Data are representative of five different experiments.

To further investigate the inhibitory functions of MDSCs in autoimmune disease I evaluated their ability to inhibit T cell proliferation in response to αCD3 and αCD3 and αCD28 antibodies. Addition of MDSCs to splenocytes from TAZ10 mice strongly inhibited T cell proliferation to αCD3 antibody. As previously observed, the addition of αCD28 was unable to reverse this inhibitory effect (Figure 3-19/A). The action of MDSCs was also dose-dependent. While the addition of MDSCs to T cells, at a MDSC:T ratio of 1:2 or 1:5, inhibited T cell proliferation in response to αCD3. Only MDSC present at a MDSC:T ratio of 1:2 were able to abrogate the stimulation provided by αCD3 and αCD28 (Figure 3-19/B).

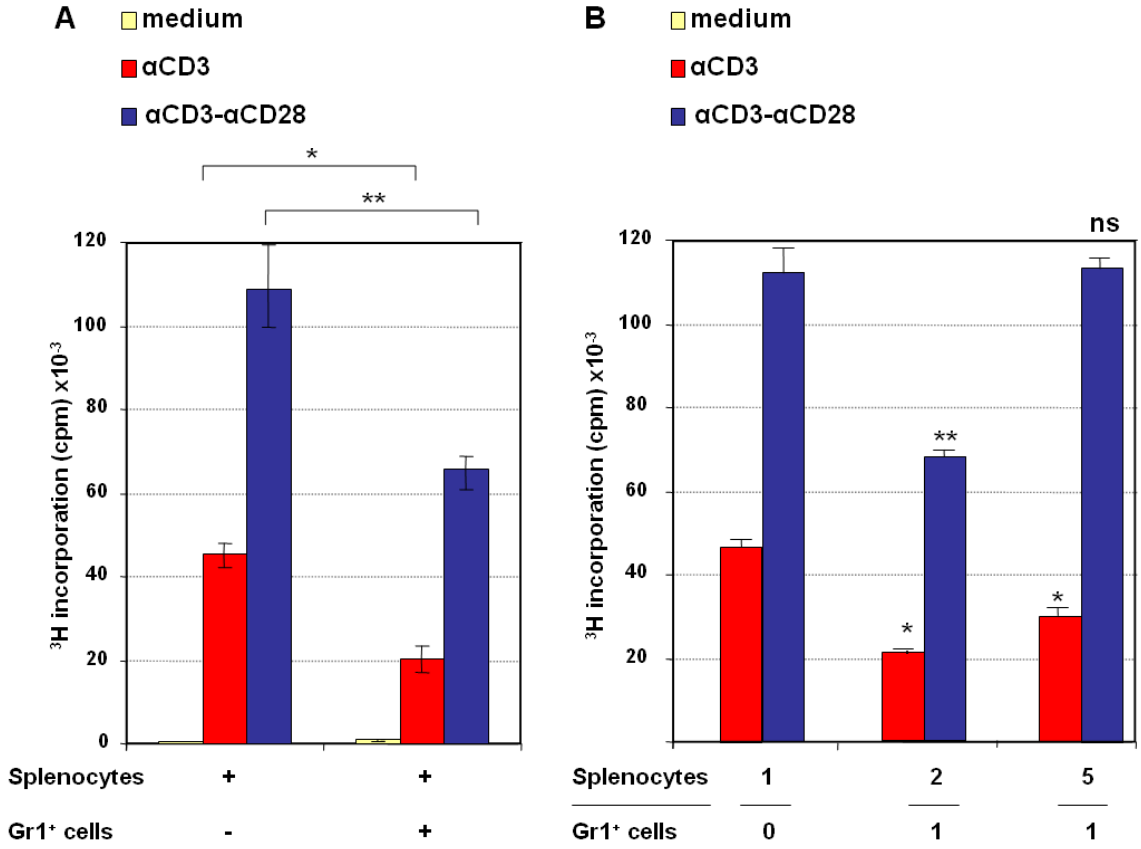


Figure 3-19: Addition of Gr1$^+$ MDSCs strongly inhibits T cell proliferation in a dose-dependent manner
(A) Gr1$^+$ myeloid derived suppressor cells were isolated and added to splenocytes from transgenic mice activated with either αCD3 or αCD3-αCD28 antibodies. Proliferation was assessed by (^{3}H) thymidine incorporation. **(B)** Gr1$^+$ cells were added to activated to T cells either at a T:MDSC ratio of 2:1 or 5:1. Data are representative of three different experiments.

MDSCs strongly inhibited T cell proliferation in response to αCD3 and αCD3-αCD28 antibodies in a dose-dependent manner. I then addressed whether their inhibitory activity could be influenced by the ongoing inflammation of the TAZ10 mice. To answer this question, I used the ability of mature bone-marrow derived DCs to provide strong

costimulatory signals to T cells when cultured in the presence of agonistic αCD3 antibody (Figure 3-10).

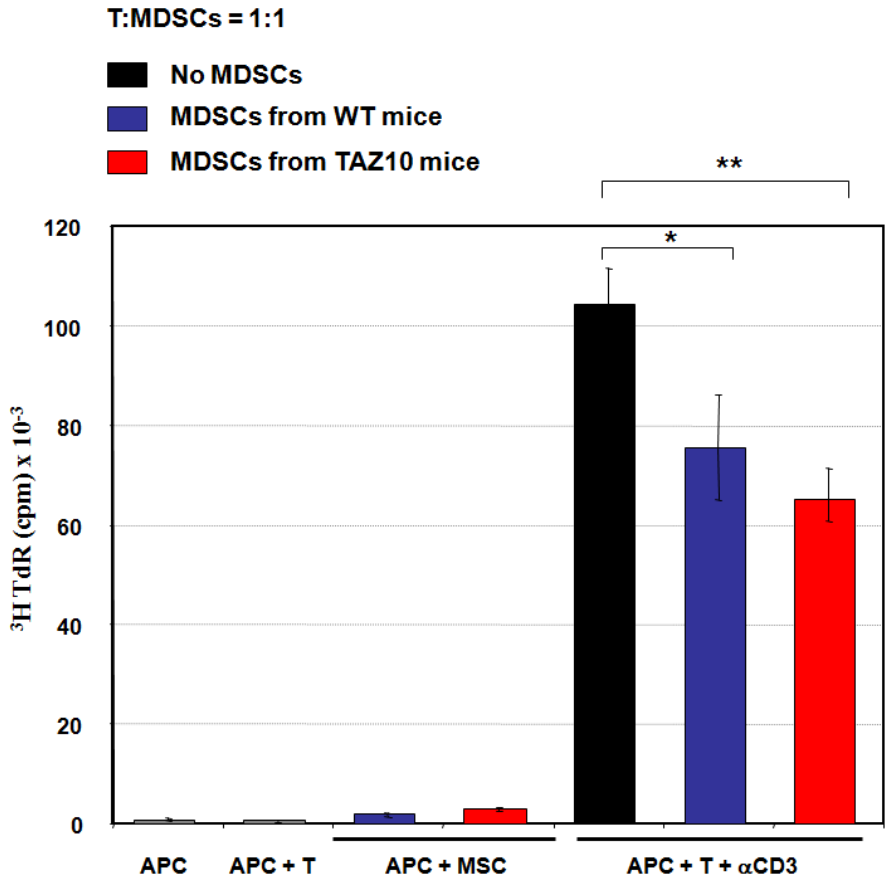


Figure 3-20: MDSCs from TAZ10 mice are more inhibitory than WT MDSCs
The activity of MDSCs from wild-type and TAZ10 mice were assessed for their ability to inhibit a strong T cell proliferation induced by the agonistic αCD3 antibody and costimulatory signals provided by mature dendritic cells (DCs). Data are representative of three different experiments.

At a T:MDSC ratio of 1 : 1, MDSCs from both WT and TAZ10 mice strongly inhibited T cell proliferation in response to αCD3 and mature DCs (Figure 3-20) thus confirming the strong inhibitory activity of MDSCs on T cell proliferation. Interestingly with the same number of cells, MDSCs from TAZ10 mice were more potent in inhibiting T cell proliferation compared to MDSCs from wild-type mice. These results may reflect the context of inflammation where MDSCs from TAZ10 mice may present a more stimulated or matured phenotype compared to wild-type mice.

3-3-4/ Myeloid Derived Suppressor Cells produce nitric-oxide (NO)

MDSCs have been shown to exert part of their inhibitory function on T cell proliferation via the products of degradation of L-arginine by inducible nitric-oxide synthase (iNOS) and Arginase I (Kusmartsev et al. 2000, Bronte et al. 2005).

To assess the mechanisms of action of MDSCs, I measured the activity of iNOS by considering the production of nitric-oxide (NO). The amount of NO in splenocytes freshly isolated from both WT and TAZ10 mice was measured by flow cytometry. MDSCs from both sets of mice produced NO. In keeping with the previous observations that MDSCs from TAZ10 mice might display a stronger inhibitory activity (Figure 3-20), the highest amounts of NO were produced by the large $Gr1^+$ $CD11b^+$ MDSCs population from transgenic mice (Figure 3-21/A). To further evaluate their ability to secrete NO, the amount of NO released in the medium with or without MDSCs was measured using the Griess method (Figure 3-21/B). In accordance with Figure 3-21/A, the amount of nitric oxide released from TAZ10 splenocytes was much higher than the amount produced by splenocytes from WT mice. In both cases, MDSCs were responsible for this secretion as their depletion abrogated the release of NO in both wild-type and TAZ10 splenocytes upon stimulation with αCD3 and αCD28 (Figure 3-21/B).

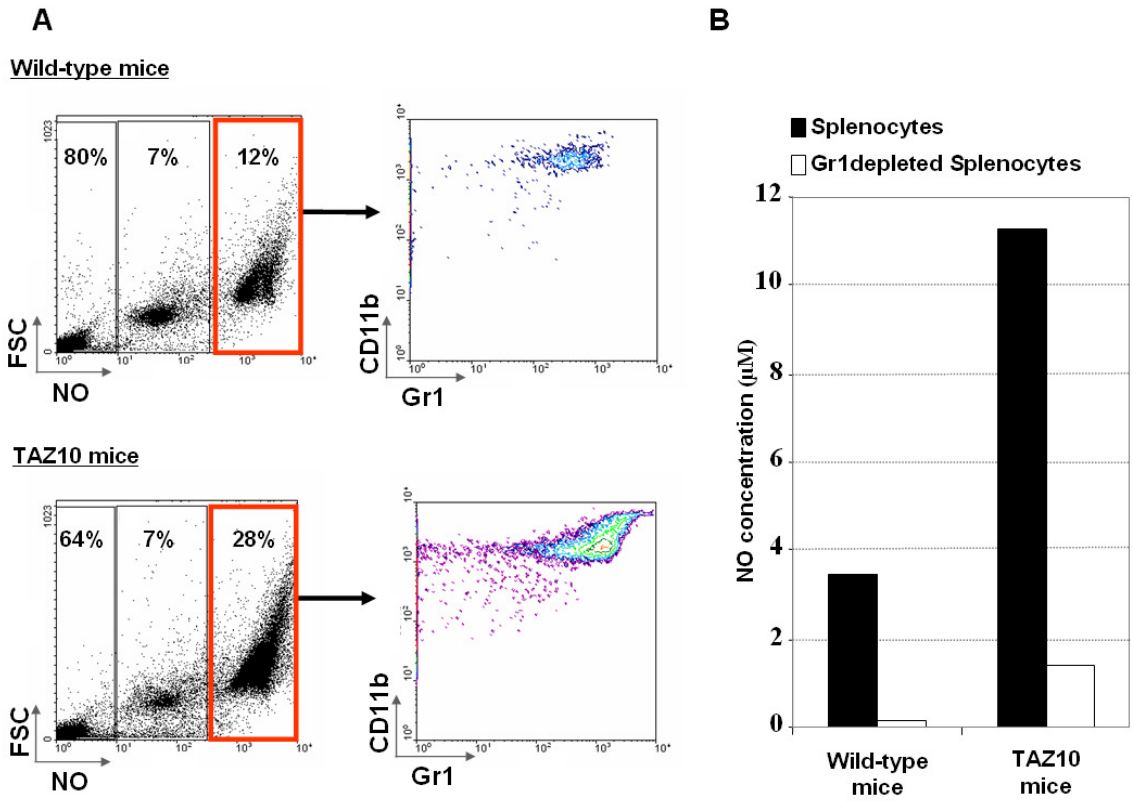


Figure 3-21: MDSCs from TAZ10 mice produce more nitric-oxide than MDSCs from WT mice
(A) Three-color FACS analysis was performed to assess the intracellular content of NO in splenocytes from both wild-type and transgenic splenocytes. Gated populations were analyzed for their level of expression of Gr1 and CD11b markers. **(B)** The amount of NO released into the medium by activated splenocytes (using αCD3 and αCD28) from WT and TAZ10 mice was measured using the Griess method. Effect of the depletion of $Gr1^+$ MDSCs on the amount of NO released was assessed. Data is representative of more than five experiments.

I have therefore shown that MDSCs are present in autoimmune disease and strongly inhibit T cell proliferation in response to αCD3 and αCD3/CD28 agonistic antibodies. Although more work need to be performed, we could suggest that this action is partially mediated via the release of nitric oxide. Results in figure 3-20 suggest that the inflammatory status of TAZ10 mice was able to promote the activity of TAZ10 MDSCs probably through the cytokine environment. This strong suppressive function on T cells along with their accumulation in TAZ10 mice (Figure 3-15) did not explain why MDSCs could not prevent the activation of self-reactive T cells as TAZ10 mice still developed the disease.

3-3-5/ MDSCs from TAZ10 mice inhibit antigen-specific T cells *in-vitro*

Stimulation of T cells using αCD3 and αCD28 does not represent the situation *in-vivo* where TAZ10 T cells are activated upon recognition of the $TPO_{536-547}$ cryptic epitope when presented in the context of MHC Class-II molecules (Quaratino et al. 2004).

The activity of MDSCs has been linked to the level of cytokines present in the microenvironment. For instance Th1 cytokines such as IFNγ will promote the transcription of iNOS and the subsequent production of NO whereas Th2 like-cytokines (such as IL-4 or IL-13) will induce the activation of ARG1 and the generation of urea amd polyamines (Reviewed in Bronte et al. 2005). We therefore hypothesized that the strong inhibition previously observed was due to the stimulation/maturation of MDSCs by the important release of cytokines resulting from the activation and proliferation of T cells induced by αCD3 and αCD28.

I first evaluated the ability of MDSCs to inhibit the proliferation of TAZ10 T cells in response to the cognate $TPO_{536-547}$ peptide (Figure 3-22).

Although T cells did proliferate when the peptide was presented by antigen-presenting cells, the addition of MDSCs at a T: MDSC ratio of 5:1 did not inhibit T cell proliferation. On the other hand, addition of MDSCs at a higher T: MDSC ratio of 1:1 led to a strong inhibition of T cell proliferation down to background levels. At this stage I had to ascertain that MDSCs were not capable of presenting the peptide to TAZ10 T cells and promoting their proliferation. I could rule out that MDSCs might induce the proliferation of Ag specific TAZ10 T cells (Figure 3-22). However, this result could not exclude the possibility that MDSCs were not expressing MHC Class-II molecules nor costimulatory molecules required for the full induction of T cell proliferation as shown for DCs (Chapter 3-2). This lack of inhibition at a T:MDSC ratio of 5:1 was then investigated as I wondered whether MDSCs were unable to inhibit antigen specific T cell proliferation or if they were not efficiently potentiated by the cytokine “milieu” as suggested from the results obtained when stimulating T cells with αCD3 and αCD28.

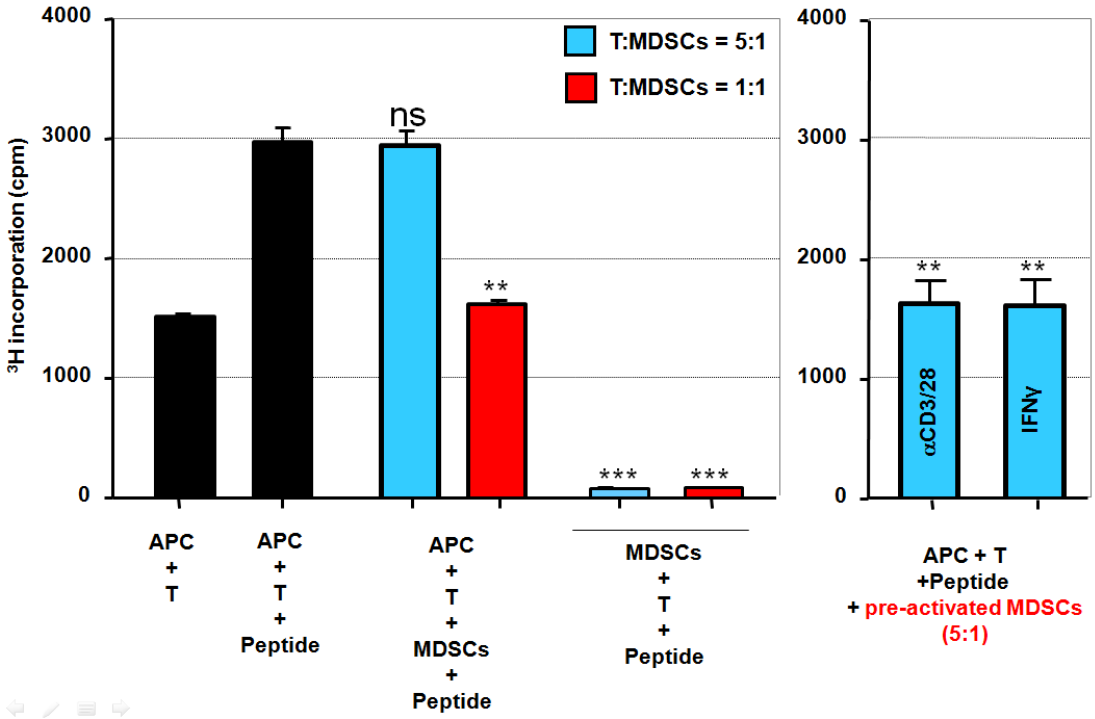


Figure 3-22: Effect of MDSCs on the proliferation of T cells in response to the $TPO_{536-547}$ cognate peptide
MDSCs were added to the culture of APCs and T cells at different T:MDSC ratio (5:1 and 1:1) to assess their inhibitory capacity. Effect of the pre-activation of MDSCs using αCD3/28 or IFNγ was evaluated (pre-activated MDSCs). Proliferation of T cells was measured by (^{3}H) thymidine incorporation. Data are representative of two different experiments.

To mimic the same stimulatory environment, MDSCs were either purified from splenocytes activated for three days with αCD3/28 or pre-activated for 3 days with IFNγ (200U/mL). After purification, pre-activated MDSCs were then added to the assay at a T: MDSC ratio of 5:1. Addition of pre-activated MDSCs strongly inhibited T cell proliferation to background. Therefore, the inability of MDSCs to inhibit T cell proliferation at a T: MDSCs ratio of 5:1 was more due to a lack of activation by cytokines (IFNγ or cytokines secreted by splenocytes upon stimulation with αCD3 and αCD28 antibodies) rather than an intrinsic inability to inhibit antigen specific T cell proliferation (Figure 4-10).

3-3-6/ Rush judgment on MDSCs: Highly inhibitory MDSCs cannot be used *in-vivo* for the treatment of autoimmune disease

The ability of MDSCs from TAZ10 mice to strongly inhibit T cell proliferation in response to αCD3 associated or not with αCD28 or costimulatory molecules, displayed by mature DCs suggested that they could be ideal candidates as a therapeutic tool to dampen the activation of T cells in autoimmune disease. As a matter of fact, numerous studies have taken advantage of the inhibitory activity of MDSCs on both NK and T cells (Li et al. 2009). Indeed, *in-vivo* depletion of MDSCs using the anti-Gr1 antibody clone RB6-8C5 abrogated the development of tumor cells in tumor bearing-mice. Similarly, it has been shown that the adoptive transfer of purified $Gr1^+$ $CD11b^+$ MDSCs promotes tumor-growth via an increased inhibition of NK cell activities (Li et al. 2009).

To assess whether MDSCs could dampen the onset and development of the disease when injected into TAZ10 mice, $Gr1^+$ MDSCs were purified using magnetic beads and adoptively transferred into TAZ10 transgenic mice. Surprisingly, despite their inhibitory function displayed *in-vitro*, the adoptive transfer of MDSCs worsened the disease as all mice died within 50days after the injections (Figure 3-23; Experiments performed by Delphine Labrousse).

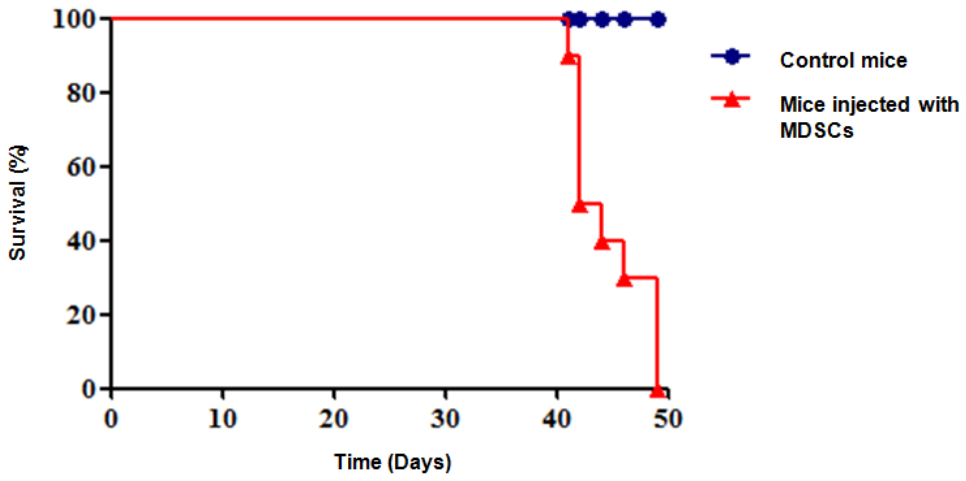


Figure 3-23: Adoptive transfer of MDSCs fails to prevent the development of the disease in TAZ10 mice
TAZ10 mice were adoptively transferred with purified $GR1^+$ MDSCs and their survival assessed over a period of 50 days. Data are representative of two independent experiments involving 3 control mice and 6 mice injected with $Gr1^+$ cells. (Experiments performed and kindly donated by Delphine Labrousse).

This result prompted us to consider the fact that MDSCs are myeloid precursors and have therefore the ability to differentiate to DCs, macrophages or granulocytes according to the

microenvironment. I consequently analyzed MDSCs *ex-vivo* for the expression of various markers of antigen presenting cells (APCs) such as CD11c, F4/80, CD86 and I-A^b (Figure 3-24) to assess whether MDSCs from TAZ10 and WT mice display a different phenotype.

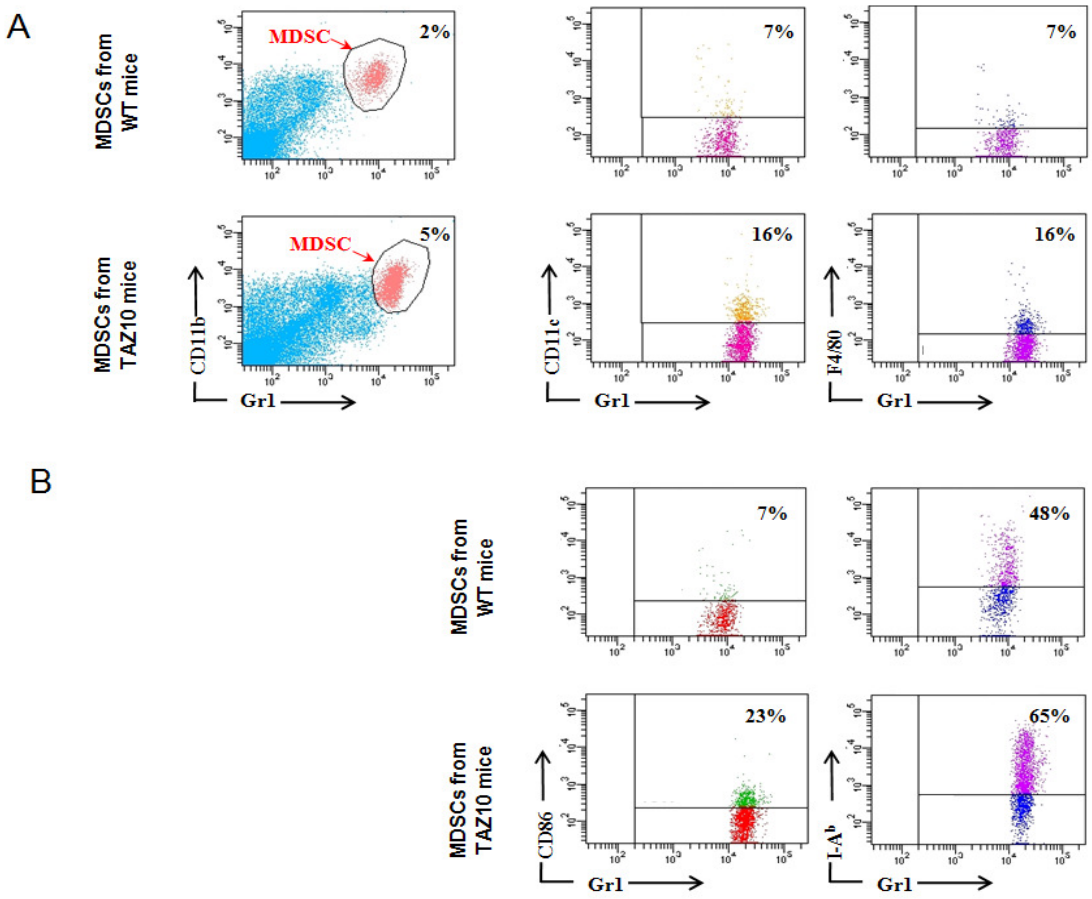


Figure 3-24: MDSCs from TAZ10 mice express markers of professional antigen presenting cells (APCs) MDSCs from the splenocytes of TAZ10 mice were assessed for the expression of markers specific of APCs such as CD11c and F4/80 **(A).** The expression of maturation markers such as CD86 and MHC-II molecules I-A^b was also evaluated by flow cytometry **(B)**. Data are representative of two independent experiments. (Experiments performed and kindly donated by Delphine Labrousse).

Interestingly, MDSCs from TAZ10 transgenic mice showed an increased proportion of cells expressing F4/80 and CD11c of over twofold compared to WT mice (Figure 3-24/A). This was associated with a higher proportion of cells expressing maturation markers such as CD86 (over threefold increase) or I-A^b molecules (about 1.5 fold increase). Together with previous results, we could hypothesize that the inflammatory status of TAZ10 mice modulating both MDSCs' functions (Figure 3-20) and phenotype (Figure 3-24).

3-3-7/ Conclusions

The inhibitory effect of MDSCs has been extensively described both in tumor-mouse models and human cancers (Gabrilovitch. 2004). Inspired by these studies, I assessed whether MDSCs could play a role in other chronic inflammatory conditions such as autoimmune diseases. To answer this question it was important to establish the presence of MDSCs in a spontaneous model of autoimmune disease, where no adjuvant was used. We therefore investigated TAZ10 mice, a humanized mouse model that spontaneously develops autoimmune hypothyroidism with the same clinical signs of disease as patients with Hashimoto's thyroiditis (Quaratino et al. 2004).

I detected an accumulation of MDSCs in different tissues in these mice depending on the status of inflammation. Young mice presenting acute inflammation (1 month old) showed greater amounts of MDSCs whereas the number decreased with age as the inflammatory processes faded, and became more chronic. The accumulation of MDSCs was detected both in the natural niche (the bone-marrow) but also in the spleen, non-draining LNs and LNs draining the thyroid.

As MDSCs have been characterized in cancer, where they strongly inhibit T cells, we assessed their functionality in our model. MDSCs strongly inhibited T cell proliferation in response to both αCD3 and αCD3/28 agonistic antibodies. We found that these actions were dose dependent. Of interest was the comparison with $CD4^+$ $CD25^+$ regulatory T cells whose inhibitory functions were abrogated when T cells were stimulated with αCD3/28 antibodies. Although MDSCs inhibited T cells in response to α-CD3, the addition of αCD28 could not abrogate their inhibitory function.

I have established that the activity of MDSCs depends on the microenvironment and, more precisely, on the level of cytokines as their activity is enhanced upon stimulation with IFNγ.

The factors leading to the recruitment of MDSCs to peripheral lymphoid organs upon inflammation remain largely unknown. G-CSF has been shown to promote both the recruitment and proliferation of myeloid precursors in mouse and human cancer via the down-regulation of CXCR4 on myeloid cells, thus removing a factor of retention in the bone-marrow (Kim et al. 2006; Gabrilovitch. 2004). Tumour cells secreting IL-1β, a major pro-inflammatory cytokine, promote the mobilization of MDSCs via the release of soluble factors by bystander cells (Song et al. 2005; Bunt et al. 2006; Tu et al. 2008). From the role of IL-1β

in inducing the recruitment of MDSCs in cancer, we suggest that the inflamed status of TAZ10 mice, shown by the activation of self-reactive T cells and their infiltration in the thyroid, explain why MDSCs accumulate at the peak of inflammation when the levels of pro-inflammatory cytokines are high.

The paradoxical situation whereby strongly inhibitory MDSCs accumulating in TAZ10 mice are unable to prevent the activation of TAZ10 self-reactive T cells and therefore the development of the disease, prompted us to address the full phenotype of MDSCs. MDSCs from TAZ10 mice expressed higher levels of markers specific for professional APCs such as CD11c and F4/80 but also the maturation markers CD86 and MHC-II molecule I-A^b. As MDSCs from TAZ10 mice were more inhibitory than MDSCs from WT mice, I could hypothesized that the acquisition of these markers was linked with an increased inhibitory function associated with the increased release of nitric oxide. The results obtained *in-vivo* in TAZ10 mice were in sharp contrast with the study of Proinsulin-NOD (PI-NOD) transgenic mice model of diabetes (Steptoe et al. 2005). Indeed, adoptive transfer of MDSC generated from bone-marrow cells cultured with a combination of GM-CSF and TGF-β were able to suppress autoimmune diabetes in these mice. This phenomenon was associated with the differentiation of injected MDSCs into resting DCs ($CD11c^+$ $MHC\text{-}II^{lo}$ $CD86^{lo}$). However the generation of MDSCs from bone-marrow precursors with GM-CSF and TGF-β could not reflect the real phenotype and functions of MDSCs present in the spleen or lymph-nodes of these mice with autoimmune diabetes.

The analysis of MDSCs in mice using only Gr1 and CD11b markers is therefore not appropriate as it does not reflect the phenotype of this inhibitory network. Although different factors such as G-CSF or All-Trans retinoic Acid (ATRA) have been used to study the effect of MDSCs in diseases such as cancer, by promoting the accumulation of MDSCs, the action of these drugs on other cellular subsets such as T cells is detrimental. On the other hand, the depletion of MDSCs *in-vivo* using the depleting antibody clone RB6-8C5 has its limits as it also depletes other cellular subsets such as granulocytes or neutrophils.

The recent acquisition of the B6-eYFP mouse model that expresses eYFP ubiquitously will be an important tool in the study of the kinetics of accumulation of MDSCs at the onset of chronic inflammatory diseases. Indeed, combining the generation of $Rag2^{-/-}$ bone-marrow chimeric mice reconstituted with the bone-marrow from B6-eYFP mice, with the adoptive transfer of naïve T cells in a $Rag2^{-/-}$ model represents the ideal tool to unravel the

mechanisms leading to the recruitment, accumulation and differentiation of MDSCs from the onset until the late chronic stages of the disease.

3-4/ Results Part 3: Characterisation of $CD34^+$ cells and MDSCs in human patients with Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE)

Although MDSCs have been largely described in tumor mouse models, their role in other chronic inflammatory diseases remains elusive. We previously highlighted the presence and inhibitory functions displayed by MDSCs in a mouse model of spontaneous thyroiditis. The study of MDSCs in human chronic inflammatory diseases is however incomplete with regard to cancer and absent in autoimmune diseases. This is in part due to the more complex nature of hematopoiesis in humans compare to mice. Also, no studies have attempted to completely describe the phenotype of MDSCs. Indeed, the markers used to characterize human MDSCs vary considerably between the different groups involved (I will discuss this issue Paragraph 3-4-4/).

In the light of our finding about MDSCs in the TAZ10 mouse model of autoimmune thyroiditis, we embarked on the characterization of human MDSCs. In this chapter, I will progressively dissect the phenotype and functions of MDSCs in patients with Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE) versus controls. These two different chronic inflammatory diseases were chosen as they differed in the level and anatomical localization of inflammation. Indeed, RA is essentially located in the joints and scoring of the disease using the DAS28 score accurately represents the stage of the disease (http://www.rheumatology.org/publications/classification /index.asp#ra and Appendix 1). On the other hand, patients affected by SLE present multiple sites of inflammation such as renal, neurological, hematologic or immunological disorders (http://www.rheumatology.org/publications/classification/index.asp#other and Appendix 2).

3-4-1/ Patients with Rheumatoid Arthritis and Systemic Lupus Erythematosus have a reduced population of monocytes

Before embarking on the description of human MDSCs, we first assessed the levels of monocytes in patients with RA and SLE. Monocytes were characterised using their morphological features by flow cytometry (Figure 3-25/A): the size (FSC or Forward Scatter) and the granulocity (SSC or Side Scatter) of the cells. By analysing Peripheral Blood Mononuclear Cells (PBMCs) obtained by passing the blood through a Ficoll Gradient, we observed that controls had ~5% monocytes whereas patients with RA and SLE both showed less than 2% (Figure 3-25/B).

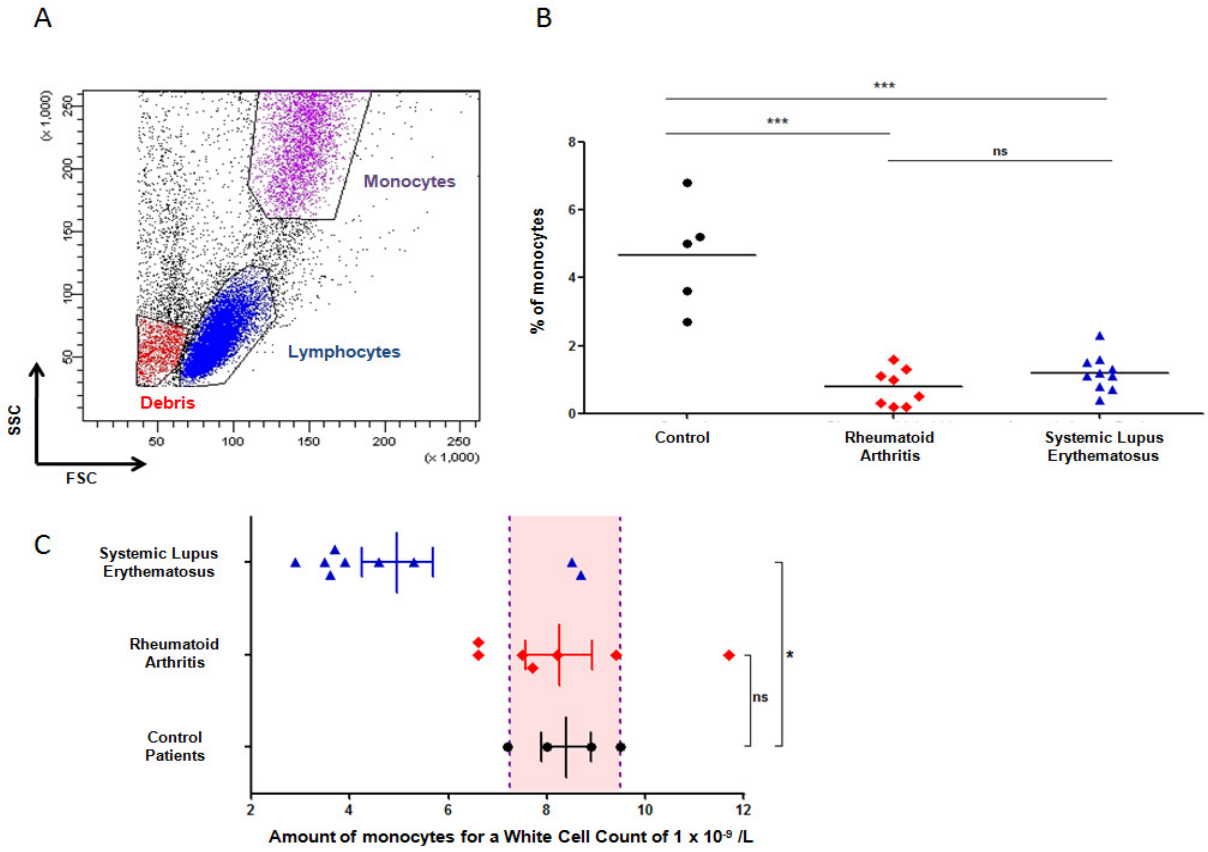


Figure 3-25: Reduction of the amount of monocytes in PBMCs from patients with Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE)
(A) Level of monocytes in PBMCs from controls and patients with RA and SLE was assessed by flow cytometry using their their size (FSC) and granulocity (SSC). **(B)** Amounts of monocytes in patients with RA (n=8), SLE (n=10) or controls (n=5) was assessed by flow cytometry as a percentage of the PBMC population. **(C)** Evaluation of the amount of monocyte for a White Cell Count of $1x10^9$/L (WCC) for each patient. The violet rectangle symbolizes the ratio for the controls. Statistics were done using the unpaired Student's t-test.

We then compared the level of monocytes in PBMCs with the White Cell Count (WCC) of controls, RA and SLE patients. The WCC includes neutrophils, eosinophils, basophils, lymphocytes and monocytes and is expressed per litre of blood. We found that although RA patients had a WCC falling within the normal range (4 to 11 $x10^9$/L), the majority of SLE patients had a low WCC (~ 5 $x10^9$/L) with 5 patients having a WCC below 4 $x10^9$/L. From this result we evaluated the ratio of the amount of monocytes present in PBMCs and the WCC to obtain the amount of monocytes for a WCC of $1x10^9$/L (Figure 3-25/C). Although the difference between the group of RA patients and controls was not significant, they ranged from 6 to 12 in RA patients compared to ~8.5 in controls. Interestingly, for the same WCC, SLE patients presented a decreased number of monocytes. Therefore, SLE and RA patients presented less monocytic cells in the PBMCs compared to controls while the amount of monocytic cells in SLE patients was severely decreased (Figure 3-25/).

I then focused on establishing a relationship between the disease activity in RA patients using the DAS28 score, and the amount of monocytes in PBMCs. The DAS28 score allows the segregation of patients into different categories as described Figure 3-26/A (Prevoo et al. 1995). This method allows the scoring of the disease activity according to various criteria: the number of swollen and tender joints for a total of 28 joints (Refer to Appendix 1); the amount of C-reactive protein; the scoring of the Overall well-being of the patient on a scale from 1 to 100 (1 being the best imaginable health state and 100 the worst imaginable health state). Below a DAS28 score of 2.6, patients are considered to be in remission. Between 2.6 and 3.2, the disease is considered to have a low activity whereas patients having a DAS28 score between 3.2 and 5.1 have a moderate disease activity. Finally, a patient presenting a DAS28 score over 5.1 present a highly chronic disease activity. This DAS28 score is essential for the establishment of therapeutic treatments. Indeed, between 2.6 and 5.1, patients can have up to 3 rounds of treatment with non-specific anti-inflammatory drugs such as methotrexate or mycophenolate. Patients presenting a DAS28 score over 5.1 and fail to respond to all the previous anti-inflammatory treatments are put on anti-TNFα therapy (Adalimumab, Etanercept or Infliximab). Failure to respond to these three separate treatments means that the patient will be put on the waiting list for treatment with Rituximab which is a monoclonal antibody targeting CD20 and that leads to the depletion of B cells (Reviewed in Smolen et al. 2007).

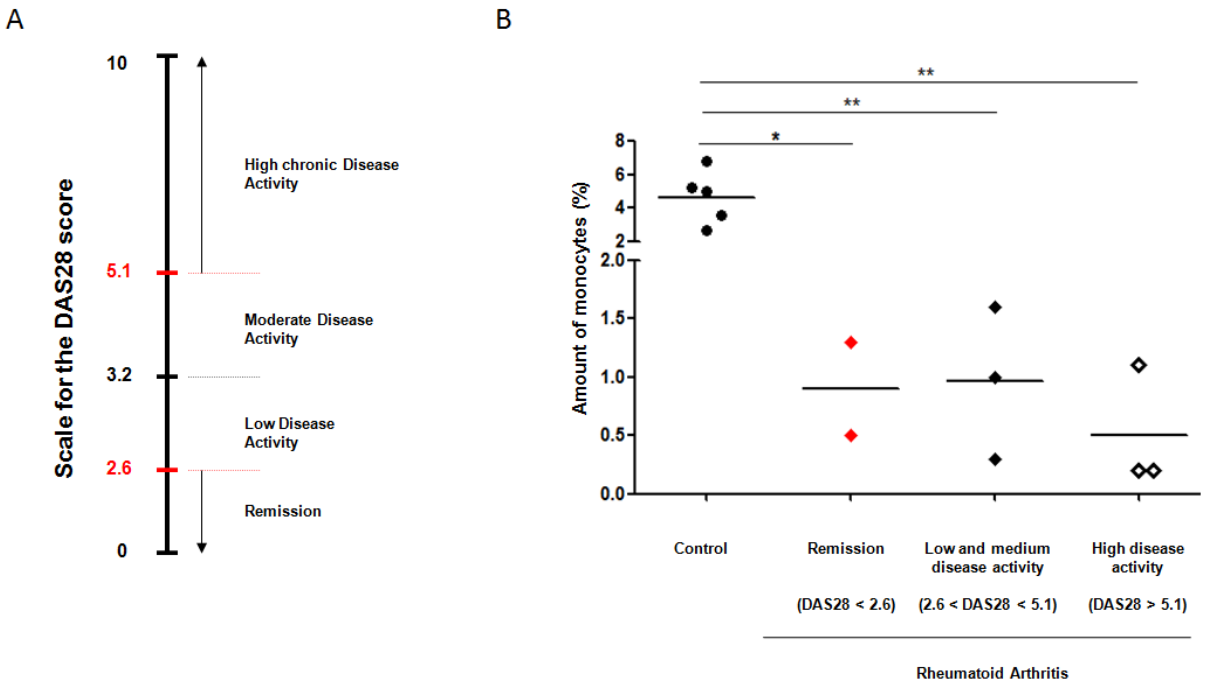


Figure 3-26: Evolution of the amount of monocytes in patients with rheumatoid arthritis with the severity of the disease (DAS28)
Description of the DAS28 scoring system in rheumatoid arthritis **(A)**. The score reflects the disease activity on a scale from 0 to 10. RA patients with a DAS28 score below 2.6 were in remission whereas DAS28 score above 5.1 presented a high disease activity. **(B)** The relation between the DAS28 score and the amount of monocytes in PBMCs was assessed for patients with rheumatoid arthritis. Statistical analysis performed using the Unpaired Student's t-test.

As described in figure 3-26/B, there seemed to be no correlation between the severity of the disease and the amount of monocytes present in patients with RA. As for patients with SLE, no relation could be established between the severity of the disease and the amount of monocytes.

3-4-2/ $CD34^+$ cells are present in patients with SLE and RA

MDSCs have been largely described in cancer where they strongly inhibit anti-tumor responses (Bronte et al. 1998 and 2000; Almand et al. 2000). Numerous studies in patients with head and neck cancer have highlighted an increased level of peripheral $CD34^+$ cells with inhibitory activity on T cell proliferation (Almand et al, 2000) where the CD34 marker is specific for hematopoietic progenitors.

Before embarking on the study of MDSCs, I first analysed whether $CD34^+$ cells were present in patients with RA and SLE and if they displayed the same inhibitory function on T cell proliferation in response to αCD3 as has been described in cancer. I analysed 8 patients

with RA, 9 with SLE and 6 controls. The majority of the SLE patients studied at the time were receiving anti-inflammatory drugs (such as methotrexate) except one patient not receiving any specific treatment. Among RA patients, 3 were recently diagnosed and were not receiving any treatments when the blood was taken, while one patient was on the anti-TNF therapy Etanercept. These specific cases will be further mentioned where appropriate.

I analysed the peripheral distribution of $CD34^+$ cells in patients and controls by flow cytometry (Figure 3-27/A). The proportion of $CD34^+$ cells in controls ranged from 0.1 to 0.2% of the total cell population. Although some patients with RA and SLE presented an increased amount $CD34^+$ cells in PBMCs (0.4 and 0.5% in RA and 0.5% in SLE), differences were overall non significant.

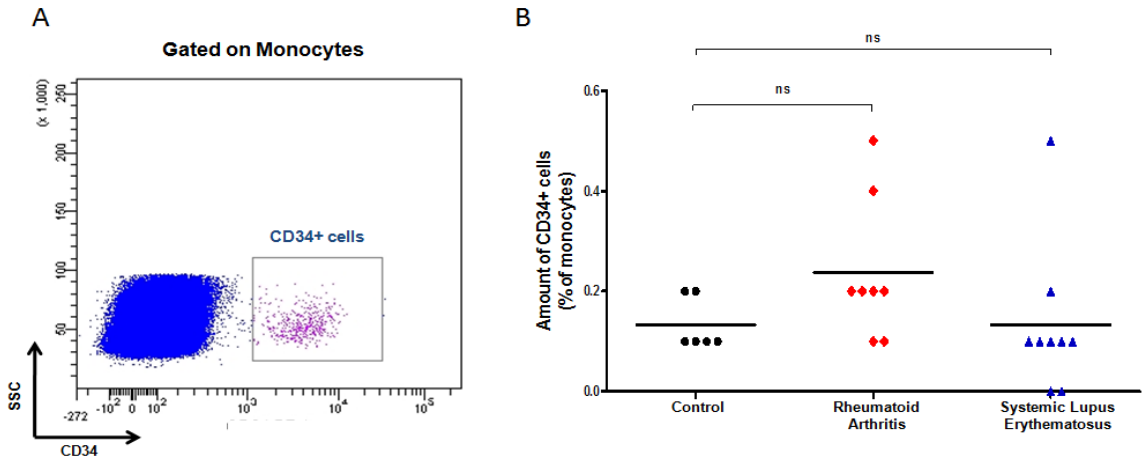


Figure 3-27: Purification and quantification of $CD34^+$ cells in PBMCs in PBMCs from controls and patients with RA and SLE
(A) FACS analysis was performed to assess the expression of the CD34 marker in PBMCs. **(B)** Amounts of monocytes as a % of monocytic cells in RA and SLE patients compared to controls. Statistical analysis performed using the Unpaired Student's t-test.

I then further explored the distribution of $CD34^+$ cells in RA and SLE patients to assess whether there was a correlation between the amount of $CD34^+$ cells and the stage of the disease (Figure 3-28). I showed that RA patients in remission (DAS28 less than 2.6) and with low disease activity (DAS28 score between 2.6 and 3.2) presented a similar proportion of $CD34^+$ cells as controls. RA patients with moderate to high disease activity (DAS28>3.2) presented an increased amount of $CD34^+$ cells. Although we had only one patient on Etanercept, they presented with a significant reduction in the amount of $CD34^+$ cells down to the levels of controls. This specific patient had a high DAS28 score of 6.91 (high disease activity), did not show any response to the ongoing treatment and failed to respond

previously to the two other anti-TNF therapy treatments available (Adalimumab and Infliximab).

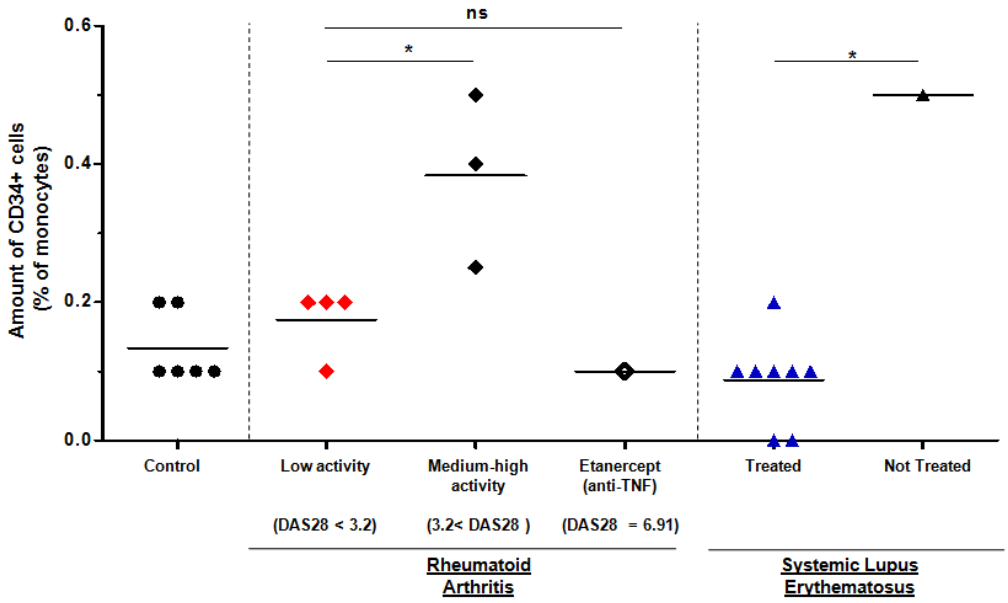


Figure 3-28: Effect of the disease activity on the amount of CD34$^+$ cells
Analysis of the amount of CD34$^+$ cells in patients with rheumatoid arthritis and systemic lupus erythematosus by flow cytometry. Amounts varied in relation to the disease activity and the treatmen received. Statistical analysis performed using the Unpaired Student's t-test.

SLE patients had from 0.1 to 0.2% of CD34$^+$ cells in the monocyte population with two patients having no CD34$^+$ cells. Interestingly, one SLE patient who was diagnosed in 2005, but was not under any treatment at the time of the study, had a high amount of CD34$^+$ cells (0.5%). Patients with RA presented an increased amount of CD34$^+$ cells with the severity of the disease cells whereas no correlation could be established in SLE patients between the amount of CD34$^+$ cells and the disease activity.

3-4-3/ CD34$^+$ cells lose their suppressive activity in patients with RA and SLE

In order to perform some functional assays, CD34$^+$ cells were isolated from 50mLs of blood from each patient and controls using anti-CD34 antibody coated magnetic beads (Myltenyi Biotech). In order to isolate this cell population representing only 0.1% of total PBMCs, the buffers and incubation times had to be optimized in order to obtain a high rate of

purity of the $CD34^+$ cell population. After purification, no cells expressing CD34 could be detected in the CD34 depleted PBMC population (Figure 3-29) and the purity reached between 75 and 90%.

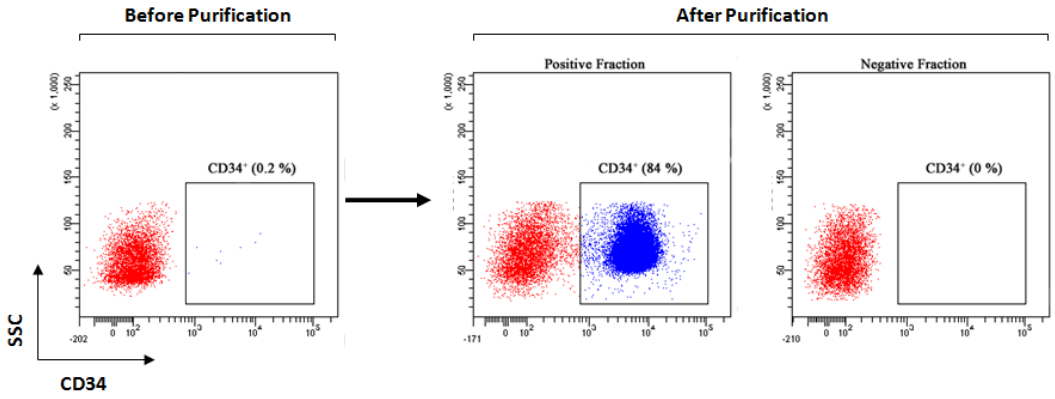


Figure 3-29: Purification of $CD34^+$ cells from PBMCs of controls and patients with RA and SLE
$CD34^+$ cells were purified from PBMCs using magnetic beads conjugated to anti-CD34 antibodies (Miltenyi Biotec). Efficiency of the purification of $CD34^+$ cells was assessed by flow cytometry. Data is representative of all patients and controls.

$CD34^+$ cells were then tested for their ability to inhibit T cell proliferation in response to agonistic anti-CD3 antibodies (αCD3, Clone OKT-3) and evaluated per 0.1% of $CD34^+$ cells. T cell proliferation was assessed using (^{3}H) thymidine incorporation assay.

Depletion of $CD34^+$ cells resulted in an important increase of T cell proliferation in response to αCD3 (Figure 3-30/A) in control individuals. To ascertain the inhibitory functions on T cells displayed by $CD34^+$ cells, we assessed the proliferative activity of PBMCs in response to αCD3 by adding $CD34^+$ cells. Addition of $CD34^+$ cells to PBMCs significantly reduced T cell proliferation to αCD3 (Figure 3-30/B). Generally, and as reported in cancer (Pak et al. 1995; Young et al. 1997 and Garrity et al. 1997), $CD34^+$ cells displayed a suppressive activity on T cells in control individuals of about 40% and ranging from 25 to 60% (Figure 3-30/C).

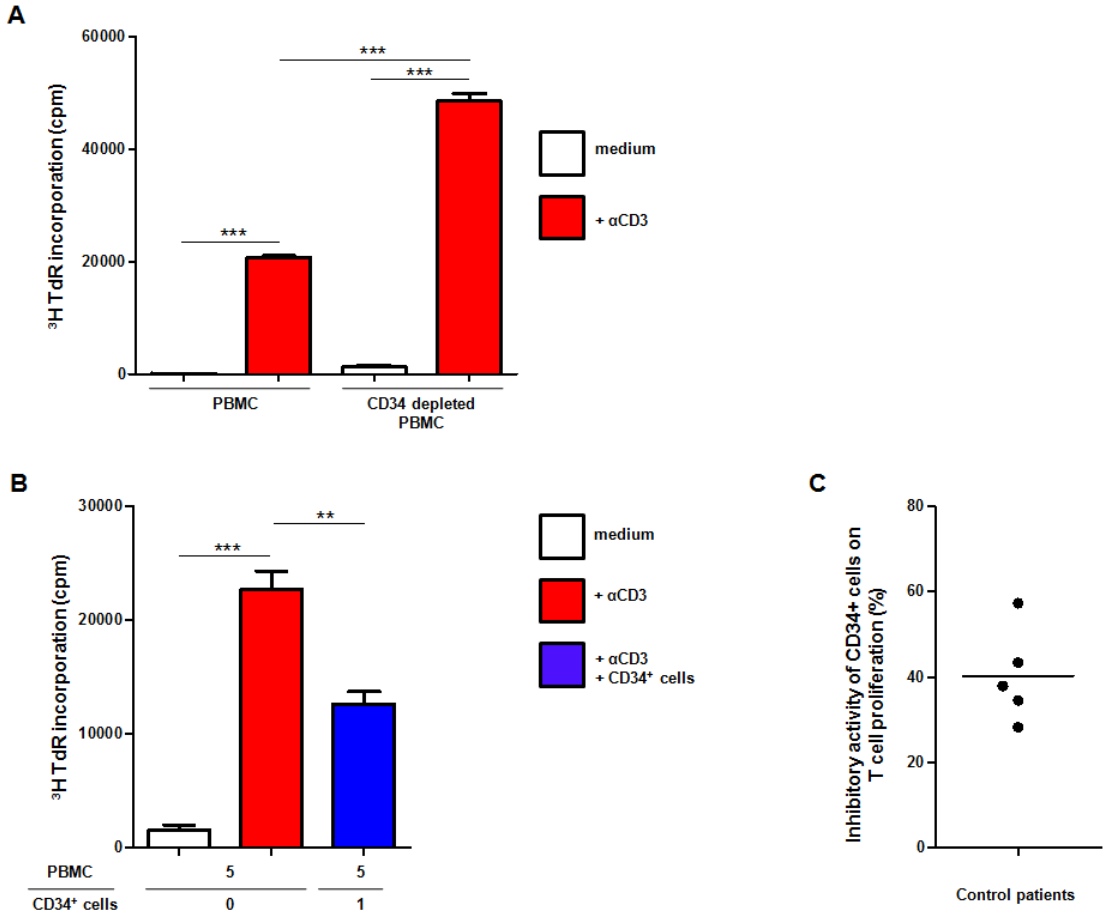


Figure 3-30: CD34$^+$ cells from controls inhibit T cell proliferation upon stimulation with αCD3
(A) Effect of the depletion of CD34$^+$ cells on PBMCs from controls and patients with RA and SLE was measured by T cell proliferation upon stimulation with αCD3 using (^{3}H) thymidine incorporation. **(B)** Pooled results of the inhibitory activity of CD34$^+$ cells. **(C)** Pooled results of the inhibitory activity of CD34$^+$ cells on T cell proliferation in response to αCD3. Data on **(A)** and **(B)** are from one individual and representative of all controls. Statistical analysis performed using unpaired Student's t-test.

The amount of CD34$^+$ cells was similar between controls and RA patients with low disease activity (DAS28 score inferior to 3.2) whereas RA patients with high disease activity had an increased amount of CD34$^+$ cells (Figure 3-28). I evaluated whether CD34$^+$ cells from RA patients could inhibit T cell proliferation as shown in controls. Removal of CD34$^+$ cells resulted in an increased T cell proliferation in response to αCD3 (Figure 3-31/A). Interestingly, this suppressive function on T cells in RA patients was lower than the inhibition provided by CD34$^+$ cells from controls (Figure 3-31/B) and ranged from 10 to 40%. No correlation between the DAS28 score and the inhibitory activity of CD34$^+$ cells could be observed. RA patients had reduced amounts of monocytes compared to controls and SLE patients (Figure 3-25/C). The amount of CD34$^+$ cells recovered after purification was unfortunately too low to perform any experiments where CD34$^+$ cells could be added to PBMCs to ascertain their inhibitory functions as described Figure 3-34/C.

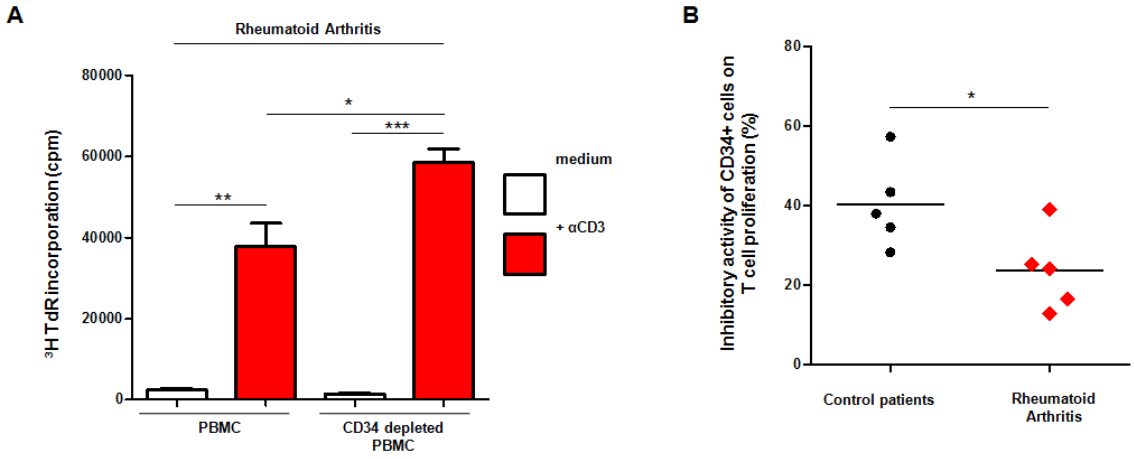


Figure 3-31: CD34$^+$ cells from patients with rheumatoid arthritis have a reduced inhibitory activity on T cell proliferation in response to αCD3
(A) Effect of the depletion of CD34$^+$ cells on PBMCs from patients with RA was measured by T cell proliferation upon stimulation with αCD3 by (^{3}H) thymidine incorporation. **(B)** Pooled results describing the inhibitory activity of CD34$^+$ cells. 5 controls and 5 patients with rheumatoid arthritis were used. Statistical analysis performed using unparired Student's t-test.

The same experiments described in figure 3-30 and 3-31 were performed on PBMCs from SLE patients. Unexpectedly, the removal of CD34$^+$ cells was detrimental to T cell proliferation to αCD3 as proliferation was reduced down to background levels (Figure 3-32/A). We could confirm the activatory function of the CD34$^+$ cells as their addition to PBMCs resulted in an increased T cell proliferation in response to αCD3. No correlation between the stage of the disease and the activatory activity of CD34$^+$ cells could be described.

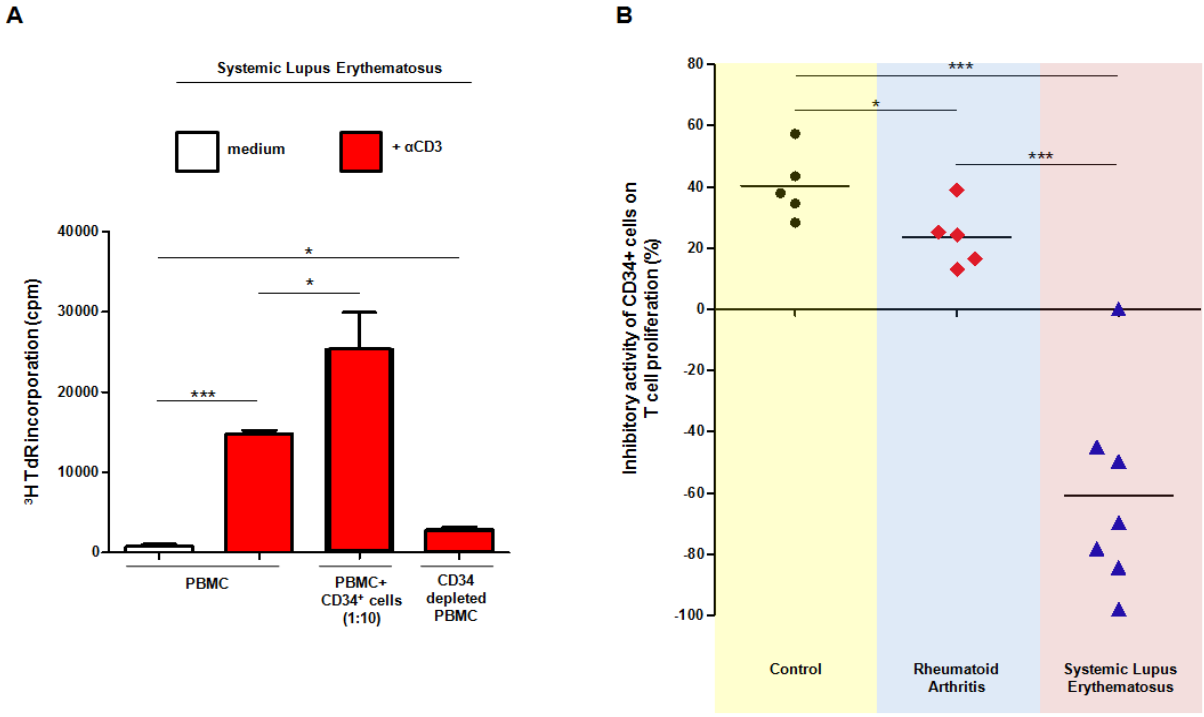


Figure 3-32: CD34$^+$ cells from patient with SLE display an activatory activity on T cells upon stimulation with αCD3

(A) Effect of the depletion and addition of CD34$^+$ cells on PBMCs from SLE patients was measured by T cell proliferation in response to αCD3 using (^{3}H) thymidine incorporation. **(B)** Pooled results of the inhibitory activity of CD34$^+$ cells from SLE patients on T cell proliferation upon αCD3 stimulation compared to RA patients and controls. Data in **(A)** is from one patient and representative of all SLE patients. Statistical analysis performed using unpaired Student's t-test.

As described in figure 3-32/B, CD34$^+$ cells from one SLE patient had no effect on T cell proliferation to αCD3. The activatory function displayed by CD34$^+$ cells from other SLE patients ranged from 40% to nearly 100%. The impact of CD34$^+$ cells on T cell proliferation upon stimulation with αCD3 was important when considering their low presence in PBMCs (0.1 to 0.5% of monocyte population). Compared to controls (Figure 3-25 and 3-26), CD34$^+$ cells from RA patients had a reduced inhibitory activity on T cell proliferation upon stimulation with αCD3. Interestingly, SLE patients present multiple sites of inflammation and autoimmune disease such as psoriasis. In this context of intense inflammation, CD34$^+$ cells lost their inhibitory function on T cells and displayed an activatory phenotype.

3-4-4/ Definition and phenotype of MDSCs in patients with Rheumatoid arthritis and controls

From these results, I wanted to address whether MDSCs had a different phenotype in patients with RA compared to controls. Although MDSCs in mice could be characterized by Gr1 and CD11b markers (Figure 3-12 and 3-14), their phenotype in humans is still not clearly defined and varies between research groups (Figure 3-33). In all these studies and despite the different phenotypes, the cells were always called MDSCs.

CD34	CD33	HLA-DR	CD11b	CD14	CD15	CD19	Lineage*	Reference
	+		+	-				Almand et al. 2001
			+	-	+			Zea et al. 2005
	+		+		+			Srivastava et al. 2008
	+	-					-	Mirza et al. 2006 Kusmartsev et al. 2008
	+	-	+				-	Diaz-Montero et al. 2009
		- / low		+				Haile et al. 2008 Filipazzi et al. 2007
		- / low		+		-		Filipazzi et al. 2007 Hoechst et al. 2008
+	+							Schmidt-Wolf et al. 1992 Sugiura et al. 1998

* Lineage = CD3, CD19, CD56, CD14 and CD15
Grey boxes: the marker is not mentioned in the studies

Figure 3-33: Markers used to characterize the phenotype of human MDSCs
Description of the different markers used to characterize the phenotype of MDSCs in human. Lineage regroups the markers for mature cells (CD3 for T cells, CD19 for B cells, CD56 for NK cells, CD14 for monocytes and CD15 for granulocytes). Boxes are grey when the specific marker is not mentioned in the studies. + and – means that the MDSCs were considered to express or not express the specific marker respectively.

First reports on the existence of progenitors of the myeloid lineage are from studies in the bone-marrow where $CD34^+$ $CD33^+$ cells were designed as Natural Suppressor Cells; with CD34 as a marker specific for stem-cell hematopoietic precursors and CD33 a marker only expressed by cells of the myeloid lineage (Schmidt-Wolf et al. 1992; Sugiura et al. 1998). I therefore investigated whether the amounts of $CD34^+$ $CD33^+$ cells varied depending on autoimmune disease. I analysed the proportion of $CD33^+$ cells within the $CD34^+$ population by flow cytometry in controls, RA and SLE patients (Figure 3-34). Control individuals had between 30 and 35% $CD33^+$ cells within the $CD34^+$ population while it was decreased in patients with RA (from 2 to 20%). Taking into account the results in figure 3-25/C, where RA

patients presented less monocytes than SLE patients and controls, we could conclude that patients with RA presented an overall reduction in the pool of monocytes, $CD34^+$ and $CD34^+$ $CD33^+$ cells. Although $CD33^+$ cells represented between 3 and 32% of $CD33^+$ cells in SLE patients, no correlation with the disease activity could be established (Figure 3-34).

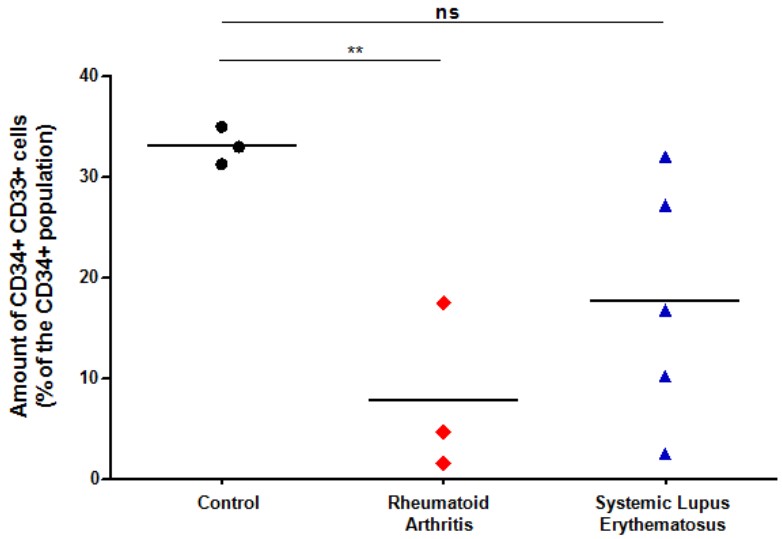


Figure 3-34: Distribution of $CD34^+$ $CD33^+$ cells in controls and patients with Rheuamatoid Arthritis (RA) or Systemic Lupus Erythematosus (SLE)
Proportion of $CD33^+$ cells within $CD34^+$ cells in controls, RA and SLE patients was assessed by flow cytometry using CD33 and CD34 cell-surface markers. Statistical analysis performed using unpaired Student's t-test.

Although first studies in mice excluded the expression of lineage specific markers by MDSCs, it is now largely admitted that they do express specific markers of APCs such as CD11c or F4/80 and markers of maturation such as MHC-II molecules and CD86 (Figure 3-24). As described in previous studies, I considered that human MDSCs could therefore express lineage markers such as CD15 (Zea et al. 2005; Srivastava et al. 2008) and CD14 (Filipassi et al. 2007; Hoechst et al. 2008).

In humans, the loss of CD34 has been linked with the maturation of CD34+ cells into cells of the myeloid lineage (Salati et al. 2007) such as DCs (Bernhard et al. 1995; Garrity et al. 1997) or $CD15^+$ cells (Tao et al. 2004; Goussetis et al. 2006). We further analysed MDSCs by analysing the levels of expression of CD15, CD14, CD11b and HLA-DR as a maturation marker. CD15 is expressed in about 90% of circulating human granulocytes and 30 to 60% of circulating monocytes. CD14 is preferentially expressed on monocytes and macrophages; it binds LPS binding protein and has recently been shown to recognize apoptotic cells. As

CD11b was commonly used to characterise MDSCs, we included this marker in our panel. CD11b is considered as a marker of inflammation (Davis et al. 2006) and is most highly expressed on neutrophils and monocytes / macrophages where it operates as a phagocytic receptor and has an important role in myeloid cell transmigration.

By analysis of $CD34^+$ $CD33^+$ cells in control patients (Figure 3-35/A), we could distinguish 5 major populations according to the expression of CD14 and CD15 (Figure 3-35/C).

Indeed, 16% of $CD34^+$ $CD33^+$ expressed high levels of CD15 (Population P1) but did not express CD11b (95% are $CD11b^-$ and HLA-DR^-). This progenitors would therefore acquire markers specific of granulocytes. The down-regulation of CD15 is a marker of maturation in granulocytes (Sackel et al. 1991; Oehler et al. 1998) and 7% of $CD34^+$ $CD33^+$ cells expressed low levels of CD15 (population P2). The low expressors of CD15 did not express HLA-DR whereas 35% upregulated the expression of CD11b.

1% of $CD33^+$ $CD34^+$ cells expressed markers of mature neutrophils; CD14, CD15, CD11b and HLA-DR (Population P5).

Finally, 12% of the cells expressed low levels of CD14 (Population P4) and were negative for the expression of HLA-DR and CD11b. This corresponded to the acquisition of markers of non-activated monocytes. On the other hand, the majority of $CD34^+$ $CD33^+$ (62%) neither expressed CD14 nor CD15 (Population P3) and the majority lacked the expression of HLA-DR (90%).

Interestingly, in all 3 controls analysed, among a small population expressing high levels of the CD34 marker 85% of the cells also expressed CD33 molecules (Population P6). Among these cells, that all expressed CD14 and CD15, the majority (90%) did not express either HLA-DR or CD11b.

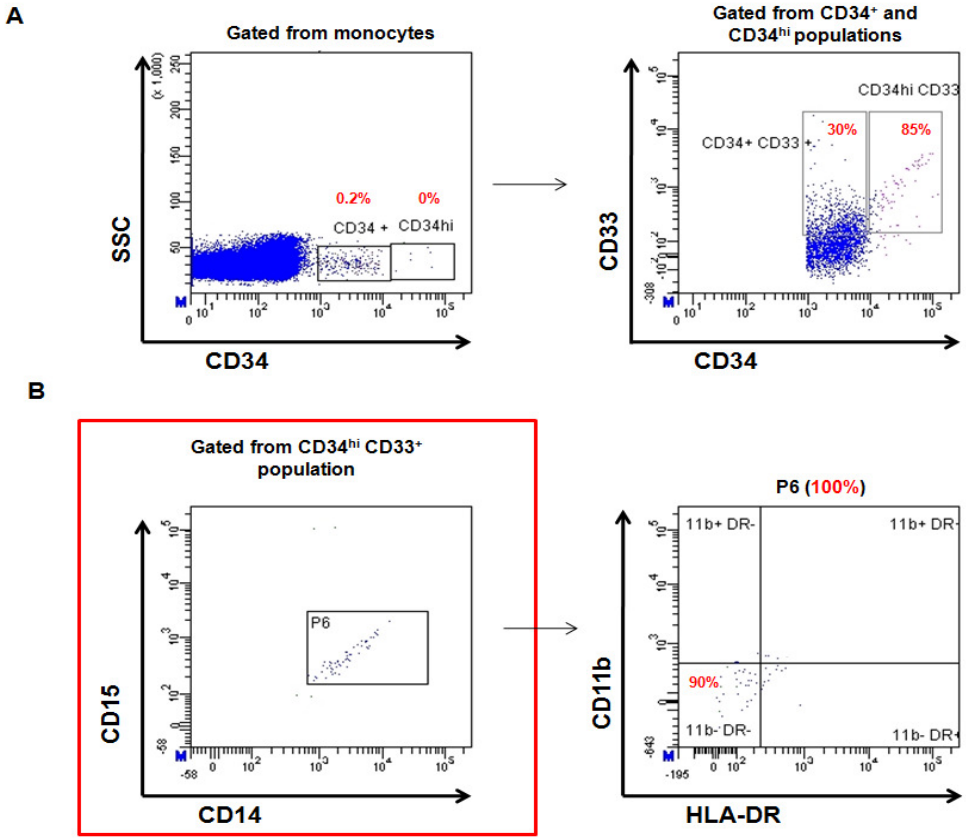
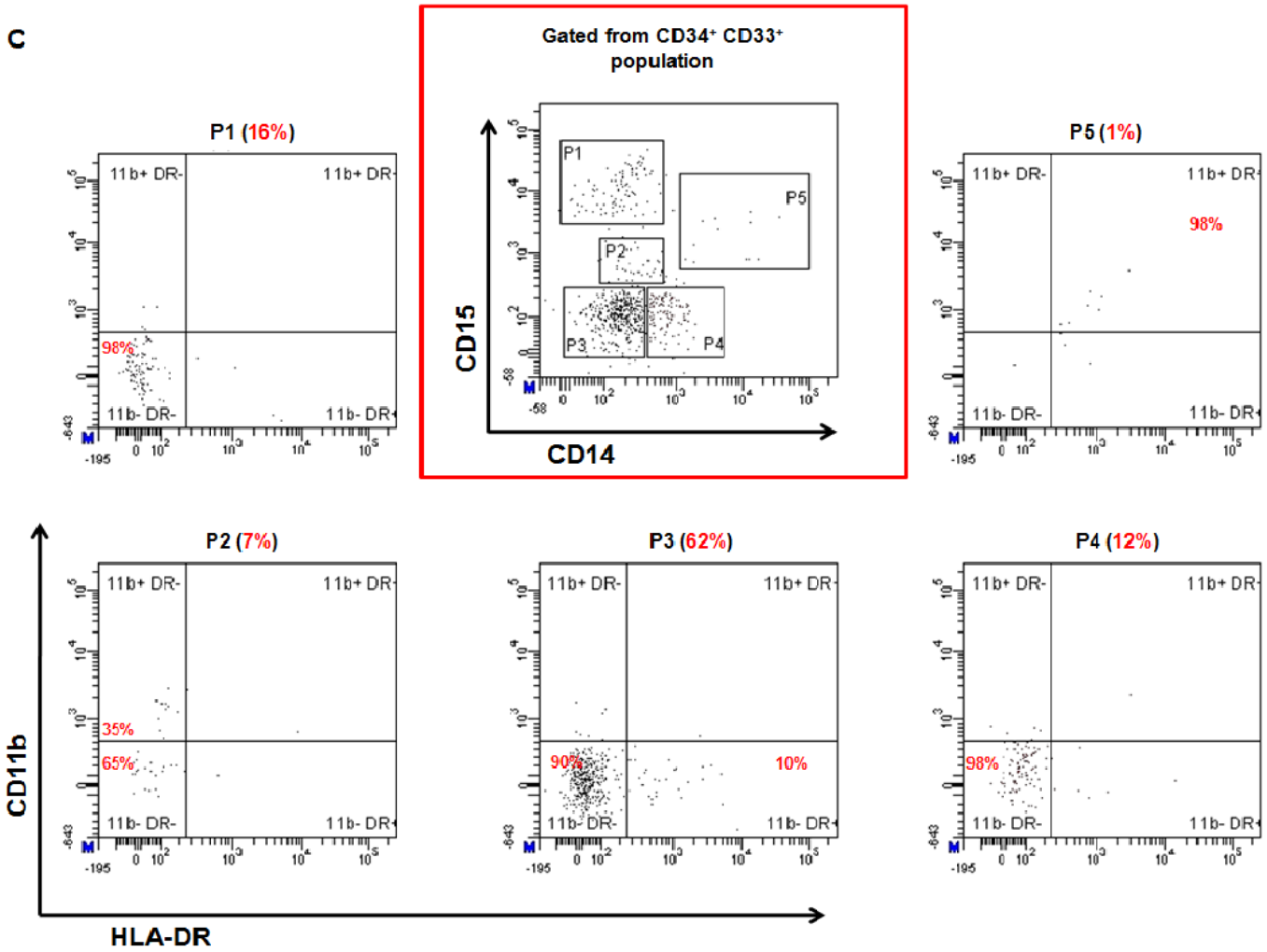


Figure 3-35: Phenotypic analysis of MDSCs from control individuals
(A) PBMCs from the blood of control individuals were analyzed for the expression of CD34 and CD33 by flow cytometry. $CD34^{hi}$ $CD33^{+}$ **(B)** and $CD34^{+}$ $CD33^{+}$ cells **(C)** were analyzed for the expression of CD14 and CD15. Each populations revealed by CD14 and CD15 was further analyzed using CD11b and HLA-DR. Data is representative of all control individuals. **For Figure 3-35/C, please see overleaf**


C
Gated from CD34+ CD33+ population
CD15
CD14
P1
P2
P3
P4
P5
P1 (16%)
P2 (7%)
P3 (62%)
P4 (12%)
P5 (1%)
11b+ DR-
11b+ DR+
11b- DR-
11b- DR+
98%
35%
65%
90%
10%
CD11b
HLA-DR

In all 3 controls analysed, we established that the description of MDSCs using CD33 and CD34 markers was ideal as CD34 is a marker for hematopoietic progenitor and CD33 a marker specific for cells of the myeloid lineage. This phenotype correlates with that of natural suppressor cells described in the bone-marrow (Schmidt-Wolf et al. 1992). I observed that MDSCs from WT mice expressed the expression of markers specific for APCs (such as CD11c and F4/80) and maturation markers (CD86 and MHC-II molecules). Increased expression was observed in a context of inflammation in the TAZ10 mouse model of autoimmune thyroiditis. In human controls, although the majority of cells did not express any markers of differentiated monocytes, $CD34^+$ $CD33^+$ cells acquired markers of neutrophils (1%), undifferentiated and differentiated granulocytes (16% and 7% respectively) and of monocytes (12%).

To assess whether human MDSCs upregulated the expression of markers of mature cells of the myeloid lineage, in models of autoimmune diseases, I assessed the phenotype of $CD34^+$ $CD33^+$ cells in patients with RA. Unfortunately, we did not have any SLE patients to perform a full phenotypic analysis on PBMCs.

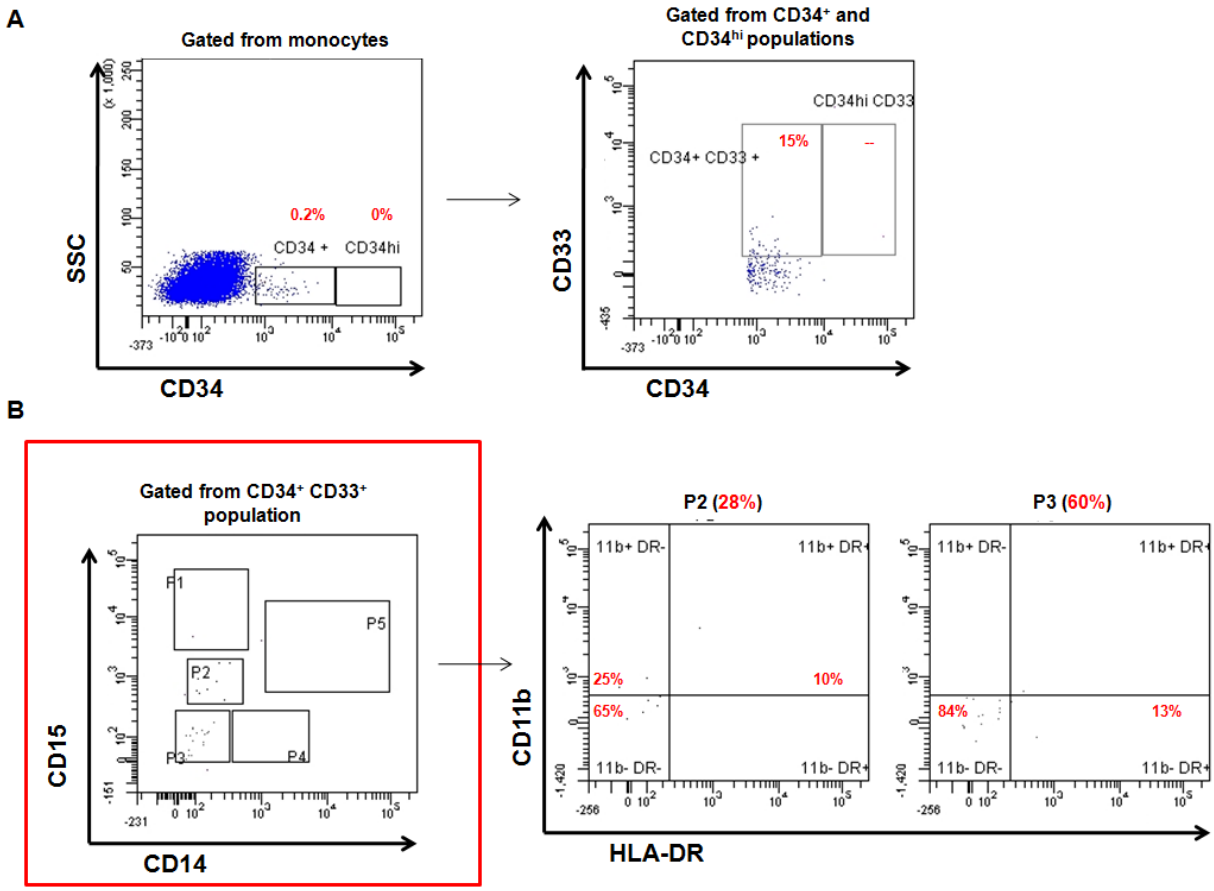


Figure 3-36: Altered phenotype of MDSCs from patient with rheumatoid arthritis
(A) PBMCs from the blood of patient with rheumatoid arthritis were analyzed for the expression of CD34 and CD33 by flow cytometry. $CD34^+$ $CD33^+$ cells **(B)** were analyzed for the expression of CD14 and CD15. Each populations revealed by CD14 and CD15 was further analyzed using CD11b and HLA-DR. Data is representative of all patients with rheumatoid arthritis.

Only 15% of $CD34^+$ cells were expressing CD33 (Figure 3-36/A) showing a twofold reduction compared to control individuals. Also, no cells expressing high levels of CD34 could be observed. Further analysis of $CD34^+$ $CD33^+$ revealed a lack of expression of CD14 and CD15. Indeed the populations of $CD14^-$ $CD15^{hi}$, $CD14^+$ $CD15^+$ and $CD14^+$ $CD15^-$ cells were not present in the PBMCs of patients with RA compared to controls. As observed in control individuals, about 60% of $CD34^+$ $CD33^+$ (Population P3) were lacking the expression of CD14 or CD15 and about 10% of the cells also expressed HLA-DR. The rest of the cells (Population P2 - 28%) expressed low levels of CD15 with 25% expressing CD11b and 10% expressing CD11b and HLA-DR.

3-4-6/ Conclusions

In this section, I analysed whether MDSCs were present in patients with autoimmune diseases such as RA and SLE. One major obstacle in this study was the undefined phenotype of MDSCs in humans as it differs between laboratories, studies and diseases.

The common expression of CD34 and CD33 ensured that the cells analysed would express the marker of hematopoietic progenitors (CD34) while expressing the common markers of the myeloid lineage (CD33). I excluded the sole use of CD33 as a marker as it does not reflect properly the definition of "progenitor" cells of the myeloid lineage. This idea is important and was the focus of three consecutive communications that were published in *Cancer Research* (Krystal et al. 2007; Yang et al. 2007 and Gabrilovitch et al. 2007). In a study on melanoma patients (Filipazzi et al. 2007), the authors excluded the use of the CD34 marker as the cells were considered to have too low a frequency in the blood. Instead, they considered $CD14^+$ $CD11b^+$ cells as the population of MDSCs. There were discrepancies between the different studies with regard to the use of markers of differentiated myeloid cells (included in the lineage markers); numerous studies considered MDSCs as not expressing any lineage specific markers. However, in a couple of studies, the markers for granulocytes-neutrophils CD15, mature myeloid cells CD13 or monocytes CD14 were included in the panels of markers used to describe the phenotype of MDSCs. It seemed clear from our analysis of PBMCs from controls and patients with RA that these markers needed to be included in order to specifically describe the nature of MDSCs that acquire markers of mature myeloid cells upon maturation / differentiation in the inflammatory context. Surprisingly, patients with RA were deficient in $CD34^+$ $CD33^+$ cells expressing the CD14 and CD15 markers compared to controls. I suggested that the lack of inhibition displayed by $CD34^+$ cells from RA patients could result from the reduced population of $CD34^+$ cells and of specific subsets of $CD34^+$ $CD33^+$ MDSCs.

The study of the phenotype of $CD34^+$ $CD33^+$ MDSCs in SLE patients would be of great interest as $CD34^+$ cells displayed a strong activatory function on T cells upon stimulation with αCD3.

3-5/ Summary

In this chapter, I have described the role and functions of MDSCs both in mouse and human chronic inflammatory diseases. $Gr1^+$ $CD11b^+$ MDSCs accumulate in a mouse model of autoimmune thyroiditis and display an inhibitory function on T cells upon stimulation with αCD3, αCD3-αCD28 or the cognate peptide. Despite their inhibitory activity on T cells *in-vitro*, MDSCs were not capable of preventing the development of the disease in mice and their use as therapeutic strategies in TAZ10 mice was detrimental, resulting in the death of the mice 50 days after adoptive transfer. By studying different methods to generate bone-marrow derived DCs, we showed that the manipulation of myeloid progenitors could influence the quality and yield of the DCs population obtained and therefore their use in cancer vaccines.

Although MDSCs from TAZ10 mice were more immunosuppressive *in-vitro* than from WT mice, the $CD34^+$ progenitor populations in RA patients had a reduced inhibitory function on T cells upon stimulation with αCD3 compared to controls. Interestingly, the population of $CD34^+$ cells in a more severe chronic inflammatory disease than RA, SLE, lost their inhibitory activity on T cells upon αCD3 stimulation to acquire a more stimulatory phenotype. We showed that patients with RA presented a reduced amount of $CD34^+$ $CD33^+$ MDSCs with an altered phenotype as revealed by the lack of expression of the CD14 marker and the important decreased expression of CD15.

Studies of MDSCs in both mice and humans highlighted the limitations in using only the Gr1 and CD11b markers (mouse) and the CD33 and CD34 markers (human). The definition of MDSCs as progenitors of the myeloid lineage implies that they acquire or upregulate markers specific for more mature myeloid cells such as CD11c and F4/80 (mouse) or CD14 and CD15 (human). I indeed established that human MDSCs could be detected as $CD34^+$ $CD33^+$ cells. However, only the addition of specific lineage markers could allow the complete study and understanding of the function of MDSCs both in mouse and human.

Interestingly, we highlighted the fundamental differences between chronic inflammatory diseases in mice and humans. MDSCs upregulate markers of professional APCs and display stronger inhibitory activity on T cells in a mouse model of autoimmune disease compared to MDSCs from WT mice. On the contrary, human MDSCs expressed fewer markers of cells of

the myeloid lineage in RA patient compared to controls. Indeed $CD34^+$ cells had a decreased inhibitory activity on T cells upon αCD3, whereas $CD34^+$ cells from SLE patients were highly activatory.

4/ Spreading of TPO cryptic epitopes initiates autoimmune disease in a spontaneous mouse model of autoimmune thyroiditis

4-1/ Introduction

Autoimmune diseases are chronic inflammatory diseases mediated by self-reactive T and B cells following a breakdown of tolerance to self-antigens. These self-reactive T cells are found in the inflamed tissues of patients with autoimmune diseases where they play a pivotal role in the destruction of the target tissue (Giordano et al. 1996; Stassi et al. 2000). Regulatory cells such as Tregs are at the forefront of peripheral mechanisms that prevent the activation of self-reactive T cells and the development of AID (Sakaguchi et al. 1995; Asano et al. 1996).

We previously described the paradoxal role displayed by cells of the myeloid lineage in modulating the activation of self-reactive T cells (Chapter 3). Despite their strong inhibitory function on T cell proliferation, MDSCs accumulate in TAZ10 mice with autoimmune thyroiditis and fail to prevent the activation of self-reactive T cells and the development of AID. Precursors of cells of the myeloid lineage, MDSCs can give rise to DCs, macrophages or granulocytes depending on the microenvironment. Indeed, we showed that MDSCs from TAZ10 mice acquire markers specific of antigen presenting cells such as DCs (Paragraph 3-1). Differentiation of bone-marrow cells also revealed that the manipulation of myeloid precursors influence the ability of mDCs to efficiently activate self-reactive T cells (Paragraph 3-2).

However, the initial mechanisms leading to the break of tolerance and the evasion of mechanisms of peripheral tolerance in autoimmune disease are still elusive.

To answer this question, I used the TAZ10 mouse model of autoimmune thyroiditis in which self-reactive T cells have been shown to be involved in the intitiation and pathogenesis of disease (Quaratino et al. 2004). TAZ10 transgenic mice express a human T cell receptor (Vβ1/Vα15) isolated from the thyroid infiltrating T cell clone 37 from a patient with Hashimoto's thyroid disease (Quaratino et al. 2004). The immunodominant T cell clone 37 is specific for thyroid peroxidase (TPO) $TPO_{535\text{-}551}$ (N-LDPLIRGLLARPAKLQ-C) (Quaratino et al. 1996). Within this autoantigen, T cell clone 37 recognizes the highly stimulatory cryptic epitope $TPO_{536\text{-}547}$ (N-DPLIRGLLARPA-C) generated upon endogenous processing of TPO by thyroid epithelial cells (Quaratino et al. 1996). Interestingly, this TCR also recognizes the naturally occurring antagonistic epitope $TPO_{537\text{-}548}$ (N-PLIRGLLARPAK-C), an altered

peptide ligand (APL) of TPO536-547. The antagonistic epitope induces anergy of T cell clone 37 when presented by DCs upon processing of exogenous TPO (Quaratino et al. 2000).

Although T cell clone 37 recognizes the TPO epitopes in the context of DQ6 in human, transgenic T cells from TAZ10 mice also recognize the murine equivalent of TPO536-547: TPO524-535 (N-DPIVRGLLARAA-C). Indeed, the homologous mouse TPO cryptic epitope induces the specific activation of TAZ10 transgenic T cells when presented by H2-A^k (Quaratino et al. 2004) and H2-A^b (Unpublished results). TAZ10 transgenic T cells display an activated phenotype *in-vivo*. TAZ10 mice spontaneously develop destructive autoimmune thyroiditis sharing the same clinical signs (gain of weight), hormonal changes (increased amounts of serological TSH and decreased levels of T4) and histological modifications (destruction of the thyroid associated with mononuclear cellular infiltrates) as patients with Hashimoto's thyroiditis (Quaratino et al. 2004).

Studies in TAZ10 mice allowed the understanding of the crucial role played by self-reactive T cells in autoimmune diseases. Interestingly, TAZ10 mice spontaneously develop thyroiditis in an environment devoid of environmental insults. In this chapter, I will investigate how the generation of the cryptic TPO524-535 epitope by thyroid epithelial cells promotes the activation of TAZ10 T cells and the initiation of thyroiditis in TAZ10 mice.

4-2/ Results Part 1: Thyroid epithelial cells (TEC) as non-professional antigen presenting cells under inflammatory conditions

TAZ10 transgenic mice develop destructive thyroid disease characterized by infiltration of monocytic cells (Quaratino et al. 2004). Thyroid autoimmune disorders have been extensively studied over the last decades and separate mechanisms involved in the destruction of the thyroid have been described such as the suicide or fratricide killing of thyroid epithelial cells (Stassi et al. 2002). The potential role of IFNγ and the upregulation of MHC-II molecules in the pathogenesis of the disease are also controversial (Fang et al. 2007). However no studies have investigated the role played by epithelial cells in the initiation of autoimmune thyroiditis. I first assessed the role played by thyrocytes in promoting the activation of TAZ10 self-reactive T cells. To answer this question, I evaluated the ability of TEC to modulate the activation of self-reactive T cells from TAZ10 mice upon inflammation.

4-2-1/ Characterization of thyroid epithelial cells in culture

To assess the effect of inflammation on the function and survival of thyroid epithelial cells (TECs) in TAZ10 transgenic mice, it was first important to optimize their culture *in-vitro* and to ensure that the preparation would not be contaminated with other cell types (fibroblasts, macrophages, dendritic cells etc.) that could alter the results obtained in further experiments. Indeed, the majority of experiments performed in this chapter required the use of thyroids from 40 to 80 wild-type or transgenic mice.

After being isolated from WT or TAZ10 mice, thyroid lobes were isolated from the trachea and cut into small pieces using a scalpel. The preparation was then digested with Collagenase I and Dispase II for 45 minutes at 37° C with gentle agitation every 10 minutes. The digestion mix obtained was gently centrifuged for 2 minutes at 200g as previously described (Jeker et al. 1999). The digestion leads to the release of partially digested thyroid lobules. The step of centrifugation is important to ensure that only partially digested thyroid lobules are isolated and cultured whereas free thyrocytes and cells (fibroblasts, dendritic cells, etc.) are discarded in the supernatant. I first assessed the morphological changes affecting partially digested lobules by light microscopy during the culture. The culture medium was changed after the first day and then every two days to ensure the constant supply

of the soluble factors (such as somatostatin and glycyl-L-histidyl-L-lysine acetate) that are essential for the growth and function of TEC. Thyroid lobules could be seen in the culture after digestion (day 0). After three days of culture, thyroid lobules lost their three-dimensional structure and flattened to form a layer of cells (Figure 4-1). Thyroid epithelial cells were proliferating by growing out from the flattened initial thyroid lobule partially digested. This result confirmed previous studies on the culture of thyrocytes (Jeker et al. 1999) and is similar to the description of primary culture of other epithelial cells such as colonocytes (Follmann et al. 2000; Birkner et al. 2004).

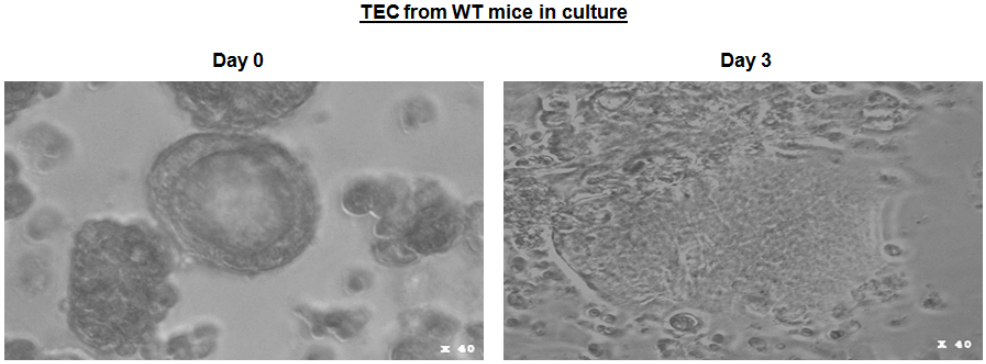


Figure 4-1: Morphological analysis of primary culture of mouse thyrocytes (TEC) from wild-type (WT) mice
Thyroid lobes from WT mice were separated from the trachea and digested in collagenase and dispase for 45 minutes at 37°C. They were then seeded in specific complete Ham'sF12-Nu Serum IV medium. Medium was changed the day after the start of the culture (day 0) and then every two days. The morphology of thyroid lobules after digestion of the thyroid and 3 days of culture was assessed using a light microscope (magnification X 40). Data shown is representative of two different experiments.

To ascertain that the culture was only constituted of TECs, it was important to assess the level of expression of markers specific of epithelial cells. Fluorescence microscopy on thyrocytes from WT mice grown for 3 days was done using a pan-cytokeratin antibody. DAPI was used to counterstain the nucleus. As revealed at magnification of X40 (Figure 4-2), the expression of cytokeratin was ubiquitous and was associated with the DAPI nuclear staining. Some patches of high intensity could be seen especially at the edge of the monolayer. Observation of the cells at higher magnification (X100) confirmed this as some cells showed intense expression whereas others only had low cytokeratin staining. Furthermore, the expression of cytokeratin was generally not diffused within the cytoplasm but more concentrated into focal points. These focal points could represent an accumulation of actin filament of cytokeratin to create the multiple tight-junctions and desmosomes as

previously observed in the primary culture of thyrocytes (Hishinuma et al. 1992; Bechtner et al. 1996) and colonocytes (Follmann et al. 2000).

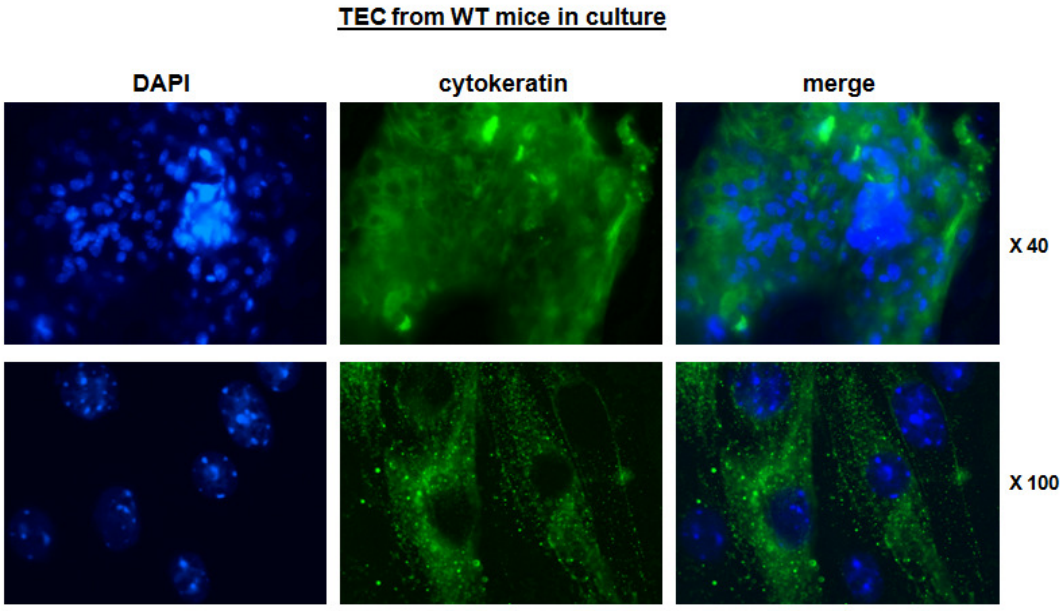


Figure 4-2: Primary culture of mouse thyrocytes (TEC) from wild-type (WT) mice
Thyroid lobes from WT mice were separated from the trachea and digested in collagenase and dispase for 45 minutes at 37°C. They were then seeded in specific complete Ham'sF12-Nu Serum IV medium. Medium was changed the day after the start of the culture and then every two days. Thyroid lobules were grown for three days and assessed for the presence of the epithelial marker cytokeratine. Immunofluoresent staining was done using a polyclonal pan-cytokeratin antibody (green) and cells were counterstained with DAPI (blue) to reveal the nucleus. Pictures were taken using a fluorescence microscope (magnification x40 and x100). Data shown is representative of two different experiments.

Studies have highlighted the loss of function of thyrocytes in culture over time. Unless mentioned, TECs were normally not cultured for more than 4 days to preserve the expression of thyroid peroxidase (TPO), the main thyroid antigen at the centre of our study (Chazenbalk et al. 1987; Bocanera et al. 1999).

4-2-2/ Thyroid epithelial cells express major histocompatibility complex (MHC) II molecules in the presence of pro-inflammatory cytokines

Induction of an adaptive immune response requires the recognition of a peptide fragment in the groove of MHC molecules by T cells via the T cell receptor. In thyroiditis and other pathological conditions, epithelial cells that normally do not express MHC-II molecules, aberrantly express these molecules suggesting their potential ability to present antigens to T cells. Indeed, early studies have highlighted the ability of TECs expressing MHC-II

molecules to present viral antigens to T cells (Londei et al. 1984). T cells cloned from the thyroid of patients with autoimmune thyroiditis were shown to be capable of recognizing autologous thyrocytes expressing MHC-II molecules (Londei et al. 1985). Following these results, Quaratino et al demonstrated that a T cell clone from a patient with autoimmune thyroiditis was capable of recognizing thyroid peroxidase (TPO), a major thyroid autoantigen (Quaratino et al. 1996). The T cell clone was capable of recognizing TPO-transfected EBV cell-line but not EBV cell-line pulsed with soluble TPO. Interestingly, this T cell-clone also recognized autologous TECs aberrantly expressing MHC-II molecules. These results suggested that cryptic epitopes endogenously generated by non-professional APCs (TECs) were playing a central role in autoimmune disease.

In TAZ10 transgenic mice, T cells express a human TCR specific for the human cryptic TPO epitope ($TPO_{536-547}$). TAZ10 T cells also recognize the murine TPO cryptic epitope $TPO_{524-535}$ (Quaratino et al. 2004). To assess the role that TEC could play at the onset of the disease, we first investigated whether TEC from TAZ10 mice could present the $TPO_{524-535}$ to TAZ10 T cells in the context of MHC-II molecules.

I first quantitated the expression of MHC-II molecules on thyroid epithelial cells by the staining of partially digested thyroid lobules with a specific antibody to $H2\text{-}A^b$. (Thyroiditis leads to the death of thyrocytes and the destruction of thyroid follicles in nearly 100% of mice at the age of 4 months (Quaratino et al. 2004)). Thyroids from young TAZ10 mice were used (2 months old) to ensure that the thyroid was not already too damaged by the ongoing inflammation. At this age the weight gain was low and similar to WT mice. ~75% $CD8^+$ and $CD4^+$ T cells presented a naïve phenotype (high levels of CD62L and no CD69) in young TAZ10 mice (Badami et al. 2005). Indeed, the extent of expression of MHC-II molecules would vary among TAZ10 mice according to their age and thereafter to the damaged inflicted on the thyroid. Thyroid from old TAZ10 mice should have a lower or no expression of MHC-II molecules compared to young TAZ10 mice, whereas TEC from WT mice devoid of inflammation in the thyroid should not express any MHC-II molecules.

Thyroid lobules from 2 months old TAZ10 mice naturally expressed high levels of MHC-II molecules as revealed by fluorescence microscopy (Figure 4-3). The neo-expression of MHC-II molecules by epithelial cells is a sign of tissue inflammation and demonstrates the important level of inflammation occurring in the thyroid of TAZ10 transgenic mice. Also,

compared to the round and follicular morphology of the partially digested thyroid lobules from wild-type mice (Figure 4-1), thyroid lobules isolated from TAZ10 mice did not show a follicular morphology hence highlighting the tissue damage that occurs in TAZ10 mice.

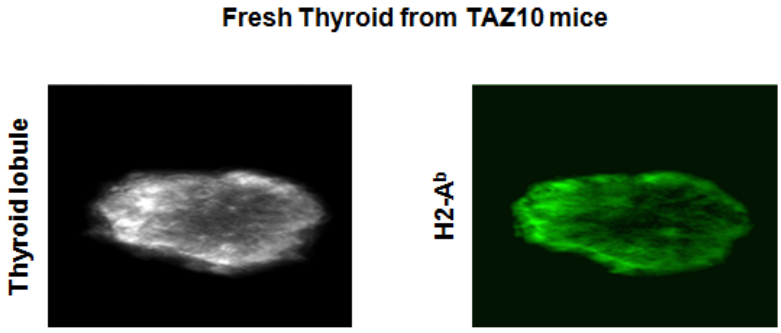


Figure 4-3: Thyroid epithelial cells from TAZ10 transgenic mice naturally express high levels of MHC-II molecules
Thyroid from young TAZ10 mice (2 months old) were isolated and digested as previously described. The morphology of the thyroid lobule was observed with a light microscope (magnification x40). Thyroid lobules were stained using a monoclonal antibody against the MHC-II molecule H2-A^b (Green). Pictures were taken using a fluorescence microscope (magnification x40). Data shown is representative of two different experiments.

Because IFNγ has been shown to promote the expression of MHC-II molecules both in mouse and human epithelial cells via the induced expression of the class-II transactivator gene CIITA (Caturegli et al. 2000), we explored the effect of IFNγ on the expression of H2-A^b molecules by cultured TECs from wild-type mice. Thyroids from wild-type mice were cultured for 3 days with or without IFNγ at 200U/mL and expression of H2-A^b MHC-II molecules assessed by fluorescence microscopy. As expected, TECs from WT mice did not express H2-A^b naturally whereas its expression was greatly enhanced when cultured with IFNγ. Expression was not uniform across the whole population as some expressed more H2-A^b molecules than others (Figure 4-4/A).

By flow cytometry, we compared the level of expression of MHC-II molecules between freshly digested thyroid epithelial cells from transgenic mice and cultured TECs from wild-type mice with or without stimulation with IFNγ (Figure 4-4/B). Addition of IFNγ increased the levels of expression of MHC-II molecules at the cell-surface in cultured TECs from wild-type mice; its expression was nearly similar to that displayed by freshly isolated TECs from TAZ10 transgenic mice (Figure 4-4/B). It was interesting to see that in this experiment, not all epithelial cells from transgenic mice expressed MHC-II molecules. Some cells showed

low or no expression of MHC-II molecules. This potentially highlighted that the damages inflicted on TECs and the subsequent destruction of the thyroid had already occurred in the young TAZ10 mice investigated.

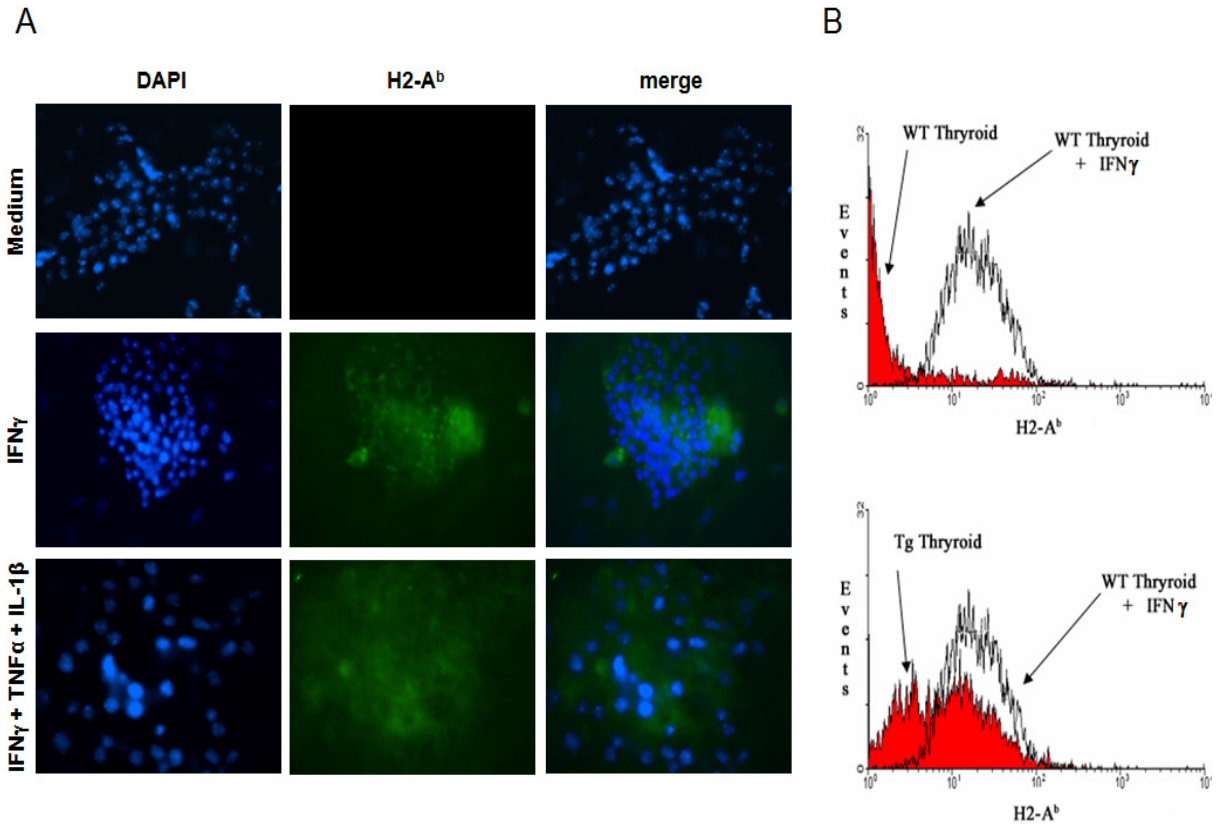


Figure 4-4: Thyroid epithelial cells from wild-type mice express MHC-II molecules H2-A^b upon exposure to pro-inflammatory cytokines

A/ Thyroid from wild-type (WT) mice were harvested and digested as previously described. Thyrocytes were grown on poly-L-Lysine coated coverslips for one day in complete medium and for a further 3 days with or without IFNγ at (200U/mL), TNFα (20ng/mL) and IL-1β (20ng/mL). Cells were then stained using a monoclonal antibody against MHC-II molecule H2-A^b and analyzed using a fluorescence microscope (magnification X 40). Thyrocytes grown with IFNγ and a mix of IFNγ, TNFα and IL-1β expressed H2-A^b molecules **(A)**. The expression of H2-A^b by thyrocytes from WT mice when cultured with IFNγ for three days was confirmed by flow cytometry. Interestingly, the level of expression was similar to that of fresh thyrocytes from young TAZ10 transgenic mice (Tg Thyroid) **(B)**. Data shown is representative of two different experiments.

The important infiltration of the thyroid by monocytic and T cells has been demonstrated both in patients with Hashimoto's thyroiditis (Giordano et al. 1997; Stassi et al. 2000), Grave's disease (Stassi et al. 2000) and in TAZ10 mice (Quaratino et al. 2004). This infiltration would be likely to lead to release of pro-inflammatory cytokines including IFNγ. We assessed whether a combination of pro-inflammatory cytokines such as IFNγ, TNFα and IL-1β would also be able to promote the neo-expression of MHC-II molecules by thyroid epithelial cells from wild-type mice *in-vitro*. Indeed, this combination of pro-inflammatory cytokines was able to promote the neo-expression of MHC-II molecules (Figure 4-4/A).

4-2-3/ TECs provide costimulatory signals to T cells in the presence of pro-inflammatory cytokines

As we previously described in Chaper 3, dendritic cells must display costimulatory molecules to efficiently activate T cells (Figure 3-10). The constitutive expression of H2-A^b MHC-II molecules by thyroid epithelial cells in TAZ10 mice prompted me to investigate whether TECs could promote the proliferation of T lymphocytes. We first assessed the costimulatory activity displayed by freshly isolated TECs from TAZ10 mice to $CD4^+$ T cells from both TAZ10 and wild-type mice in the presence of the agonistic αCD3 antibody (Figure 4-5).

Both $CD4^+$ T cells purified from the spleen of WT and TAZ10 mice proliferated strongly to αCD3 and mature bone-marrow derived DCs as antigen presenting cells (black bars; Figure 4-5). Interestingly, T cells did not proliferate in response to αCD3 and TEC from WT mice (*blue bars*; Figure 4-5). I could exclude that this was due to the lack of expression of MHC-II molecules by TEC from WT mice as previously shown (Figure 4-4) as αCD3 bypassed the presence of MHC-II molecules. However, T cells from TAZ10 mice proliferated in the presence of αCD3 and TECs from TAZ10 mice (*red bars*; Figure 4-5). $CD4^+$ T cells from WT mice strongly proliferated when co-cultured with TECs from TAZ10 mice in the presence of αCD3. From this result, I could rule out that the proliferation of TAZ10 T cells previously observed was due to its transgenic nature or any eventual activation by thyroid antigens presented by TECs from TAZ10 mice (Figure 4-5).

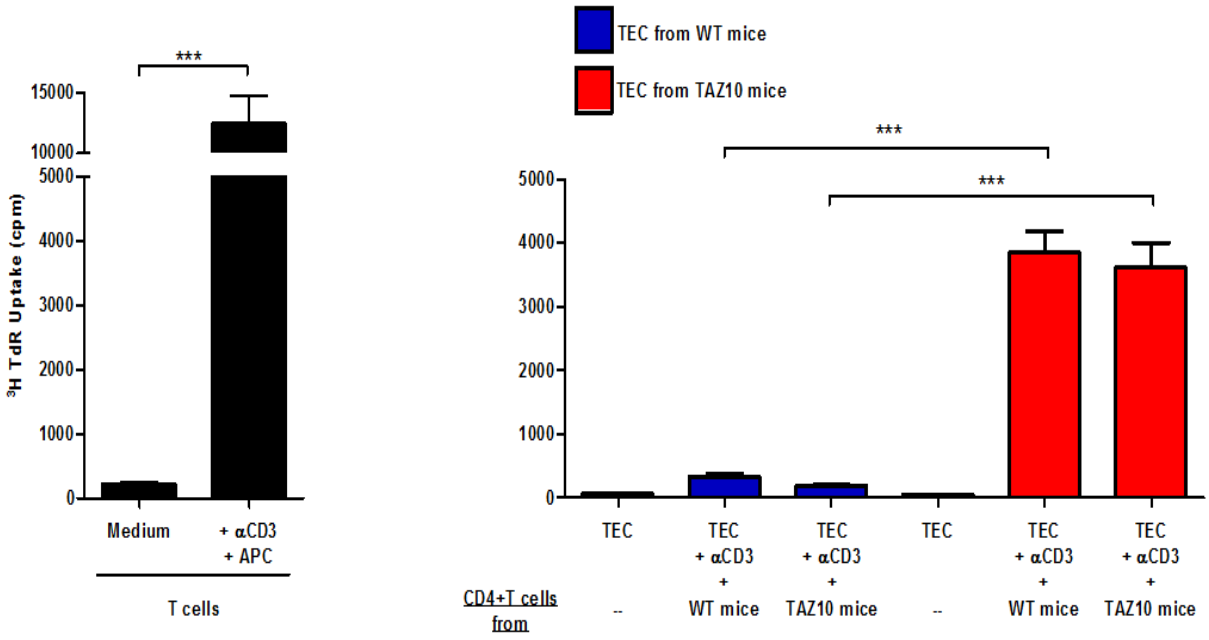


Figure 4-5: Costimulatory activity of TECs from TAZ10 transgenic mice on T cells
The proliferation of T cells from WT and TAZ10 mice assessed by (^{3}H) thymidine incorporation was used as readout of the costimulatory activity of TECs from TAZ10 transgenic mice. TECs were digested and directly co-cultured with purified CD4$^+$T cells from wild-type and TAZ10 mice. The addition of αCD3 antibodies mimicked the TCR:MHC engagement whereas TECs would eventually provide co-stimulatory signals to T cells. The level of costimulation provided by TEC was assessed by measuring the T cell proliferative activity using (^{3}H) thymidine incorporation. Statistics were performed using the unpaired Student's t-test. Data shown is representative of two different experiments

Thyrocytes from WT mice were unable to provide costimulatory activity to CD4$^+$ T cells from both WT and TAZ10 mice (Figure 4-5). As we previously showed that TECs from WT mice express high levels of MHC-II molecules upon stimulation with IFNγ (Figure 4-4), we wondered whether IFNγ could also promote the expression of costimulatory molecules. TEC from TAZ10 mice provided costimulatory signals to T cells from WT mice (*red bars*, Figure 4-6) whereas TEC from WT mice did not (blue bars, Figure 4-6). Stimulation of TEC from WT mice with IFNγ led to an increased proliferation of WT T cells in the presence of agonistic αCD3 antibody (*green bars*, Figure 4-6). We could rule out that IFNγ was involved in the activation of T cells as TECs were thoroughly washed with medium prior to the addition of T cells from WT and TAZ10 mice. The costimulatory activity displayed by TEC from WT mice upon stimulation with IFNγ was however inferior to the one provided by TECs from TAZ10 mice.

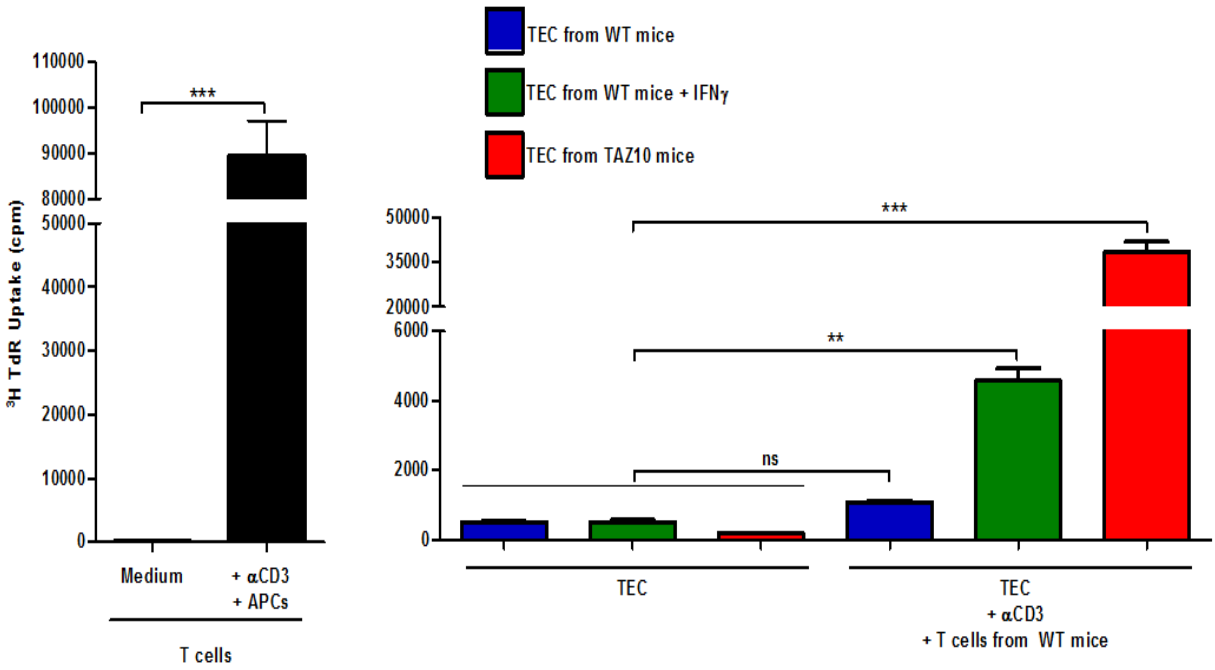


Figure 4-6: TECs from WT mice provide costimulatory signals to T cells upon stimulation with IFNγ
TECs were digested and cultured for 3 days in the presence or absence of IFNγ. $CD4^{+}$T cells from wild-type mice were added to the culture and the costimulatory activity of TECs was assessed by (^{3}H) thymidine incorporation. The addition of αCD3 antibody was mimicking the TCR:MHC engagement whereas TECs would eventually provide co-stimulatory signals to T cells. Statistics were performed using the unpaired Student's t-test. Data presented is representative of two different experiments.

Although I have not identified the costimulatory molecules expressed by thyrocytes, this result is consistent with previous studies showing that TECs upregulate the expression of costimulatory molecules such as B7-1 (Battifora et al. 1998) and B7-2 (Peterson et al. 1999) in an inflammatory context (Reviewed in Salmaso et al. 2002). Alongside the high level of expression of MHC-II molecules by TECs from TAZ10 mice, this result highlights the potential that the inflammatory environment in the thyroid of TAZ10 mice has to induce a T cell response via TECs.

The ability of epithelial cells to upregulate the expression of MHC-II and costimulatory molecules suggest they can act as non-professional antigen presenting cells. We then wondered whether TECs from TAZ10 mice and TECs from WT mice challenged with IFNγ were able to present the TPO cryptic epitope to transgenic T cells.

4-2-4/ Thyroid epithelial cells from TAZ10 transgenic mice expressing MHC II molecules present the cryptic TPO524-535 epitope to TAZ10 transgenic T cells.

TAZ10 T cells proliferate in response to the human and mouse TPO cryptic epitope (respectively TPO536-547 and TPO524-535) when presented by antigen presenting cells in the context of MHC-II molecules (Quaratino et al. 1996, Quaratino et al. 2004). Interestingly, the T cell clone 37 isolated from the thyroid of the patient with thyroiditis could recognize the cryptic epitope presented by allogeneic thyroid epithelial cells. I wanted to assess whether thyroid epithelial cells from TAZ10 mice could present the TPO524-535 cryptic epitope to TAZ10 T cells bearing the same TCR than the human T cell clone 37.

Thyroid lobes were isolated from young TAZ10 mice to ensure they would not be destroyed and would express high levels of MHC-II and costimulatory molecules (Figure 4-3 and 4-5). Thyroid lobules were digested in collagenase and dispase as previously described and partially digested thyroid lobules washed and co-cultured with $CD4^+$ T cells from TAZ10 or WT mice.

Although I previously demonstrated that thyroid epithelial cells from TAZ10 mice naturally express MHC-II molecules (Figure 4-3), we evaluated its expression using the superantigen *Staphylococcus-aureus* Enterotoxin D (SED) at 10ng/mL. In human, SED promotes the proliferation of T cells by the simultaneous binding of TCR through the Vβ3 region (Choi et al. 1989) and the binding of dimerized MHC-II molecules induced by SED (Chintagumpala et al. 1991; Al-Daccak et al. 1998). T cells from TAZ10 mice bearing the TCR from the human T cell clone 37 (Vβ1), also proliferate in response to SED in the presence of murine H2-A^b MHC-II molecules whereas T cells from WT mice do not (Unpublished results).

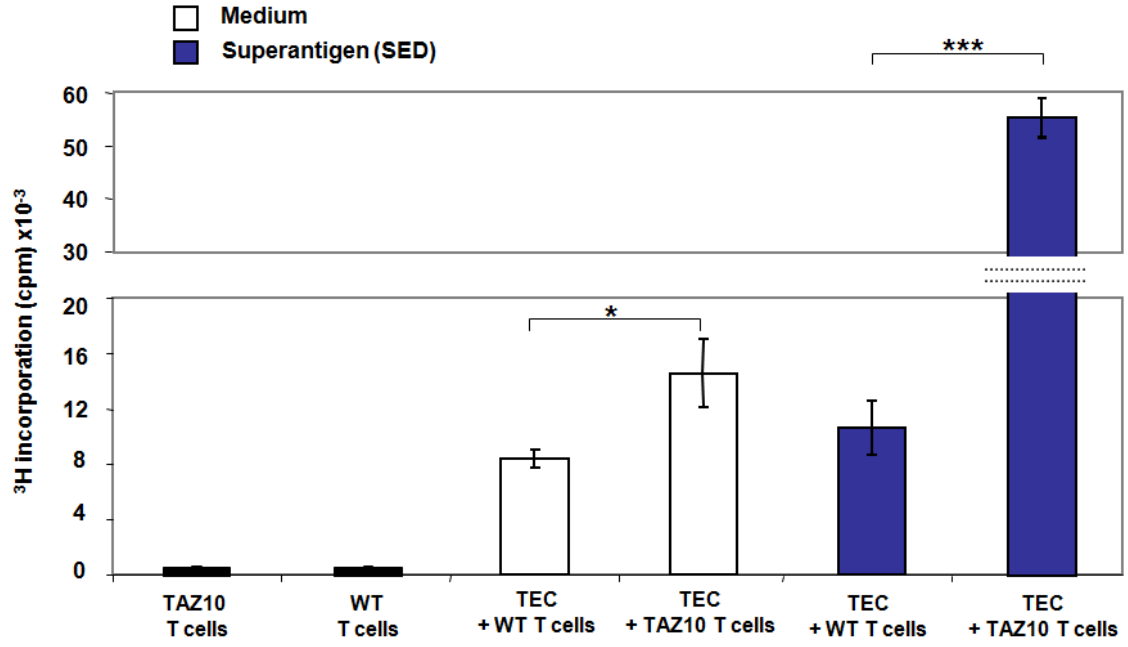


Figure 4-7: Thyroid epithelial cells (TECs) from TAZ10 transgenic mice present the TPO$_{524-535}$ cryptic epitope to T cells from TAZ10 mice
The ability of TECs from TAZ10 transgenic mice to present the TPO cryptic epitope to T cells was assessed by (^{3}H) thymidine incorporation. Purified CD4$^+$ T cells from TAZ10 or WT mice were co-cultured with TECs from TAZ10 mice for 3 days. Proliferation of TAZ10 T cells in response to the superantigen SED (*Staphylococcus aureus* Enterotoxin D) was taken as readout for the expression MHC-II molecule as SED is only recognized by T cells from TAZ10 mice. Statistics were done using the unpaired Student's t-test. Data shown is representative of two different experiments.

T cells from TAZ10 mice proliferated in response to the superantigen SED (blue bars, Figure 4-7), thus confirming the aberrant expression of MHC-II molecules by TECs from TAZ10 mice. Transgenic T cells were able to proliferate when cocultured with TEC from transgenic mice in the absence of the superantigen SED. CD4$^+$ T cells from WT mice however slightly proliferate, thus suggesting that TECs from transgenic mice presented the TPO$_{524-535}$ cryptic epitope to TAZ10 T cells (white bars, Figure 4-7).

From these results, I wanted to assess whether TECs from WT mice were able to present the TPO cryptic epitope to transgenic T cells when expressing MHC-II molecules upon stimulation with IFNγ.

4-2-5/ In a context of inflammation, thyroid epithelial cells from wild-type mice can present the cryptic TPO epitope to transgenic T cells from TAZ10 mice

I previously showed that TECs from TAZ10 mice naturally expressing MHC-II molecules are efficient in activating TPO-specific transgenic T cells. TECs from WT mice exposed to IFNγ strongly upregulated the expression of MHC-II molecules. TECs from WT mice also provided costimulation to T cells when pre-exposed to IFNγ. I wanted to determine whether presentation of the endogenous TPO$_{524-535}$ cryptic epitope can be initiated by IFNγ in TEC from WT mice.

To optimize the results, I needed to carefully titer the numbers of TAZ10 T cells for the number of TEC after the thyroid lobules were partially digested. The major problem we encountered was that in order to count the cells, TECs had to be harvested using EDTA and trypsin. Unfortunately, after re-seeding, the majority of thyroid epithelial cells died within 2 days. I also avoided harvesting TECs before co-culturing them with T cells so as to prevent the removal of eventual peptides present at the surface of the cells. Indeed, the thyroid preparation was seeded in term of volume and not in term of cell-number directly in wells of 96 well-plates. I estimated that in a volume of 25µL of partially digested thyroid lobules, we had 60,000 thyroid epithelial cells.

TECs from WT mice were cultured with or without IFNγ for 3 days. To assess the eventual presentation of the TPO cryptic epitope by TECs, $2x10^5$ $CD4^+$ T cells from TAZ10 mice were co-cultured with TECs from WT mice and their proliferation assessed by (^{3}H) thymidine incorporation 3 days later (Figure 4-8).

As expected, the proliferation of TAZ10 T cells was negligible when co-cultured with TECs from WT mice previously cultured for 3 days in medium (blue bars; Figure 4-8).

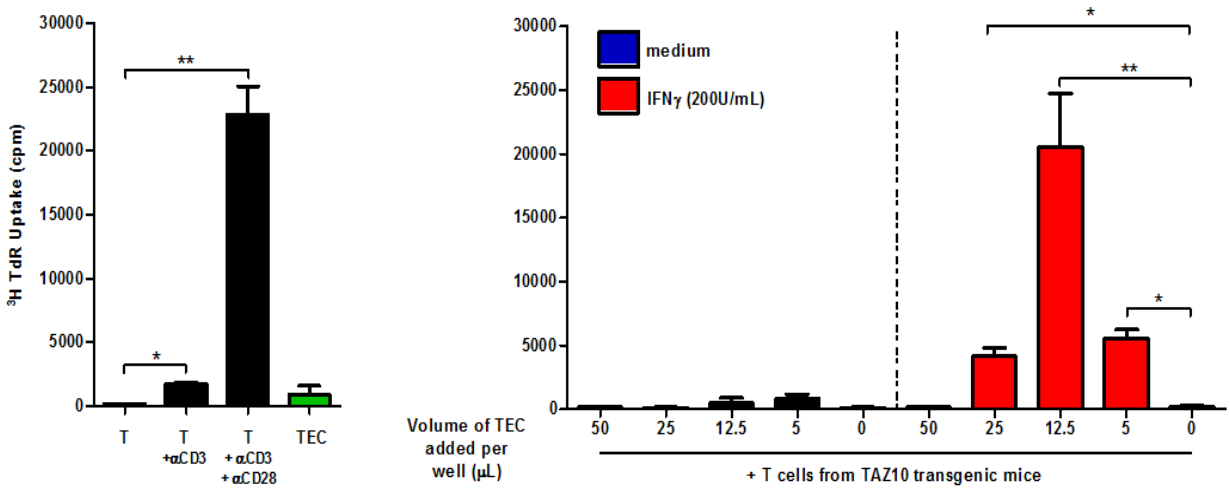


Figure 4-8: Titration of the ratio WT TECs:T cells from TAZ10 mice
TECs from WT mice were digested and seeded at different concentration for 3 days with or without IFNγ at 200 units/mL. $CD4^+$ T cells from TAZ10 mice were seeded at $2x10^5$ per well for 3 days and their proliferation assessed by (^{3}H) thymidine incorporation. Statistics were performed using the unpaired Student's t-test.

The addition of IFNγ to the thyroid culture strongly enhanced the proliferation of T cells from TAZ10 mice when 12.5µL of the digested TEC was used as APCs (red bars; Figure 4-8). Lower T cell proliferations were detectable when using 5 and 25µL of partially digested thyroid lobules. As a result, in all subsequent experiments, 40 thyroids (80 thyroid lobes) were digested and resuspended in 8.8mL of medium. From this mix 12,5µL was used per well of a 96 well-plate and TECs were co-cultured with $2x10^5$ $CD4^+$ T cells from TAZ10 or wild-type mice.

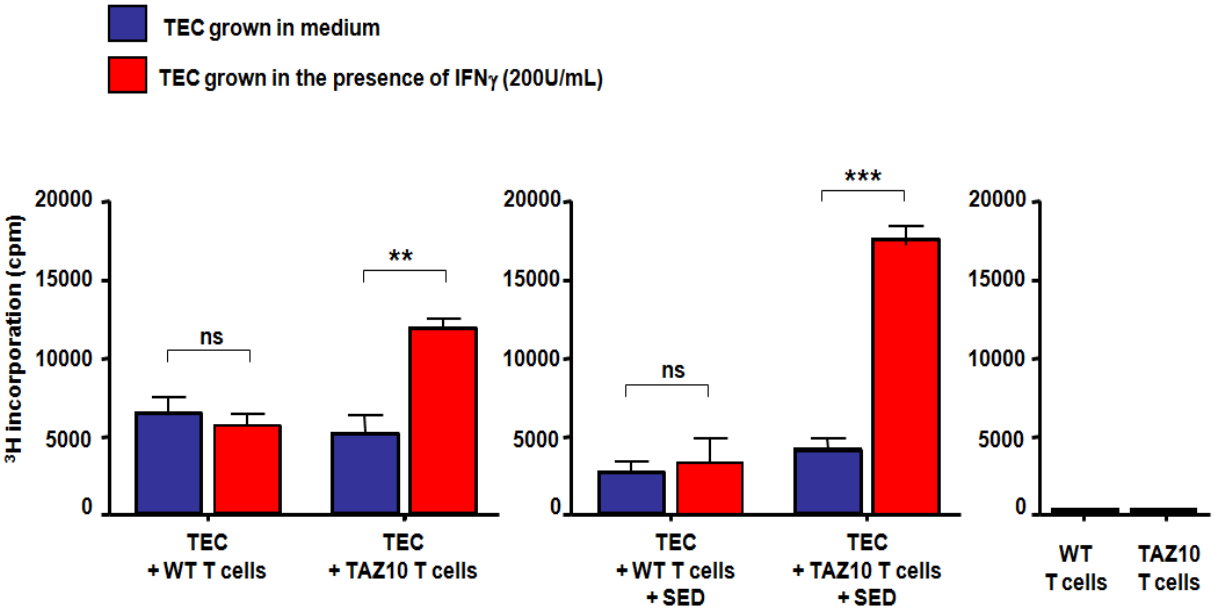


Figure 4-9: Thyroid epithelial cells from WT mice act as non-professional APCs when stimulated with IFNγ

Thyroids from WT mice were digested and TECs seeded in 96 well-plates for 3 days with or without IFNγ at 200 units/mL. $CD4^+$ T cells from TAZ10 or wild-type mice were co-cultured with TEC for a further 3 day and their proliferation assessed by (^3H) thymidine incorporation. Addition of the superantigen (SED) was used as readout for the expression of MHC-II molecule and is only recognized by TAZ10 T cells. Data shown is representative of two different experiments. Statistics performed using the unpaired Student's t-test.

I wanted to ascertain that the proliferation of TAZ10 T cells observed Figure 4-8 was the result of the recognition of the TPO cryptic epitope presented by MHC-II molecules to transgenic T cells. We assessed the proliferative activity of $CD4^+$ T cells from WT and TAZ10 mice when co-cultured with TECs from WT mice with or without stimulation with IFNγ. The proliferation of T cells was assessed by (^3H) thymidine incorporation.

The vigorous proliferation of T cells from TAZ10 mice in the presence of SED confirmed that TECs from WT mice stimulated with IFNγ expressed high levels of MHC-II molecules. TECs from WT mice grown without IFNγ did not express MHC-II molecules and therefore were not able to induce the proliferation of the TAZ10 T cells in response to SED. This result confirmed the upregulation of MHC-II molecules upon inflammation as previously obtained by fluorescence microscopy and flow cytometry (Figure 4-4).

TECs from TAZ10 transgenic mice promote the proliferation of T cells from TAZ10 mice (Figure 4-9). $CD4^+$ T cells from wild-type mice did not proliferate in the presence of TECs from WT mice pre-stimulated or not with IFNγ. T cells from TAZ10 mice were not able to proliferate when co-cultured with TECs from WT mice not challenged with IFNγ. The upregulation of MHC-II and costimulatory molecules induced by IFNγ on WT TECs allowed

TAZ10 T cells to proliferate. We could conclude that the cryptic epitope TPO$_{524-535}$ was therefore presented to TAZ10 T cells in the context of MHC-II molecules by TECs from TAZ10 mice and TECs from WT mice challenged with IFNγ (Figure 4-7).

IFNγ was therefore capable of partially mimicking the effect of the general inflammation occurring in the thyroid of the diseased mice; at least at the levels of MHC-II molecule expression and presentation of the TPO cryptic epitope.

The effects of IFNγ on the induction of the ability of thyrocytes to display antigen presenting capacities to T cells are confirmed by other studies on various cell types such as mesenchymal stem cells (Chan et al. 2006), Schwann cells (Kingston et al. 1989) and bronchial epithelial cells (Suda et al. 1995).

The upregulation of MHC-II molecules by the combination of pro-inflammatory cytokines (IFNγ, TNFα and IL-1β) suggest that cytokines secreted by activated T cells or antigen presenting cells present at the site of inflammation could trigger the up-regulation of MHC-II molecules and the subsequent antigen presenting function of thyroid epithelial cells. This phenomenon however was independent of the recognition of thyroid epithelial cells by T cells from TAZ10 mice.

I highlighted the central role played by IFNγ in the induction of the antigen presenting capacities of thyrocytes. A study on Schwann cells (SC) confirmed the important role of IFNγ (Kingston et al. 1989). If IFNγ alone could induce the expression of MHC-II molecules in 50% of SC, the addition of TNFα had a synergetic effect as 80% of the cells were expressing MHC-II molecules. Also, addition of activated T cells was capable to promote the neo-expression of MHC-II molecules by SC and their ability to promote a T cell response. This phenomenon was abrogated by the blocking of IFNγ, suggesting the important role of this pro-inflammatory cytokine in the upregulation of MHC-II by epithelial cells. Although I excluded the presence of APCs such as DCs and macrophages in TEC culture *in-vitro*, I wondered whether APCs that are not TEC could present the cryptic epitope from exogenous source of TPO antigens. This will be explored Paragraph 4-3/.

The destruction of thyrocytes by cytotoxic MHC-I molecule restricted - $CD8^+$ T lymphocytes has been shown in autoimmune diseases and thyroiditis (Sugihara et al. 1995).

However, in TAZ10 mice, $CD8^+$ T cells are not MHC-I restricted and recognize the TPO cryptic epitope when presented in the context of MHC-II molecules (Quaratino et al. 2004). Therefore, we could rule out any role played by $CD8^+$ TAZ10 T cells in the recognition and killing of thyrocytes. Indeed, we hypothesized that activated TAZ10 T cells initially infiltrating the thyroid would induce thyrocyte damage by the release of pro-inflammatory cytokines.

4-2-6/ Control of the survival of T cells and thyrocytes in inflammation

Although thyrocytes from patients with Grave's disease do not undergo apoptosis, Hashimoto's thyroiditis is characterized by the death of thyrocytes and the destruction of thyroid follicules. Both CD95 (Fas) and CD95L (Fas-L) are involved in the destruction of thyrocytes by apoptosis (Stassi et al. 2000). The destruction of thyroid epithelial cells in autoimmune thyroiditis has been described and includes the direct lysis of TECs by $CD8^+$ CTLs, thyrocytes fratricide or suicide (Reviewed in Stassi et al. 2002). To address the effect of pro-inflammatory cytokines on the induction of apoptosis in TECs, thyrocytes from WT mice were grown with or without a combination of pro-inflammatory cytokines: IFNγ at (200U/mL), TNFα (20ng/mL) and IL-1β (20ng/mL) for 3 days. Staining for TUNEL showed that TECs grown in medium only did not undergo cell-death. If TECs challenged with IFNγ showed some cell-death, the combination of IFNγ, TNFα and IL-1β drastically enhanced the amount of thyrocytes undergoing apoptosis (Figure 4-10).

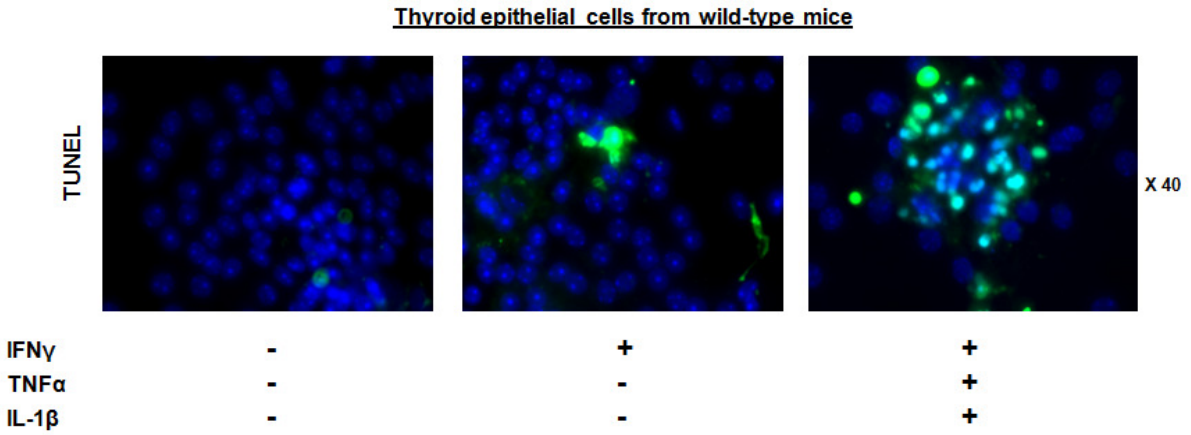


Figure 4-10: Thyroid epithelial cells from WT mice undergo apoptosis upon inflammation
Thyroid from wild-type (WT) mice were harvested and digested as previously described. Thyrocytes were grown on poly-L-Lysine coated coverslips for one day in complete medium and for a further 3 days with or without IFNγ (200U/mL), TNFα (20ng/mL) and IL-1β (20ng/mL). Apoptosis was detected by TUNEL (green) staining using a fluorescent microscope and nucleus was counterstained with DAPI (blue). Data shown is representative of two different experiments.

Interestingly, staining with TUNEL was not immediate and only occurred after 2- days of exposure with pro-inflammatory cytokines highlighting that thyrocytes are particularly resistant to apoptosis (Stassi et al. 2000).

The role of nitric-oxide (NO) has been largely documented in chronic inflammatory diseases and is associated with tissue destruction. Indeed, increased production of NO is associated with myelin damage in multiple sclerosis (Hill et al. 2004) and is associated with the destruction of pancreatic islets in a mouse model of diabetes (non-obese diabetes mouse model or NOD mice) (Lakey et al. 2001; Suarez-Pinzon et al. 2001).

I measured the amounts of NO released by thyrocytes upon challenge with IFNγ and a combination of IFNγ, TNFα and IL-1β (*yellow bars*; Figure 4-11). Thyrocytes grown in medium did not release NO. Addition of IFNγ led to a 5 fold increase of the production of NO while IFNγ, TNFα and IL-1β increased NO concentration by 20 fold. I then assessed whether there was any link between the amount of nitric-oxide secreted and the survival of thyrocytes using the MTT assay. In all cases, the iNOS inhibitor L-NMMA abrogated the release of NO by TECs. L-NMMA was able to decrease the apoptosis of TECs induced by IFNγ (*blue bars*; Figure 4-11) and to prevent the apoptotic effect on TEC mediated by IFNγ, TNFα and IL-1β. Together, these results confirm a small number of studies performed on human thyrocytes (Kasai et al. 1995; van den Hove et al. 2002).

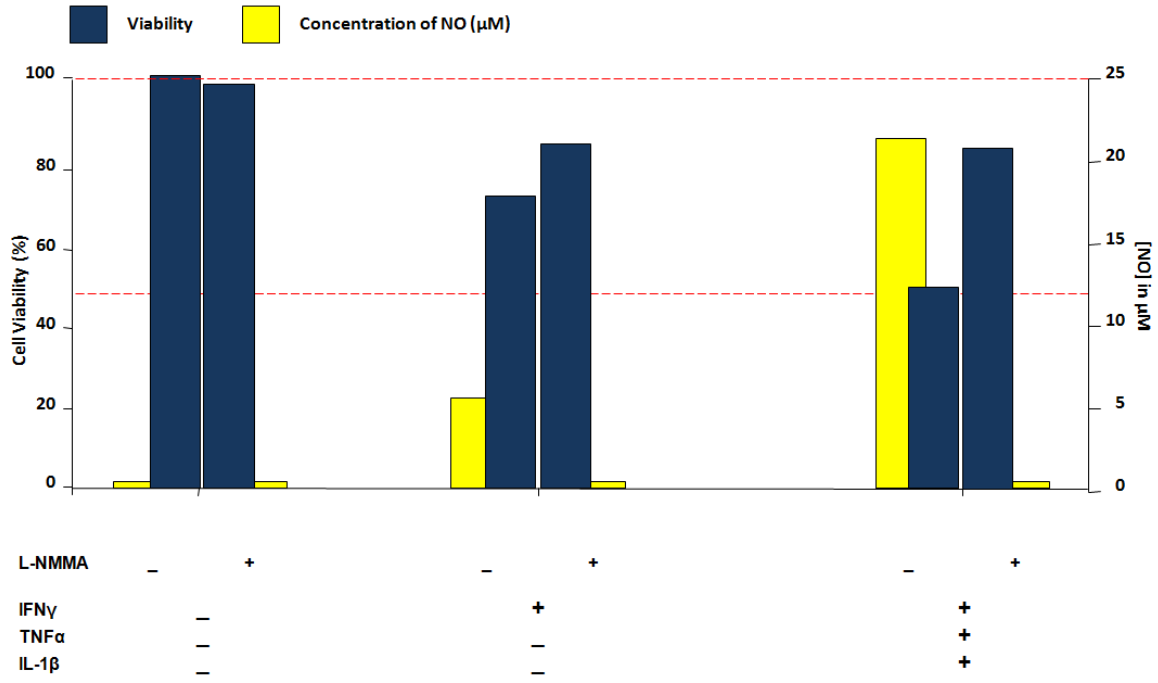


Figure 4-11: Thyroid epithelial cells from WT mice undergo apoptosis upon inflammation: Role of nitric-oxide (NO)
Thyroid from wild-type (WT) mice were harvested and digested as previously described. Thyrocytes were seeded in 96 well-plates for one day in complete medium and for a further 3 days with or without a combination of IFNγ (200U/mL), TNFα (20ng/mL) and IL-1β (20ng/mL). Cell survival was detected by mitochondrial respiration or MTT assay (blue bars). Amounts of NO released in the supernatant were measured using the Griess method (yellow bars). The cell viability in medium only was taken as the reference (100%). Data shown is representative of two different experiments.

Studies in experimental mice of autoimmune uveitis (EAU) have also highlighted the important role played by NO. Indeed, treatment of the mice with the iNOS inhibitor (L-NAME) delayed the onset while reducing the clinical signs of the disease (Hoey et al. 1997). This mechanism was IFNγ dependent. Interestingly, the apoptosis of cells from the retina by NO was shown to be mediated by Fas/Fas-L interactions and signaling pathways (Liversidge et al. 2002). I therefore assessed whether there was any link between the amount of NO released by thyroid epithelial cells and the levels of expression of Fas and Fas-L by flow cytometry analysis (Figure 4-12).

Constitutive expression of FasL on thyrocytes in human (Giordano et al. 1997; Stassi et al. 1999 and 2000) confers an immune privileged status to the thyroid by promoting the deletion of Fas-expressing activated T cells (Stassi et al. 1999). Therefore, the expression of Fas-L by human thyrocytes prevents the occurrence of autoimmune diseases. In mice however, although thyrocytes express high amounts of FAS mRNA (Tang et al. 2000; Wei et al. 2001 and 2003), they do not express either Fas or Fas-L at the protein level (Batteux et al.

1999, 2000; Tourneur et al 2001; Wang et al. 2002; Wei et al. 2001, 2003, 2004). Indeed, the transgenic expression of Fas-L on thyrocytes in mice restores immune privilege and prevents the occurrence of autoimmune thyroiditis (Batteux et al. 1999 and 2000; Tourneur et al. 2001). Therefore, the thyroid in mouse is not an immune-privileged tissue. I first assessed whether primary culture of TECs from WT mice expressed Fas and Fas-L.

In WT mice, I found that the majority of thyrocytes did not express Fas and Fas-L (Figure 4-12) molecules. 20% of TEC did however express Fas *in-vitro* (*red histograms*; Figure 4-12) and I suggested that the expression was the result of stress imposed on TEC during the culture. The majority of the cells did not however expressed Fas-L (*blue histograms*; Figure 4-12) and did not undergo apoptosis as described Figure 4-10 and 4-11.

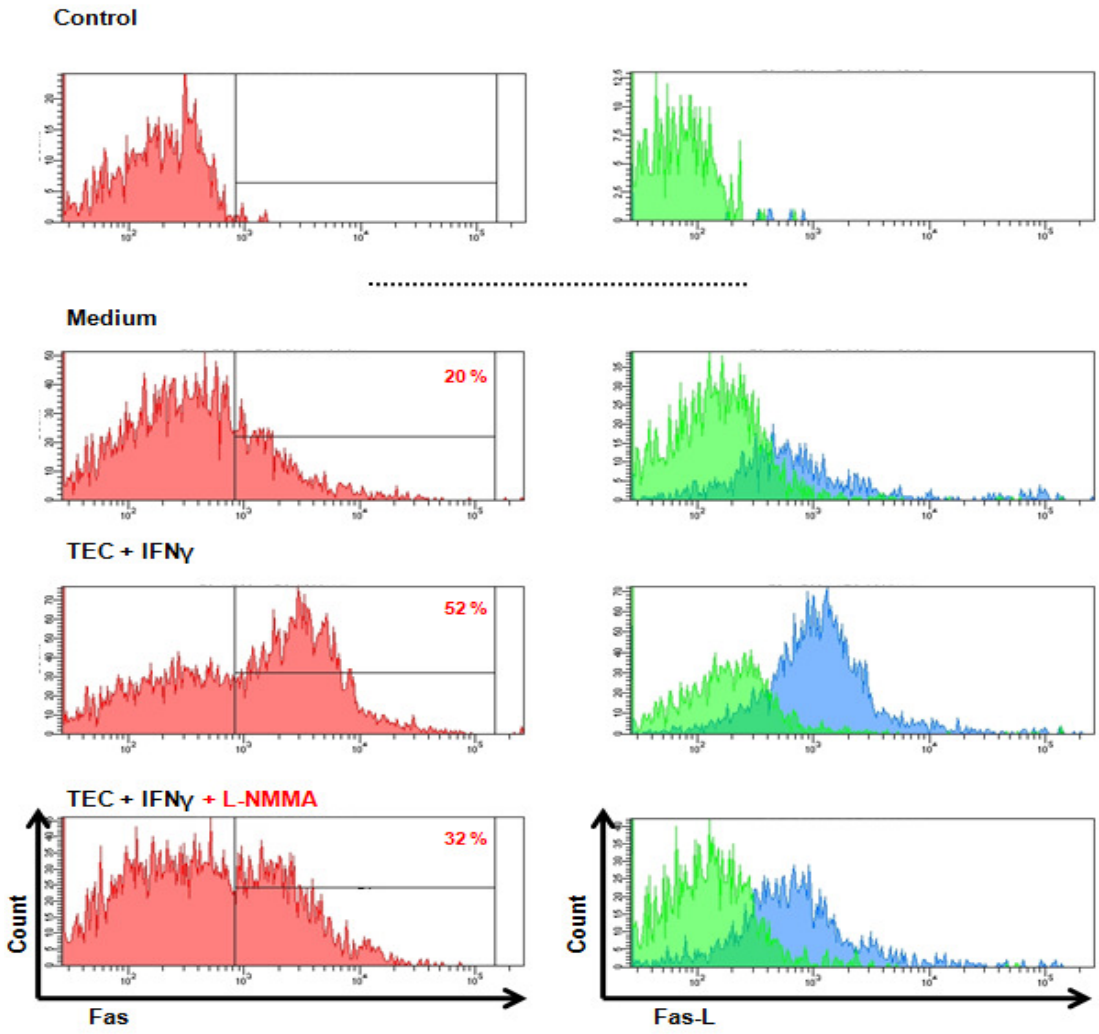


Figure 4-12: Upregulation of Fas and Fas-L by TEC upon challenge with IFNγ is dependent in the production of nitric oxide (NO)
Thyroids from wild-type (WT) mice were harvested and digested as previously described. Thyrocytes were seeded in 96 well-plates for one day in complete medium and for a further 3 days with or without a combination of IFNγ at 200U/mL. The level of expression of Fas was assessed by flow cytometry analysis (red histograms). Fas^+ and Fas^- TECs were further analysed for their expression of Fas-L (blue and green histograms respectively). To assess the role of nitric oxide we used L-NMMA, an inhibitor of NO synthase (5µM). Data shown is representative of two different experiments.

Upon challenge with IFNγ, 52% of TECs expressed high levels of Fas and Fas-L markers. Inhibition of the production of NO by TECs using the NOS inhibitor L-NMMA strongly decreased the expression of Fas by TECs down to 32%. This was associated with a decreased staining for Fas-L.

The destruction of thyrocytes by suicide or fratricide has been shown to be mediated by Fas / Fas-L interaction (Giordano et al. 1997; Stassi et al. 2000). Expression of Fas-L by TECs in human thyroid is the basis of the definition of immunoprivilege. Indeed Fas

molecules expressed by activated T cells binding Fas-L expressed by TECs leads to the deletion of the activated T cells; thus preventing the occurrence of AID. We wondered whether the increased expression of Fas and Fas-L led to an increased death of T cells from TAZ10 and WT mice (Scwab et al. 2005). Thyroid epithelial cells were cultured with or without IFNγ for three days and washed with medium to remove any traces of IFNγ. Target cells ($CD4^+$ T cells from TAZ10 mice) were labeled with the membrane dye PKH26 and co-cultured with TECs for 6 hours. At the end of the assay, the cells were harvested and labeled with the DNA probe YO-PRO at 1μM for 30 minutes on ice. Unlike the Chromium release assay, PKH26 stained both proliferating and non-proliferating cells. YO-PRO penetrated cells demonstrating some permeability and could therefore label dying and dead cells. These cells would not be all detected using the classical staining using AnnexinV (AnV) and propidium-iodine (PI). A second population expressing high amounts of YO-PRO was considered as dead cells that would be double positive for PI and AnV. Samples were analysed by flow cytometry. T cells were distinguished from TECs by their staining with PKH26 (Figure 4-13/A). We could exclude any contamination of TEC cells with PKH26 labelled T cells. $CD4^+$ T cells did not contain detectable amounts of dying cells (Figure 4-13/A) thus excluding a potential involvment of T cell suicide or T cell / T cell fratricide that have been previously described (Reviewed in Stassi et al. 2002).

Although histology analysis excluded the expression of Fas and Fas-L by murine TECs, the primary culture was leading to an increased expression of Fas by TEC. Indeed, after 6 hours of incubation, about 2.3-2.5% of WT and TAZ10 T cells stained for moderate levels of YO-PRO-1 demonstrating that TECs could induce the death of T cells within hours. Interestingly, TECs challenged with IFNγ for 3 days increased importantly the amount of dying T cells to 3% and 4.1% for WT and TAZ10 T cells respectively (Figure 4-13/B and C). We did not detect the death of thyrocytes after 6 hours thus confirming that they are more resistant to cell death than T cells (Stassi et al. 2000).

We showed that TECs upregulating Fas and Fas-L when challenged with pro-inflammatory cytokines are able to trigger the depletion of activated T cells. However, the expression of Fas and Fas-L by TECs leads to fratricide and thyrocyte destruction.

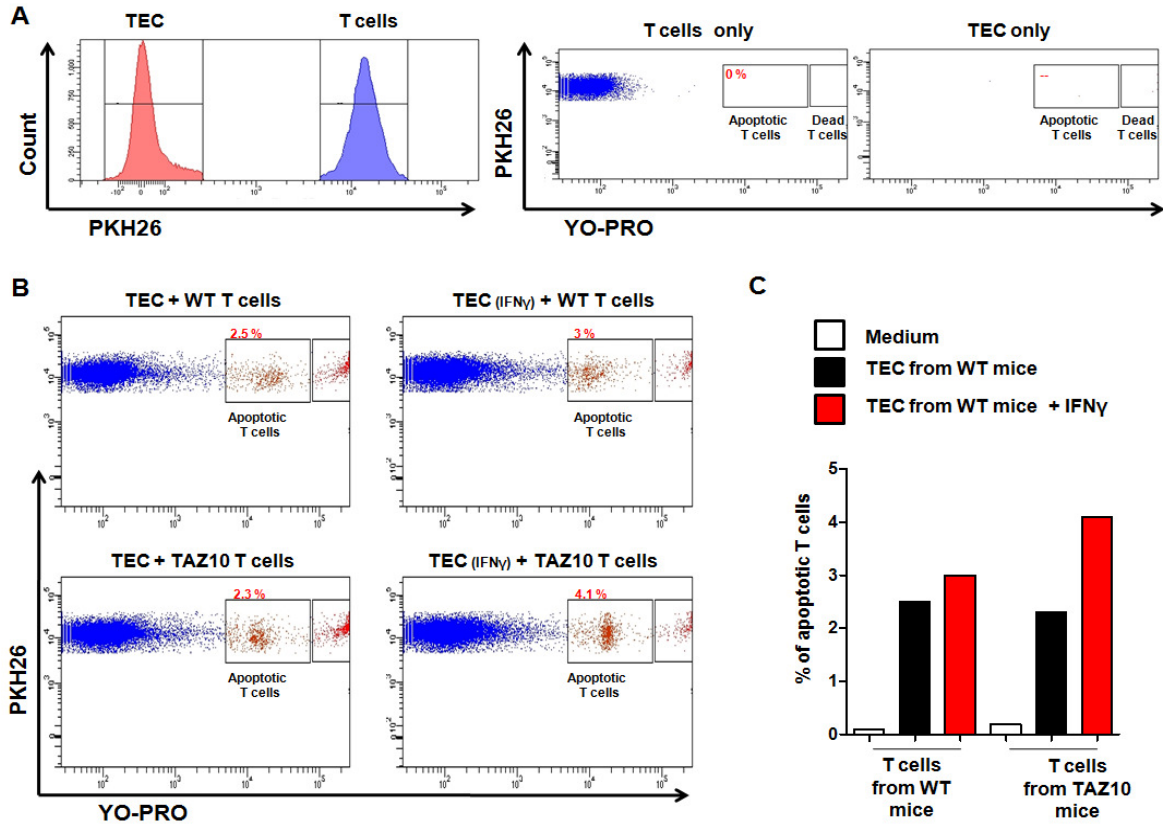


Figure 4-13: Thyrocytes promote the death of T cells from wild-type and transgenic mice
Thyroid from wild-type (WT) mice were harvested and digested as previously described. Thyrocytes were seeded in 96 well-plates for one day in complete medium and for a further 3 days with or without a IFNγ at 200U/mL. $CD4^+$ T cells from WT and TAZ10 mice were stained with PKH26 **(A)** and added to TECs for 6 hours. At the end of the assay, thyrocytes and T cells were harvested and apoptotic cells revealed by the staining with the nuclear probe YO-PRO **(C)**. TECs and T cells alone were used as negative controls **(A)**. Data were analysed by flow cytometry and analysed with BD FACS Diva software. Graphic representation of the evolution of the percentage of apoptotic T cells **(C)**. Data shown is representative of two different experiments.

We could observe an important infiltration of TAZ10 T cells in the thyroids of 3-4 months old TAZ-10 mice. Similarly, thyrocytes had undergone extensive apoptosis as revealed by the important TUNEL staining. The death of TAZ10 T cells was however not observed in histology in TAZ10 mice of 4 months of age (Quaratino et al. 2004) thus suggesting that the induction of T cell death in the thyroid could have occurred earlier in the development of thyroiditis. Indeed, these TAZ10 mice had extensive thyroid damage as the majority of TEC had undergone apoptosis.

4-3/ Results Part 2: Initiation of the T cell response in TAZ10 mice *in-vivo*

Transgenic T cells from TAZ10 mice recognize the human self peptide $TPO_{535-551}$ (Quaratino et al. 2004). Within this peptide, two different epitopes can be recognized by the transgenic TCR; the agonist epitope $TPO_{536-547}$ (Quaratino et al. 1996) and the antagonist Altered Peptide Ligand (APL) $TPO_{537-548}$ (Quaratino et al. 1995). The agonist epitope $TPO_{536-547}$ is the cryptic epitope resulting from the endogenous processing of the TPO protein and is processed and presented by TECs expressing MHC-II molecules (Quaratino et al. 1996). The antagonist epitope $TPO_{537-548}$ induces T cell-anergy and is generated by dendritic cells having uptaken TPO (Quaratino et al. 1995).

TAZ10 trangenic T cells also recognize the murine cryptic epitope $TPO_{524-535}$ and the antagonist $TPO_{525-536}$ APL (Quaratino et al. 2004). In a context of inflammation, thyroid epithelial cells are capable of presenting the $TPO_{524-535}$ cryptic epitope to transgenic T cells (Figure 4-7 and 4-9). However, I still wondered if TECs naturally produce the cryptic epitope from endogenous processing.

4-3-1/ Dendritic cells that have captured necrotic thyroid epithelial cells induce proliferation of both TAZ10 and wild-type T cells

To answer this question, I used the ability of professional antigen presenting cells to efficienty uptake, process and present exogenous antigens in the context of MHC-II molecules (Chapter 3-2). I first assess whether iDCs can mature when stimulated with necrotic TECs from WT mice. Semi-mature DCs obtained at day 11 using "Lutz's method" (Figure 3-2) were stimulated with LPS from *E.Coli* or necrotic TECs from WT mice at a DC:TEC of 2:1.

The addition of LPS increased the levels of expression of MHC-II and CD86 molecules (Figure 4-14/A) and the MHC-II^{hi} DCs represented 30% of all CD11c expressing mDCs (Figure 4-14/B). Interestingly, challenge of semi-mature DCs with necrotic TECs induced a much stronger maturation as MHC-II^{hi} DCs expressing high levels of the costimulatory molecule CD86 represented half of the CD11c+ DCs (Figure 4-14/A and B). Indeed, necrosis results in the release of proinflammatory signals such as dsDNA or HSP molecules able to activate an immune response via Toll-like receptors (Vabulas et al. 2001 and 2002). MHC-II^{hi}

DCs promote an efficient T cell response compared to MHC-II^{lo} DCs (Masurier et al. 1999). The large population of MHC-II^{hi} DCs obtained after maturation of semi-mature DCs using necrotic TECs from WT mice, led us to investigate their ability to induce the activation of transgenic T cells from TAZ10 mice.

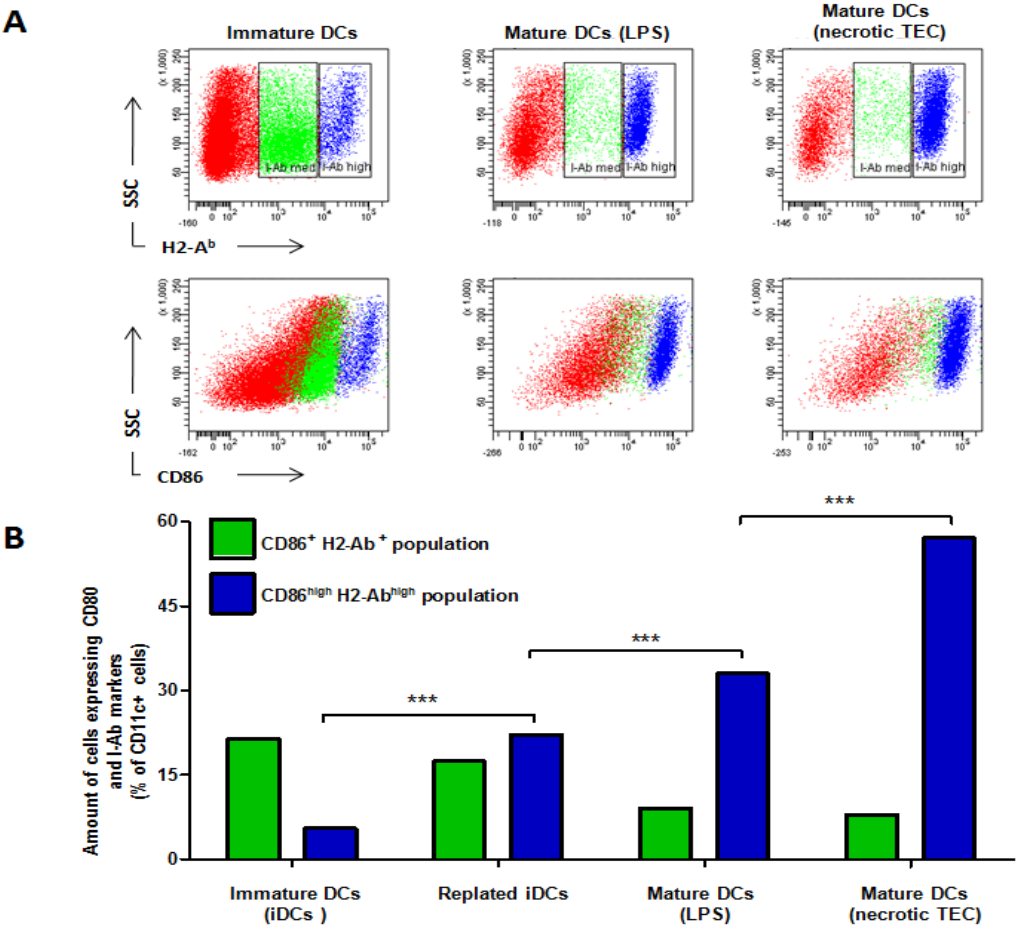


Figure 4-14: Necrotic thyroid epithelial cells (TEC) induce the full maturation of dendritic cells
Thyroid lobes from WT mice were digested as previously described and partially digested thyroid lobules seeded in 96 well-plates for 3 days. Plates were then spundown for 5 minutes at 1300 rpm and supernatant quickly harvested before they were stored for a couple of days at -80°C to mimick necrosis. mDCs generated as described previously following the "Lutz's method". Replated iDCs (semi-mature DCs) were matured overnight in the presence of LPS from *E.Coli* (0.5 µg/mL) or necrotic TECs at a DC:TEC ratio of 2:1. Cells were stained for H2-A^b and CD86 and analysed by flow cytometry **(A)**. Analysis of the proportion of CD86$^+$ H2-Ab$^+$ (green population) and CD86^{hi} H2-Ab^{hi} (blue population) populations **(B)**. Statistics were performed using the unpaired Student's t-test.

To assess the ability of dendritic cells to activate TAZ10 T cells after taking-up necrotic TECs, bone-marrow derived mDCs were fed overnight with necrotic thyroid epithelial cells from WT mice. CD4$^+$ T cells from WT and TAZ10 mice were added to the culture for a further three days and their proliferative activity measured by (^{3}H) thymidine activity (Figure 4-15).

DCs matured with necrotic TECs induced the proliferation of $CD4^+$ T cells from WT mice suggested the presence of a population of self-reactive T cells in WT mice. This response was inhibited when TECs were challenged with a combination of pro-inflammatory cytokines prior to the necrosis being induced (Figure 4-15). Indeed, several studies have highlighted the presence of self-reactive T and B cells in the T cell repertoire of a wild-type mouse where resolution of apoptosis is deficient and leading to secondary necrosis.

Transgenic $CD4^+$ T cells from TAZ10 mice proliferated when co-cultured with DCs fed with necrotic TECs. This response was further enhanced when TECs had previously been challenged with IFNγ (200U/mL), TNFα (20ng/mL) and IL-1β (20ng/mL) prior to the induction of necrosis (Figure 4-15). I could rule out that the proliferation of T cells from both WT and TAZ10 mice could be triggered by the necrotic TECs or DCs matured with LPS from *E.Coli* as they could not.

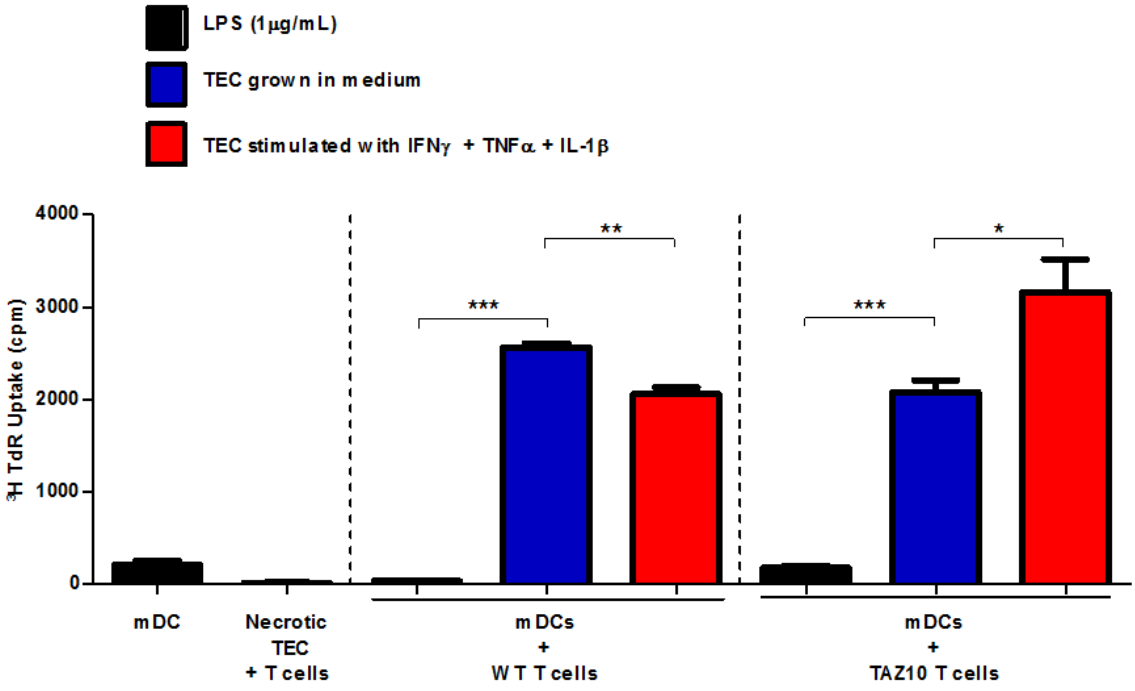


Figure 4-15: Mature dendritic cells (mDCs) present the TPO crypic epitope to TAZ10 T cells while activating the proliferation of T cells from WT mice
TECs from WT mice were digested and seeded in 96 well-plates for 3 days with or without a combination of IFNγ at (200U/mL), TNFα (20ng/mL) and IL-1β (20ng/mL). Plates were then spundown for 5 minutes at 1300 rpm and supernatant harvested before they were stored for a couple of days at -80°C to mimic necrosis. mDCs generated as described previously following the "Lutz's method". Replated iDCs (semi-mature DCs) were matured overnight in the presence of LPS from *E.Coli* (0.5 µg/mL) or necrotic TECs at a DC:TEC ratio of 2:1. $CD4^+$ T cells from WT and TAZ10 mice were added to the culture for a further 3 days and T cell proliferation was assessed by (^{3}H) thymidine incorporation for the last 8 hours of culture. Statistics were performed using the unpaired Student's t-test.

Although I demonstrated the ability of TECs to present the cryptic TPO epitope to T cells upon inflammation and subsequent upregulation of MHC-II and costimulatory molecules, the origin of the generation of the cryptic epitope remained elusive. The proliferation of TAZ10 T cells when co-cultured with mDCs matured with necrotic TECs from WT mice suggested that mDCs had presented the cryptic $TPO_{524-535}$ epitope to TAZ10 T cells. From this result, we showed that the cryptic $TPO_{524-535}$ is naturally produced by TECs. Therefore, the ability to induce the proliferation of TAZ10 T cells depends on the level of MHC-II molecules expressed in inflammatory conditions by TECs. This allowed mDCs to uptake dying TECs and present their antigens (including $TPO_{524-535}$) to T cells from WT and TAZ10 mice.

DCs taking-up necrotic TECs could be expected to take-up TPO protein and therefore to generate the antagonistic $TPO_{525-536}$ epitope to T cells (Quaratino et al. 1996). However, DCs were still able to induce the proliferation of TAZ10 T cells. Quantitative analysis of the two TPO peptides is under-way to correlate the forces present of anergy versus activation. This latest point will be considered and more detailed in the summary (Paragraph 4-4). Although TECs challenged with pro-inflammatory cytokines displayed antigen-presenting capacities, it seemed that IFNγ also enhanced the ability of TECs to generate the cryptic $TPO_{524-535}$ epitope as T cells from TAZ10 mice proliferated more.

In Chapter 3, we described the distribution of MDSCs in the spleen of TAZ10 and WT mice over time. Unfortunately, as mice were not weaned before 1 month of age, we could not evaluate the level of MDSCs in TAZ10 mice at the onset of the disease. This raised the question on the development of the disease and we needed to establish when TAZ10 T cells are activated, promote the destruction of the thyroid and the generation of autoimmune thyroiditis. Although the results previously obtained on the origin of the TPO cryptic epitope were encouraging, it was important to assess *in-vivo* the anatomical locations where the cryptic epitope is present in WT mice. This would allow the characterization of the triggers in TAZ10 mice models of autoimmune thyroiditis.

4-3-2/ The cryptic epitope $TPO_{524-535}$ is present in the thyroid and lymph-nodes draining the thyroid in WT mice

We first investigated whether we could define where the cryptic $TPO_{524-535}$ epitope was in wild-type mice. Indeed, it was important to assess whether the first activation of TAZ10 T cells occurred in the thyroid, the lymph-nodes draining the thyroid or other lymphoid or non lymphoid tissues. The thyroids, LN draining and not draining the thyroid, spleen and other tissues such as the trachea or the gut were isolated from 80 WT mice. After tissues were passed through a cell-strainer, cells were lysed in formic acid and boiled. Lysis-mixes were centrifuged to pellet the membranes and supernatant was passed through a 30 kDa cutoff filter by centrifugation. The different peptide extracts obtained were then tested for their ability to promote the proliferation of TAZ10 T cells with BM-DCs generated following "Lutz's method" used as APCs (Figure 4-16).

The peptide extraction requiring both lysis of the cells and the use of a 30 kDa Cutoff filter, we assessed the level of the TPO cryptic peptide that would be lost in the process. The Caco-2 colon carcinoma cell line was used as a control cell line and peptide extracts were done with or without the addition of the cryptic epitope at 1µg/mL. The proliferation of T cells from TAZ10 mice was assessed by (^{3}H) thymidine incorporation (*black and blue bars*; Figure 4-16/A and 4-17). During the peptide extraction, we lost some peptide as the proliferation decreased from about 60 to 80 % between mDCs pulsed with 1µg/mL of TPO peptide and mDCs pulsed with the peptide extracts of CaCo-2 cells supplemented with 1µg/mL of TPO peptide.

The presence of the cryptic epitope was then assessed in peptide extracts from the spleens, thyroids and LN draining the thyroid. As expected from the results previously obtained (Figure 4-15), TAZ10 T cells proliferated in response to mDCs pulsed with the peptide extract from the thyroid. Interestingly, extracts from the draining lymph-nodes also revealed the presence of the cryptic epitope thus confirming our previous results (Figure 4-15) that mDCs uptaking dying TEC and migrating to the peripheral lymphoid tissue were presenting the TPO cryptic epitope (Figure 4-16/A; *red bars*).

Although we could not exclude that intra-thyroidal APCs uptaking dying TECs would generate the antagonistic TPO peptide (Quaratino et al. 1996), these results confirmed that the TPO cryptic epitope is naturally generated by thyroid epithelial cells. To further assess the amount of the TPO cryptic epitope present in the thyroids or DLN, I evaluated the

proliferation of TAZ10 T cells per 1,000,000 cells used in the peptide extracts (Figure 4-16/B). For the same number of cells used to generate peptide extracts, thyroid cells did not induce a stronger proliferation of TAZ10 T cell than cells from the DLN.

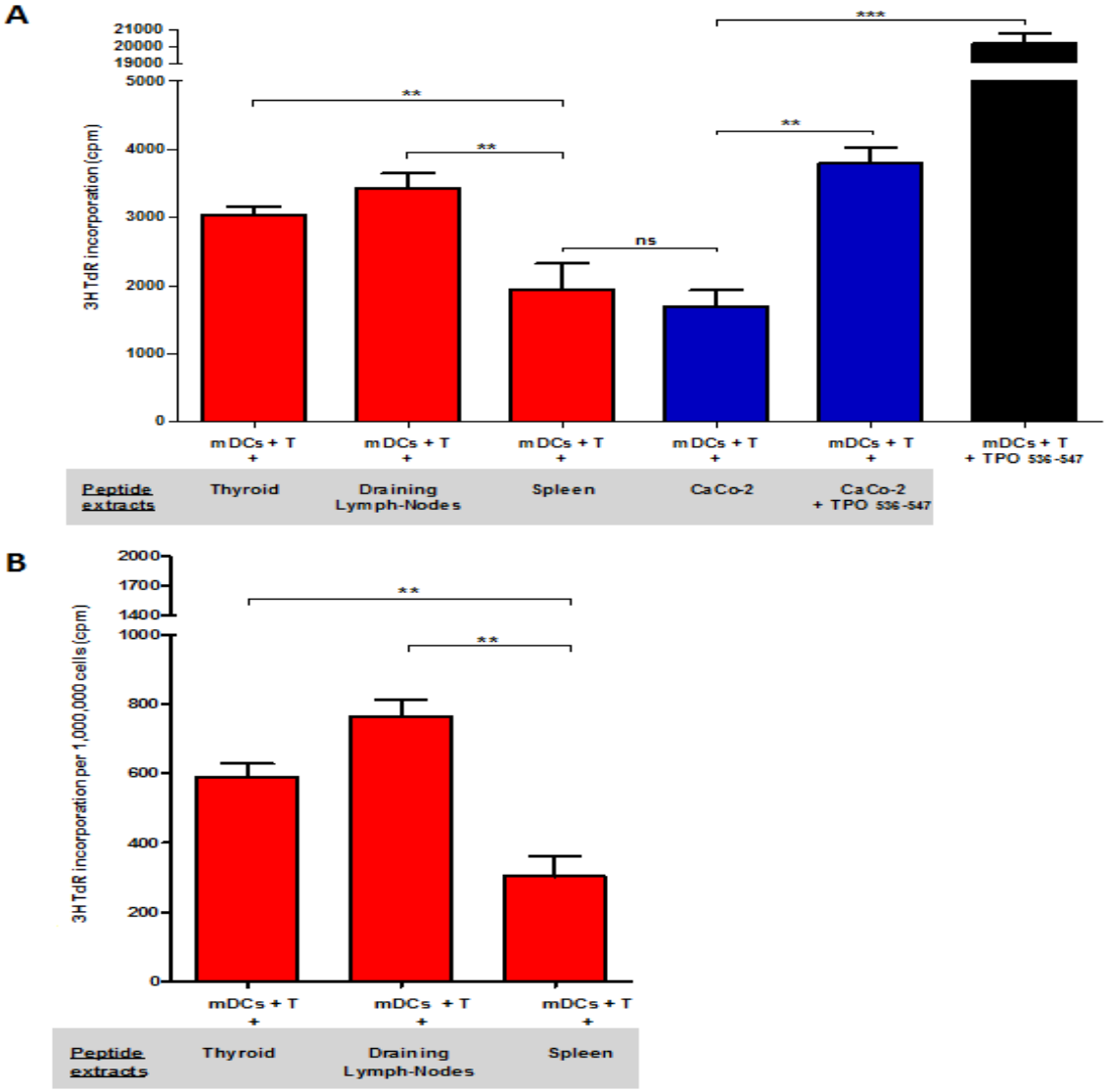


Figure 4-16: The cryptic epitope TPO$_{524-535}$ is present in the thyroid and lymph-nodes draining the thyroid of wild-type (WT) mice
Thyroids, cervical lymph-nodes draining the thyroid and spleens mice were isolated from up to 80 WT mice and peptides extraction performed. Proliferation of $CD4^+$ T cells from TAZ10 mice in response to the peptide extract was assessed by (^{3}H) thymidine incorporation. Mature bone-marrow derived dendritic cells from WT mice (mDCs) and expressing high levels of MHC-II molecules mice were used as APCs. Peptide extracts from Caco-2 and Caco-2 cells supplemented with the cryptic epitope at 1µg/mL were used as controls **(A)**. Proliferation of $CD4^+$ T cells from TAZ10 mice was evaluated per $1x10^6$ cells used to extract peptides **(B)**. Statistics were performed using the unpaired Student's t-test. Data shown is representative of two different experiments.

To ascertain that the proliferation of TAZ10 T cells I observed Figure 4-16 was in response to the cryptic TPO epitope and not the result of non-specific activation, I assessed the proliferative activity of TAZ10 T cells in response to mDCs pulsed with peptide extracts from the trachea and gut of WT mice (Figure 4-17). TAZ10 T cells did not proliferate in

response to mDCs pulsed with peptide extracts from spleens, guts and tracheas from WT mice thus demonstrating the specific T cell response to TPO524-535 observed Figure 4-16.

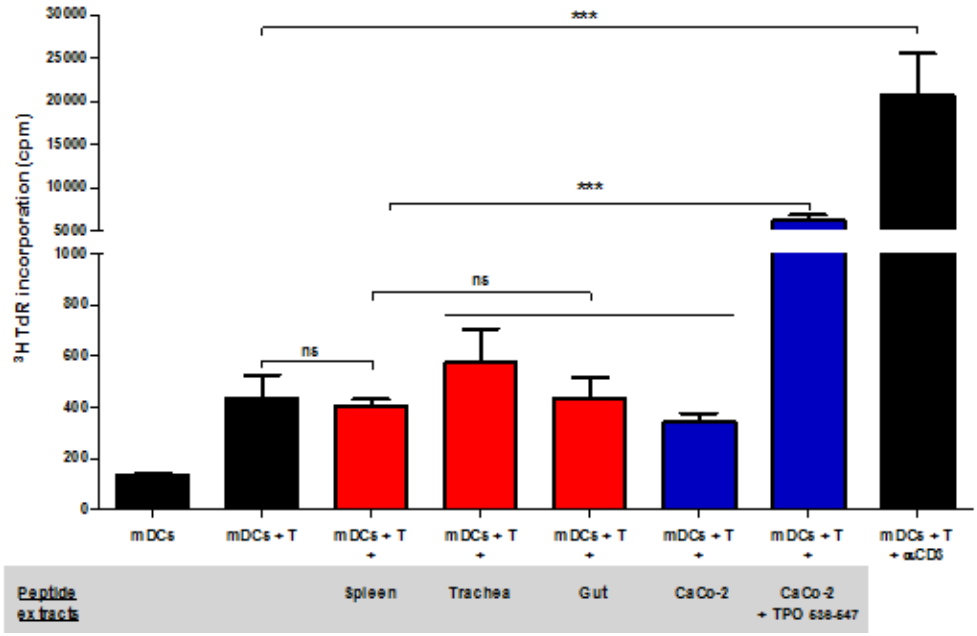


Figure 4-17: The cryptic epitope TPO524-535 is not present in the gut and trachea of WT mice
Spleens, trachea and guts from 80 wild-type (WT) mice were harvested and peptides extraction performed. Proliferation of $CD4^+$ T cells from TAZ10 mice in response to the peptide extract was assessed by (^3H) thymidine incorporation. Mature bone-marrow derived dendritic cells from WT mice were used as APCs. Peptide extracts from Caco-2 and Caco-2 cells supplemented with the cryptic epitope at 1µg/mL were used as controls. Statistics were performed using the unpaired Student's t-test.

In light with the results obtained previously on the origin of the cryptic TPO epitope, we ascertained that the cryptic epitope is generated by thyroid epithelial cells and presented to TAZ10 T cells by thyroid epithelial cells upon challenge with pro-inflammatory cytokines and by dendritic cells having taken up dying TECs. It therefore seemed that the physiological turnover of thyroid epithelial cells and their clearance by dendritic cells allows the spreading of the TPO cryptic epitope to cervical lymph nodes draining the thyroid.

Although the cryptic TPO524-535 epitope is naturally present in the thyroid and cervical lymph-nodes of WT mice, we wondered whether this epitope spreading naturally mediated by APCs could trigger the activation of TAZ10 T cells and the development of autoimmune thyroiditis.

4-3-3/ Spontaneous activation of self-reactive T cells *in-vivo*

TAZ10 T cells are reactive to the cryptic epitope $TPO_{524-535}$ present in the thyroid and cervical LN draining the thyroid of WT mice. We transferred CFSE labeled naïve T cells ($CD4^+$ $CD62L^{hi}$) from TAZ10 mice (TAZ10-T_N) into $Rag2^{-/-}$ mice. $Rag2^{-/-}$ mice were used as recipient as they are devoid of any lymphocytes and would allow for the important expansion of the $CD4^+$ T cells that may encounter the cryptic epitope $TPO_{524-535}$. Naïve T cells from WT mice (WT-T_N) were used as a control and transferred into $Rag2^{-/-}$ mice.

Seven days after adoptive transfer occurred, blood, spleen, thyroids, DLN and NDLN were analysed for the presence of donor T cells and CFSE dilution (Figure 4-18). Interestingly, T cells from WT mice could still be seen in the blood whereas transgenic T cells from TAZ10 mice were absent. This suggested that TAZ10-T_N had all migrated to peripheral lymphoid organs where they would be activated. Indeed even if T cells from WT and TAZ10 mice were detected in the spleens, DLN and NDLN of recipient mice, only transgenic T cells strongly proliferated as shown by the important CFSE dilution. However, a bigger number of transgenic T cells were proliferating in the DLN compared to NDLN and spleens. Transgenic T cells were present in the thyroid of $Rag2^{-/-}$ recipient after adoptive transfer with TAZ10-TN while no T cells from wild-type mice could be detected (Figure 4-18).

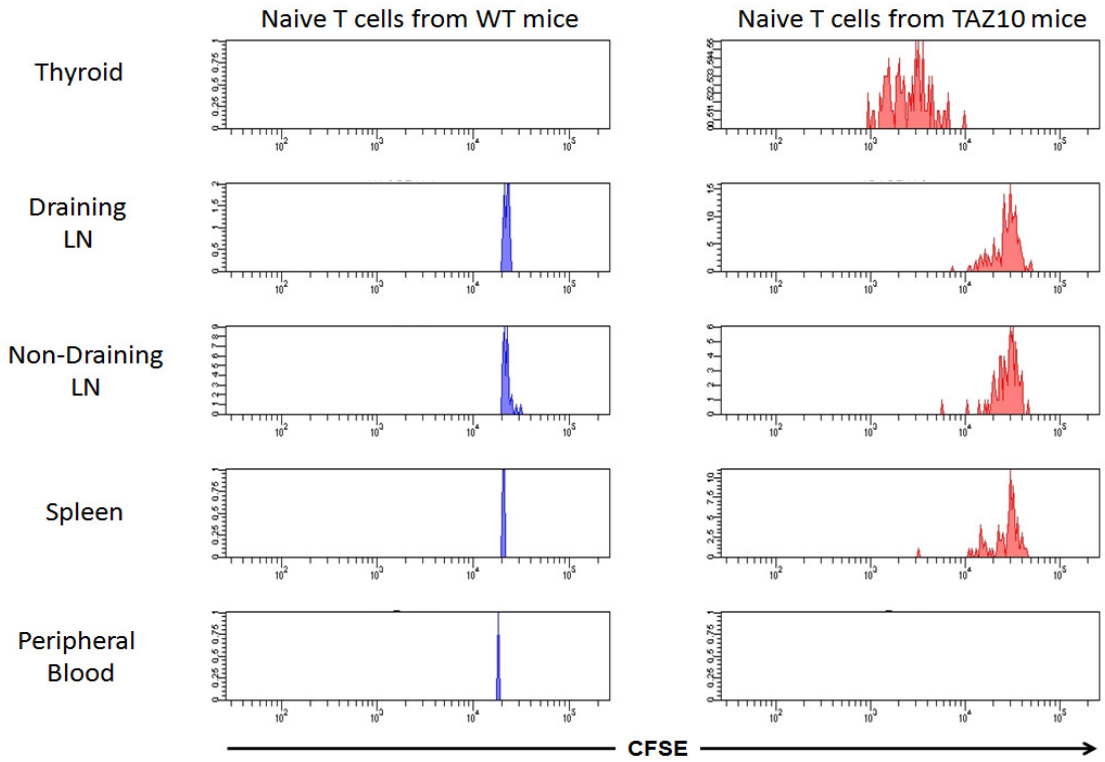


Figure 4-18: Naïve CFSE-labeled TAZ10 T cells proliferate in lymphoid organs of Rag2$^{-/-}$ mice
Rag2$^{-/-}$ mice were immunized with 2x10^6 naïve T cells from WT and TAZ10 mice. 7 days after the adoptive transfer occurred, thyroids, spleens, lymph-nodes draining and not draining the thyroid were isolated from the mice. Cells were stained for CD4, Vβ1 and analysed for the presence of T cells expressing CFSE by flow cytometry. Data shown is representative of 7 and 4 different mice for the adoptive transfer of naïve T cells from TAZ10 and WT mice respectively.

Interestingly, T cells from TAZ10 mice were detected in tissues other than the thyroid and DLN, suggesting that not all primarily activated CD4$^+$ T cells migrated to the thyroid but instead redistributed to TPO cryptic epitope-free lymphoid organs (Figure 4-16 and 4-17) such as the spleen and NDLN. This phenomenon suggested that the generation of memory TAZ10 T cells occurs early after initiation of the antigen immune response. This is consistent with other studies which have shown that CD8$^+$ T cell expansion can be detected as early as 3 to 4 days after the initiation of the immune response (Brinkman et al. 2008).

I then explored whether TAZ10-TN and WT-TN presented an activated phenotype by the analysis of the CD25 activation marker (Figure 4-19). T cells from wild-type mice that did not proliferate in recipient mice (Figure 4-18) did not express CD25 thus confirming their naïve phenotype (Figure 4-19). Upregulation of the CD25 marker indicated that the transgenic T cells present in peripheral lymphoid organs (Spleen, DLN and NDLN) had an activated phenotype (25 to 50%). Interestingly, the expression of CD25 on Transgenic T cells

was higher in DLN than NDLN (Figure 4-19/B) in all Rag2$^{-/-}$ recipient mice (7 in total) that had received TAZ10-TN.

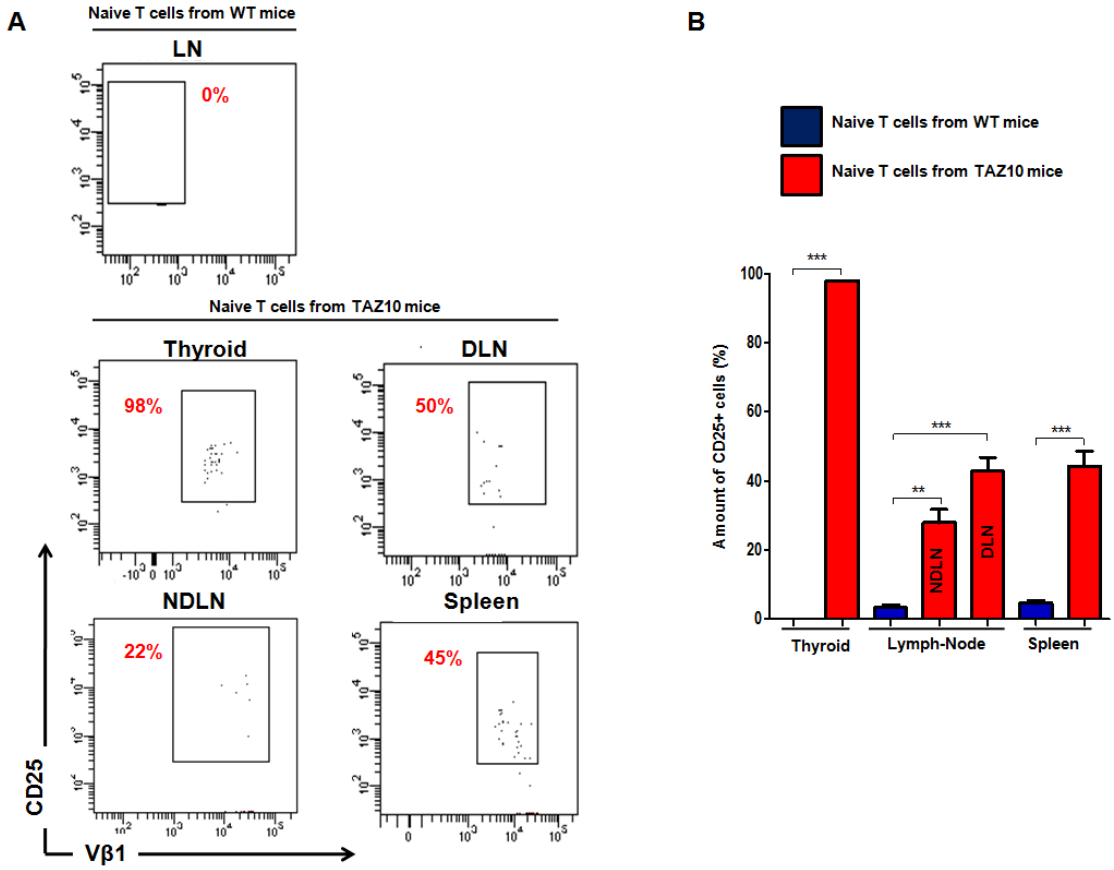


Figure 4-19: TAZ10 trangenic T cells have an activated phenotype in the thyroid and lymphoid organs of Rag2$^{-/-}$ mice

Rag2$^{-/-}$ mice were immunized with 2x10^{6} naïve T cells from WT and TAZ10 mice. 7 days after the adoptive transfer occurred, thyroids, spleens, lymph-nodes draining (DLN) and not draining (NDLN) the thyroid were isolated from the mice. Cells were stained for CD4, TCR-Vβ1 and analyzed for the levels of expression of CD25 by T cells expressing CFSE by flow cytometry. Data shown is representative of 7 and 4 different mice for the adoptive transfer of naïve T cells from TAZ10 and WT mice respectively **(A)**. Cumulative result showing the percentage of T cells expressing CD25 in the different tissues 7 days after adoptive transfer. Statistics were performed using the unpaired Student's t-test **(B)**.

The absence of CFSE^{+} cells in the thyroid suggested that WT-T$_N$ cells did not migrate to the thyroid (Figure 4-18). Only seven days after adoptive transfer, T cells from TAZ10 mice were present in the thyroid of Rag2$^{-/-}$ mice where they had clearly proliferated according to CFSE dilution. Interestingly, the lower fluorescence intensity of CFSE displayed by TAZ10 T cells from the thyroid suggested they did not migrate directly to the thyroid to proliferate. From this result, we could suggest that the activation of TAZ10-TN occurred primarily in the cervical lymph nodes draining the thyroid where the cryptic epitope TPO$_{524-535}$ is present. Activated transgenic T cells would then migrate to the thyroid where they undergo intensive proliferation. We previously demonstrated that the TPO cryptic epitope was present both in

the thyroid and cervical LN draining the thyroid (Figure 4-16 and 4-17). We could hypothesize that the spreading of the TPO cryptic epitope that occurs first in the thyroid allows the activation of TPO self-reactive T cells and the initiation of thyroiditis in TAZ10 mice

4-4/ Summary

In this chapter, we investigated the initial events leading to the activation of self-reactive T cells in the TAZ10 mouse model. Interestingly, this model spontaneously develops autoimmune thyroiditis in an environment devoid of any environmental insults (Quaratino et al. 2004). This point is of importance as infectious agents are sometimes considered as key in the development of autoimmune diseases by molecular mimicry, bystander activation, epitope spreading or crypticity (Paragraph 1-5/; Vandergult et al. 2002).

4-4-1/ Physiological turnover of TECs and epitope spreading initiate autoimmune thyroiditis

We first established the origin of the $TPO_{524-535}$ cryptic epitope homologous to the human cryptic epitope $TPO_{536-547}$. Although TECs from TAZ10 mice and TECs from WT mice challenged with IFNγ could induce the specific activation of T cells from TAZ10 mice, we did not know whether TECs were naturally generating the $TPO_{524-535}$ cryptic epitope.

The role of IFNγ in the pathogenesis of thyroiditis is controversial (Fang et al. 2007). Indeed, G-EAT mice with a defect in IFNγ develop thyroiditis characterized by an important eosinophil infiltration while G-EAT mice with functional IFNγ genes show no involvement of eosinophils when developing the disease (Tang et al. 1998). WT mice constitutively expressing IFNγ in the thyroid also develop hypothyroidism (Caturegli et al. 2000). This resulted from the lack of expression of the sodium iodide transporter gene induced by IFNγ and confirmed previous studies showing that IFNγ downregulates thyroid specific genes (Mori-Aoki et al. 2000; Tang et al. 1995; Gerard et al. 2006).

However, constitutive overexpression of IFNγ in NOD.H-2h4 mice susceptible to thyroiditis upon immunization with mouse thyroglobulin, promoted the expression of Class-II molecules by TECs without the development of the disease. $IFN\gamma^{-/-}$ NOD.H2h4 mice immunized with mouse thyroglobulin had a suppressed B and T cell response that was restored with blockade of IFNγ *in-vivo* using a monoclonal Ab (Barin et al. 2003). Despite this negative effect on lymphocytes, the action of IFNγ on thyrocytes was essential for the development of the disease in NOD.H-2h4 mice upon immunization with mouse thyroglobuline (Yu et al. 2006).

Histological studies have highlighted the presence of IFNγ expressing lymphocytes in the thyroid of patients with thyroiditis (Hamilton et al. 1991). IFNγ induces the expression of MHC-II molecules in epithelial cells such as hepatocytes (Herkel et al. 2003), bronchial cells (Suda et al. 1995), mesenchymal stem cells (Chan et al. 2006), scwann cells (Kingston et al. 1989) and thyrocytes (Todd et al. 1995; Maile et al. 2000). Transgenic mice constitutively expressing MHC-II molecules in the thyroid do not develop thyroiditis (Li et al. 2004). This suggested that the expression of MHC-II molecules was not sufficient to induce the disease but increased the severity of the disease after onset (Kinura et al. 2005).

TECs from WT mice do not express MHC-II molecules whereas TECs from young TAZ10 mice do. We could induce the expression of MHC-II molecules by TECs from WT mice upon challenge with IFNγ or a combination of IFNγ, TNFα and IL-1β. This suggested that MHC-II upregulation was a secondary phenomenon resulting from the presence of activated lymphocytes secreting cytokines in the thyroid of TAZ10 mice (Yue et al. 1998). If the induction of the antigen-presenting capacities of TECs by IFNγ was necessary for the efficient stimulation of transgenic T cells from TAZ10 mice, TECs constitutively generate the cryptic epitope $TPO_{524-535}$ (Figure 4-20/A). We could confirm this as T cells from TAZ10 mice could proliferate to peptide extracts from the thyroids of WT mice when presented by dendritic cells in the context of MHC-II molecules. In a context of inflammation, TECs upregulate the expression of costimulatory and MHC-II molecules allowing the presentation of the cryptic epitope $TPO_{524-535}$ to T cells from TAZ10 mice (Figure 4-20/B).

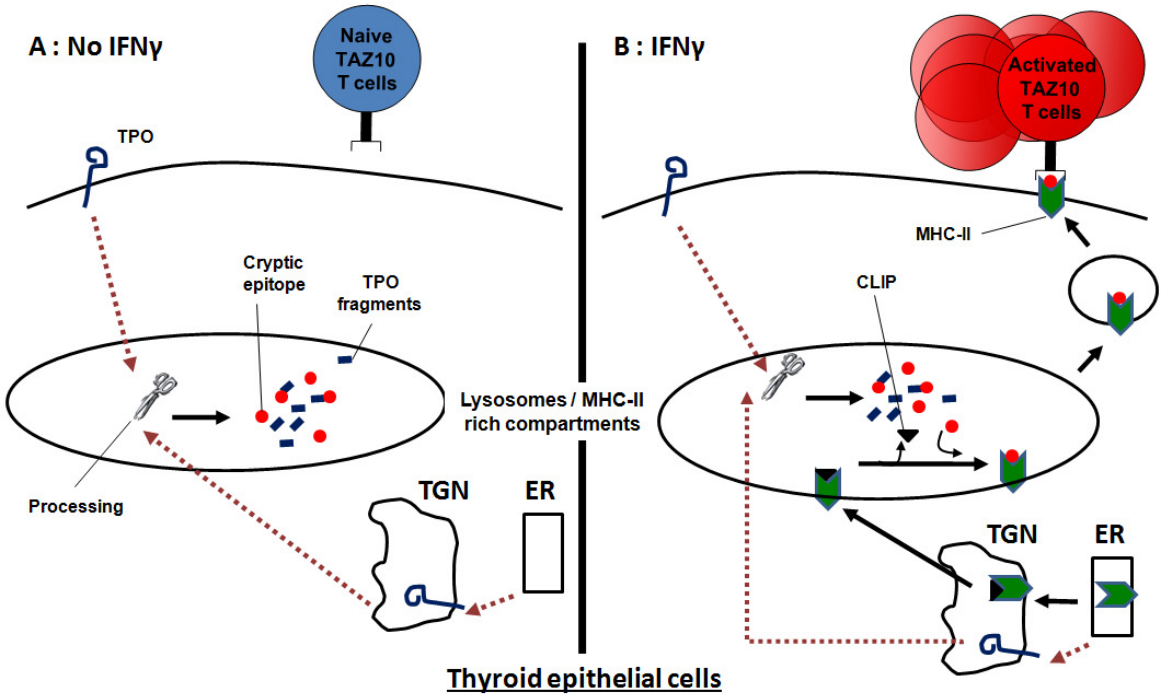


Figure 4-20: Thyrocytes naturally generate the cryptic epitope TPO524-535
(A) Thyroid epithelial cells (TECs) express TPO at their apical membrane. Turnover of the TPO protein leads to its degradation where the cryptic TPO epitope TPO524-535 (red dots) is generated. The absence of MHC-II molecules by TECs does not allow the cryptic epitope to be presented to T cells from TAZ10 mice. **(B)** Upon challenge with pro-inflammatory cytokines such as IFNγ, TECs express MHC-II molecules in the endoplasmic reticulum (ER) before being exported to the lysosomes/MHC-II rich compartment via the trans-Golgi network (TGN). MHC-II molecules exchange the CLIP peptide (black triangle) with the cryptic epitope. MHC-II molecules associated with the cryptic epitope are exported at the cell surface where they are recognized by T cells from TAZ10 mice.

Several studies have stressed the role of dendritic cells in the development of the disease. Immunization of mice models of thyroiditis with dendritic cells loaded with necrotic TEC have been shown to considerably accelerate the disease (Ma et al. 2005; Li et al. 2006). It is now established that immature dendritic cells are present in healthy thyroid and they regulate the growth of TECs via different cytokines such as IL-6, IL-1β or TNFα. This cross-talk between TECs and DCs has also been shown to be important in maintaining DCs in their immature state (Croizet et al. 2000 and 2001; Mooij et al. 1994, Simons et al. 1998). We suggested that the physiological turnover of TECs in the thyroid results in the engulfment of dying TECs by intra-thyroidal DCs. The break of the cross-talk between TECs and DCs resulting from the death of thyrocytes would lead to the maturation and the migration of dendritic cells to the cervical DLN. mDCs would then present the cryptic epitope and process the glycoprotein TPO that are naturally present in dying TEC (Figure 4-21).

Because wild-type mice do not develop thyroiditis, the essential difference with the transgenic model was the presence of T cells expressing the TCR specific for the TPO cryptic epitope. Interestingly, the presence of TPO524-535 was also detected in the cervical LN

draining the thyroid but not in the non-draining lymph-nodes, the spleen or other tissues such as the gut or the trachea of WT mice. We could suggest that TECs dying as a result of physiological turnover were taken-up by intra-thyroidal dendritic cells that would present the cryptic epitope to T cells after migrating to the cervical lymph-nodes.

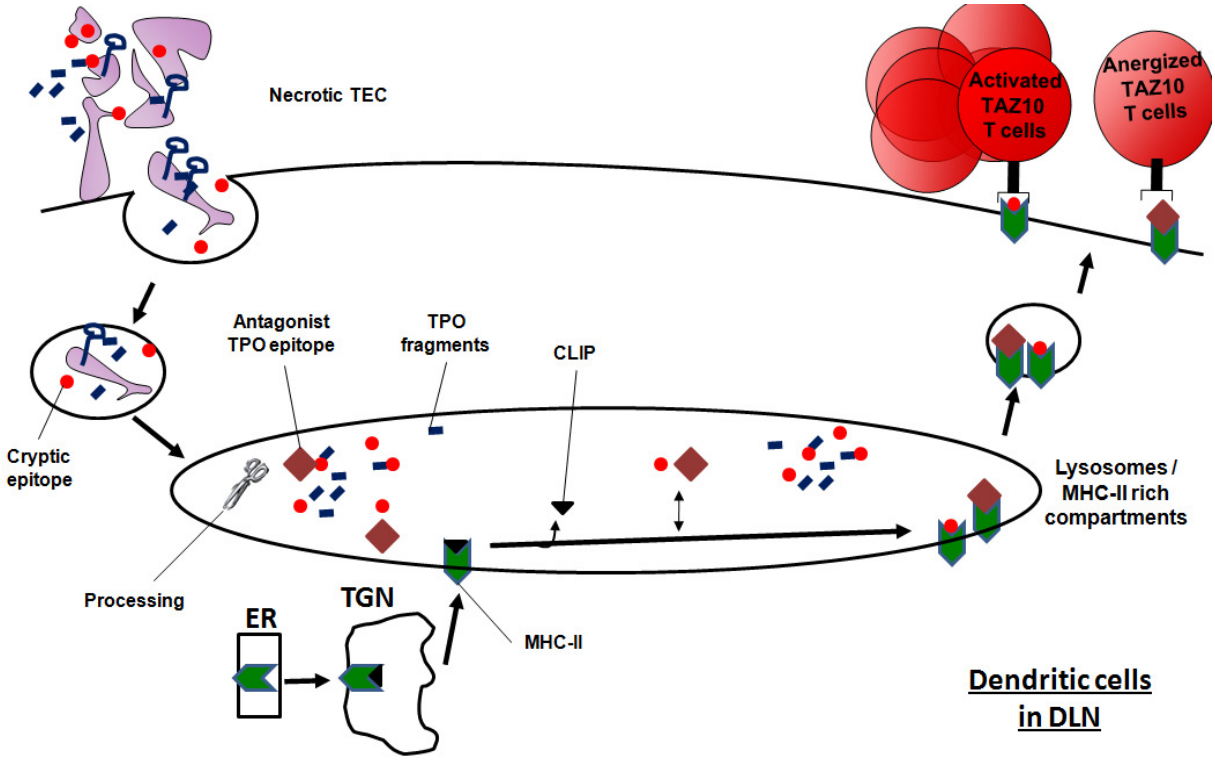


Figure 4-21: Dendritic cells taking-up dying thyrocytes present the antagonistic epitope TPO$_{525-536}$ and the cryptic epitope TPO$_{524-535}$ in the context of MHC-II molecules
Dying thyroid epithelial cells (TECs) containing the cryptic epitope TPO$_{524-535}$ (red dots) and the TPO glycoprotein are engulfed by resident dendritic cells. These dendritic cells migrate to the lymph node draining the thyroid (DLN) where they present the cryptic epitope to TAZ10 T cells promoting their differentiation and proliferation. DCs also process the TPO glycoprotein to generate the antagonistic epitope TPO$_{525-536}$ (brown square).

Dendritic cells migrating to the DLN allow the spreading of the TPO cryptic epitope to induce the activation and proliferation of T cells from TAZ10 mice. Activated transgenic T cells expressing high levels of CD25 then migrate to the thyroid where they undergo high proliferation. Although we are currently investigating the different subsets of helper T cells generated following the initial priming of naïve transgenic $CD4^+$ T cells, the vigorous proliferation of transgenic T cells is associated with the important release of pro-inflammatory cytokines. In this environment, TECs express high levels of Fas and Fas-L leading to the fratricide and suicide of TECs via in part on the release of nitric-oxide (Wang et al. 2002; Bretz et al. 1999; Hoey et al. 1997). TECs expressing Fas-L also promote the

death of the activated lymphocytes (expressing high levels of Fas molecules) that have infiltrated the thyroid.

Histological analysis of the thyroid in 4 months old TAZ10 mice reveals the extensive damages inflicted on the thyroid. All TECs have undergone apoptosis and thyroids present an important infiltrate of $CD4^+$ and $CD8^+$ T cells (Quaratino et al. 2004). The absence of T cells undergoing apoptosis in these mice does not exclude that this phenomenon does not occur in the first few weeks after transgenic T cells are first invading the thyroid. We still need however to assess the level of the antagonistic versus cryptic epitope present in the thyroid and DLN to clearly define the forces of anergy versus activation facing transgenic T cells.

4-4-2/ Mechanisms of peripheral tolerance fail to prevent the development of autoimmune thyroiditis

Naïve T cells from TAZ10 mice showed extensive proliferation 7 days after $Rag2^{-/-}$ mice were immunized. The rapid spontaneous development of AID in TAZ10 mice raises the question on the failure of mechanisms of peripheral tolerance to prevent the activation of self-reactive T cells from TAZ10 mice.

No detrimental effects on the expression of the cryptic epitope $TPO_{524-535}$ by TEC upon challenge by IFNγ was observed in our assays as TECs from WT mice challenged with IFNγ could present the cryptic TPO epitope to T cells from TAZ10 mice. While the activation of CIITA following stimulation with IFNγ promoted the expression of MHC-II and costimulatory molecules (Figure 4-4 and 4-6) it has been reported that IFNγ also suppressed the expression of thyroid specific genes such as TSH-R, thyroglobulin and TPO both in-vitro (Mori-Aoki et al. 2000; Tang et al. 1995; Gerard et al. 2006) and in-vivo (Caturegli et al. 2000, Gerard et al. 2006). TAZ10 mice have been shown to have increased serological amounts of TSH that was associated with a decreased amount of T4 (Quaratino et al. 2004). In contrast to IFNγ, TSH has been shown to decrease the expression of MHC-I and MHC-II molecules while promoting the expression of thyroid specific genes (Tang et al. 1995. Gerard et al. 2006). Interestingly, the upregulation of TPO mediated by TSH can be inhibited by IFNγ (Ashizawa et al. 1989; Asakawa et al. 1992) and other pro-inflamatory cytokines such as IL-1β and IL-6 (Chiovato et al. 1991). These mechanisms would therefore have a protective role to control the presentation of self-antigens to T cells in the context of both

MHC-I and MHC-II molecules; thus highlighting its importance for the maintenance of peripheral tolerance in the thyroid.

However, this process is not immediate and human thyroid epithelial cells have been shown to co-express for some time both MHC-II molecules and TPO thus promoting the presentation of cryptic epitope in the context of MHC II molecules (Chiovato et al. 1994). Indeed, we have shown that infiltration, proliferation and activation of T cells from TAZ10 mice occur in the thyroid within a week of the adoptive transfer of naïve T cells from TAZ10 mice. Also, within three weeks of age, TAZ10 mice had a high serological concentration of TSH; this was abolished by the *in-vivo* depletion of $CD4^+$ Tcells (Ester Badami PhD Thesis, University of Southampton, UK).

The control of expression of both MHC-II molecules and TPO by epithelial cells in relation to the increased concentration of TSH and inflammatory cytokines such as IFNγ in TAZ10 mice has therefore two consequences. During the early stages of the disease TECs would express both MHC-II molecules and TPO thus favoring the presentation of the cryptic epitope to T cells thereby promoting the inflammatory process. This mechanism would however fade away with the concentration of pro-inflammatory cytokines (such as IFNγ) increasing in the thyroid as a result of the important infiltration of the thyroid with activated transgenic T cells. Increased concentration of pro-inflammatory molecules favors the expression of MHC-II molecules by TECs while abrogating the TSH induced up-regulation of TPO expression. However, we do not yet know the impact of these mechanisms on the development of thyroiditis in TAZ10 mice.

In human, the thyroid is considered to be an immune-priviledged tissue. Indeed human thyrocytes constitutively expressing Fas-L molecules induce the apoptosis of infiltrating activated self-reactive T cells that express Fas (Stassi et al. 1999). Thyrocytes in mouse do not constitutively express Fas or Fas-L (Batteux et al. 1999, 2000; Tourneur et al 2001; Wang et al. 2002; Wei et al. 2001, 2003, 2004). We showed that TECs from WT mice stimulated with IFNγ upregulate Fas and Fas-L and induce the death of T cells from TAZ10 mice (Figure 4-13). This however implies that the activation of self-reactive T cells and their migration to the thyroid have previously occurred. Furthermore, the death of thyrocytes is significant when challenged with pro-inflammatory cytokines such as IFNγ, TNFα and IL-1β (Figure 4-10). Histological analysis of the thyroid of 4-months old TAZ10 mice reveals that T cells are not in contact with TECs and do not undergo apoptosis in 4 months-old TAZ10

mice (Quaratino et al. 2004). This suggest that the induction of apoptosis of T cells from TAZ10 mice has occurred earlier but could not overcome the strong accumulation of highly activated self-reactive T cells.

We previously showed that highly inhibitory MDSCs accumulate in TAZ10 mice with thyroiditis (Chapter 3). Although we are still investigating whether the recruitment of MDSCs in the DLN occurs as soon as T cells from TAZ10 mice are activated in the DLN or at the first signs of thyroid damage, MDSCs are not capable of preventing the activation of self-reactive T cells. It could be possible that the inhibitory activity on T cells displayed by MDSCs is overpowered by the strong activation of T cells from TAZ10 mice upon recognition of the cryptic epitope.

Previous work (Badami et al. 2005 and Badami E, Cexus O, Londei M and Quaratino S. Manuscript in preparation) has revealed that $CD4^+$ $CD25^{hi}$ $Foxp3^+$ Tregs are not present at the site of inflammation (the thyroid) and the cervical lymph nodes. Indeed, Tregs die by AICD upon recognition of TPO epitopes when presented by mature DCs. Interestingly, only mDCs pulsed with the TPO protein could induce the death of Tregs. mDCs pulsed with the TPO cryptic epitope resulted in the loss of expression of Foxp3 that was not associated with the death of the cells. It was therefore suggested that mDCs unmasking the antagonistic $TPO_{525-536}$ epitope would induce the conversion of Tregs into Teff. This phenomenon has been previously observed with the reprogrammation of antigen-specific $Foxp3^+$ Tregs to Th17 cells effector cells (Radhakrishnan et al. 2008). Interestingly mDCs uptaking dying TECs from WT mice previously challenged with IFNγ are able to present the $TPO_{524-535}$ cryptic epitope to T cells from TAZ10 mice. This was confirmed as we showed that the cryptic epitope $TPO_{524-535}$ is naturally present in the thyroid and cervical lymph nodes of WT mice but not in other tissues. However, it is likely that mDCs fed with dying TECs generate the antagonist epitope $TPO_{525-536}$ along with the cryptic epitope $TPO_{524-535}$ (Quaratino et al. 1996; Quaratino et al 2000). Therefore mDCs migrating from the thyroid to the cervical lymph-nodes could either induce the death or Tregs or induce their convertion to Teff. To prove this, we need to assess the levels of both $TPO_{524-535}$ and $TPO_{525-536}$ both in the thyroid and DLN by functional assays and mass-spectometry on peptide extracts from thyroids and DLN.

5/ Role of tissue transglutaminase 2 (TG2) in chronic inflammatory diseases

5-1/ Introduction

I have previously highlighted the important role played by professional and non-professional antigen presenting cells in the pathogenesis of thyroiditis in the TAZ10 transgenic mouse model spontaneously developing autoimmune thryoiditis (Chapter 4). The physiological turnover of TECs is at the origin of the development of autoimmune thyroiditis in the DLN. Indeed, professional APCs are responsible for the spreading of the cryptic epitope $TPO_{524-535}$ from the thyroid to the DLN resulting in the strong activation of transgenic T cells from TAZ10 mice. The infiltration of the thyroid with highly proliferative activated transgenic T cells promotes the death of thyrocytes. Activated lymphocytes in the thyroid releasing pro-inflammatory cytokines such as IFNγ trigger the destruction of the thyroid that is achieved in almost 100% of TAZ10 mice at 4 months of age (Quaratino et al. 2004).

It is now established that an impaired clearance of apoptotic cells by APCs such as macrophages is an important factor involved in the pathogenesis of autoimmune diseases such as SLE (Gaipl et al. 2007). I previously highlighted the important role played by MFG-E8 in the engulfment of apoptotic bodies by APCs. Indeed impaired MFG-E8 function promotes the activation of self-reactive T and B cells associated with the production and deposition of antibodies to dsDNA into the kidney and the development of autoimmune diseases (Hanayama et al. 2002 and 2004; Asano et al. 2004). Interestingly a recent study showed that MFG-E8 binds tissue transglutaminase 2 (TG2) (Toth et al. 2009), a pleiotropic enzyme expressed in many cell-types and upregulated upon inflammation (Lorand et al. 2003; Ientile et al. 2007). Indeed, the formation of a complex between TG2 and MFG-E8 is necessary for the efficient recognition and phagocytosis of apoptotic bodies by macrophages (Toth et al. 2009)

TG2 has been linked with the development and resolution of apoptosis in normal physiological conditions. TG2 promotes the formation of apoptotic bodies by cross-linking cytoplasmic actin (Nemes et al. 1997; Melino et al. 1994) and the exposure of phosphatidyl-serine by apoptotic cells (Sarang et al.2007). TG2 also has an important role in regulating the anti-inflammatory response (Falasca et al. 2005) while being involved in the engulfment of apoptotic bodies by macrophages (Szondy et al. 2003; Toth et al. 2009).

In this respect, treatment with the TG2 inhibitor cystamine (Jeitner et al. 2005) has been shown to be beneficial in the NZB/W F1 mouse model of SLE. The downregulation of TG2 activity in treated mice was coupled with a reduced expression of pro-inflammatory cytokines such as TNFα involved in the pathogenesis of SLE (Hsu et al. 2007). Mice injected with cystamine also presented a reduced activity of matrix metalloproteinase 9 (MMP-9) and decreased level of autoantibodies in the serum (Hsu et al. 2007). Treatment of NZB/W F1mice with cystamine led to a reduction of apoptosis in the liver and the brain which was associated with a decreased expression of important pro-inflammatory proteins such as COX-2 or iNOS (Hsu et al. 2008; Tzang et al. 2008; Wang et al. 2009).

These results prompted me to start analyzing the potential role played by TG2 in TAZ10 transgenic mice characterized by the destruction of thyrocytes (Quaratino et al. 2004). I previously highlighted the role of pro-inflammatory cytokines such as IFNγ in inducing the apoptosis of thyrocytes via the release of NO and the induced-expression of Fas and Fas-L (Figure 4-9, 4-10 and 4-11). By fluorescence microscopy, I analyzed the impact of inflammation on the expression and activity of TG2 by thyrocytes. TECs from WT mice presented a basal expression level of TG2 *in-vitro*. Upon challenge with IFNγ for 3 days, TECs presented an increased TG2 expression (Figure 5-1/A) and activity. To ascertain this, we analyzed the expression of TG2 mRNA in different tissues from WT and TAZ10 mice (Figure 5-1/C). If no difference in the expression of TG2 mRNA could be detected in the lungs, guts and brains of WT and TAZ10 mice, thyroids from TAZ10 mice showed a higher TG2 mRNA expression compared to WT mice (Figure 5-1/B).

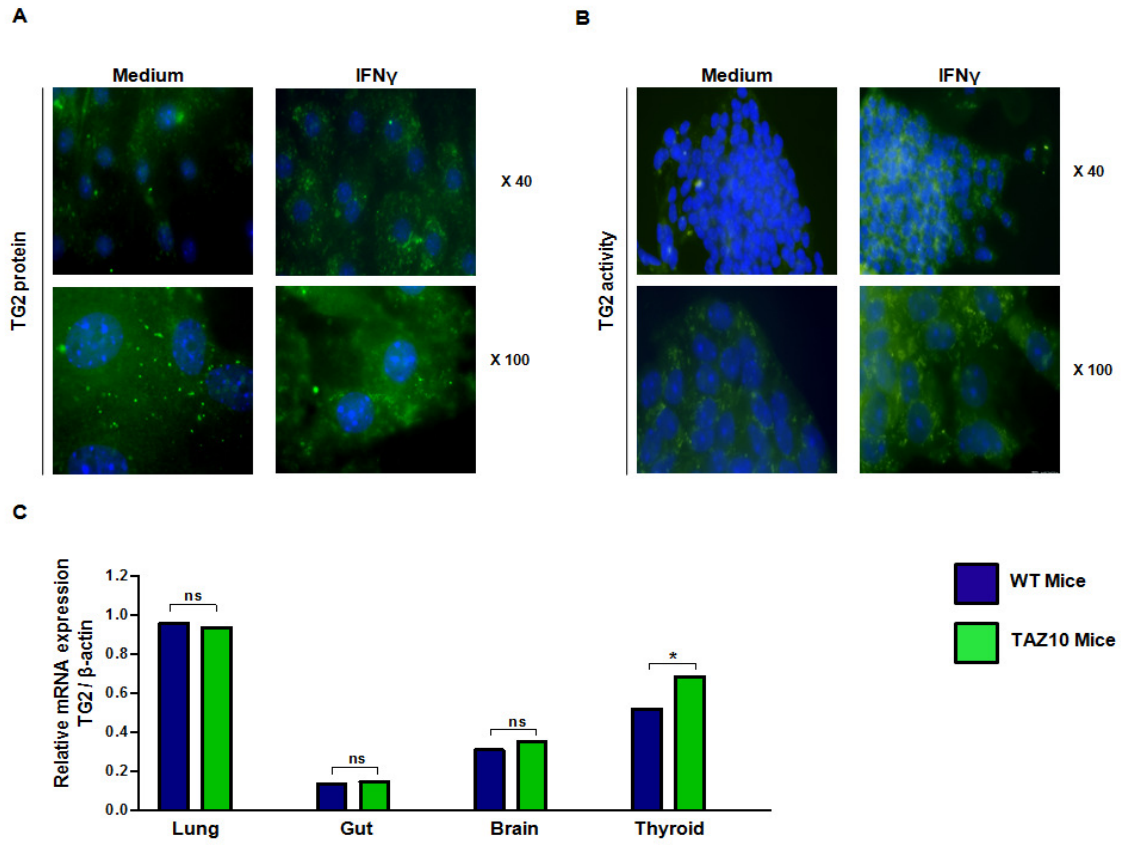


Figure 5-1: Increased expression and activity of tissue transglutaminase (TG2) by TECs upon inflammation

(A) Thyroids were isolated from wild-type (WT) mice and digested as previously described. Thyrocytes were grown on poly-L-Lysine coated coverslips for one day in complete medium and for a further 3 days with or without IFNγ at 200U/mL. Cells were then stained for tissue transglutaminase (TG2, green). **(B)** The effects of IFNγ on the activity of TG2 was assessed by the incorporation of biotin monodansyl-cadaverine revealed with streptavidin-Alexa488 (green). **(A, B)** Slides were analyzed by fluorescence microscopy (magnification x40 and x100). DAPI (blue) was used to counterstain the nucleus. **(C)** The relative expression of TG2 mRNA versus β-actin mRNA was measured in wild-type and 2 months-old TAZ10 mice. Data shown are representative of two different experiments.

TECs from TAZ10 mice were able to present the cryptic TPO epitope to T cells from TAZ10 mice (Figure 4-6). The ability of TECs from WT mice stimulated with IFNγ to present the TPO cryptic epitope to transgenic T cells was associated with the neo-expression of MHC-II molecules (Figure 4-3 and 4-8). While I suggested that this phenomenon was not involved at the onset of the disease, we wondered whether the upregulation of TG2 by TECs upon challenge with IFNγ had any effect on the expression of MHC-II molecules. We therefore assessed the expression of MHC-II molecules ($H2\text{-}A^b$) by thyrocytes co-cultured with IFNγ in the presence or absence of the TG2 specific inhibitor cystamine. As observed by fluorescence microscopy, cystamine was able to reduce the effect of IFNγ-induced MHC-II expression by thyrocytes (Figure 5-2).

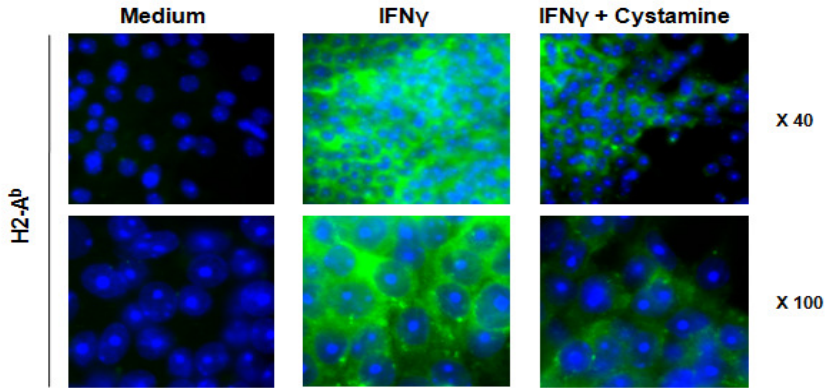


Figure 5-2: Inhibition of TG2 controls the upregulation of MHC-II molecules on thyrocytes induced by IFNγ

Thyroids were isolated from wild-type (WT) mice and digested as previously described. Thyrocytes were grown on poly-L-Lysine coated coverslips for one day in complete medium and for a further 3 days with or without IFNγ at 200U/mL. Cells were then analysed for the level of expression of MHC-II molecules ($H2\text{-}A^b$, green). The effect of TG2 on the expression of MHC-II molecules by thyrocytes was assessed using the TG2 specific inhibitor cystamine. Slides were analyzed using a fluorescence microscope (magnification x40 and x100). DAPI (blue) was used to counterstain the nucleus. Data shown are representative of two different experiments.

I then decided to measure the impact of the inhibition of TG2 on inflammation by assessing the proliferative activity of T cells from the spleen of TAZ10 mice treated daily with cystamine for 7 only days. As expected, T cells from untreated TAZ10 mice strongly proliferated in response to αCD3. Surprisingly, the treatment of TAZ10 mice with cystamine led to a dramatic reduction of T cell proliferation in response to αCD3 (Figure 5-3/A). Indeed, if αCD3 increased T cell proliferation by about 750 fold compared to unstimulated splenocytes, treatment with cystamine led to a dramatic reduction of the ability of T cells to proliferate in response to αCD3 down to about 70 fold (Figure 5-3/B).

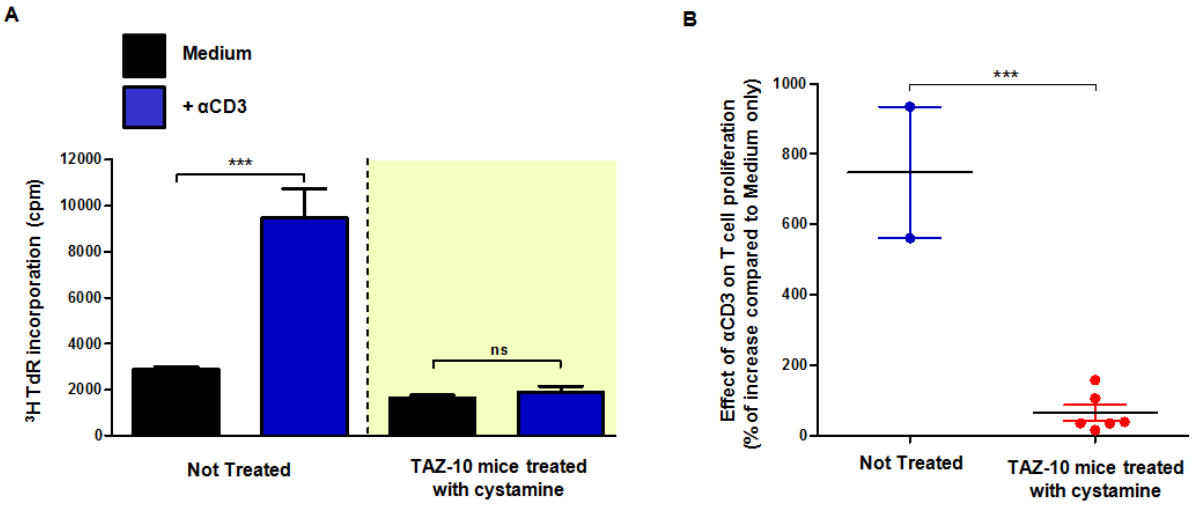


Figure 5-3: Treatment of TAZ10 mice with the TG2 inhibitor cystamine abrogate the proliferative activity of splenic T cells

6 TAZ10 mice were treated for 7 days with 100µL of 0.01M daily injection of cystamine dihydrochloride dissolved in PBS. Spleens were isolated and the proliferative activity of splenocytes in response to αCD3 was assessed using (^{3}H) thymidine incorporation (cpm). 3 untreated TAZ10 mice were used as controls. Data in **(A)** represents one mouse of each group representative of all the mice in the same group. **(B)** Pooled analysis on the effect of αCD3 on the proliferation of transgenic T cells assessed as a percentage of increase compared to the basal proliferation of splenocytes in medium only (no αCD3). Statistical analysis was performed using the unpaired Student's t-test.

I previously showed that MDSCs efficiently inhibit the proliferation of TAZ10 T cells in response to the cognate peptide (Figure 3-22) or strong stimuli such as αCD3 (Figure 3-18) and accumulate in lymphoid and non-lymphoid organs in TAZ10 mice (Figure 3-15). As MDSCs accumulate in peripheral organs in response to inflammation and proinflammatory cytokines such as IL-1β (Gabrilovitch. 2004; Song et al. 2005; Bunt et al. 2006 ; Kim et al. 2006; Tu et al. 2008), I wondered whether TAZ10 mice treated with cystamine would present an altered distribution of MDSCs that could explain the results previously obtained (Figure 5-3).

By flow cytometry analysis, I revealed that $Gr1^{+}$ $CD11b^{+}$ MDSCs accumulated in the spleen of cystamine-treated TAZ10 mice from about 2.5% to 4% of the total splenocyte population (Figure 5-4/A, *left panel*). Although this accumulation of MDSCs in cystamine treated splenocytes could explain the decreased proliferation of T cells from TAZ10 mice, it did not confirm the increased accumulation of MDSCs in inflammation I observed in TAZ10 mice (Chapter3/). I then investigated the proportion of MDSCs in the DLN of TAZ10 mice. Indeed we previously showed that LN from WT mice do not contain MDSCs (Figure 3-13/B and 3-15/B) while they accumulate in a context of cancer (Figure 3-13/B) or autoimmune disease (Figure 3-15/B). Surprisingly, the amount of $Gr1^{+}$ $CD11b^{+}$ MDSCs was nearly halved

in the DLN of cystamine-treated mice (Figure 5-4/A, *right panel*); thus confirming the beneficial effects of the treatment in TAZ10 mice.

I also took this opportunity to analyse the proportion of effector $CD4^+$ T cells in the tissues previously analysed. Effector T cells were characterized by the lack of expression of CD62L (L-selectin) and CD127 (α subunit of the IL-7 receptor) (Stemberger et al. 2007) by flow cytometry. To our surprise, we also found a significant reduction in $CD4^+$ $V\beta1^+$ $CD62L^-$ $CD127^-$ effector T cells both in the spleen (Figure 5-4/B, *left panel*) and the DLN (Figure 5-4/B, *righ panel*) of TAZ10 mice treated for 7 days with cystamine. We could rule out that this difference was due to a reduction in the pool of $CD4^+$ T cells in these tissues (Figure 5-4/C).

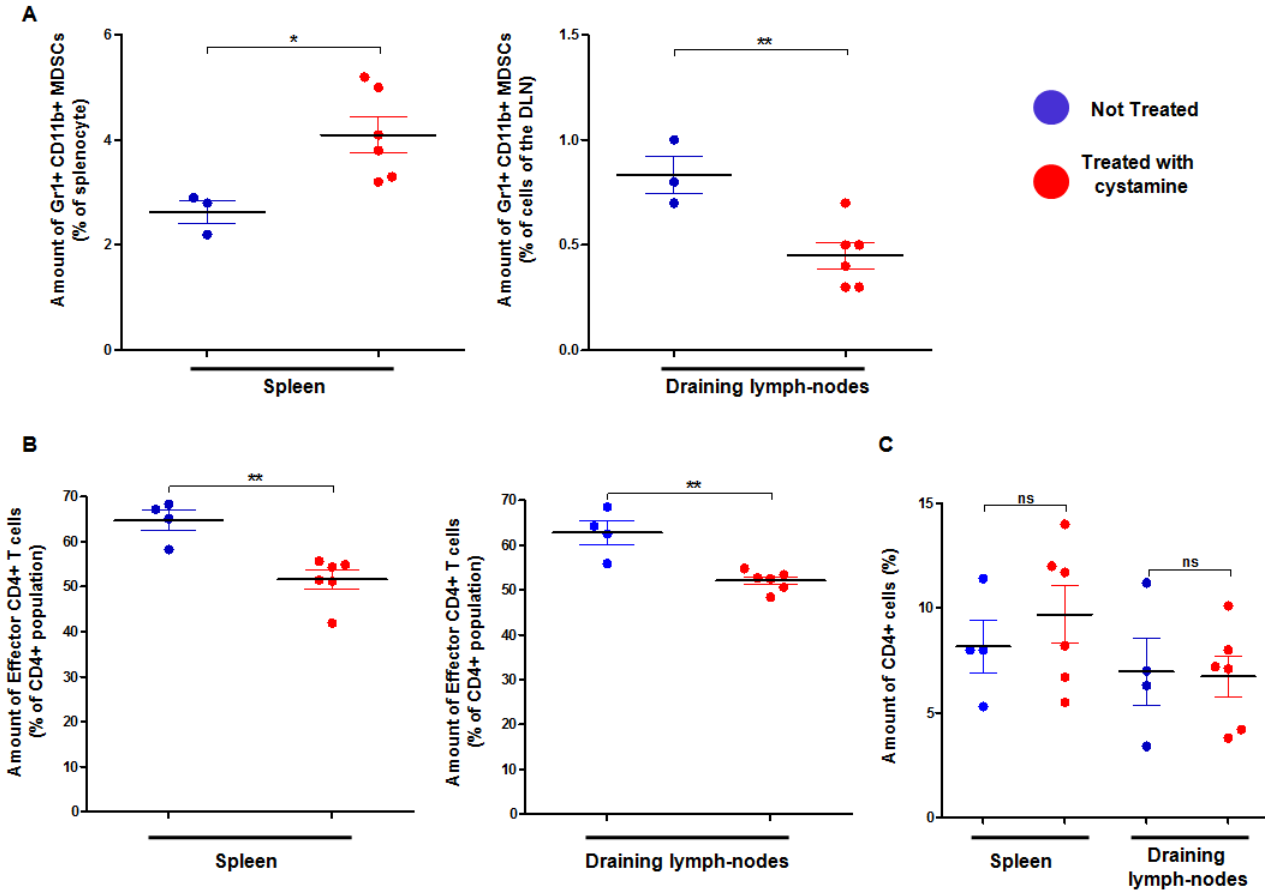


Figure 5-4: Treatment of TAZ10 mice with the TG2 inhibitor cystamine alters the proportion of MDSCs and CD4$^+$ effector T cells

(A) 6 TAZ10 mice were treated for 7 days with 100µL of 0.01M daily injection of cystamine dihydrochloride dissolved in PBS. Spleens (*left panel*) and draining lymph nodes (*right panel*) were isolated and cells analysed for the presence of Gr1$^+$ CD11b$^+$ MDSCs. The proportions of CD4$^+$ T cells **(C)** and CD4$^+$ CD62L$^-$ CD127$^-$ effector T cells were assessed in the spleens (*left panel*) and draining lymph nodes (*right panel*) of cystamine-treated and untreated TAZ10 mice **(B)**. Blue and red dots represent untreated and cystamine-treated TAZ10 mice respectively. Statistical analysis was performed using the unpaired Student's t-test. Red and blue bars represent SEM.

Although experiments are still being performed to study in depth the mechanisms by which the inhibition of TG2 activity seems to reduce inflammation in TAZ10 mice with spontaneous thyroiditis, the results obtained are encouraging.

Chronic inflammatory diseases affect a significant percentage of the population and are the cause of considerable suffering as well as large economic burden. Spanning from cancer to autoimmune diseases (such as thyroiditis, rheumatoid arthritis or systemic lupus erythematosus) and chronic disorders of the gut (celiac disease) or the lung (cystic fibrosis), chronic inflammatory diseases have in common the infiltration of tissues by monocytic immune cells, the release of soluble factors such as cytokines (IL-1β, IL-6, TNFα) or nitric-

oxide and the activation of p38 MAPK and NF-κB. Interestingly, patients with autoimmune thyroiditis have an increased chance of developing celiac disease and vice versa (Naiyer et al. 2008; Duntas, 2009). In celiac disease (CDis), TG2 is the main autoantigen recognized by autoantibodies (Dieterich et al. 1997; Marsh, 1997). TG2 also plays a fundamental role in the pathogenesis of the disease by operating a crucial deamidation of glutamine to glutamic acid of immunodominant gliadin epitopes that drive the adaptive immune response (van de Wal et al. 1998; Arentz-Hansen et al. 2000).

However, a recent study has highlighted a new unexpected role of TG2 in celiac disease where the inhibition of TG2 activity controlled the activation of gliadin-specific T cells. While the non-immunodominant gliadin peptide p31-43 induced the death of epithelial cells of the gut via Fas engagement (Maiuri et al, 2001; Maiuri et al. 2003), these effect were abrogated by incubating the cells with a monoclonal antibody blocking TG2 expressed at the cell-surface (Maiuri et al. 2005). Our close interactions with pediatric endoscopists provided an opportunity to investigate the role of TG2 in inflammatory bowel diseases (such as Crohn's and celiac disease) as well as cystic fibrosis (CF) with a view to establishing whether other chronic inflammatory diseases in humans might share common mechanisms with other autoimmune pathologies such as that displayed in the TAZ10 transgenic mouse model of thyroiditis.

5-2/ Human CFTR-defective airway epithelial cells are constitutively stressed

In Cystic Fibrosis, persistent infections produce a state of chronic inflammation in the lungs that is associated with biliary and pancreatic disorders (Didierlaurent et al. 2007; Ratjen et al. 2003). The inability of the clearance of infections such as *Pseudomonas Aeruginosa* in these patients is exacerbated both by the inactivation of neutrophils in the lungs (Hartl et al. 2007) and the failure to efficiently clear the mucus from the airways. The impaired function of the CFTR channel consequential to mutation(s) of the CFTR gene, results in reduced chloride permeability and an increased sodium and water absorption (Boucher et al. 1988; Reddy et al. 1999). This consequently leads to the cilia being unable to efficiently clear the thickened mucus (Ratjen et al. 2003). While few studies have suggested that the decreased airway mucociliary clearance and airway mucosal inflammation occur before any airway infection (Zahm et al. 1997), the impact of CFTR mutations on lung epithelial cells is still elusive.

5-2-1/ Upregulation of the common markers of inflammation in CF airway epithelial cells

I investigated the consequences of CFTR mutations in the human CF bronchial cell line bearing the common ΔF508/W1282X CFTR mutation (IB3-1 cell-line) and the isogenic C38 cell-line rescued with functional CFTR (Egan et al. 1992). I first assessed the level of activation of the two-different cell-lines in sterile tissue culture. To ensure that our system was free of any infections, I regularly carried out RT-PCR to assess for the presence or absence of *mycoplasma*. Culture media were also supplemented with antibiotics and low endotoxin FCS was used. By fluorescence microscopy, we evaluated the level of phosphorylated tyrosines, a marker of inflammation in airway epithelial cells upon challenge with LPS (Raia et al. 2005). As shown Figure 5-5/A, IB3-1 cells showed a high staining for phospho-tyrosine compared to the control C38 cells thus highlighting that the cells present displayed some level of inflammation and cellular signaling.

NF-κB has been shown to be at the centre of inflammatory responses by inducing the expression of pro-inflammatory genes such as IL-1β, TNFα or COX-2 (Li et al. 2002; Youssef et al. 2006; van Loo et al. 2006). By fluorescence microscopy, I could see that IB3-1 cells bearing the defective CFTR protein presented a higher amount of the phosphorylated

form of p65 subunit of NF-κB that was mainly colocalizing in the nucleus whereas staining for C38 cells was scarce (Figure 5-5/B). This confirms previous studies describing that NF-κB translocation to the nucleus is important in airway epithelial cells of CF patients (Tabary et al. 1999).

To assess the impact of this increased NF-κB activation, I evaluated the expression of IL-1β and TNFα at messenger (mRNA) and protein levels. CF cells presented high amounts of IL-1β and TNFα mRNAs while their expression in C38 cells was negative and very low respectively (Figure 5-5/C). By ELISA, I could see that IB3-1 cells secreted high amounts of TNFα and IL-1β cytokines (Figure 5-5/D) compared to C38 cells. This confirmed previous results highlighting a marked increase in the expression of pro-inflammatory cytokines such as IL-1β, TNFα, IL-6 or IL-17 associated with the CF phenotype (Osika et al. 1999).

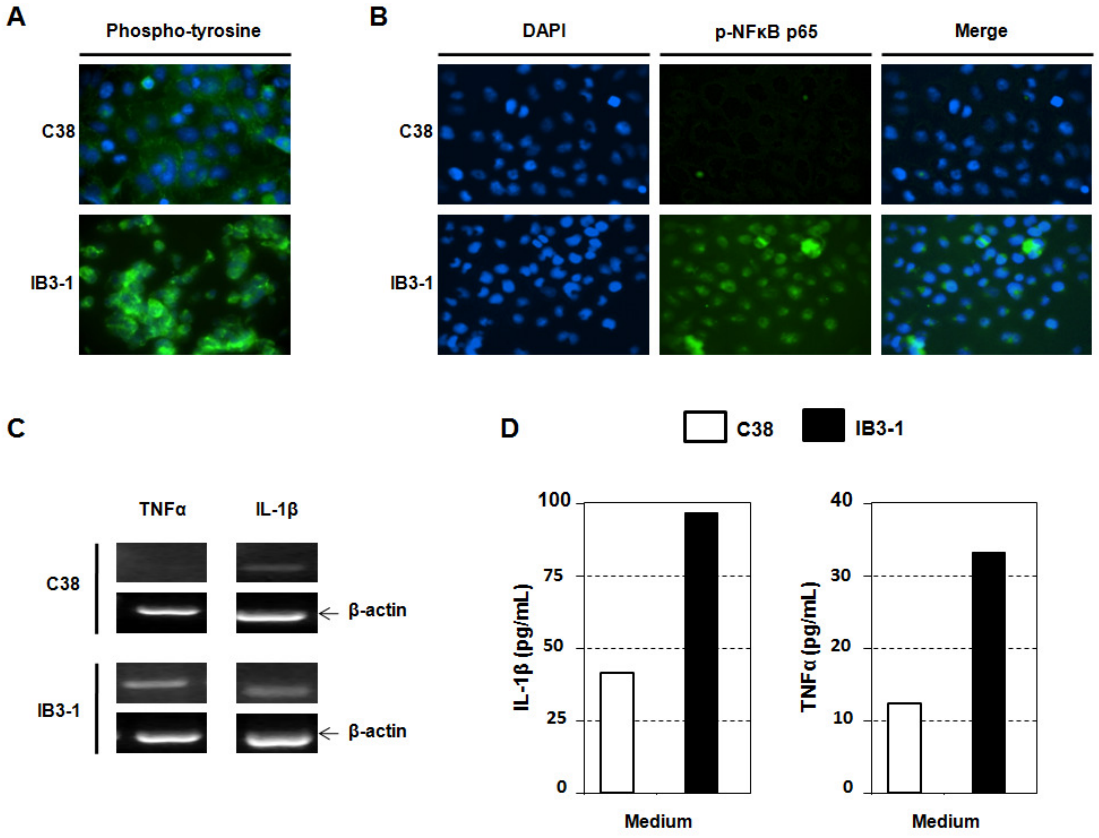


Figure 5-5: Up-regulation of inflammatory markers in human CFTR-defective airway epithelial cells
Presence of phospho-tyrosine residues **(A)** (green) and the phosphorylated form of p65 subunit of NF-κB (green) **(B)** in human CFTR-defective cell-line (IB3-1 cells) and the isogenic cell-line rescued with functional CFTR (C38 cells). DAPI was used to counterstain the nucleus. Cells were analysed by fluorescence microscopy (magnification x40). **(C)** mRNA profile of C38 and IB3-1 cell-lines for TNFα and IL-1β. mRNA expression for β-actin was used as control. **(D)** Amount of TNFα and IL-1β secreted by C38 and IB3-1 cell-lines by ELISA. Data shown are representative of at least two different experiments.

Lungs of patients with CF are characterized by the recruitment of neutrophils (Ratjen et al. 2003; Downey et al. 2009). Both ICAM-1 (Inter-Cellular Adhesion Molecule 1) and IL-8 have been shown to be important factors involved in the recruitment and activation of neutrophils (Nathan et al. 2006, Downey et al. 2009).

Prompted by the expression of TNFα, IL-1β and the phospho-p65 subunit of NF-κB, we evaluated the levels of expression of ICAM-1 and IL-8 by fluorescence microscopy analysis and ELISA respectively. IB3-1 cells expressed high levels of ICAM-1 while this expression was weak in C38 cells (Figure 5-6/A). This was associated with the release of IL-8 that was further increased upon challenge with LPS from *Pseudomonas aeruginosa* (PA-LPS) at 1µg/mL (Figure 5-6/B).

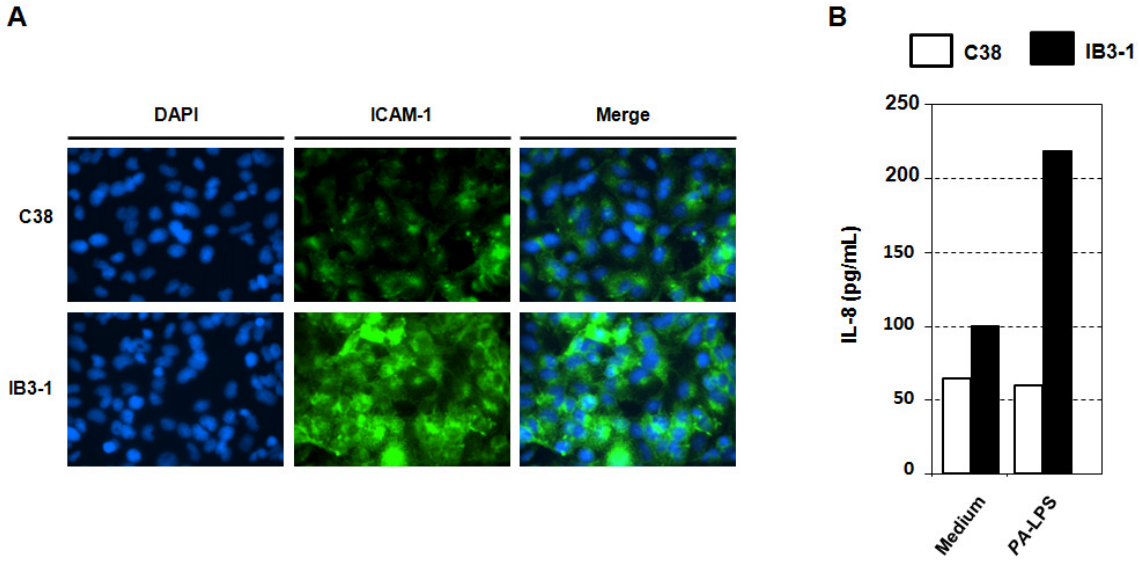


Figure 5-6: Increased expression of neutrophils attractant by human CFTR-defective airway epithelial cells
(A) Expression of ICAM-1 (green) in human CFTR-defective cell-line (IB3-1) and the isogenic cell-line rescued with functional CFTR (C38). DAPI was used to counterstain the nucleus. Cells were analysed by fluorescence microscopy (magnification x40). **(B)** IL-8 ELISA in C38 and IB3-1 cell-lines with or without challenge with LPS from *Pseudomonas-aeruginosa* (PA-LPS). Data shown are representative of at least two different experiments.

p38 Mitogen Activated Protein Kinase (MAPK) is highly activated in cystic fibrosis airways and its inhibition has been shown to dampen inflammation in CF airway (Raia et al. 2005). Interestingly, IL-8 over-expression has been shown to depend of MAPK and NF-κB signaling pathways in CF epithelial cells (Carrabino et al. 2006; Muselet-Charlier et al. 2007). Also, the adherence of neutrophils in CF airways is increased and depends on the

activation of NF-κB-induced expression of ICAM-1 (Melotti et al. 2001; Tabary et al. 2006). Together these results suggested that cells bearing a defective CFTR protein as a result of gene mutation presented an increased pro-inflammatory phenotype favoring the important recruitment of neutrophils as shown by the high expression of IL-8 and ICAM-1.

5-2-2/ Increased TG2 expression in CFTR-defective airway epithelial cells

Celiac disease (CDis) is a common pathology caused by an inappropriate immune response to wheat and gluten and is characterised by local (small intestine) and systemic manifestations (Schuppan et al. 2000). Important histological features of untreated patients with CD are the activation of gliadin specific T cells, the infiltration of intraepithelial lymphocytes and the flattening of the mucosa of the small intestine (Schuppan et al. 2000).

Tissue transglutaminase 2 (TG2) is essential in the pathogenesis of celiac disease (CDis) as operating an important deamidation of glutamine to glutamic acid of the gliadin immunodominant epitopes (Molberg et al. 2008). However, the enzymatic activity of TG2 is not its only function as it displays other multiple biological activities (Akimov et al. 2000 and 2001) and is upregulated upon inflammation (Lorand et al. 2003; Ientile et al. 2007). TG2 is a calcium-dependent enzyme with a transamidating activity that also results in the cross-linking of proteins via ε(γ-glutamyl) lysine bonds (Fesus et al. 2002). More recent studies have highlighted the important role played by TG2 in the pathogenesis of numerous neurodegenerative diseases. Indeed, in Parkinson's disease, TG2 mediates the cross-linking and aggregation of α-synuclein in Lewy inclusion bodies (Junn et al. 2003).

CDis is triggered by a non-immunodominant gliadin peptide which stimulates the innate immune response and induces epithelial damage and death by apoptosis (Maiuri et al. 2003). This non-immunodominant peptide induces TG2 upregulation whose inhibition controls the activation of epithelial changes and damages and the subsequent activation of pathogenic gliadin-specific T cells (Maiuri et al. 2005 and Submitted).

Prompted by this unexpected role of TG2 in the modulation of inflammation in CDis, we wanted to address whether the increased inflammatory status displayed by IB3-1 cells was associated with an increased expression of TG2. Indeed, IB3-1 cells had an increased expression in TG2 mRNA compared to the control C38 cell-line (Figure 5-7/A). This was

associated with the expression of TG2 protein as shown by immunofluorescence (Figure 5-7/B, *top panel*) and western-blot analysis (Figure 5-7/B, *bottom panel*). By confocal microscopy, we also observed an increased TG2 activity in IB3-1 cells as revealed by the incorporation of monodansyl-cadaverine by TG2 (Figure 5-7/C).

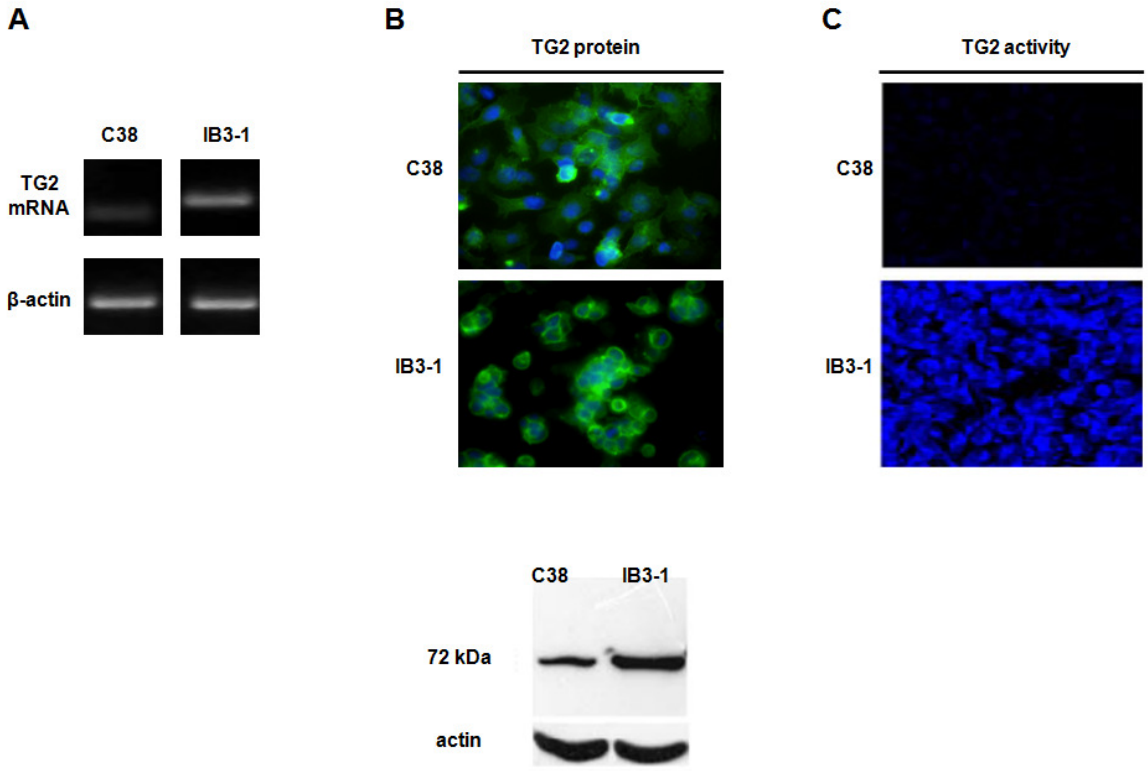


Figure 5-7: CFTR-defective airway epithelial cells have an increased TG2 expression and activity
(A) mRNA profile of C38 and IB3-1 cell-lines for the expression of TG2 mRNA. mRNA expression of β-actin was used as control. **(B)** The level of expression of TG2 (greeb) by C38 cells and the CFTR-defective IB3-1 cells was assessed by fluorescence microscopy (magnification x40) (*top-panel*) and western-blot (*bottom-panel*). DAPI was used to counterstain the nucleus **(C)** The enzymatic activity of TG2 (blue) in C38 and IB3-1 cells was assessed by the incorporation of monodansyl cadaverine and analysed by fluorescence microscopy (scale bar, 10μm). Data shown are representative of at least two different experiments. Figure B (western-blots) and C were kindly provided by Prof. Luigi Maiuri; figures from (Maiuri et al. 2008).

Mutation of the CFTR gene resulting in a defective CFTR protein confers an increased inflammatory status to bronchial epithelial cells. Although we expected an increased expression of the common markers of inflammation such as NF-κB, TNFα and IL-1β, we wanted to investigate whether the increased expression of TG2 had any impact in IB3-1 compared to C38 cells.

5-3/ PPARγ colocalize in perinuclear aggresomes in CFTR-defective airway epithelial cells

We first analyzed whether CF cells presented an altered expression of PPARγ, a key regulator involved in dampening signs of inflammation in many inflammatory diseases (Bailey et al. 2005; Pascual et al. 2005). By fluorescence microscopy, we could see that the cellular distribution of PPARγ was atypical in IB3-1 cell-line. While PPARγ was distributed evenly in the cytoplasm of C38 cells, its distribution in IB3-1 cells suggested that PPARγ had aggregated (Figure 5-8/A). To investigate this hypothesis, we analysed the distribution of PPARγ in C38 and IB3-1 cells by subcellular confocal microscopy (Figure 5-8/B). Interestingly, we could confirm that PPARγ proteins were accumulating essentially in the proximity of the nucleus while PPARγ was evenly distributed in the cytoplasm of C38 cells.

Numerous neurodegenerative diseases have in common the intracellular accumulation and aggregation of proteins such as huntingtin in Huntington's disease (Karpuj et al. 1999) or α-synuclein in Parkinson's disease (Junn et al. 2003) disease. Different types of inclusions of aggregated proteins have been described according to the cellular localization, the solubility and the level of ubiquitination and aggregation of the proteins. Indeed, soluble ubiquitinated proteins form perinuclear aggregates where proteasomes are abundant. These juxta-nuclear quality control compartments or JUNQ are associated with the endoplasmic reticulum associated protein degradation or ERAD (Plemper et al. 1999; Kaganovitch et al. 2008). On the other hand, aggregated proteins can form perivacuolar inclusions named insoluble protein deposits or IPOD associated with high amounts of Hsp104. Interestingly, amyloid proteins such as huntingtin and prion are associated with IPOD and their enhanced ubiquitination target them to JUNQ perinuclear compartments (Kaganovitch et al. 2008).

Finally, unfolded or misfolded proteins not degraded by the proteasome can form aggregates that are transported along microtubules via dynein proteins to the microtubule organizing center (MTOC) (Kopito, 2000; Garcia-Mata et al. 2002). These perinuclear inclusions called aggresomes bind dynein via the histone deacetylase 6 (HDAC6) recognizing and binding poly-ubiquitin chains on aggregated proteins (Kawaguchi et al. 2003). We wanted to ascertain whether the accumulation of PPARγ in IB3-1 cells was associated with one of these inclusions. By confocal microscopy analysis, we found that PPARγ was

colocalizing with both ubiquitin (Figure 5-8/C, *top panel*) and HDAC6 (Figure 5-8/C, *bottom panel*) this confirming that PPARγ accumulated in aggresomes.

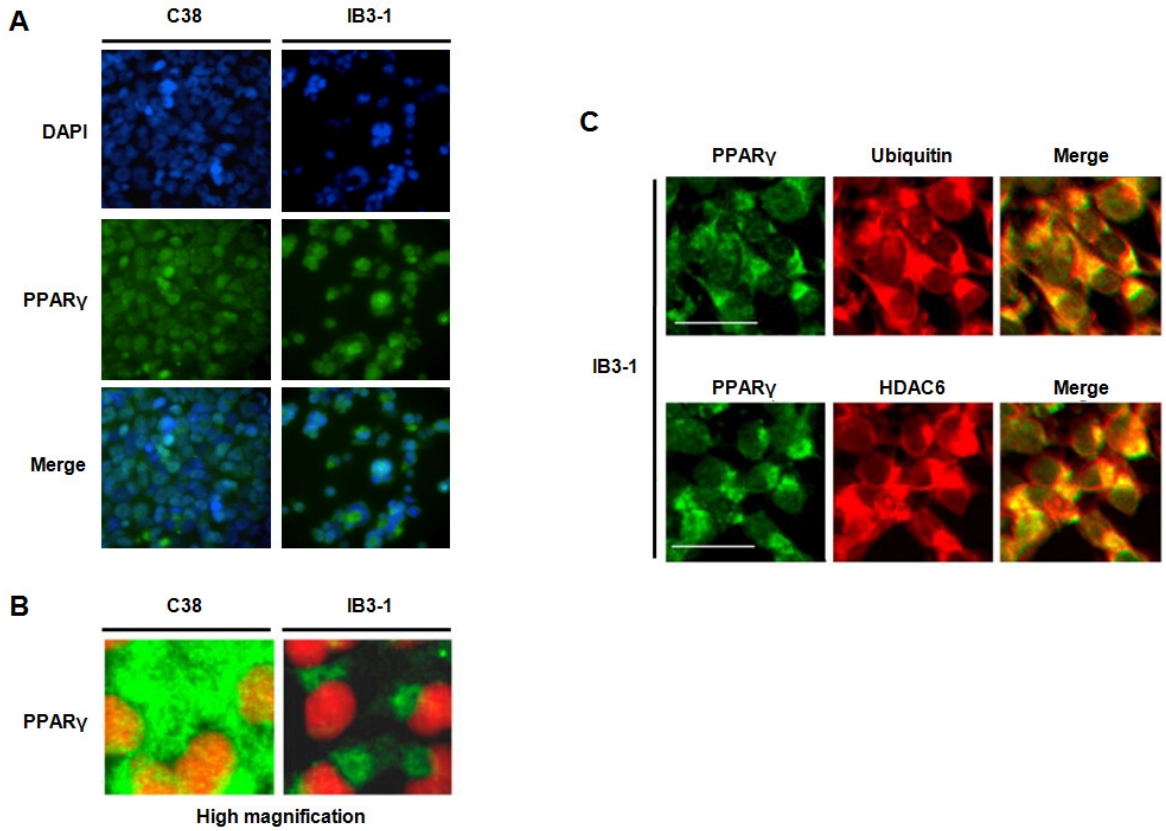


Figure 5-8: PPARγ colocalizes in perinuclear aggresomes in CFTR-defective airway epithelial cells
(A) Expression of PPARγ (green) in the human CFTR-defective cell-line (IB3-1) and the isogenic cell-line rescued with functional CFTR (C38 cell-line) was analysed by fluorescence microscopy (magnification, x40). DAPI was used to counterstain the nucleus. **(B)** Expression of PPARγ in IB3-1 and C38 cell-lines (green) was analysed by confocal microscopy at high magnification. CyTRAK (orange) was used to counterstain the nucleus. **(C)** Co-localization of PPARγ (green) and ubiquitin (red, *top panel*) or HDAC6 (red, *bottom panel*) was assessed by confocal microscopy (scale bar, 10μm). Data shown are representative of at least two different experiments. Figure B and C were kindly provided by Prof. Luigi Maiuri; figures from (Maiuri et al. 2008).

Perinuclear inclusions have been shown to be associated with proteasomes to allow for the degradation of ubiquitinated proteins (Kaganovitch et al. 2008). Indeed, treatment of IB3-1 cells with the proteasome inhibitor MG132 resulted in an increased level of PPARγ protein as revealed by western-blot analysis (Figure 5-9/A, *left panel*) and confocal microscopy (Figure 5-9/B). Purified PPARγ by immunoprecipitation also revealed that inhibition of the proteasome increased its ubiquitination as shown by the increased size obtained by western-blot (55 kDa for normal PPARγ protein and around 72kDa for ubiquitinated forms of PPARγ) (Figure 5-9/A, *right panel*).

HDAC6 dependent processing of misfolded protein can involve two mechanisms (Kawaguchi et al. 2008). Indeed ubiquitinated proteins can undergo degradation mediated by the proteaseome. Misfolded proteins not being degraded by the proteasome form toxic aggregates that are recognized by HDAC6 through the polyubiquitin chains (Kawaguchi et al. 2006). We wanted to assess this by inhibiting the degradation of PPARγ by the proteaseome. Indeed, incubation of IB3-1 cells with MG132 resulted in higher amounts of polyubiquitinated forms of PPARγ as detected by western-blot (Figure 5-9/A, *right panel*) and confocal microscopy analysis (Figure 5-9/C, *top panel*). Interestingly, treatment of the cells with MG132 dramatically increased the amount of HDAC6 expressed (Figure 5-9/C, *bottom panel*).

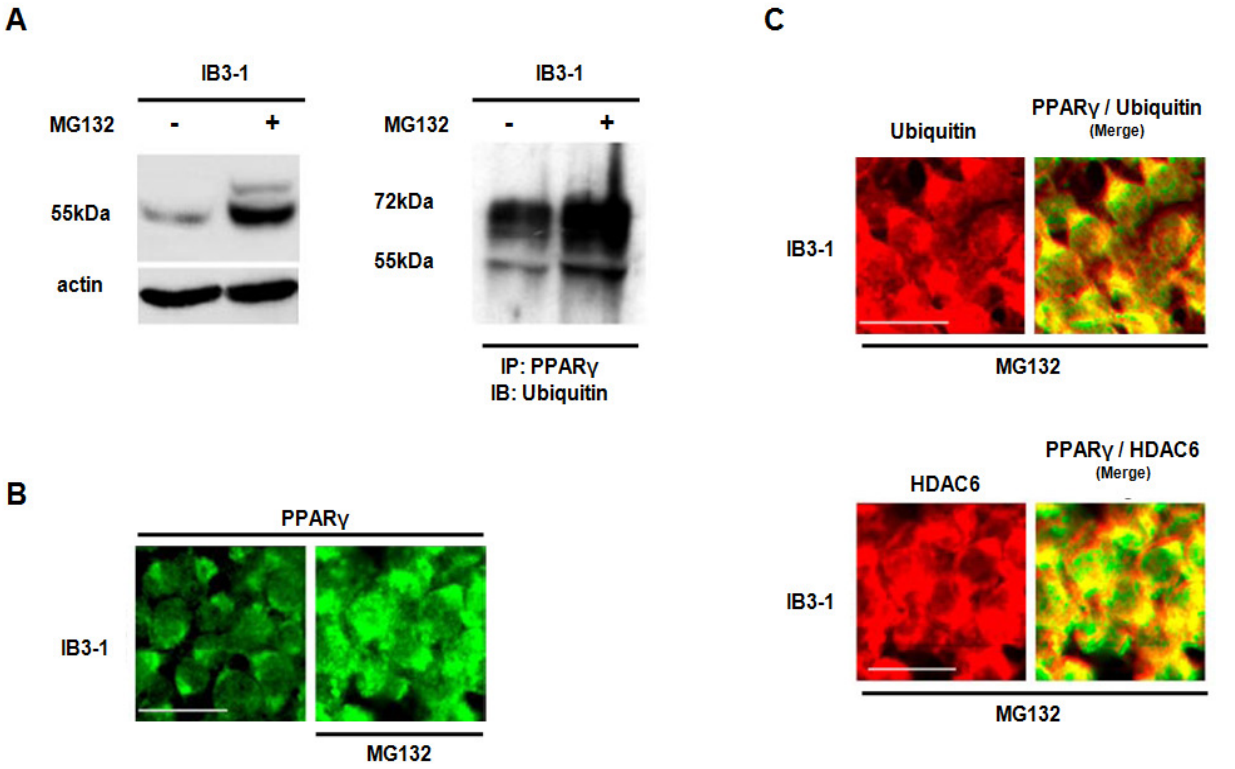


Figure 5-9: PPARγ proteins colocalizing in aggresomes of IB3-1 cells are degraded by the proteasome
(A) *Left panel,* Presence of PPARγ in the human CFTR-defective cell-line (IB3-1) with or without the proteasome inhibitor MG132 was assessed by western-blot analysis. *Right panel,* Immunoprecipitated (IP) PPARγ proteins are assessed for their immunoreactivity (IB) to ubiquitin in the presence or absence of MG132. **(B)** Effect of the inhibition of the proteasome activity on the cellular distribution of PPARγ (green) in IB3-1 cells was analysed by confocal microscopy (scale bar, 10μm). **(C)** Confocal analysis of the co-localization of PPARγ (green) with ubiquitin (red, *top panel*) and HDAC6 (red, *bottom panel*) in IB3-1 cells. Data shown are representative of at least two different experiments. Figures were kindly provided by Prof. Luigi Maiuri; figures from (Maiuri et al. 2008).

By inhibiting the degradation of PPARγ by the proteasome, we therefore increased the amount of aggregates of ubiquitinated form of PPARγ hence favoring the formation of aggregates as revealed by the increased expression of HDAC6.

5-4/ Up-regulation of TG2 promotes inflammation in CFTR-defective airway epithelial cells

Once synthesized, proteins must acquire a structure that will allow them to exert their specific structural, enzymatic and/or signaling functions. The appropriate folding of proteins is mediated by chaperones and prevents the formation of misfolded proteins and their aggregation that could become detrimental and toxic to the cells (Hartl et al. 2002). Despite the abundance of molecular chaperones, protein-folding is not efficient and it has been assumed that nearly 30% of newly synthesized proteins are defective products that are normally rapidly ubiquitinated and degraded by the proteasome (Schubert et al. 2000). However, the degradation of several proteins is defective. Indeed, ΔF508-CFTR mutant proteins are not efficiently degraded by the ubiquitin-proteaseome pathway and have been shown to accumulate in aggresomes (Kawaguchi et al. 2003). Although it has been suggested that initial aggregation of proteins could occur while translation is still ongoing and nascent proteins are generated (Kopito. 2000; Garcia-Marta et al. 2002), this could not explain the aggregation of PPARγ in cells bearing the ΔF508 mutation of the CFTR gene.

5-4-1/ Up-regulation of TG2 leads to the Sequestration of PPARγ in the CFTR-defective airway epithelial cells

We previously observed an increased expression of TG2 proteins in the IB3-1 CFTR-defective cell-line (Figure 5-7). Interestingly, PPARγ sequence contains two specific sites (QG and QXXP) that are recognized by TG2 mediating its cross-linking activity through the glutamine (Q) residues (Lorand et al. 2003). At this point, TG2 was an ideal candidate in promoting the aggregation of PPARγ proteins.

We first assessed whether TG2 would co-localize with PPARγ by confocal microscopy in C38 and IB3-1 cell-lines using a cross-link isopeptide specifically recognized by TG2 (Figure

5-10/A). TG2 proteins colocalized with PPARγ-containing aggresomes in IB3-1 cells while PPARγ was diffused in the cytoplasm of C38 cells as observed Figure 5-8.

We then wondered whether the inhibition of TG2 using the irreversible TG2-specific inhibitor R283 (kind gift from M. Griffin, Aston University, Birmingham, UK) would restore the levels of PPARγ in IB3-1 cells back to levels present in C38 cells (Figure 5-8 and 5-9). Indeed, levels of 55kDa PPARγ were low in IB3-1 cells compared to C38 cells (Figure 5-10/B, *left panel*) and restored upon incubation of the cells with R283 (Figure 5-10/B, *right panel*) as shown by western-blot analysis. We still needed to ascertain that TG2 was promoting the cross-linking of PPARγ proteins. To do this we used an isopeptide bearing a recognition sequence for TG2: TG2 is capable of cross-linking the isopeptides that can be identified using an isopeptide-specific antibody. We therefore assessed whether TG2 would promote protein cross-linking between the TG2 specific isopeptide and PPARγ by immunobloting purified PPARγ proteins previously immunoprecipitated for the isopeptide. Several high molecular weight forms of PPARγ ranging between 72kDa and 250kDa could be detected with the anti-isopeptide antibody, with major bands at 95kDa and 130kDa (Figure 5-10/C, *left panel*). Addition of R283 promoted a major reduction of PPARγ-isopeptide aggregates (Figure 5-10/C, *right panel*) while restoring normal levels of 55kDa PPARγ (Figure 5-10/B, *right panel*).

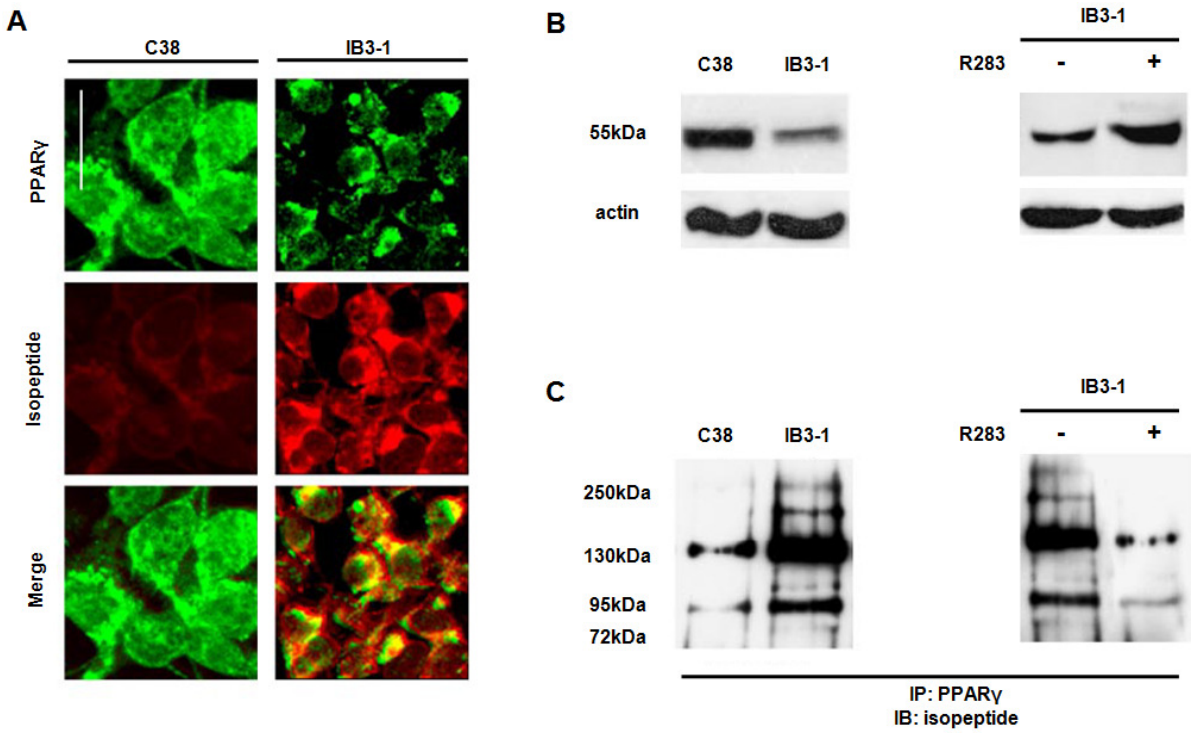


Figure 5-10: PPARγ co-localize with TG2 in CFTR defective airway epithelial cells
(A) Confocal analysis of the co-localization of PPARγ (green) with TG2 as revealed by the staining for TG2 isopeptide (red) in C38(*left panel*) and IB3-1 (*right panel*) cell-lines (scale bar, 10μm). **(B)** The detection of PPARγ in C38 and IB3-1 cells (*left panel*) and the effect of the TG2 specific inhibitor R283 on the level of expression of PPARγ in IB3-1 cells (*right panel*) were assessed by western-blot analysis. **(C)** *Right panel,* Immunoprecipitated (IP) PPARγ proteins are assessed for their immunoreactivity (IB) to the TG2 specific isopeptide in C38 and IB3-1 cells. The effect of the inhibition of TG2 by R283 in IB3-1 cells is also assessed (*right panel*). Data shown are representative of at least two different experiments. Figures were kindly provided by Prof. Luigi Maiuri; figures from (Maiuri et al. 2008).

We observed that the inhibition of TG2 activity restored the cytoplasmic distribution of PPARγ (Figure 5-11/A) by confocal microscopy analysis in both nasal polyp mucosa from CF patients and IB3-1 cells. This redistribution of PPARγ in IB3-1 cells promoted by the TG2 specific inhibitor R283 was similar to the action of the proteasome inhibitor MG132 we previously observed (Figure 5-9/B). Rosiglitazone, a potent PPARγ agonist was capable of inducing the nuclear translocation of PPARγ in C38 cells. However, this could only be achieved in IB3-1 cells when TG2 activity was also inhibited by R283 (Figure 5-11/B).

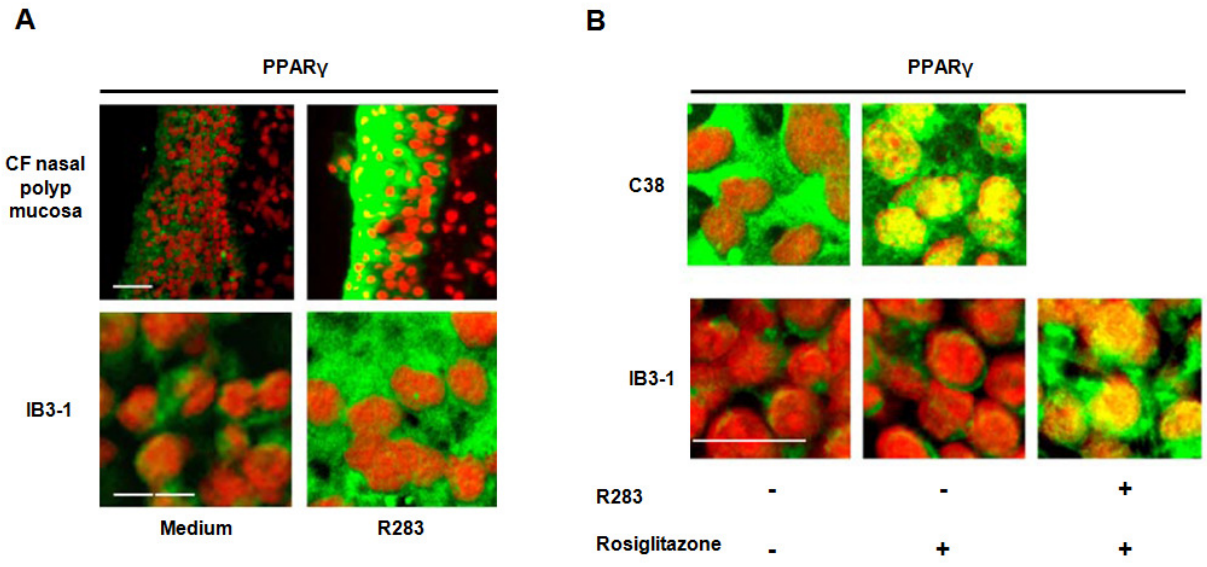


Figure 5-11: Inhibition of TG2 promotes a redistribution of PPARγ in CFTR-defective cells
(A) Detection of PPARγ (green) in the human CFTR-defective cell-line (IB3-1) and nasal polyp mucosa from CF patients with or without the TG2 inhibitor R283. **(B)** Effect of the potent PPARγ agonist rosiglitazone on the cellular distribution of PPARγ (green) with or without the TG2 inhibitor R283 in C38 and IB3-1 cells. Slides were analysed by confocal microscopy (scale bar, 10µm). CyTRAK (orange) was used to counterstain the nucleus. Data shown are representative of at least two different experiments. Figures were kindly provided by Prof. Luigi Maiuri; figures from (Maiuri et al. 2008).

Altogether these results showed that in CFTR-defective cells, PPARγ proteins are ubiquitinated and degraded via the proteasome. Also, the lack of functional CFTR leads to an increased expression of TG2 which promotes the cross-linking of PPARγ into aggresomes.

PPARγ has been shown to "transrepress" inflammatory responses mediated by NF-κB (Bailey et al. 2005; Pascual et al. 2005). Interestingly, we previously showed that IB3-1 cells presented an overexpression of the phosphorylated p65 subunit of NF-κB whereas presenting an inflammatory phenotype as described by the increased expression of IL-1β, TNFα or IL-8 (Paragraph 5-2/). This prompted us to investigate whether we could reduce inflammation in CFTR-defective cells by abrogating the aggregation and degradation of PPARγ mediated by TG2.

5-4-2/ TG2 mediates inflammation in CFTR-defective cells

Calcium (Ca^{2+}) homeostasis has been shown to be abnormal in airway epithelial cell-lines bearing the CFTR-ΔF508 mutation (Antigny et al. 2008) with CF cells presenting a high mobilization of Ca^{2+}. As prolonged increase of intracellular Ca^{2+} promotes the upregulation

of TG2 whose activity also depends on the level of Ca^{2+} (Yoo et al. 2005; Lorand et al. 2005), we wondered whether the increased TG2 activity we previously observed in IB3-1 cells was driven by the increased Ca^{2+} concentrations in these cells.

Indeed, TG2 activity and p42/44 (also called extracellular signal-regulated kinase or ERK-1 and ERK-2 respectively) phosphorylation was drastically reduced in IB3-1 cells treated with the calcium chelator BAPTA-AM (Figure 5-12/A, *left panel*). To mimic the Ca^{2+} environment present in IB3-1 cells into C38 cells, we used the Ca^{2+} ionophore ionomycin inducing an important influx of Ca^{2+} in the cells. Treatment of isogenic CFTR-corrected C38 cells with ionomycin considerably increased both TG2 activity and the phosphorylation of p42/44 (Figure 5-12/A, *right panel*).

TG2 activity also depends on the intracellular content of reactive oxygen species (ROS) (Lee et al. 2003) whose production is associated with an efflux of calcium from intracellular organelles (Yi et al. 2006; Leo et al. 2008). Interestingly, CF patients and CF cells present an increased production of ROS. We wondered whether increased ROS production in CF cells was also responsible for promoting TG2 activity (Cowley et al. 2002; Velsor et al. 2006). Indeed, IB3-1 cells treated with the ROS scavenger NAC (N-acetylcysteine) had a slight reduction of TG2 expression while TG2 activity was strongly reduced (Figure 5-12/B) as shown by confocal microscopy. We could also see a strong reduction in p42/44 phosphorylation (Figure 5-12/B) and TNFα secretion (Figure 5-12/C) in IB3-1 cells treated with NAC.

Together, these results showed that increased Ca^{2+} concentration and ROS production promoted by the mutation of the CFTR gene was driving the activation of TG2 and therefore the inflammation in CF cells.

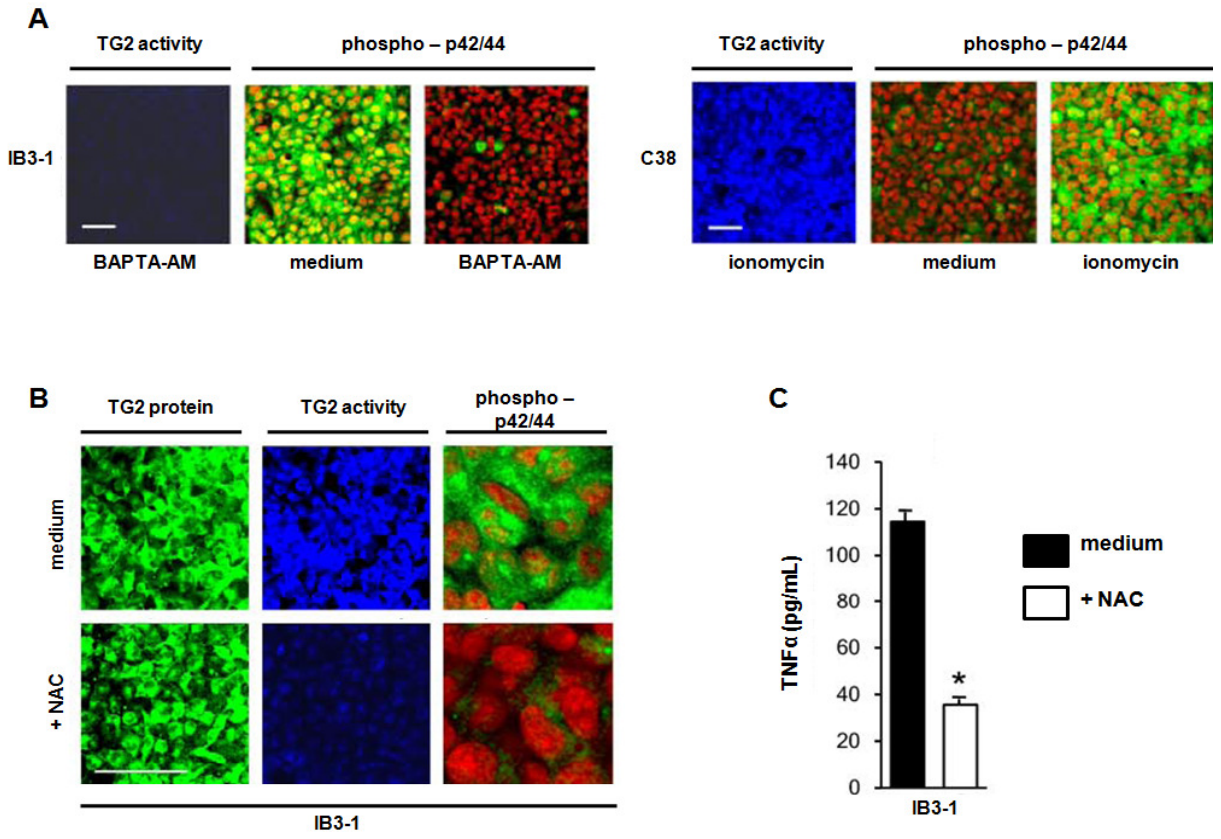


Figure 5-12: TG2 activity is regulated by calcium and reactive-oxygen species (ROS) in CFTR-defective airway epithelial cells
(A) *Left panel,* Effect of the calcium chelator BAPTA-AM on TG2 activity (blue) and phosphorylation of p42/44 (green) in CFTR-defective IB3-1 cells. *Right panel,* Analysis of the effect of the calcium ionophore ionomycin on TG2 activity (blue) and the level of phosphorylation of p42/44 (green) in C38 cells. **(B)** Expression (green, *left panel*) and activity (blue) resulting from the action of the ROS chelator NAC was analysed in IB3-1 cells. Effect of NAC on the level of phospho-p42/44 in IB3-1 cells was also analysed (green, *right panel*). **(A, B)** The slides were analysed by confocal microscopy (scale bar, 10μm). CyTRAK (orange) was used to counterstain the nucleus. Data shown are representative of at least two different experiments. **(C)** The amount of TNFα secreted by IB3-1 cells with or without the ROS chelator NAC was evaluated by ELISA. Data shown are representative of at least two different experiments. Figures were kindly provided by Prof. Luigi Maiuri; figures from (Maiuri et al. 2008).

We went on to investigate whether we could dampen the signs of inflammation in CF airway epithelial cells by inhibiting TG2 activity. Indeed, R283 induced a significant reduction of the level of phosphorylation of p42/44 proteins both in nasal polyps from CF patients (Figure 5-13/A, *top panel*) and IB3-1 cells (Figure 5-13/A) as shown by confocal microscopy and western-blot analysis. The action of R283 on the level of phospho-p42/44 in IB3-1cells could also be mimicked by silencing TG2 expression using TG2 siRNA while control siRNA did not have any effect (Figure 5-13/A, *bottom panel*). IB3-1 cells presented high levels of phospho-tyrosine that was absent in C38 cells (Figure 5-5/A). The specific PPARγ antagonist GW9662 could abrogate the important decrease in tyrosine-phosphorylation induced by the inhibition of TG2 activity in nasal polyp mucosa from CF

patients (Figure 5-13/ B). Finally, we could prevent the release of TNFα by IB3-1 cells by inhibiting TG2 expression with TG2 siRNA and TG2 activity with R283 (Figure 5-13/C)

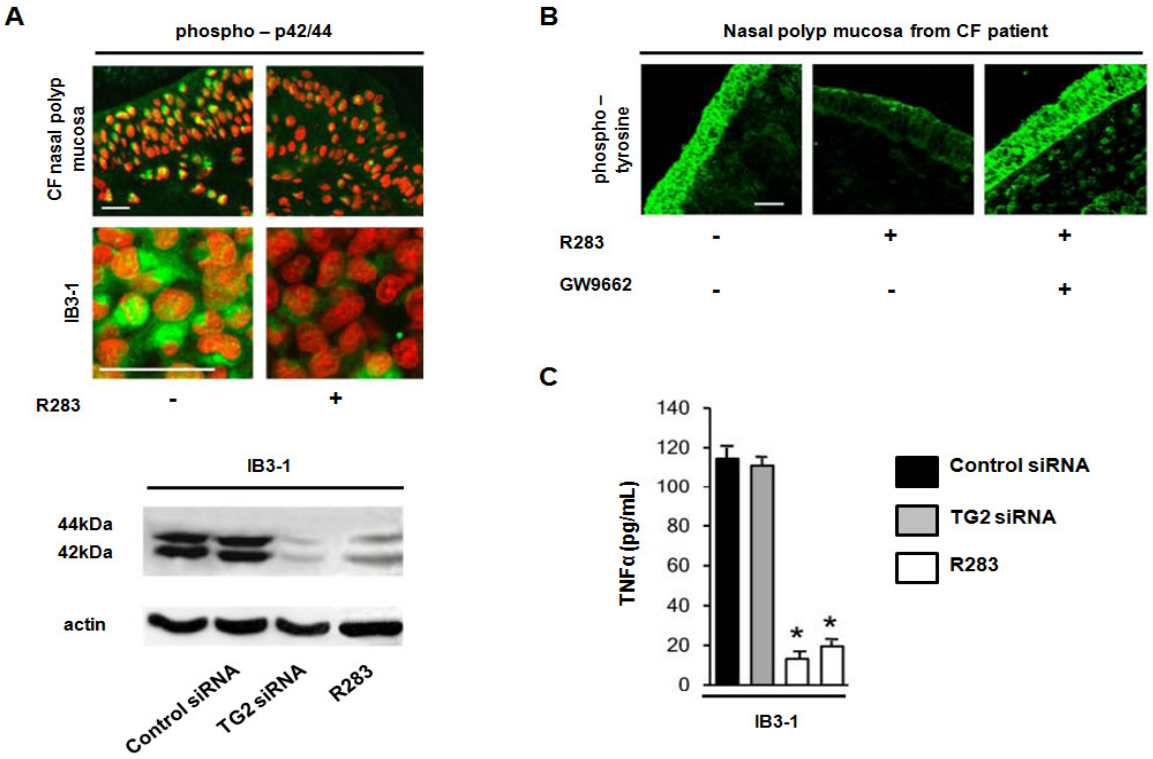


Figure 5-13: TG2 promotes inflammation in CFTR-defective airway epithelial cells
(A) Detection of the level of phosphorylation of p42/44 (green) in nasal polyp mucosa from CF patients and IB3-1 cells upon incubation with the TG2 specific inhibitor R283 by confocal microscopy (*top panel*) and western-blot (*bottom panel*). CyTRAK (orange) was used to counterstain the nucleus. (Scale bar, 10μm) **(B)** Expression of phospho-tyrosines (green) by nasal polyp mucosa from CF patients upon incubation with the TG2 inhibitor R283 and followed by the PPARγ antagonist GW9662. **(C)** The effect of the inhibition of TG2 by TG2 siRNA and TG2 inhibitor R283 on the expression of TNFα by IB3-1 cells was assessed by ELISA. **(A**, **B**, **C)** The slides were analysed by confocal microscopy (scale bar, 10μm).Data shown are representative of at least two different experiments. Figures were kindly provided by Prof. Luigi Maiuri; figures from (Maiuri et al. 2008).

Together, these results showed that increased Ca^{2+} concentration and ROS production in CF cells was driving the activation of TG2 and therefore the inflammation.

To demonstrate that the induction of inflammation was mediated by TG2 promoting the functional sequestration of PPARγ, we used the CFTR-selective inhibitor CFTRinh-172 to induce a CF phenotype in the normal bronchial 16HBE epithelial cell-line. As previously reported (Velsor et al. 2006), the inhibition of CFTR led to an increased production of ROS in 16HBE cells as shown by confocal microscopy (Figure 5-14/A, *top panel*) and fluorescence (Figure 5-14/A, *bottom panel*) analysis using the ROS specific probe CM-H_2DCFDA (5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester).

Similarly, addition of CFTRinh-172 to 16HBE cells promoted an increased phosphorylation of p42/44 proteins and nuclear translocation as revealed by confocal microscopy (Figure 5-14/B, *top panel*) and western-blot (Figure 5-14/B, *bottom panel*) analysis.

Interestingly, by blocking the functional CFTR in 16HBE bronchial cells, we induced TG2 protein expression and activity (Figure 5-14/C) while the expression of PPARγ was drastically decreased (Figure 5-14/D).

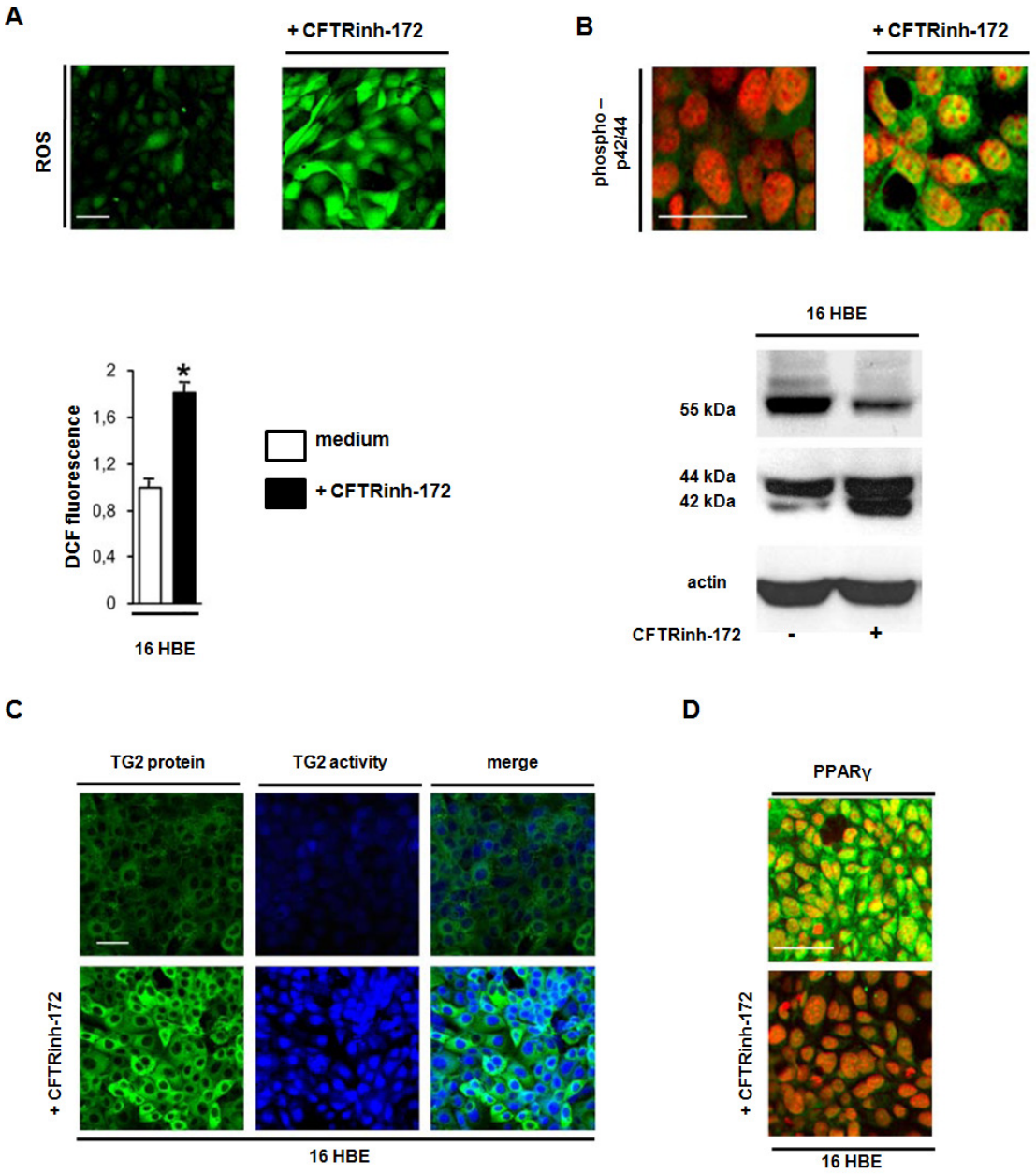


Figure 5-14: Inhibition of CFTR promotes inflammation in airway epithelial cells
(A) The effect of CFTR inhibition using CFTRinh-172 on the amount of reactive oxygen species (ROS, green) produced by bronchial 16HBE cells was assessed by confocal microscopy (*top panel*) and fluorescence (*bottom panel*) using the ROS specific probe CM-H_2DCFDA. **(B)** The level of phosphorylation of p42/44 (green) in 16HBE cells upon inhibition of CFTR with CFTRinh-172 was analysed by confocal microscopy (*top panel)* and western-blot analysis (*bottom panel*). CyTRAK (orange) was used to counterstain the nucleus. **(C)** Effect of the inhibition of CFTR by CFTRinh-172 in 16HBE cells on TG2 expression (green) and activity (blue) was assessed by confocal microscopy. **(D)** Detection of PPARγ (green) in the human 16HBE cell-line with or without the inhibition of CFTR with CFTRinh-172 was assessed by confocal microscopy. CyTRAK (orange) was used to counterstain the nucleus. (Scale bar, 10µm) Data shown are representative of at least two different experiments. Figures were kindly provided by Prof. Luigi Maiuri; figures from (Maiuri et al. 2008).

Altogether, these results specify the central role played by the defective CFTR protein in the induction of inflammation in CF.

5-5/ Summary

Tissue transglutaminase 2 (TG2) is the major player in the pathogenesis of celiac disease (CDis) as operating an important deamidation of glutamine to glutamic acid of the gliadin immunodominant epitopes (Molberg et al. 2008). In patients with CDis, a humoral immune response is also mounted against TG2 (presence of TG2 specific autoantibodies) making it the major autoantigen in CDis (Dieterich et al. 1997; Marsh, 1997; van de Wal et al. 1998; Arentz-Hansen et al. 2000). The unexpected role of TG2 in the modulation of inflammation in CDis (Maiuri et al. 2005) together with the results exposed in this chapter, confirm the central role played by TG2 in controlling inflammation in epithelial cells during chronic inflammatory diseases. In this study, we demonstrated that CFTR-defective epithelial cells present an increased expression of TG2 whose activity is responsible for the sequestration of functional PPARγ by promoting their aggregation into aggresomes.

PPARγ is a major anti-inflammatory protein that repressess inflammatory responses generated by NF-κB (Bailey et al. 2005; Pascual et al. 2005). Its downregulation in CF patients is therefore responsible for the increased inflammatory status of epithelial cells. Compared to wild-type mice, $CFTR^{-/-}$ mice are defective in PPARγ expression (Ollero et al. 2004). While patients with cystic fibrosis upregulate the expression of IL-8 and have a reduction of PPARγ expression, they also suffer from osteoporosis (Dif et al. 2004; Hecker et al. 2004; Shead et al. 2006) Interestingly, increased levels of IL-8 have been associated with bone resorption in cancer patients (Bendre et al. 2003) while PPARγ regulate bone formation in mice and favors bone progenitors to the osteoclast lineage (Wan et al. 2007). The important roles played by PPARγ in lung diseases have been at the centre of the therapeutic strategies aiming at reducing inflammation in chronic diseases of the airways (Huang et al. 2005).

We previously highlighted (Paragraph 1-2-2-1/) the importance of the degradation of proteins by the ubiquitin-proteasome system. Indeed an alteration of this system promotes the aggregation of unfolded / misfolded proteins and functional proteins that are destined for degradation. The accumulation of these proteins in the cells being toxic, they aggregate into inclusion bodies generally situated close to the nucleus. The aggregation of proteins into inclusions bodies is a common feature of various neurodegenerative diseases such as Huntington's or Parkinson's disease (Karpuj et al. 1999; Junn et al. 2003). We have shown

that PPARγ aggregates in perinuclear aggresomes in CFTR-defective airway epithelial cells as they colocalize with HDAC6 and ubiquitin. In C38 cells, misfolded PPARγ and folded PPARγ at the end of their "life" are ubiquitinated and degraded by the proteasome (Figure 5-15).

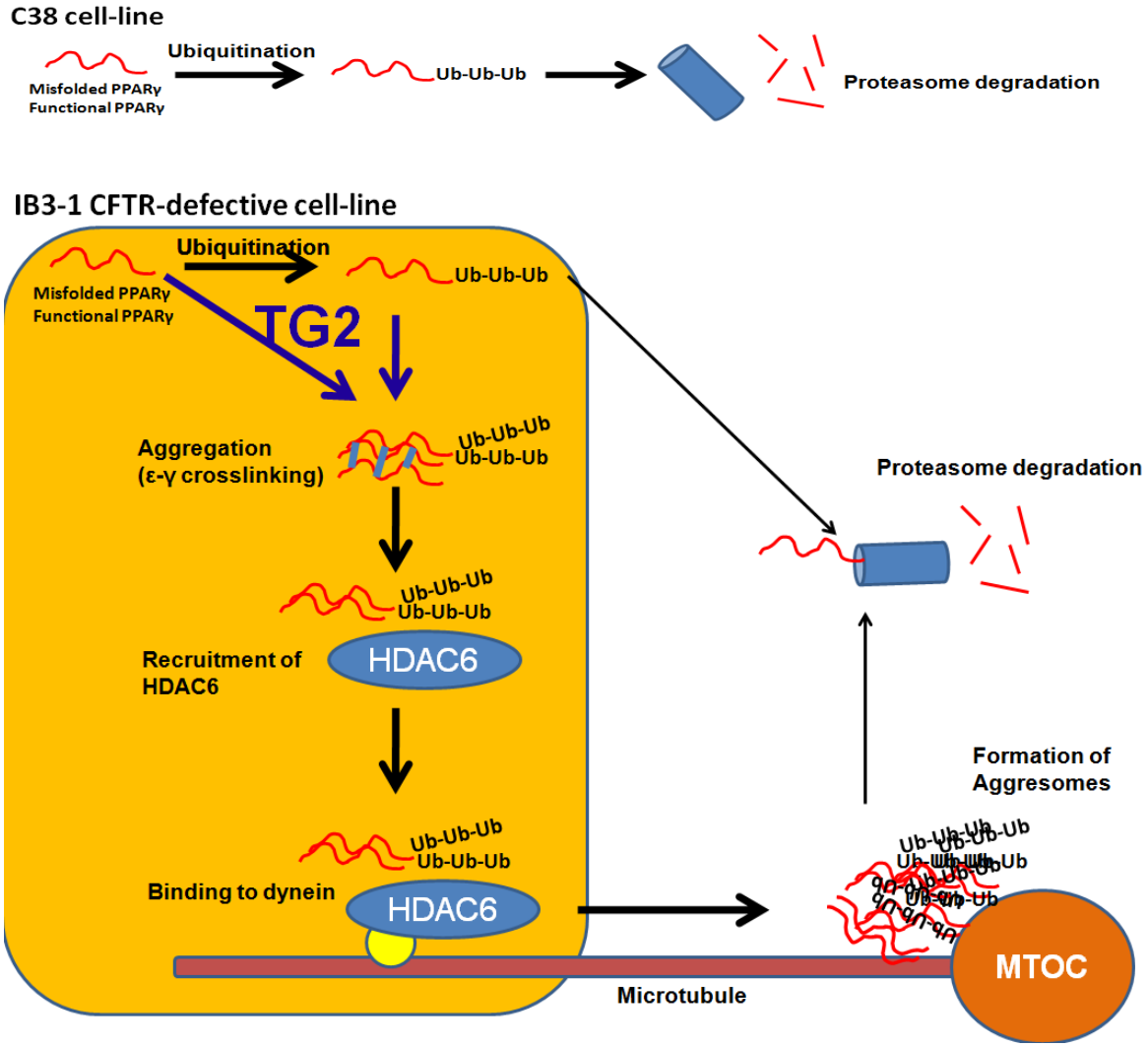


Figure 5-15: Increased TG2 expression and activity in CFTR-defective airway epithelial cells promotes the aggregation of PPARγ into aggresomes
In C38 cells, PPARγ is an anti-inflammatory protein that is commonly degraded by the ubiquitin (Ub) / proteasome system after ubiquitination.
Airway epithelial cells presenting a defective CFTR protein (Cystic Fibrosis Transmembrane Conductance Regulator) upregulate the expression and activity of tissue transglutaminase (TG2). PPARγ proteins ubiquitinated or not are recognized by through the two TG2 specific sites (QG and QXXP) by TG2 which mediates their aggregation by ε-γ crosslinking. PPARγ aggregates are recognized by histone deacetylase 6 (HDAC6) via the poly-ubiquitin chain. This complex then binds dynein that transports the aggregates along the microtubules to the microtubule organizing centre (MTOC) to form aggresomes. The proteasome can still degrade single and aggregated ubiquitinated PPARγ proteins.

Inhibition of the proteasome with MG132 prevents the degradation of PPARγ proteins thus leading to its accumulation in airway epithelial cells. Upon MG132 exposure CFTR-defective airway epithelial cells increase the expression of HDAC6, the ubiquitination status

of PPARγ and its aggregation into aggresomes. By contrast, inhibition of TG2 prevents the aggregation of PPARγ and the formation of aggresomes in CF cells. Ubiquitinated PPARγ proteins can therefore be degraded by the proteasome as observed in CFTR competent cells. This increase of TG2 expression is dependent on the increased concentration of calcium and ROS as a result of the lack of functional CFTR proteins.

This is the first report describing an intrinsic mechanism promoting inflammation in airway epithelia of cystic fibrosis patients.

Upregulation of TG2 has also been associated with metastasis in cancer. Indeed, increased TG2 expression confers resistance to chemotherapy and is linked with the overexpression of drug multi resistance genes (Herman et al. 2006; Verma et al. 2007; Cao et al. 2008). Tissue transglutaminase promotes metastasis by favoring cell attachment, tissue invasion and cell survival. This is partially mediated via the activation of NF-κB resulting from the depletion of functional IκBα proteins (Mehta et al. 2004; Mann et al. 2006; Mann et al. 2007). These studies further strengthen the role played by TG2 in inflammation in chronic inflammatory diseases and cancer. Our study on the consequences of TG2 upregulation in the modulation of inflammation may therefore provide new therapeutic strategies for patients with chronic inflammatory diseases.

6/ Summary – Induction of autoimmune disease in TAZ10 transgenic mice

The general aim of this work was to understand and highlight the mechanisms leading to chronic inflammatory diseases and the effects of some regulatory pathways/networks that control these mechanisms. I have addressed these issues using the humanized TAZ10 transgenic mouse model of spontaneous autoimmune thyroiditis (Quaratino et al. 2004). Here I have investigated the initial events involved in the activation of self-reactive $CD4^+$ T cells which mediate the destruction of the thyroid. TAZ10 transgenic mice express a human TCR specific for a cryptic epitope ($TPO_{524-535}$) of thyroid peroxidase (TPO) generated upon endogenous processing by thyroid epithelial cells (TEC) and a naturally occurring antagonistic epitope ($TPO_{525-536}$) presented by APC upon exogenous processing of TPO.

I found that TPO cryptic epitopes are present in the thyroid and DLN of WT mice. Thus, APCs such as dendritic cells are at the origin of the spreading of TPO cryptic epitopes initiated in the thyroid from the physiological turnover of TECs (Figure 6-1; **Point 1**). I also showed that TECs naturally generate the TPO cryptic epitope from the processing of TPO, although the exact processing pathway is still unknown. Indeed, as shown in figure 4-20, TPO could be degraded after synthesis in the ER and/or upon endocytosis. This issue is currently being investigated. In WT mice, naive T cells do not recognize the cryptic epitope presented by mDCs in the DLN (Figure 6-2; **Point 2**). In TAZ10 mice, T cells bear the human TCR Vβ1/Vα15 specific for the TPO cryptic epitope. T cells from TAZ10 mice are able to recognize the cryptic epitope presented in the context of MHC-II molecules by mDCs in the DLN (Figure 6-2; **Point 3**).

From this point, I showed by adoptive-transfer that transgenic T cells are rapidly activated and proliferate in the DLN within a week. We suggest that the onset of autoimmune disease is gradual and results from a cumulative response of activated transgenic T cells infiltrating the thyroid. At onset, most regulatory networks are absent from DLN of TAZ10 mice. Indeed, MDSCs are not present (similar to the situation observed in LN from WT mice) and Tregs undergo apoptosis upon encounter of the cryptic epitope presented by APCs. APCs taking-up dying TEC in the thyroid, take-up both TPO cryptic epitopes and TPO proteins from which they generate the antagonist TPO epitope (Quaratino et al. 1996; Quaratino et al. 2000). We are currently investigating the different concentrations of $TPO_{524-535}$ and $TPO_{525-536}$ by mass-spectometry analysis of peptide extracts from the thyroid and DLN from WT mice.

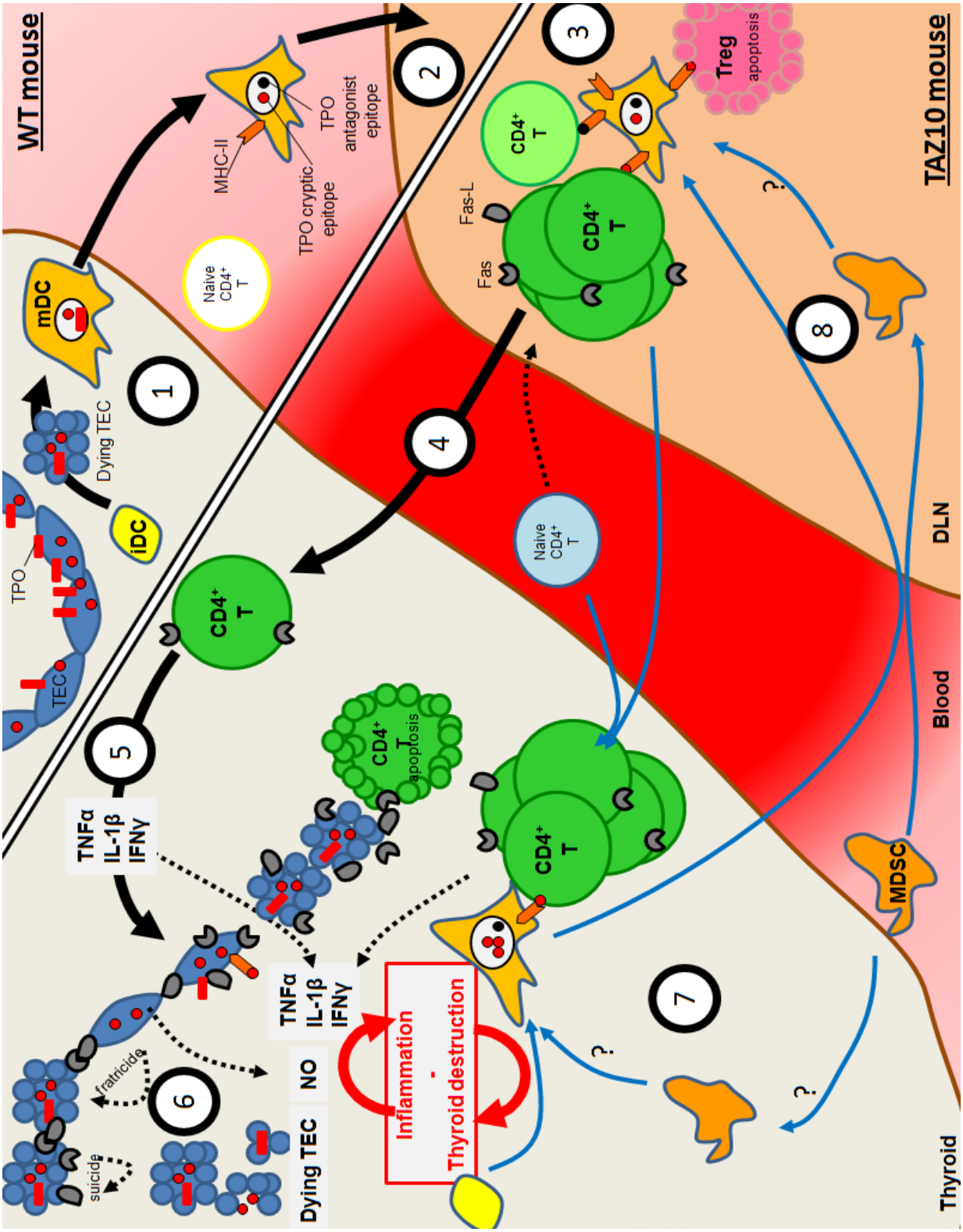


Figure 6-1: Initial events leading to the development of autoimmune disease in TAZ10 transgenic mice
Figure is detailed in the text of this chapter. **iDC**: immature dendritic cell. **mDC**: mature dendritic cell; **MDSC**: myeloid derived suppressor cell; **TEC**: thyroid epithelial cell; **TPO**: thyroid peroxidase; **NO**: nitric oxide; **MHC-II**: MHC class II molecule; **DLN**: cervical draining lymph nodes; **?**: unknown.

From this study, we will be able to investigate the forces dictating T cell activation versus T cell anergy occurring both in DLN and thyroid of TAZ10 mice at onset.

While our study focussed on $CD4^+$ T cells from TAZ10 mice, $CD8^+$ T cells are MHC-II restricted and recognize the TPO cryptic epitope presented in the context of MHC-II by APCs (Quaratino et al. 2004). This important point excluded the possibility that the disease could be induced by $CD8^+$ T cells recognizing MHC-I restricted peptides.

Activated $CD4^+$ T cells migrate and accumulate into the thyroid of TAZ10 mice (Figure 6-1; **Point 4**). The release of cytokines such as IFNγ, TNFα and IL-1β by activated transgenic T cells in the thyroid (Figure 6-1; **Point 5**) is the first event leading to the death of thyrocytes.

Indeed, TECs secrete nitric-oxide and upregulate the expression of Fas and Fas-L upon stimulation with inflammatory cytokines (Figure 6-1; **Point 6**). This triggers a destructive cascade of events leading to the death of thyrocytes. TEC expressing Fas and Fas-L die by suicide or fratricide and promote the death of activated transgenic Fas^+ $CD4^+$ T cells. At this stage, an amplification loop of inflammation is in place as more T cells are recruited. These T cells can come from the DLN and be activated. They can also be activated in the thyroid as mature APCs present the TPO cryptic epitope in the context of MHC-II molecules. I showed that TECs expressing MHC-II upon stimulation with inflammatory cytokines can present the TPO cryptic epitope to T cells from TAZ10 mice. It is unlikely that the expression of MHC-II by TEC is involved at onset of disease; it could however be involved in the amplification of inflammation after first thyroid damages have occurred. Other cells of the immune system are infiltrating the thyroid such as circultating Tregs that undergo apoptosis in the thyroid upon recognition of TPO cryptic epitopes.

The increasing inflammation leads to the recruitment of MDSCs in the thyroid (Figure 6-1; **Point 7**) and DLNs (Figure 6-1; **Point 8**). We do not know yet the precise role of these cells in the pathogenesis of the disease. It is clear that they are not present at onset of disease and they accumulate in these tissues as inflammatory processes amplify. Despite their immunosuppressive functions, they are not able to prevent the progression of the disease. However, they express specific markers of APCs (such as CD11b and F4/80) and activation markers such as MHC-II and costimulatory molecules. It could be possible that MDSCs differentiate into immunogenic APCs thus contributing to the pathogenesis of the disease. We are currently investing this point.

I also highlighted the potential role played by TG2 in the pathogenesis of autoimmune thyroiditis. Treatment of TAZ10 mice with the TG2 specific inhibitor cystamine drastically reduced markers of inflammation such as the expression of MHC-II by TEC. TAZ10 mice treated with cystamine had reduced amounts of MDSC and effector $CD4^+$ T cells in the DLN. TG2 promotes inflammation in celiac disease (CDis) and we showed that up-regulation of TG2 in cystic fibrosis leads to inflammation mediated by the functional sequestration of anti-inflammatory PPARγ proteins in perinuclear aggresomes (Maiuri et al. 2008; Chapter 5).

It is now admitted that patients with autoimmune thyroiditis are more susceptible to develop CDis (Duntas, 2009). By analysing the diets of TAZ10 mice, we realised that food-pellets used to feed TAZ10 and WT mice contained wheat, gliadin and barley. More interestingly, $Rag^{+/+}$ TAZ10 mice have diarrhea, a common symptom of CDis, while $Rag^{-/-}$ TAZ10 mice do not. We are currently assessing whether $Rag^{+/+}$ TAZ10 mice develop celiac disease. If this is the case, the study of TG2 in TAZ10 mice could unravel interesting mechanisms leading to the generation of CDis. As $Rag^{-/-}$ TAZ10 only have transgenic $CD4^+$ and $CD8^+$ T cells, we intend to address whether CDis in TAZ10 mice with autoimmune thyroiditis is mediated by T cells, B cells or a combination of the two.

From this work, there are a number of questions that have emerged. Future work could investigate these questions as follows:

- The precise sequence of the TPO peptides remains to be determined. The nature of the different TPO peptides (*ie*. $TPO_{524-535}$ and $TPO_{525-536}$ and their human equivalent) has so far been determined using synthetics peptides (Quaratino et al. 1996 and 2000). The nature of these different peptides would be determined by HPLC and Mass Spectrometry analysis. To investigate the endogenous processing of TPO, we will generate an immortalized thyroid epithelial cell-line using the pSV3-neo expression vector (ATCC, USA). To investigate the exogenous processing, we are planning to use BM-DCs generated following the Lutz's method.

- The different mechanisms and proteases involved in the processing of endogenous and exogenous TPO by TEC and BM-DC respectively, could be analysed using the different cellular systems above.
- We also aim to characterize the quantity of the TPO cryptic and APL peptides from peptide extracts that will allow us to measure the forces of anergy (TPO525-536) versus activation (TPO524-535). This will be performed using proliferation assays by flow cytometry and also by studying the different signalling pathways following TCR engagment.
- Finally, we will analyse the fate of naive $CD4^+$T cells after adoptive transfer into Rag-/- and WT mice to assess which $CD4^+$ T cell subsets are generated following recognition of the TPO cryptic epitope in the draining LNs. We will then assess the kinetic of recruitment of MDSCs in TAZ10 mice at the onset of the disease.

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8/ Appendices

Appendix 1: 1987 Criteria for the Classification of Acute Arthritis of Rheumatoid Arthritis

Criterion	Definition
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides fo the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

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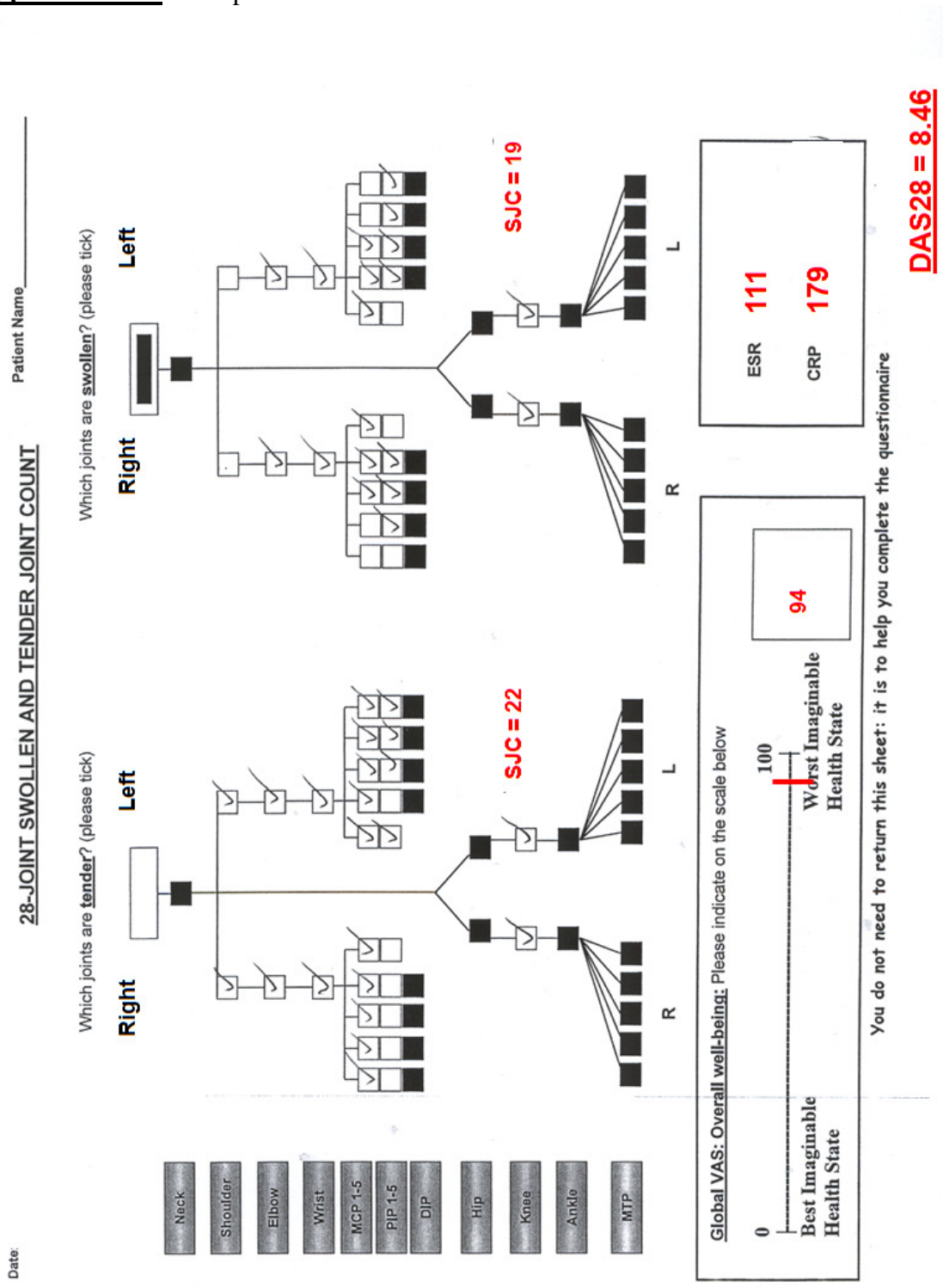
* For classification purposes, a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least 4 or these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks. Patients with 2 clinical diagnoses are not excluded. Designation as classic, definite, or probable rheumatoid arthritis is *not* to be made.

Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315---24.

Appendix 1-bis: Example of DAS28 score



Appendix 2: The 1997 Update of the 1982 American College of Rheumatology Revised Criteria for Classification of Systemic Lupus Erythematosus (http://www.rheumatology.org/publications/classification/SLE/1997UpdateOf1982RevisedCriteriaClassificationSLE.asp?aud=mem)

Criterion	Definition
1. Malar Rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician
5. Nonerosive Arthritis	Involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
6. Pleuritis or Pericarditis	a) Pleuritis--convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion *OR* b) Pericarditis--documented by electrocardigram or rub or evidence of pericardial effusion
7. Renal Disorder	a) Persistent proteinuria > 0.5 grams per day or > than 3+ if quantitation not performed *OR* b) Cellular casts--may be red cell, hemoglobin, granular, tubular, or mixed
8. Neurologic Disorder	a) Seizures--in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance *OR* b) Psychosis--in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance
9. Hematologic Disorder	a) Hemolytic anemia--with reticulocytosis *OR* b) Leukopenia--< 4,000/mm^3 on ≥ 2 occasions *OR* c) Lyphopenia--< 1,500/ mm^3 on ≥ 2 occasions *OR* d) Thrombocytopenia--<100,000/ mm^3 in the absence of offending drugs
10. Immunologic Disorder	a) Anti-DNA: antibody to native DNA in abnormal titer *OR* b) Anti-Sm: presence of antibody to Sm nuclear antigen *OR* c) Positive finding of antiphospholipid antibodies on: **1. an abnormal serum level of IgG or IgM anticardiolipin antibodies,** **2. a positive test result for lupus anticoagulant using a standard method, or** **3. a false-positive test result for at least 6 months confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test**
11. Positive Antinuclear Antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs