**UNIVERSITY OF SOUTHAMPTON**

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

# Functional analysis of TL1A/DR3 interactions during T cell-mediated immune responses

by

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ABSTRACT
FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES
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FUNCTIONAL ANALYSIS OF TL1A/DR3 INTERACTIONS DURING T CELL-MEDIATED IMMUNE RESPONSES
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Members of the tumour necrosis factor superfamily (TNFSF) are important regulators of inflammation and immunity. TL1A (TNFSF15), ligand for death receptor 3 (DR3), is the most recently discovered member of this superfamily and full understanding of its structure and the role in T cell-mediated immune responses is currently incomplete. DR3 expression is strongly up-regulated on activated T cells, although it also is present on resting $CD4^+$ T cells, while the expression of TL1A is rapidly and transiently up-regulated on activated cells of the immune system such as dendritic cells, monocytes and T cells. The research published to date shows that TL1A/DR3 interaction is involved in the pathogenesis of several autoimmune diseases and enhances activation of $CD4^+$ T cells, however very little is known about its role in co-stimulation of $CD8^+$ T cells. Several studies showed that TL1A also acts as a polarizer of the immune response by inducing secretion of IFN-γ, IL-4, IL-10 and/or IL-17A from activated $CD4^+$ T cells, although the results vary depending on the conditions of a given experiment. The research presented in this thesis identifies Toll-like receptors 3 and 4 as the inducers of TL1A expression on dendritic cells. Furthermore, different binding patterns of anti-TL1A monoclonal antibody (raised against the homotrimeric form of TL1A) and DR3.Fc construct to cells transfected with TL1A cDNA and cells naturally expressing TL1A suggest that TL1A may exist as a homo- and heterotrimeric protein. Ecotopic expression of TL1A on J558L tumour cells promotes their elimination in a $CD8^+$ T cell-dependent manner and renders mice immune to a subsequent challenge with tumour cells. Moreover, TL1A promotes the proliferation and accumulation of antigen-specific $CD8^+$ T cells both *in vitro* and *in vivo* as well as their activation and differentiation into cytotoxic T cells *in vivo*. It also enhances the secondary expansion of endogenous antigen-specific memory $CD8^+$ T cells. The studies presented here also show that TL1A/DR3 interaction enhances the proliferation and activation of $CD4^+$ T cells. CD11c-TL1A transgenic and CD2-TL1A transgenic mice that constitutively express TL1A on dendritic cells and T cells, respectively, show elevated levels of IL-13 and IL-17A in the secondary lymphoid organs suggesting that in this setting TL1A skews the immune response toward Th2 and Th17 type. Furthermore, CD11c-TL1A transgenic mice develop a striking goblet cell hyperplasia in the ileum. TL1A also enhances regulatory T cell accumulation *in vivo*. The findings presented in this thesis show that TL1A may have the potential for enhancing vaccines that aim to elicit $CD8^+$ T cell responses and also identify mechanisms by which sustained expression of TL1A could promote pathogenesis in inflammatory bowel disease.

**LIST OF CONTENTS**

## LIST OF TABLES AND FIGURES

**Academic Thesis: Declaration of Authorship**

I, Tomasz Jerzy Ślebioda, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

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I confirm that:

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## ABBREVIATIONS

| | |
|---|---|
| **A-GalCer** | A-Galactosylceramide |
| **Ab** | Antibody |
| **ACAD** | Activated T cell autonomous death |
| **AICD** | Activation-induced cell death |
| **ALPS** | Autoimmune lymphoproliferative syndrome |
| **AP1** | Activator protein 1 |
| **APC** | Antigen presenting cell |
| **BSA** | Bovine serum albumin |
| **BMDC** | Bone marrow-derived dendritic cell |
| **CCR** | CC-chemokine receptor |
| **CD** | Cluster of differentiation |
| **CDK** | Cyclin-dependent kinase |
| **CDR** | Complementarity-determining region |
| **CLIP** | Class II invariant chain peptide |
| **CFSE** | 5, (6)-carboxyfluorescein diacetate succinimidyl ester |
| **CRD** | Cysteine-rich domain |
| **CTL** | Cytotoxic T lymphocyte |
| **CTLA-4** | Cytotoxic T lymphocyte antigen-4 |
| **DC** | Dendritic cell |
| **DC-SIGN** | DC-specific ICAM-3 grabbing non-integrin |
| **DD** | Death domain |
| **DMSO** | Dimethyl sulfoxide |
| **DR** | Death receptor |
| **ELISA** | Enzyme-linked immunosorbant assay |
| **FADD** | Fas-associated protein with death domain |
| **FCS** | Foetal calf serum |
| **FITC** | Fluorescein isothiocyanite |
| **GITR** | Glucocorticoid-induced TNFR family receptor |
| **GS** | Glutamine synthetase |
| **h** | Hours |
| **HRP** | Horseradish peroxidise |
| **HVEM** | Herpes virus entry mediator |
| **IBD** | Inflammatory bowel disease |
| **ICAM** | Intracellular adhesion molecule |
| **ICOS** | Inducible co-stimulator |
| **IFN** | Interferon |
| **Ig** | Immunoglobulin |
| **IKK** | IκB kinase |
| **IL** | Interleukin |
| **i.p.** | Intraperitoneal |
| **IRF** | Interferon regulatory transcription factor |
| **ITAM** | Immune tyrosine-based activation motif |
| **i.v.** | Intravenous |
| **JNK** | c-Jun N-terminal kinase |
| **L** | Ligand |
| **LFA-1** | Lymphocyte function-associated antigen-1 |
| **LPS** | Lipopolysaccharide |
| **LT** | Lymphotoxin |

| | |
|---|---|
| **mAb** | Monoclonal antibody |
| **MAPK** | Mitogen-activated protein kinase |
| **MFI** | Mean fluorescence intensity |
| **MHC** | Major histocompatibility complex |
| **ML** | Mesenteric lymph node |
| **MMP** | Matrix metalloproteinase |
| **MSX** | Methionine sulfoximine |
| **NFAT** | Nuclear factor for activated T cells |
| **NF-κB** | Nuclear factor κB |
| **NK** | Natural killer cell |
| **NKT** | Natural killer T cell |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **Pam3CSK4** | (S)-(2,3-bis(polmitoyloxy)-(2RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-$Lys_4$-OH trihydrochloride |
| **PAMP** | Pathogen-associated molecular pattern |
| **PBS** | Phosphate-buffered saline |
| **PCR** | Polymerase chain reaction |
| **PE** | Phycoerithrin |
| **PKB** | Protein kinase B |
| **PKC** | Protein kinase C |
| **PLAD** | Pre-ligand assembly domain |
| **Poly(I:C)** | Polyriboinosinic:polyribocytidylic acid |
| **PRR** | Pattern recognition receptor |
| **s.c.** | Subcutaneous |
| **SCID** | Severe combined immunodeficiency syndrome |
| **SDS** | Sodium dodecyl sulfate |
| **SE** | Standard error |
| **SF** | superfamily |
| **TAP** | Transporter associated with antigen processing |
| **$T_{CM}$** | Central memory T cell |
| **TCR** | T cell receptor |
| **$T_{EM}$** | Effector memory T cell |
| **Th** | Helper T cell |
| **TLR** | Toll-like receptor |
| **TNF** | Tumour necrosis factor |
| **TNFR** | Tumour necrosis factor receptor |
| **TRAF** | TNFR-associated factor |
| **TRAPS** | TNF receptor-associated periodic syndrome |
| **Treg** | Regulatory T cell |

# CHAPTER 1

# INTRODUCTION

## 1.1. Overview of the immune system

The role of the immune system is to protect organisms from invasion by pathogenic agents and growth of tumour cells. There are two main types of the immune response – the innate and the adaptive immunity. Evolutionarily older is the innate immunity, present from higher plants to mammals, which acts as the first line of defence against infection. The innate immune system is less precise than the adaptive one but responds faster, without prior exposure to pathogens. The elements of the innate immune system comprise anatomic and physiological barriers separating organisms from the outside environment, chemotactic and pro-inflammatory cytokines and antimicrobial proteins secreted by epithelial cells, phagocytes, such as macrophages and neutrophils and cytotoxic cells, such as natural killer cells or eosinophils. The adaptive immune system, present in all vertebrates, responds slower than the innate system, because it depends on selection and multiplication of T and B lymphocytes precisely targeted to specific pathogens. The initiation of the adaptive immune response depends, however, on the innate system which first detects the presence of the infection. T cells arise from lymphoid progenitor cells which have differentiated from haematopoietic stem cells. T and B cells mature in the thymus and bone marrow, respectively. Naive mature lymphocytes circulate in blood and lymphatic system. They are activated and acquire the ability to migrate to peripheral tissues in secondary lymphoid organs such as lymph nodes or spleen which have distinct regions for the activation of T and B cells. There are also other, less structured forms of secondary lymphoid tissue such as mucosal-associated lymphoid tissue or skin-associated lymphoid tissue. The adaptive immune response consists of B cell-mediated humoral response and T cell-mediated cellular response which complement each other. There are two major types of T lymphocytes involved in the cellular arm of this response – cytotoxic T lymphocytes (CTLs) which kill target tumour cells or cells infected with pathogens and helper T cells which regulate the function of other lymphocytes. The adaptive immune response allows the generation of specialized memory lymphocytes that survive for years providing the host with faster adaptive response if it is exposed to the given pathogen for the second time.

## 1.2. Development of T cells

The greater part of T cell development occurs in the thymus. Even though the thymus undergoes atrophy and is partially replaced by adipose tissue in mature individuals, the development of new T cells is still maintained (although slows down) and T cell numbers are maintained through cell divisions and survival of mature T cells in the periphery. T cells differentiate from a common lymphoid progenitor that gives rise also to B cells. Stimulation of Notch-1 receptors on the progenitor cells destins them to differentiate into T cells, leave the bone marrow and migrate to the thymus (1).

Progenitor cells that enter the thymus, lack most of the markers characteristic of T cells. During the initial phase of differentiation, they gain distinctive markers of the T cell lineage (e.g. CD2) but do not express the T cell receptor (TCR) or CD4 and CD8 molecules, therefore are called "double-negative" thymocytes (2). A fraction of these cells that expresses FoxP3 gives rise to natural T regulatory cells (nTregs) (3). In mice, nTregs migrate from the thymus into the periphery after the first 3 days of life (4). Double negative thymocytes undergo screening for successful rearrangement of the T cell receptor (TCR) β chain which is expressed together with the pre-TCRα chain. This process is called β-selection. The correct assembly of this pre-TCR leads to intensive cell proliferation, the arrest of further rearrangements of β, γ and δ chains, and induces the expression of both CD4 and CD8 molecules. The cells which do not manage to correctly rearrange the β chain undergo apoptosis. Rearrangement of genes encoding the chains comprising TCR is the main source of T cell diversity. It is still very unclear which signals induce the differentiation of Tγδ cells. However, it is believed that this process begins before the rearrangement of TCR genes and Tγδ cells arise before Tαβ cells during thymic ontogeny (2, 5). After the double positive cells cease to proliferate, they express recombination activating genes (RAG), rearrange the TCRα locus and down-regulate the expression of the pre-TCRα chain. Thymocytes, expressing the mature TCRαβ complex that does not bind to self-peptide-MHC complexes present on epithelial cells of the thymic cortex, die by apoptosis while the cells that are able to interact with self-peptide-MHC survive. Depending on whether the latter interaction is with MHC class I or class II, cells differentiate to either CD8 or CD4 single positive mature lineages (6). Cells migrate subsequently into thymic medulla where they undergo functional maturation and negative selection to eliminate self-reactive clones (7).

### 1.3. Subsets of T cells

T cells are divided into two main subsets based on the presence or absence of the co-receptors CD4 and CD8. After activation, naive $CD4^+$ T cells differentiate into functional subsets called T helper 1 (Th1), T helper 2 (Th2) or T helper 17 (Th17) which produce defined and specific sets of cytokines (8, 9). Another subset of $CD4^+$ T cells are regulatory T cells (Tregs) which are further divided into natural Tregs (nTregs) that develop in the thymus and inducible T regs (iTregs) that are induced from naive $CD4^+$ T cells in the periphery. There is, however, no reliable marker that would allow to distinguish nTregs from iTregs (9). Differentiation of $CD4^+$ T cells into different subsets is regulated primarily by cytokines secreted by activated antigen presenting cells. Signalling molecules, transcription factors and cytokines produced by different $CD4^+$ T cell subsets prevent the differentiation of other subsets of these cells (10, 11).

Th1 cells are protective against intracellular microbes and play a pathogenic role in chronic inflammatory disorders. These cells produce high levels of IFN-γ, IL-2 and TNF, which are responsible for the activation of macrophages and play an important role in cell-mediated immunity. However, it is not correct to say that Th1 cells are responsible only for cell-mediated immunity since they also provide help for B cells in production of antibodies – they allow the production of IgG2a antibodies in mice and of IgM, IgA, IgG1, IgG2 and IgG3 in humans (10). IFN-γ and IL-12 are powerful inducers of Th1 differentiation. IFN-γ activates the signal transducer and activator of transcription-1 (STAT-1) in naive $CD4^+$ T cells which up-regulates the Th1 master regulator, transcription factor T-bet. T-bet is responsible also for induction of IFN-γ production and IL-12Rβ2 expression on $CD4^+$ T cells which can now directly respond to IL-12. STAT-4, an IL-12 signal transducer, is important for amplifying Th1 responses; it directly induces IFN-γ production in activated Th1 cells which initiates the positive feedback loop in which IFN-γ acting through T-bet induces more IFN-γ. In this way collaboration between IFN-γ and IL-12 induces full Th1 differentiation. Furthermore, IL-18 which is not involved in Th1 differentiation, synergizes with IL-12 in inducing IFN-γ, implying that this cytokine also plays an important role in Th1 responses (10, 11).

Th2 cells mediate host defence against extracellular pathogens. Cytokines produced by Th2 cells mediate IgE class switching in B cells (IL-4 and IL-13), activate eosinophils (IL-5) and mast cells (IL-9), they also induce proliferation and antibody production by B

cells (IL-4, IL-5, IL-10 and IL-13), notably IgG1 and IgE in mice and IgM, IgG4 and IgE in humans (10). Th2 cells produce also IL-17E which amplifies Th2-mediated responses (9-11). Differentiation of Th2 cells depends on IL-4 which activates STAT-6 which in turn up-regulates the transcription factor GATA-3 which is the master regulator of Th2 genes expression. Another important regulator of Th2 differentiation is IL-2-induced STAT-5. Collaboration between GATA-3 and STAT-5 allows for full differentiation of Th2 cells (10, 11).

Th1 and Th2 cells are fully differentiated after 3-4 cell divisions. After that time, they cannot change their phenotype to the opposite one when they are treated with the appropriate set of Th1 or Th2-polarizing cytokines (12). Th cells are very heterogeneous, only a small proportion of cells expresses the full set of cytokines of a given subpopulation (9). Moreover, some Th1 cells can produce IL-10 while IFN-γ production is maintained (9).

Th17 cells mediate immune responses against fungi and extracellular bacteria. They also participate in the induction of many autoimmune disorders such as multiple sclerosis or rheumatoid arthritis. Th17 cells produce IL-17A, IL-17F, IL-21 and IL-22. IL-17A induces the expression of many pro-inflammatory cytokines. Both IL-17A and IL-17F recruit and activate neutrophiles during immune responses. Th17 differentiation is induced by TGF-β and IL-6 which induce the expression of IL-21, IL-23R and the transcription factor RORγt which is responsible for the induction of IL-17A and IL-17F production. IL-21 can replace IL-6 in inducing RORγt, therefore acts as an amplification cytokine for Th17 differentiation. Another important cytokine is IL-23 which is critical for survival and maintaining the function of Th17 cells, although it does not induce their differentiation. IL-6, IL-21 and IL-23 act via common signal transducer, STAT-3, which is indispensable for Th17 differentiation (11).

Tregs are critical for regulating immune responses and maintaining self-tolerance by suppressing a variety of both adaptive and innate immunity cells, including $CD4^+$ and $CD8^+$ T cells, dendritic cells (DCs), mast cells and NK cells. Tregs do not rely on only one mechanism for their function but instead use many alternate mechanisms to control immune responses. They inhibit degranulation of mast cells via OX40/OX40L interaction (13) and directly kill DCs (14), B cells (15) and NK cells (16) via perforin and granzymes; the same mechanism is used for killing of effector $CD4^+$ and $CD8^+$ T cells (17). Interestingly, $CD8^+$, but not $CD4^+$ T cells, are killed by Tregs also by Fas/FasL interaction (18). Tregs suppress the activity of $CD4^+$ and $CD8^+$ T cells also by secretion of TGFβ

which has an anti-proliferative effect on T cells due to its ability to block the upregulation of high affinity IL-2 receptors on T cells and is therefore able to block the ability of T cells to respond to IL-2 stimulation (19). TGFβ upregulates also cell cycle inhibitors in T cells (20). Other cytokines secreted by Tregs, which interfere with T cell activation, are IL-10 and IL-35. There are reports that stimulation of TCR-activated T cells with IL-10 results in their anergy (17, 20) while IL-35 enhances proliferation of Tregs but suppresses the proliferation of TCR-activated $CD4^+$ $CD25^-$ T cells (21, 22). Finally, due to the constitutive expression of CD25, a high affinity receptor for IL-2, Tregs remove IL-2 from the nearby environment and thereby prevent proliferation and differentiation of T cells (23). It is worth to emphasise that although the suppressive activity of Tregs requires their prior activation via TCR, once activated, they suppress in a non antigen specific way. Thus, Tregs with one antigen-specificity can suppress effector T cells with many other distinct antigen specificities (23). Tregs inhibit also maturation and cytokine production of DCs and in this way suppress indirectly also the activity of T cells. IL-10 and TGFβ, secreted by Tregs, inhibit the production of pro-inflammatory cytokines and downregulate the production of a number of costimulatory molecules on DCs, including CD80 and CD86; they also downregulate the expression of MHC class II by DCs which profoundly affects the ability of DCs to activate effector T cells (20). Tregs also modulate the metabolism of target cells via expression of nucleoside triphosphate diphosphohydrolase-1 (CD39) and ecto-5'nucleotidase (CD73) (11, 24). During the normal course of an immune response, Tregs expand at the rate parallel to the effector cells (25), at the peak of the response, their relative accumulation can even exceed that of effector T cells (26). Homeostatic proliferation of Tregs auguments their ability to suppress target cells (27); therefore they gain full suppressive activity right after the peak of the response. Both in mice and humans, Tregs are defined as $CD4^+CD25^+FoxP3^+$ cells. They constitutively express high levels of CD25 (IL-2Rα), whereas the expression of this molecule on conventional $CD4^+$ T cells is much lower and transient (induced after the activation). The distinguishing feature of iTreg differentiation is its dependence on TGF-β (28). High concentration of TGF-β in the absence of pro-inflammatory cytokines (e.g. IL-6) activates Smad-3, while TCR stimulation activates the transcription factor NFAT. NFAT cooperates with Smad3 in induction of FoxP3 which is a transcription factor crucial for iTreg differentiation (11). Differentiation of nTregs in the thymus depends on TCR signalling of increased strength which results in up-regulation of CD25 (IL-2Rα) and increased responsiveness of IL-2; the other important factor is CD28 signalling. Activation of these

pathways results in recruitment of NFAT, cRel, Creb and STAT-5 and consequently leads to the induction of FoxP3 expression in developing thymocytes (28).

$CD8^+$ T cells become cytotoxic T cells (CTLs) which recognize antigens presented by MHC class I molecules and kill tumour cells or cells infected with pathogens. They mediate killing of target cells through the secretion of serine proteases (granzymes), perforin or molecules belonging to the tumour necrosis factor superfamily, such as FasL (29, 30). Perforin is a pore-forming protein that is capable of membrane permeabilization (29). It causes little death at low concentrations, although at high concentrations, it can cause necrotic death of a cell due to the loss of membrane integrity (31). The main role of perforin, however, is delivery of granzymes into the cytosol of the target cell where they independently of each other trigger apoptosis (29, 31). For example, granzyme B mediates detachment of cells and causes cell death by anoikis (death due to the lack of extracellular contact). It also cleaves Bid protein to its truncated form (tBid) which activates mitochondrial pathway of apoptosis. Other target proteins for granzyme B are caspase-8 and caspase-3 which cleavage also activates apoptosis (29). Granzyme A, as well as granzyme B, directly cleave lamin B which maintains the structural integrity of the nuclear envelope and consequently induce apoptosis independently of caspases (32). FasL-mediated apoptotic death of target cells for CTLs is the consequence of triggering of Fas receptor on the surface of target cells. CTLs can release FasL in its membrane-bound form into the synaptic cleft between a CTL and a target cell in a process mediated by secretory lysosomes. The transmembrane form of FasL is 1000 times more biologically active than the cleaved soluble form (30).

$CD8^+$ T cells are divided into conventional Tαβ and Tγδ cells depending on the structure of their TCR; additionally $CD8^+$ Tγδ usually express CD8αα homodimer instead of CD8αβ heterodimer expressed on conventional $CD8^+$ Tαβ cells (33).

Another subset of CTLs are natural killer T cells (NKT cells). Most of them are $CD4^-$ and $CD8^-$, although a small proportion of NKTs is $CD4^+$. NKT cells express TCRαβ and markers typical for natural killer (NK) cells (such as NK1.1 or CD49b in mice or CD16 and CD56 in humans) and recognize glycolipids presented by antigen presenting cells by CD1 molecules (2, 34). Activation of NKT cells leads to rapid secretion of IFN-γ, IL-4, IL-2, TNF and other cytokines that regulate the immune response. They also up-regulate expression of co-stimulatory molecules, such as CD40L, thus contribute to activation of dendritic cells (35) and can directly kill target cells through cytolytic activity – by secretion of perforin and expression of FasL (2, 34).

## 1.4. T cell-mediated immune response

T cells are recruited from blood vessels to secondary lymphoid organs such as the lymph nodes or the spleen where they interact with antigen presenting cells (e.g. dendritic cells) and become activated. The site of T cell activation is located in the paracortex region of the lymph nodes and in the white pulp surrounding the branches of the splenic arteries. Most cytotoxic T cells are $CD8^+$ lymphocytes that recognize antigens presented on MHC class I molecules. For activation, these cells require help provided by $CD4^+$ T helper lymphocytes and dendritic cells. Dendritic cells not only present antigens which are recognized by T cell receptors (TCRs) cells but also express co-stimulatory molecules that are indispensable for activation of T cells. The process of activation of $CD4^+$ and $CD8^+$ T cells is very similar although $CD4^+$ cells recognize antigens presented in the context of MHC class II while $CD8^+$ T cells in the context of MHC class I (2).

The interaction between T cells and dendritic cells occurs in three distinct phases. During the first phase, T cells migrate in random directions after entering the lymph node and undergo multiple short encounters with dendritic cells. In this way, T cells scan dendritic cells to locate the one that is presenting the antigen recognised by a given clone of T cells. During the second phase, T cells form stable antigen-dependent conjugates with dendritic cells and secrete IFN-γ and IL-2. This phase is responsible for activation of T cells and promotion of their proliferation, survival and effector functions. Finally, during the last phase, T cells resume rapid migration and their proliferation is significantly increased (36).

The initial, antigen-independent interaction between T cells and dendritic cells is facilitated by the interaction between adhesion molecules DC-specific ICAM-3 grabbing integrin (DC-SIGN) and ICAM-3 expressed on dendritic cells and T cells, respectively (37). After the engagement of TCR, cell to cell contact is stabilised through adhesion between integrins (e.g. LFA-1) on T cells and ICAM molecules on antigen-presenting cells. The next step is formation of an immunological synapse which leads to the development of a ring of LFA-1/ICAM complexes surrounding a central region of increased density of TCR/antigen-MHC complexes which auguments TCR occupancy by antigen-MHC complexes and enhances cross-talk between the clustered receptors, thus promotes sustained signalling (38).

Help provided by $CD4^+$ T cells is required by $CD8^+$ T cells for efficient priming and memory development. There are reports showing that $CD8^+$ T cells can be successfully

primed without $CD4^+$ T cell-help, although they are incapable of mounting efficient secondary response in the absence of help provided by $CD4^+$ T cells during the initiation of the immune response (39, 40). $CD4^+$ T cells do not require direct contact to provide help for $CD8^+$ T cells. They provide an indirect help by secreting cytokines that increase their proliferation (e.g. IL-2) inducing activation of dendritic cells which in turn up-regulate the expression of co-stimulatory molecules.

Activation of T cells results in their rapid expansion initiated in the secondary lymphoid organs and development of effector functions (2). Effector $CD8^+$ T cells reduce the expression of L-selectin (CD62L), up-regulate chemokine receptors CCR2 and CCR5 and gain the ability to migrate into inflamed tissues (41) where they kill target cells that express antigens recognised by them in the context of MHC class I molecules. Non-specific proliferation (i.e. proliferation which is not specific for a given clone) of T cells may be induced by mitogens – substances that trigger mitotic cell division (e.g. phytohaemagglutinin (PHA) or concavalin A (ConA)). Other class of substances that can trigger non-specific proliferation of T cells are superantigens, produced by pathogenic microorganisms, which induce also a massive cytokine release from T cells. They bind directly to a specific β chain of a TCR or to the outer surface of MHC class II molecules. It is estimated that superantigens are capable of activating up to 20% of T cells present in the body (2).

The immune response is followed by a contraction phase during which ~90% of activated T cells undergo apoptosis. This is the result of activation-induced cell death (AICD) mediated by the interaction between TNF receptor superfamily members expressed on T cells and TNF superfamily members expressed on the same or neighbouring cells (FasL/Fas, CD30L/CD30, TNFR1/TNF and TNFR2/TNF) (42-44). This process is described in more detail in the section 1.8.2. The other mechanism that leads to death of activated T cells is activated T cell autonomous death (ACAD), also termed "cytokine withdrawal", which is controlled by the balance of pro- and anti-apoptotic members of the $Bcl_2$ family that regulate cell death by inducing or inhibiting the mitochondrial pathway of apoptosis. Limitation of IL-2 in the environment during the contraction phase results in the decrease of anti-apoptotic protein $Bcl_2$ inside the cell. Activated cells show also elevated levels of a pro-apoptotic protein Bim which, together with Bax and Bak, induces the release of apoptogenic factors from mitochondria, downstream activation of caspase-9 and eventual cell death (43).

Around 10% of T cells survive the contraction phase of the immune response. They form a pool of memory cells that persists for years. In the absence of antigens, homeostatic proliferation of $CD8^+$ memory T cells depends on IL-15, whereas continual division of $CD4^+$ memory T cells appears to be IL-12-dependent (45). Memory T cells provide faster immune response when the host organism is exposed to the antigen for the second time (2). Compared to naive T cells, memory cells divide after a shorter lag time, have increased division rate and show more rapid and efficient development of effector functions (46).

Memory T cells established after infection can be divided into two subsets – effector memory T cells ($T_{EM}$) and central memory T cells ($T_{CM}$). $T_{EM}$ cells are terminally-differentiated cells, present in peripheral tissues, that lack lymph node-homing receptors (CD62L and CCR7) but express receptors that enable these cells to migrate to inflamed tissues, such as CD44, CCR4 or CCR9. $T_{EM}$ cells exert immediate effector functions without the need for further differentiation. $T_{CM}$ cells circulate through the secondary lymphoid organs (and show expression of both CD62L and CCR7), they lack immediate effector functions but can develop the phenotype and function of $T_{EM}$ cells after restimulation with antigen. After pathogen clearance, the antigen-specific memory cell pool consists mostly of $T_{EM}$ cells which convert into $T_{CM}$ cells over time (45, 47).

## 1.5. Structure and signalling of the T cell receptor

T cell receptors (TCRs) are type I transmembrane dimeric proteins belonging to the immunoglobulin superfamily; the average number of TCRs present on a single T cell is $5x10^4$. Over 90% of peripheral blood T lymphocytes have TCRs composed of α and β (TCRαβ) chains, while in 1-10% of peripheral blood lymphocytes the receptor is composed of γ and δ chains (TCRγδ). There is also a very small proportion of T cells bearing TCRs composed of two γ chains, two β chains or β and γ chain (2). The dimeric structure of a TCR is stabilised by disulfide bridges formed between the two chains of the receptor. Each polypeptide chain consists of a membrane proximal constant domain and a membrane distal variable domain which is composed of three complementarity-determining regions (CDRs) that recognise antigens in the context of MHC molecules. The hypervariable CDR3 is particularly important for antigen specificity of TCR, while CDR1 and CDR2 seem to play a less important role in this process (2, 38). The cytoplasmic domains of TCRs are devoid of any signalling motifs, therefore TCRs are expressed in conjunction with CD3 molecules which contain immune tyrosine-based activation motifs (ITAMs) and mediate signalling from TCRs (38). CD3 molecules are organized in dimeric units of CD3γε, CD3δε and CD3ζζ which form a complex associated with two TCRs (48). TCRs are associated also with CD4 or CD8 molecules which bind to MHC class II or MHC class I molecules respectively (2).

During the interaction between TCR and antigen-MHC complex, CD4 or CD8 co-receptors bind to invariant regions of MHC class II or MHC class I, respectively. This interaction activates Lck kinase which is associated with the cytoplasmic tail of CD4 and CD8. Lck kinase phosphorylates tyrosine residues in the ITAM motifs of CD3ζ chains which subsequently bind ξ-chain-associated protein kinase 70 (ZAP70) that is indispensable for activation of T cells. ZAP70 is then activated by phosphorylation by Lck and in turn phosphorylates itself and the ITAM motifs in other CD3 chains which now can also bind ZAP70. This kinase phosphorylates also adaptor proteins LAT and SLP76 which activate the transcription factor NF-AT via other proteins such as Grb2, Grap and Vav. TCR engagement leads also to phosphorylation of phospholipase C-γ (PLC-γ) which catalyses degradation of phosphatidylinositol-4,5-biphosphate (PIP2) to inositol-1,4,5-triphophate (IP3) and diacylglycerol (DAG). IP3 releases $Ca^{2+}$ ions from the endoplasmic reticulum which activates calcineurin via calmodulin. Calcineurin is a serine phosphatase that can activate NF-AT. DAG directly activates protein kinase C θ (PKCθ) which,

together with Ras and mitogen-actived protein kinase (MAPK), takes part in activation of the transcription complex AP-1 (2, 49). PKCθ activates also a trimolecular complex CARMA1-BCL10-MALT1 which promotes ubiquitinylation of NEMO – the regulatory subunit of I-κB (inhibitor of κB) kinase (IKK) complex. This results in the activation of IKK complex, phosphorylation of I-κB and activation of the transcription factor NF-κB (50). Activation of AP-1 and NF-AT promotes the transcription of genes involved in proliferation and effector function of T cells. Additionally, the engagement of TCR induces polymerisation of actin which leads to changes in the cytoskeleton that result in the decrease of T cell motility and consequently stronger contact between the T cell and the antigen-presenting cell ("stop signal") (49).

## 1.6. Presentation of antigens

### *1.6.1. Antigen presenting cells*

All nucleated cells can present endogenous antigens in the context of MHC class I molecules. In this way, tumour cells or cells infected with pathogens can be recognised and killed by cytotoxic T lymphocytes. There are also several cell types, called antigen presenting cells, that express also MHC class II molecules and can initiate the adaptive immune response by presenting antigens to both $CD4^+$ and $CD8^+$ T lymphocytes. These cell types include B cells, macrophages and dendritic cells (DCs) (2). Following the antigenic stimulation of a B cell receptor, B cells process and present antigens in the context of MHC class II to recruit specific $CD4^+$ T helper cells in order to obtain their help in promoting proliferation and effector function (51). Macrophages are resident phagocytic cells present in both lymphoid and non-lymphoid tissues. Unlike B cells and dendritic cells, they constitutively express very little MHC class II. Instead, they up-regulate them following stimulation with IFN-γ (2, 52).

Dendritic cells are called professional antigen presenting cells. In contrast to B cells and macrophages, they express co-stimulatory molecules required for full activation of T cells, although B cells and macrophages can also act as antigen presenting cells for T cells that have been previously primed with DCs. Furthermore, B cells and macrophages (unlike DCs) are not present in the T cell areas of the secondary lymphoid organs and express 10-100-fold lower level of MHC molecules than DCs (53). Dendritic cells are present in most tissues or can migrate to the sites of inflammation, where they capture antigens by phagocytosis or endocytosis and subsequently process them and present in the context of MHC class II or MHC class I. Following the antigen uptake and stimulation with pathogen-associated molecular patterns or other factors that induce maturation of DCs (for example CD40L), DCs migrate to the secondary lymphoid organs, such as lymph nodes or spleen. During the migration, the cells undergo maturation which results in a reduced capacity to process antigens, up-regulation of co-stimulatory molecules, redistribution of MHC class II molecules from the intracellular endocytic compartments and secretion of cytokines that induce proliferation and effector function of T cells, and also may skew the immune response towards Th1, Th2 or Th17 type. They also regulate the differentiation and proliferation of regulatory T cells. Resting, immature DCs may take part in activation of T cells, although they are much less efficient compared to mature DCs. In the presence

of negative co-stimulatory signals (e.g. CTLA-4 or PD-1), dendritic cells induce tolerance to the presented antigens (54).

### *1.6.2. Presentation of antigens in the context of MHC class I molecules*

MHC class I molecules are composed of a light chain ($\beta_2$-microglobulin; 12 kDa) and a heavy chain (α chain; 40-45 kDa) which are bound by non-covalent association. The extracellular part of the α chain has three 90 amino acids-long domains – α1, α2 and α3. The polymorphic α1 and α2 domains form a cleft which binds 8-10 amino acids-long peptides. The α3 domain is non-polymorphic and binds $\beta_2$-microglobulin (2).

MHC class I molecules present antigens derived from the breakdown of proteins synthesised within the cell (endogenous antigens), although in some cases exogenous peptides can be diverted to the MHC class I pathway in a process termed "cross-presentation". *In vivo*, only dendritic cells are capable of cross-presentation (55). A single MHC class I molecule can bind many peptides that have different sequence but identical "docking amino acids" that take part in binding to the cleft of MHC class I. A normal, healthy cell presents normal cellular antigens on MHC class I molecules which does not induce killing by cytotoxic T lymphocytes (CTLs). However, presentation of foreign or altered antigens will result in activation of CTLs. Furthermore, reduction of the normal level of MHC class I expression, which occurs in tumour or virus-infected cells, may result in killing of the cell by natural killer cells.

Proteins synthesised within a cell and destined for degradation are marked with ubiquitin which directs them to proteasomes. Proteasomes are 26S multisubunit complexes consisting of a 20S catalytic core and two 19S regulatory subunits which role is proteolysis of proteins. Their main function is degradation of unneeded or damaged proteins by proteolysis. They produce short peptides which can be then be further trimmed by additional amino peptidases. These peptides are subsequently translocated from the cytosol into the lumen of the endoplasmic reticulum and loaded onto MHC class I molecules. This process is mediated by the MHC class I loading complex which consists of MHC class I molecules, the transporter associated protein (TAP), calnexin, calreticulin, the thiol oxidoreductase ERP57 and tapasin. TAP is a heterodimeric polypeptide situated in the membrane of the endoplasmic reticulum which transports the peptides into its lumen. Tapasin recruits MHC class I molecules to TAP and facilitates loading of MHC class I with antigenic peptides. Calnexin acts as a chaperone for the folding of the α chain of

MHC class I and later is replaced by calreticulin which assists in further assembly of MHC class I-peptide complex. Correctly assembled MHC class I-peptide complexes are subsequently exported to the cell surface (2).

In the process of cross-presentation, exogenous peptides are translocated to the cytosol from early phagosomes where they are degraded in proteasomes and follow the endogenous route for MHC class I molecules. Alternatively, following the degradation in proteasomes, they may re-enter the endocytic compartment for loading on MHC class I molecules. However, the drop in pH value in maturing endosomes and phagosomes results in the decrease of endoplasmic reticulum-derived proteins and these compartments become incompetent for cross-presentation. Instead, they acquire characteristic features required for loading peptides onto MHC class II molecules (55).

### *1.6.3. Presentation of antigens in the context of MHC class II molecules*

MHC class II molecules are composed of α (33 kDa) and β (29 kDa) chains which are connected with each other by non-covalent bonds. The external, polymorphic domains of each chain (α1 and β1) form a cleft similar to the one present in MHC class I molecules that can bind peptides up to the length of 20 amino acids. Similarly to MHC class I, MHC class II molecules can bind broad spectrum of peptides. The specificity of binding is limited by the presence or absence of amino acid motifs in the peptide, required for binding of the peptide to a given allele of MHC class II (2).

MHC class II molecules present exogenous peptides that are taken up by the cell by phagocytosis or pinocytosis. The proteins are degraded in endosomes by a variety of proteases. The peptide products are loaded onto MHC class II molecules in exchange for a fragment of an invariant chain peptide (CLIP) which prevents binding of endogenous peptides to MHC class II molecules in a process catalysed by the MHC class II homologue DM (2, 56). MHC class II-peptide complexes are transported to the cell surface via late endosomes and lisosomes. On the way, the peptide present in the cleft of MHC class II may still be trimmed by preoteases but the cleft protects it from full degradation (2).

### *1.6.4. Presentation of antigens in the context of CD1 molecules*

CD1 molecules are encoded by five non-polymorphic genes (*CD1A – CD1E*). Their structure is very similar to MHC class I molecules. CD1a, b and c molecules are present on

monocytes, B cells and some dendritic cells, whereas CD1d molecules are expressed on intestinal epithelial cells. CD1 molecules present endogenous lipid and glycolipid antigens (e.g. α-Galactosylceramide) in a process which is independent of TAP and DM proteins (2, 56).

## 1.7. Costimulation of T cells

The interaction between an antigen presented by an MHC molecule and a T cell receptor (TCR) is not sufficient to drive the activation of naive T cells. In fact, this interaction, termed signal 1, is inactivating when delivered alone. Naive T cells that are stimulated only through TCR are unable to sustain proliferation and become unresponsive (anergic) to stimulation or undergo apoptosis (57, 58). Costimulatory molecules provide additional signals (signal 2) which are indispensable for proliferation, survival and differentiation of T cells. Full activation of T cells is achieved only by integration of signals 1 and 2. Costimulatory signals may also be defined as any interaction that enhances antigen receptor signalling, proliferation and/or effector function of T cells (59). Furthermore, differentiation into effector cells is generally dependent on a signal supplied by antigen presenting cells in soluble form (e.g. IL-12, IL-4 or IFN-γ) (59, 60). There are also molecules known as negative costimulators which inhibit responses of T cells (59, 61). Thus, costimulation plays a critical role in determining whether the outcome of antigen stimulation would be activation and anergy. The threshold for T cell activation is set by the balance of negative and positive costimulatory signals which is critical for aspect of regulation of T cells function (62).

Costimulatory signals act on particular aspects of T cell activation such as survival, proliferation and differentiation to effector or memory cells. The function of costimulatory molecules is related to the timing of its action; therefore their expression is tightly regulated and depends on the activation status of the cell (60). Costimulatory molecules fall into two major families (Table 1.1.) – the immunoglobulin (Ig) superfamily (including B7.1, B7.2, CD28, CD2 or ICOS) and TNF/TNFR superfamily (59) which includes costimulatory molecules such as TL1A/DR3, CD70/CD27, OX40L/OX40 or 4-1BBL/4-1BB and is described in more detail in the next section.

| Receptor | Ligand | Superfamily | Positive / negative costimulation |
|---|---|---|---|
| CD28 | CD80 (B7.1), CD86 (B7.2) | Ig SF | Positive |
| CTLA-4 (CD152) | CD80 (B7.1), CD86 (B7.2) | Ig SF | Negative |
| ICOS (CD278) | ICOS-L (CD275, B7-H2) | Ig SF | Positive |
| PD-1 (CD279) | PD-L1 (CD274, B7-H1), PD-L2 (CD273, B7-DC) | Ig SF | Negative |
| LFA-2 (CD2) | LFA-3 (CD48) | Ig SF | Positive |
| LFA-1 (CD11a) | ICAM1, ICAM2, ICAM3 | Ig SF | positive |
| VLA-4 | VCAM1 | Ig SF | positive |
| TLT-2 | B7-H3 | Ig SF | Negative |
| Unknown | B7S1 | Ig SF | Negative |
| Unknown | B7S3 | Ig SF | Negative |
| Unknown | BTNL2 | Ig SF | Negative |
| CD27 (TNFRSF7) | CD70 (TNFSF7) | TNF/TNFRSF | Positive |
| 4-1BB (TNFRSF9) | 4-BBL (TNFSF9) | TNF/TNFRSF | Positive |
| OX40 (TNFRSF4) | OX40L (TNFSF4) | TNF/TNFRSF | Positive |
| GITR (TNFRSF18) | GITRL (TNFSF18) | TNF/TNFRSF | Positive |
| CD30 (TNFRSF8) | CD30L (TNFSF8) | TNF/TNFRSF | Positive |
| HVEM (TNFRSF14) | LIGHT (TNFSF14) | TNF/TNFRSF | Positive |
| DR3 (TNFRSF25) | TL1A (TNFSF15) | TNF/TNFRSF | Positive |
| CD40 (TNFRSF5) | CD40L (TNFSF5) | TNF/TNFRSF | Positive |

**Table 1.1.** Costimulatory molecules.

A classical example and the best-characterized pathway of T cell costimulation is B7/CD28 system which is critical for T cell activation. CD28 is a disulfide-linked homodimeric glycoprotein expressed on resting T cells and up-regulated after their activation. Ligands for CD28 – B7.1 (CD80) and B7.2 (CD86) are expressed on antigen presenting cells activated with pathogen-associated molecular patterns. Signal transduction from CD28 results in the activation of the transcription factor NF-κB and AP-1 transcription complex which are important for promoting cytokine expression and proliferation (63). Moreover, CD28 forms clusters together with TCR, recruits protein kinase C θ (PKCθ) and retains it in the immunological synapse which results in sustained signals for T cell activation (59). Stimulation of CD28 on T cells enhances their

proliferation via mediation of cell cycle progression and induction of IL-2 production. *In vitro*, IL-2 stimulation can even reverse the state of anergy (64). Also long-term antigen stimulation can bypass the requirement for CD28 costimulation (65). CD28 prevents apoptosis by inducing the expression of an anti-apoptotic molecule Bcl-$x_L$ (63). Furthermore, CD28 costimulation reduces by approximately 7-fold the threshold number of TCRs required for activation which may be the consequence of synergy between the TCR and CD28 signalling pathways (66). It has also been shown that CD28 takes part in formation of mature immunological synapse by inducing cytoskeleton rearrangement (67).

Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), a homolog of CD28, is a higher affinity binding partner for B7.1 and B7.2 molecules when compared to CD28 (Kd values for B7.1 binding to CTLA-4 and CD28 are 12 and 200 nM, respectively). In contrast to CD28, CTLA-4 is not constitutively expressed on T cells (with the exception of regulatory T cells). Instead, it is up-regulated in response to TCR ligation and, in particular, to TCR/CD28 costimulation (63, 65). Despite its structural similarity to CD28, CTLA-4 has a dampening effect on T cell activation which protects against autoimmunity or autoproliferative diseases. CTLA-4 signalling inhibits cell cycle progression and activation of transcription factors NF-κB, NF-AT and AP-1. On the other hand, it also activates JNK and PKB which are known to be involved in promoting cell survival and proliferation but, on the contrary to CD28, the overall effect of CTLA-4 signalling is induction of non-responsiveness or anergy of T cells. Coligation of TCR and CTLA-4 blocks the formation of ξ-chain-associated protein kinase 70 (ZAP70)-containing clusters in T cells which inhibits TCR signalling. CTLA-4 also increases T cell adhesion and motility, thereby can reverse the TCR-induced "stop signal" needed for strong contact between T cells and antigen presenting cells. The CTLA-4-induced limitation of contact between T cells and antigen presenting cells results in elevated threshold for TCR signalling (65).

Productive antigen recognition still occurs in mice deficient for CD28, indicating that there are other costimulatory molecules that can replace it (63). ICOS, a molecule structurally similar to CD28, is another crucial positive regulator of T cell activation. $CD4^+$ and $CD8^+$ T cells deficient for both ICOS and CD28 are severely impaired in proliferation (68). Expression of ICOS on T cells is induced following stimulation of TCR and CD28, although TCR signalling alone is sufficient for induction of ICOS expression. ICOS ligand (ICOS-L) shows 20% homology with B7 molecules and is constitutively expressed on B cells, dendritic cells, macrophages, fibroblasts and endothelial cells (61). Function of ICOS

is very similar to that of CD28. Both of these molecules induce production of IL-4, IL-5, IL-6 and IFN-γ from T cells and enhance their proliferation. However, unlike CD28, ICOS does not increase the production of IL-2. The reason for that is the inability of ICOS to bind growth factor receptor-bound protein 2 (Grb2) which is required for activation of transcription factors required for the synthesis of IL-2 (61, 68).

There are also several negative regulators of T cell activation belonging to the immunoglobulin family which inhibit activation, proliferation or effector function of T cells and are expressed on T cells either constitutively or are induced after the stimulation of TCR. Their ligands are expressed on antigen presenting cells or also on T cells. One of the negative regulators is PD-1 which binds PD-L1 (B7-H1) and PD-L2 (B7-DC) ligands. There are, however, contradictory reports showing that PD-L1 and PD-L2 can either enhance or inhibit responses of T cells (69, 70). Most probably PD-L1 and PD-L2 bind also to an unidentified receptor that induces T cell activation. This system may act similarly to the B7.1/B7.2:CD28/CTLA-4 system (71, 72). Other negative regulators of T cell responses include TLT-2, B7S1, B7S3 and BTNL2 (68).

Other molecules that take part in T cell activation are adhesion molecules such as LFA-1. These molecules facilitate the interaction between a T cell and an antigen presenting cell but do not act as direct co-stimulatory molecules therefore do not provide a complete signal 2 (73).

## 1.8. The TNF and TNFR superfamilies

### *1.8.1. Expression, structure and signalling*

Majority of proteins belonging to the tumour necrosis factor (TNF) superfamily (TNFSF) and their receptors, constituting the TNF receptor superfamily (TNFRSF), are expressed on a variety of cells of the immune system including antigen presenting cells, NK cells, $CD4^+$ and $CD8^+$ T cells. There are 20 TNFSF and 32 TNFRSF members identified to date (Figure 1.1.) (74). Their expression was detected also on other cells, including epithelial cells, endothelial cells and muscle cells (74-76). TNF/TNFRSF members such as TL1A, CD70, OX40L and OX40 or 4-1BBL and 4-1BB, are not constitutively expressed or are expressed at very low levels. Instead, their expression is induced on the cells of the immune system following their activation (74).

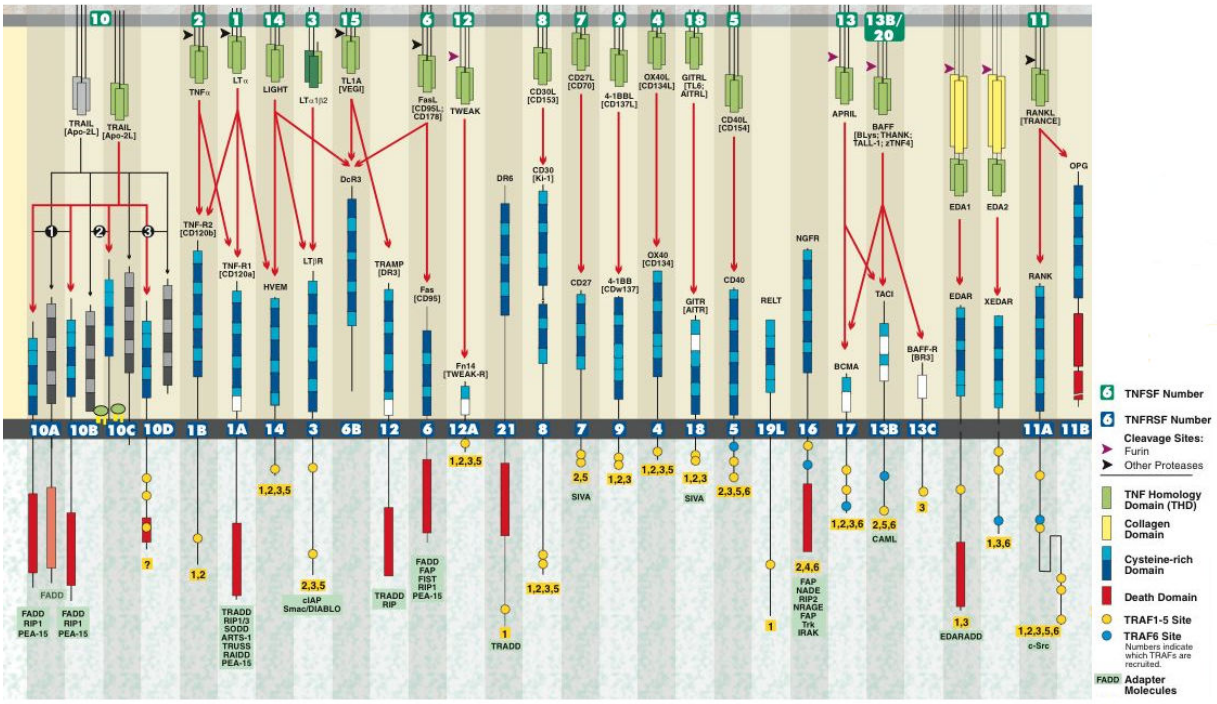



**Figure 1.1.** Tumour necrosis factor superfamily members and their receptors. Figure taken from (77).

The ligands are type II transmembrane proteins with intracellular N-terminus and extracellular C-terminus but many of them can be processed into a soluble form by a proteolytic cleavage by metalloproteinases (75, 78). The only known exception is lymphotoxin α (LTα) which exists only as a secreted protein. Most ligands are biologically active as homotrimers; only lymphotoxin β (LTβ) is a heterotrimer consisting of one α

chain (monomer of LTα) and two β chains or one β and two α chains. The TNFSF members share a 150 amino acid long, conserved C-terminal TNF homology domain (THD) which is responsible for their trimerization and binding to the receptors. It contains a conserved framework of aromatic and hydrophobic residues and shows 20%-30% amino acid sequence homology between the members of TNFSF and a similar tertiary structure (75, 76, 78). External surfaces of the ligands show little similarity which accounts for their specificity (75, 76).

Most receptors are type I transmembrane proteins (with intracellular C-terminus and extracellular N-terminus), although some of them can be enzymatically cleaved from the cell surface and some of them can be directly expressed as soluble isoforms lacking the transmembrane domain as the result of either alternative splicing (e.g. Fas, 4-1BB) or lack of this domain in the encoding gene (e.g. DcR3) (75, 78). The unique features of the receptors are 1-6 cysteine-rich domains (CRDs) present in their extracellular parts. They form intrachain disulphide bonds and are involved in binding to the THD domain present in the ligands (75, 76, 78, 79). The receptors bind trimeric ligands and are biologically active as trimers, although for some receptors, including Fas, TNFR2 and TRAIL, higher order oligomers may be required (80). Some members of the TNFRSF (e.g. Fas, TNFR1 and TNFR2) pre-assemble on the cell surface prior to ligand binding. This process requires an N-terminal region, including a part of the first CRD, termed the pre-ligand assembly domain (PLAD) which binds to another PLAD present on the other monomer (75).

Members of the TNFRSF can be divided into two groups based on the presence or absence of the death domain (DD). It is a 60 amino acid-long structure consisting of 6 conserved α-helices present in the intracellular part of the receptor.

Multimerization of DD-containing receptors leads to the recruitment of intracellular adaptor proteins, such as Fas-associated death domain (FADD) and TNF receptor-associated death domains (TRADD), that contain DD homology regions and take part in activation of the caspase cascade resulting in apoptosis of a cell. These receptors also recruit tumour necrosis factor receptor-associated proteins (TRAFs) which serve as adaptor proteins and bind to TRADD. TRAF proteins initiate signal transduction pathways that lead to activation of transcription factors, such as AP-1 and NF-κB; they also trigger MAP kinase cascades that lead to activation of JNK pathway. In addition, TRAF proteins directly bind inhibitors of apoptosis, such as A20 and c-IAP which results in inhibition of caspase activation (81).

A classical example of a DD-containing receptor is TNFR1; it is also the best studied member of the TNFRSF (Figure 1.2.). Following the activation of the receptor, silencer of death domain (SODD) dissociates from TNFR1 allowing formation of a signalling complex with TRADD – an adaptor protein which binds to the DD present in the intracellular part of the receptor (82, 83). TRADD recruits two different factors – FADD and the TNF receptor-associated factor 2 (TRAF2), although Jin and El-Deiry (84) show that FADD is dispensable for TNFR1-induced apoptosis. Following the binding to TRADD, FADD recruits pro-caspase-8 via homotypic interaction between the death effector domain (DED) present in pro-caspase-8 and another DED present in FADD. Pro-caspase-8 undergoes subsequent self-cleavage leading to its activation which initiates the caspase cascade. Active caspase-8 cleaves inactive pro-forms of effector caspases including caspase-3, -6 and -7, thereby activates them. Effector caspases, in turn cleave other protein substrates within the cell which triggers apoptotic death of the cell. Caspase-8 also processes Bid protein to its truncated form (tBid) which inhibits anti-apoptotic BCL-2 family members and induces the release of cytochrome c from mitochondria. This leads to activation of initiator caspase-9 and effector caspases (85). TRAF2 recruits receptor interacting protein (RIP), although this protein may also bind directly to the death domain of the receptor (86). RIP activates I-κB kinase (IKK) complex which is composed of two catalytic subunits with kinase activity (IKK-α and IKK-β) and one structural and regulatory subunit (NEMO, also known as IKK-γ). This process is most probably mediated by MEKK3 kinase (85). IKK complex phosphorylates the inhibitor of κB (IκB) which leads to its ubiquitinylation and degradation in proteasomes. This process releases the transcription factor NF-κB which translocates to the nucleus of the cell and activates transcription of several genes. Caspase-8 appears to play a role also in activation of NF-κB – nuclear translocation of NF-κB p65 subunit was delayed in caspase-$8^{-/-}$ B cells after the stimulation of TLR4 (87). Activation of NF-κB prevents apoptosis and induces proliferation or differentiation of cells. However, NF-κB also induces expression of pro-apoptotic genes such as several death receptors and their ligands, p53 and c-Myc (82). This adds complexity to the role of NF-κB in the regulation of apoptosis. DD-containing receptors can induce either cell death or proliferation and activation of cells. Their functional activity depends on the cellular context and the balance between pro- and anti-apoptotic factors inside the cell.

The TNFRSF members that do not contain the DD, signal through TRAF proteins only which bind directly to the cytoplasmic domains of the receptors. Despite the lack of

the DD, these receptors are also involved in both pro-survival and pro-apoptotic signalling (81). Some TRAF proteins which are involved in signal transduction from these receptors can not only induce activation of transcription factors but also activate caspases. For example C-terminal TRAF domain of TRAF6 interacts with death effector domain of pro-caspase-8 and allows for its activation (88).

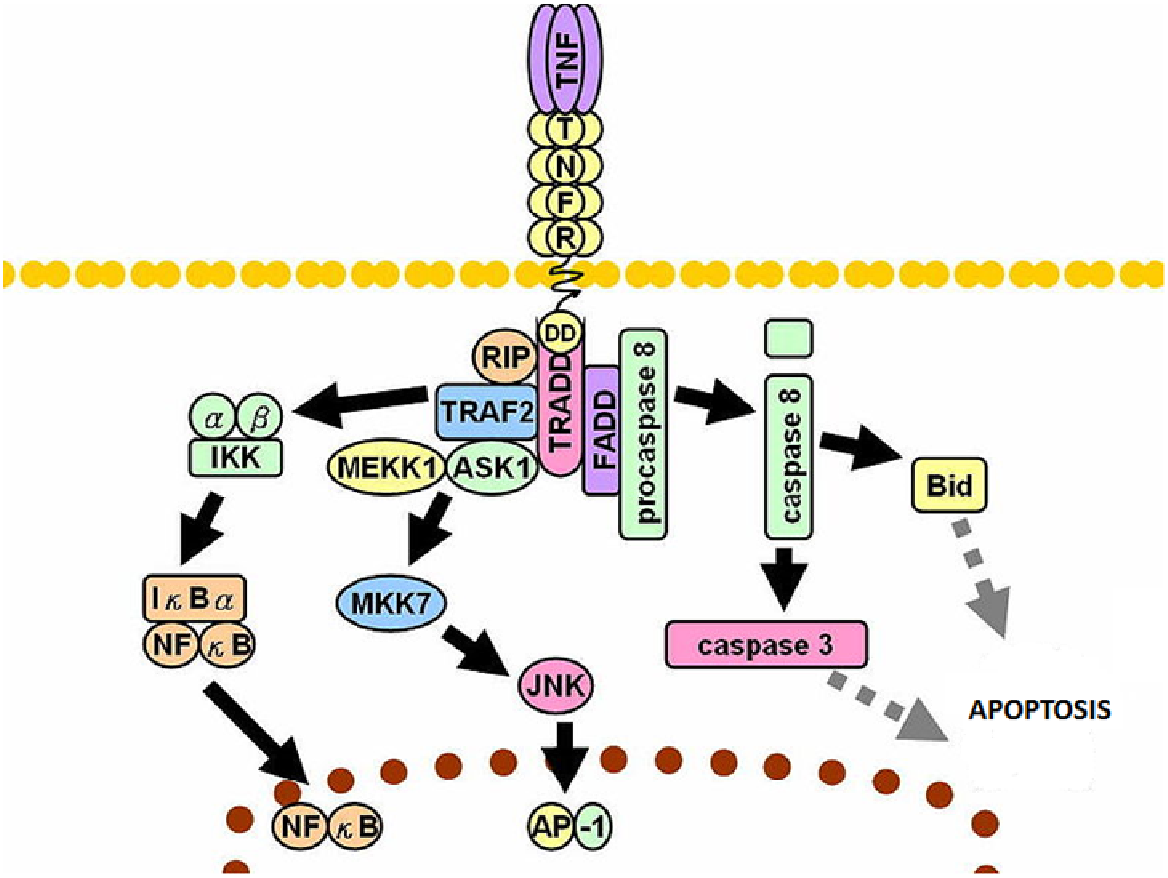



**Figure 1.2.** TNFR1 signalling

### *1.8.2. The role of TNFSF/TNFRSF members in T cell responses*

Structural attributes of the receptors and the ligands of the TNF superfamily link them to signalling pathways for survival, proliferation, differentiation or apoptosis. They play an important role in maintaining the equilibrium of the immune system whilst promoting immune responses to pathogens by exerting influence on innate immunity, organogenesis of secondary lymphoid organs, activation of antigen presenting cells and acting as pro-inflammatory cytokines (75, 76, 79, 83, 89). TNF/TNFRSF members regulate

T cell responses by providing direct signals required for full activation of T cells, regulation of their expansion, contraction of the effector pool and survival of the memory cells.

CD70/CD27, 4-1BBL/4-1BB, OX40L/OX40 and GITRL/GITR interactions enhance proliferation of $CD4^+$ and $CD8^+$ T cells when combined with TCR engagement and CD28 signalling (89). It has been shown also that 4-1BB, GITR and OX40 signalling can replace CD28, although in the presence of CD28 the effect of co-stimulation is more profound (89, 90). CD27 signalling alone is neither required nor sufficient to induce effector cell formation, it also is not required for the initial activation of T cells which is the consequence of the expression pattern of CD70 which is transiently induced on activated T cells but is not present on naïve cells. Instead, CD27 triggering contributes to the formation of the effector cells by inducing proliferation and survival of activated T cells during the primary response by collaborating with signals transduced from CD28 (60). Furthermore, co-administration of CD70 together with an antigen prevents the induction of the tolerance which is observed when an antigen is administered alone (91). CD27 signalling induces Th1 gene expression profile in $CD4^+$ T cells (60). OX40L/OX40 interaction directly promotes the proliferation of both $CD4^+$ and $CD8^+$ T cells although this effect is more profound in $CD4^+$ T cells which can be explained by the fact that expression of OX40 is much more transient on activated $CD8^+$ T cells compared to $CD4^+$ T cells. OX40 signalling does not control the initial activation of T cells; instead, it maintains their late proliferation and prolongs their survival. Engagement of OX40 $CD4^+$ T cells leads also their differentiation into Th2 cells which is driven by autocrine production of IL-4 induced by OX40. However, the type of antigen and exogenous IL-12 signals can overcome this effect and lead to generation of Th1 cells (92). LIGHT/HVEM interactions enhance proliferation and effector function of $CD8^+$ T cells (75, 89, 93). LIGHT stimulates also expansion of $CD4^+$ T cells and induces their differentiation into Th1 cells (94). HVEM, however, acts as a bi-directional switch of T cell activation. Binding of B- and T-lymphocyte attenuator (BTLA) or CD160 (members of the immunoglobulin superfamily, expressed mostly on NK, NKT and T cells) to HVEM present on T cells results in inhibition of activation of these cells. BTLA and CD160 bind to a different CRD region of HVEM (CRD1) than LIGHT (95).

Survival signals from TNFSF/TNFRSF members are also required to maintain T cell memory. In physiological conditions, 4-1BBL expands $CD8^+$ memory T cells, however its overexpression in mice results in an increased frequency of both $CD4^+$ and

$CD8^+$ memory T cells. CD30L and OX40L are required for maintaining of $CD4^+$ memory T cells. Expression of receptors for 4-1BBL (4-1BB) on $CD8^+$ memory T cells and CD30L (CD30) and OX40L (OX40) on $CD4^+$ memory T cells is induced by IL-15 and IL-7 present in $CD8^+$ T cell or $CD4^+$ T cell memory niches, respectively (96). Moreover, OX40 is involved in formation $CD4^+$ T effector memory cells but not of $CD4^+$ T central memory cells. It also takes part in activation of memory $CD4^+$ T cells and therefore is required for the initiation of the recall response. OX40 is critical for generation of both central and effector memory $CD8^+$ T cells; it also enhances their proliferation (92). Another TNFSF member involved in maintaining T cell memory is CD70 which increases frequency of both $CD4^+$ and $CD8^+$ memory T cells (97).

The detailed mechanism of integration of TCR and TNFRSF members signalling pathways remains unknown but TCR signalling and signalling of TNFRSF members involved in costimulation leads to activation of the same transcription factors (NF-κB and AP-1), therefore signal transduction elements involved in their activation are prime candidates for the integration of signals derived from TCR and TNFRSF members. A recent study suggests that one such protein is TRAF3 (98). TRAF3 deletion in T cells results in defective IgG1 response and impaired antigen-specific $CD8^+$ and $CD4^+$ T cell responses to infection with *Listeria monocytogenes* in mice. TRAF3 is recruited to TCR/CD28 signalling complex but it binds also to cytoplasmic domains of almost all of TNFRSF members that do not contain the death domain, including the ones that are involved in costimulation (4-1BB, CD27, OX-40, GITR, TNFR2 and CD30), therefore it remains possible that the role of TRAF3 in signalling of TNFRSF members also contributes to the defective T cell immunity in TRAF3-deficient mice (98).

TRAF3 binds directly to cytoplasmic domains of TNFRSF that do not contain the death domain and are involved in costimulation (4-1BB, CD27, TNFR2, GITR, OX-40 and CD30).

The interaction between TNFSF members and their receptors affects also the activity of regulatory T cells (Tregs). Stimulation of GITR, OX40 and 4-1BB present on Tregs results in loss of their suppressive activity and development of autoimmunity (74, 99). Furthermore, OX40 down-regulates the expression of FoxP3 and IL-10 in $CD4^+$ T cells and in this way contributes to inhibition of Treg differentiation (74).

FasL/Fas, CD30L/CD30, TNF/TNFR1 and TNF/TNFR2 interactions are involved in activation-induced cell death of T lymphocytes (42-44). In this process, the majority of activated T cell undergo apoptosis following the peak of an immune response. FasL,

CD30L or TNF bind to their receptors on the same or a neighbouring cell and trigger apoptosis (42, 44, 100). In activated cells, high levels of IL-2 sensitizes cells to Fas-induced apoptotic death by decreasing levels of c-FLIP during S phase of the cell cycle (101). C-FLIP is a protease-deficient homologue of caspase-8 that competes with it for the recruitment to FADD and acts as an inhibitor of apoptosis. Also CD30L/CD30 interaction triggers AICD *in vitro*, in the presence of IL-2 (100). FasL/Fas interaction plays a minor role in the death of activated T cells responding to a conventional antigen, instead it controls mostly self-reactive T cells in which repetitive stimulation with systemic antigen leads to increased levels of IL-2 and reduced levels of c-FLIP (43).

### *1.8.3. TNFSF/TNFRSF members in autoimmune diseases and tumours*

Members of the TNF superfamily are involved in the pathogenesis of a number of autoimmune diseases. Interactions between OX40L and OX40, 4-1BBL and 4-1BB, and TL1A and DR3 increase the severity of inflammatory bowel disease (IBD), asthma, arthritis and experimental autoimmune encephalomyelitis (EAE) which is a mouse model of multiple sclerosis. Depletion of the ligands or blockade of the receptors by specific antibodies results in a reduced disease score (74, 102). Also TNF plays a role in the pathogenesis of several autoimmune diseases. Currently there are five TNF antagonists registered in the USA and in the European Union – infliximab (mouse/human chimeric α-TNF monoclonal antibody), adalimumab (human α-TNF monoclonal antibody), etanercept (fusion protein of TNFR2 and the Fc fragment of human IgG1), certolizumab (PEGylated Fab' fragment of a humanized IgG1 α-TNF monoclonal antibody) and golimumab (human α-TNF monoclonal antibody). They are used in therapies against rheumatoid arthritis, ankylosing spondylitis, ulcerative colitis, Crohn's disease and other autoimmune disorders (103, 104). Neutralization of TNFSF/TNFRSF interactions results in suppression of symptoms of an autoimmune disease but the possible side effect of this kind of therapy is increased susceptibility to infectious agents and tumours.

The death receptor Fas is a key player in the induction of lymphocyte apoptosis. It maintains the homeostasis of lymphocytes by taking part in AICD (42, 43). Mutations in Fas, FasL or caspase-10 gene leading to a defective Fas-induced apoptosis of lymphocytes can result in autoimmune lymphoproliferative syndrome (ALPS) which is characterized by autoimmunity, chronic, non-malignant lymphoproliferation, although ALPS patients are susceptible to malignancy. ALPS is associated with a presence of peripheral T cell

population bearing TCRαβ but lacking the expression of CD4 or CD8 molecules (105-107). These cells are believed to originate from aging peripheral $CD4^+$ or $CD8^+$ T cells that lost the co-receptor expression and can reach up to 40% of lymphocytes in ALPS patients (compared to 1% in healthy individuals) (105, 107). Other abnormalities include elevated levels of $CD5^+$ B cells, $CD57^+$ $CD8^+$ T cells and TCRγδ cells and a decrease in the frequency of $CD4^+$ $CD25^+$ T cells (105). Most of the ALPS treatment strategies remain targeted at the disease manifestations (108).

Systemic triggering of TNFR1 results in a massive inflammatory response, high levels of C-reactive protein, IL-1, IL-6 and neutrophilia, (2, 109). Mutations in the gene of TNFR1 can lead to TNF receptor-associated periodic syndrome (TRAPS) characterized by prolonged episodic fevers and inflammation which can affect multiple organs with an average duration of 21 days (109, 110). Autosomal dominant mutations leading to TRAPS occur predominantly in CRD1 and CRD2 while there are not any known TRAPS mutations in the transmembrane and intracellular domains of TNFR1. The mutations in CRD1 and CRD2 are thought to render the receptor constitutively active, increase its affinity for TNFR1 or to impair cleavage of the receptor leading to reduced serum levels of soluble TNFR1. TRAPS is treated with etanercept and infliximab which neutralize TNF (109).

Therapy for cancer should promote activity of T cells and antigen presenting cells. Ideally, it should also inhibit generation and function of Tregs. It may lead to the development of autoimmune diseases which is an inherent risk of this type of therapy. However, the benefits can probably outweigh the risk for most patients with tumours. Members of the TNFSF can be used in therapy of a wide range of tumours. There are three methods of using TNFSF members in anti-tumour therapy. The first is administration of a TNFSF member known to enhance T cell response (e.g. OX40L, 4-1BBL or CD70), or to induce apoptosis of tumour cells (e.g. LIGHT). This method can be combined with a co-administration of an immunostimulatory adjuvant, such as granulocyte-macrophage colony stimulating factor (GM-CSF). Another method is increasing the immunogenicity of tumour cells by transfecting them with cDNA encoding the appropriate TNFSF ligand. This can be achieved by *in vivo* injection of viral vectors into the tumour. The third method is injecting dendritic cells engineered to express the appropriate TNFSF ligand into the organism of a patient. A very effective method is also a combined approach (74).

## 1.9. TL1A (TNFSF15)

### *1.9.1. Structure and expression pattern of TL1A*

TL1A (TNF superfamily member 15, TNFSF15) is the most recently discovered member of the TNFSF, cloned for the first time in 2002. In humans, the gene of TL1A has the length of 17 kbp, contains 4 exons (111, 112) and is situated in the chromosome 9q32 (111). In humans, there are three different isoforms of the protein generated from this gene as a result of alternative splicing – VEGI-174, VEGI-192 and the full length product – VEGI-251 (TL1A). They have the length of 174, 192 and 251 amino acids, respectively (112, 113) and share the same C-terminal 151-amino acid long fragment (113). Migone *et al.* (111) have noticed that VEGI-174 cDNA completely matches a continous TL1A cDNA region that contains both exons and introns and therefore suggest that VEGI-174 might be a cloning artefact.

The main receptor for VEGI-174 and VEGI-192 has not been identified yet, although it is known that it is different than death receptor 3 – the main receptor for TL1A (111, 112). Human VEGI-174 and VEGI-192 were identified as endothelium-derived factors that take part in regulation of angiogenesis by inducing apoptosis of proliferating endothelial cells or growth arrest of $G_0$-$G_1$ endothelial cells (111-115). Mouse and rat TL1A have the length of 252 amino acids and show 63.7% and 66.1% sequence homology to the human counterpart respectively (111). TL1A contains a predicted hydrophobic transmembrane region near the N terminus (111) but exists either in a transmembrane (116, 117) or soluble form (118, 119). Recombinant human soluble TL1A forms a homotrimer that resembles the trimeric structure other TNF superfamily members (120), however still little is known about the quaternary structure of the native form of TL1A.

In humans, TL1A mRNA was detected in endothelial cells, kidney, prostate, liver, skeletal muscles and lungs (111, 113). Its expression is up-regulated by TNF and IL-1α (111) and is dependent on NF-κB which binds to the promoter region of the TL1A gene and activates its expression (121, 122). In human dendritic cells and monocytes, expression of TL1A mRNA is rapidly (4-6 h) and transiently up-regulated following *in vitro* stimulation with plate-bound immune complexes which stimulate Fcγ receptors. These cells start to secrete soluble TL1A after 6-12 h of *in vitro* stimulation with plate-bound IgG, while expression of the transmembrane form of TL1A was detected only on human monocytes stimulated *in vitro* with plate-bound IgG for 16 h (119). In murine bone

marrow-derived dendritic cells, the level of TL1A mRNA peaks after 3 hours of *in vitro* stimulation with immune complexes or toll-like receptor (TLR) ligands, notably lipopolysacharide (LPS, ligand for TLR4) and soluble tachyzoite antigen (STAg, ligand for TLR11) (123). TL1A was detected also in chicken blood samples and spleens 2 - 4h after injection of LPS (124). Shih *et al.* (121) identified several other TLR agonists as inducers of TL1A mRNA in human monocytes – synthetic bacterial lipoprotein Pam3CSK4 (ligand for TLR2), heat-killed *Listeria monocytogenes* (recognized by TLR2), polyinosinic-polycytidylic acid (poly(I:C), ligand for TLR3), lipopeptide FSL (ligand for TLR2), LPS, single-stranded RNA (ligand for TLR7) and unmethylated CpG DNA sequence (ligand for TLR9). Several bacteria strains, including both Gram negative and Gram positive organisms, induce the expression of TL1A on human monocytes and monocyte-derived dendritic cells. The level of TL1A mRNA in these cells peaks after 4h (monocytes) and 8h (monocyte-derived dendritic cells) of *in vitro* stimulation with live bacteria. Furthermore, expression of TL1A mRNA induced by bacteria is higher than induced by single TLR ligands, suggesting that TLRs act synergistically to up-regulate the expression of TL1A (121). Transient expression of TL1A, peaking after 24h, was detected also on *in vitro* activated $CD4^+$ and $CD8^+$ T cells (123, 125). Very little expression of TL1A was found on non-activated cells of the immune system (111, 125).

### *1.9.2. Death receptor 3 (DR3)*

Death receptor 3 (DR3) is the main receptor for TL1A, it is a death domain-containing receptor belonging to the TNFRSF. In humans there are at least 12 different splice variants of this receptor. The full length product contains both the death domain and the transmembrane domain, while the others are potentially secreted proteins (126, 127). Mouse gene of DR3 is situated in the chromosome 4E1, contains 10 exons and shows 55% homology to the human counterpart. At the protein level the homology is 63% with 94% homology in the death domain and 52% in the extracellular domain. There are three different splice variants of mouse DR3 –the full length transmembrane receptor, soluble receptor lacking the transmembrane domain and transmembrane receptor lacking one of the CRD regions in the extracellular domain (128).

Signalling pathways for TNFR1, presented in the previous section, also function downstream of DR3. The death domain of DR3 shows 47% amino acid sequence similarity

to the death domain of TNFR1 (129). Furthermore, only TNFR1 and DR3 associate constitutively with SODD (75, 130). Several research groups showed that, like TNFR1, DR3 may induce both apoptosis and activation of NF-κB (129, 131, 132). DR3 forms signalling complex comprising TRADD, TRAF2, RIP, FADD and caspase-8 (132, 133), although Wen *et al.* (132) showed that FADD does not take part in signal transduction from DR3. This inconsistency can be explained by the fact that Wen and colleagues (132) were investigating DR3 signalling on erythroleukaemic TF-1 cells which naturally express DR3, while other researchers were using transient systems in which DR3 molecule was overexpressed (133).

Expression of DR3 was detected on activated human monocytes (134), resting mouse NKT cells, $CD4^+$ T cells and subpopulations of $CD11c^+$ and $NK1.1^+$ cells (125). DR3 mRNA present in resting human $CD4^+$ T cells and monocytes is strongly up-regulated in activated $CD4^+$ and $CD8^+$ T cells (135). Expression of DR3 on human $CD56^+$ T cells and NK cells is slightly increased by *in vitro* stimulation with IL-12; this effect is more significant when the cells are treated with IL-18 alone (136). Since IL-12 up-regulates the receptor for IL-18, these cytokines act in synergy to increase the expression of DR3. Combination of both IL-12 and IL-18 up-regulates expression of DR3 on human $CCR9^+$ $CD4^+$ T cells, $CD56^+$ T cells and NK cells, although it has minimal effect on other subsets of human T cells which do not constitutively express receptors for IL-12 and IL-18 (136, 137).

### *1.9.3. Soluble decoy receptor 3 (DcR3)*

The second receptor for TL1A, not present in mice, is DcR3 (soluble decoy receptor 3). It is a soluble receptor lacking the transmembrane domain; it belongs to the TNFRSF and acts as a decoy receptor also for FasL and LIGHT (118, 138).

DcR3 blocks the interaction between its ligands and their membrane-bound receptors and therefore can neutralize their biological effects (118). DcR3 is overexpressed by several tumours (139), notably malignant tumours arising from gastrointestinal tract, lungs (138, 140) and virus-associated lymphomas (118, 141). Expression of DcR3 by tumour cells helps them to gain survival advantage by preventing Fas-mediated apoptosis and by interfering with LIGHT-mediated T cell activation (139). DcR3 is also able to induce *in vitro* angiogenesis in human umbilical vein endothelial cells (HUVEC cells) by promoting their proliferation and migration. These effects may be caused by the

interference with biological activity of TL1A, since it has been shown that they can be mimicked by α-TL1A and α-DR3 antibodies. Furthermore, human aortic endothelial cells, which do not express TL1A, do not respond to DcR3 treatment (138). DcR3-treated DCs suppress $CD4^+$ T cell proliferation and up-regulate IL-4 production by naïve $CD4^+$ $CD45RA^+$ T cells (142). Solid phase (i.e. membrane- or plate-bound) DcR3 can trigger reverse signalling of FasL or LIGHT which results in T cell costimulation (143). In this process, the signal is transduced by ligands which serve as receptors while receptors play roles of ligands. However, the question whether TL1A is capable of reverse signalling remains unanswered.

### *1.9.4. Function of TL1A/DR3 interactions in immune response*

The soluble form of TL1A exhibits growth inhibition of *in vitro* cultured HUVEC cells and overexpression of TL1A by tumour cells retards the tumour growth due to inhibition of angiogenesis (113). In TF-1 cells, TL1A can induce apoptosis only in the presence of caspase inhibitors or cycloheximide (CHX) – an inhibitor of protein synthesis (111, 132) which prevents synthesis of proteins induced by NF-κB and consequently only the signalling pathway leading to apoptosis remains active. Stimulation of activated T cells with TL1A does not lead to their apoptotic death even in the presence of CHX (111) suggesting that TL1A plays a role in costimulation of T cells.

*In vitro* studies show that the interaction between TL1A and DR3 in T cells triggers proliferative and costimulatory signals through activation of NF-κB-mediated pathways. TL1A increases proliferation of $CD4^+$ T cells which is the direct consequence of increased production of IL-2 (123). Furthermore, T cells up-regulate the expression of IL-2 receptor in response to TL1A stimulation (111). The effect of increased proliferation of $CD4^+$ T cells in response to TL1A is most evident for $CD45^{low}$ $CD4^+$ memory T cells while it is minimal for naïve $CD45^{high}$ $CD4^+$ cells, which is the consequence of up-regulation of DR3 on activated T cells (144). Pappu *et al*. (145) showed that TL1A selectively enhances proliferation of activated Th17 cells but not Th1 cells.

TL1A also induces production of IL-6 from $CD4^+$ T cells isolated from gut-associated lymphoid tissue from mice with chronic colitis. This effect is less significant in $CD4^+$ T cells isolated from healthy mice (146), which is the consequence of much lower expression of DR3 on non-activated T cells. The increase of IL-6 production is much higher when TL1A acts in synergy with IL-23 (146).

TL1A acts as a polarizer of the immune response by regulating secretion of cytokines that skew the immune response towards Th1 (IFN-γ), Th2 (IL-4, IL-10 and IL-13) or Th17 (IL-17) type. *In vitro* stimulation with TL1A enhances production of IL-4 and IFN-γ from mouse $CD4^+$ T cells activated with anti-CD3 and anti-CD28 antibodies (123). On the other hand, splenocytes from mice overexpressing DcR3, stimulated with an antigen show elevated production of IL-4 and IL-10 but downregulate the production of IFN-γ. Stimulation of these cells with an antigen together with TL1A but not LIGHT restored the production of IFN-γ (142). $CD4^+$ T cells grown in the presence of DcR3-treated dendritic cells show reduced proliferation and secretion of IFN-γ while they up-regulate the production of IL-4. Stimulation of the dendritic cells with Fas.Fc and LTβR.Fc does not result in a similar effect showing that it is the consequence of binding TL1A to DcR3 (142, 147). Prehn *et al.* (117) showed that TL1A slightly increases production of IL-4 from human peripheral blood monocytes but does not up-regulate production of IL-10 from these cells. Also stimulation of DR3 on activated NK/NKT cells increases the production of IL-4 and IL-13, but has no effect on the production of IFN-γ (125). When TL1A is administered to mice intranasally together with an antigen, it strongly increases the level of serum $IgG_1$ and the production of IL-4 and IL-5 from splenocytes (148).

TL1A alone or in synergy with IL-23 enhances production of IL-17 from activated Th17 cells (145, 146, 149), however it has no effect on IFN-γ production by Th1 cells which is the consequence of high level of DR3 expression on Th17 cells but not on other subsets of T helper cells (145). In the absence of IL-2 signalling (which prevents Th17 differentiation), TL1A enhances Th17 differentiation from naïve $CD4^+$ T cells (145).

Taken together, the results of different research groups presented above vary depending on the conditions of a given experiment and show that the role of TL1A in polarization of the immune response towards Th1, Th2 or Th17 type is still not clear and requires careful investigation.

Production of IFN-γ by activated mouse $CD4^+$ T cells is enhanced by stimulation with TL1A alone or in synergy with IL-12 (146). TL1A increases production of this cytokine also from non-activated mouse $CD4^+$ T cells in synergy with both IL-12 and IL-18 (144) and from activated human peripheral blood mononuclear cells, lamina propria mononuclear cells and T cells independently of, but in synergy with IL-12 and IL-18 (111, 117). A similar effect is observed in human peripheral blood NKT cells, NK cells and activated $CD4^+$ T cells stimulated with TL1A together with IL-12 and IL-18, although in NK cells this effect is due to stimulation of proliferation, not to increased IFN-γ production

per cell (136). The up-regulation of IFN-γ production in response to stimulation with TL1A together with IL-12 and IL-18 is most evident for $CCR9^+$ $CD4^+$ T cells which represent 4% of peripheral blood T cells and are involved in small intestinal inflammatory process (137).

TL1A has been shown to enhance IL-12 and IL-18-induced cytotoxicity (150) and proliferation of human NK cells (136)

TL1A affects also differentiation and maturation of dendritic cells which show reduced expression of CD11c, CD40, CD54 and MHCII when are cultured *in vitro* in the absence of TL1A/DR3 signalling. These studies were conducted using DcR3, however the described effect is due to TL1A, because stimulation of the dendritic cells with Fas.Fc and LTβR.Fc (receptor for LIGHT) constructs did not give similar results. (147, 151).

Treatment of human macrophage-like cells, THP-1, with TL1A in combination with IFN-γ induces production of TNF, macrophage chemoattractant MCP-1 and IL-8 which is a pro-inflammatory cytokine and a neutrophile chemoattractant (134). TL1A induces production of IL-8 also in human peripheral blood macrophages (152).

### *1.9.5. Role of TL1A in autoimmune diseases*

TL1A is involved in pathogenesis of several autoimmune diseases mediated by Th1, Th2 or Th17 cells. TL1A protein and mRNA is up-regulated in inflammatory bowel disease (IBD) and its production is localized to lamina propria macrophages, monocytes, dendritic cells, $CD4^+$ and $CD8^+$ T cells in patients with Th1- and Th17-mediated Crohn's disease (146, 149, 153) as well as plasma cells isolated from patients with Th2-mediated ulcerative colitis (116). Furthermore, patients with IBD show a higher proportion of DR3-expressing intestinal lamina propria T cells than healthy subjects (116, 117). The amount of TL1A protein and the number of TL1A positive cells correlates positively with the severity of this disease (116) and administration of anti-TL1A antibodies prevents development of experimental bowel inflammation (134) and attenuates chronic colitis (146) in mice.

Increased level of TL1A was detected also in blood and synovial fluids of patients with Th1-mediated rheumatoid arthritis (RA) (154, 155). High expression of TL1A was detected in atherosclerotic plaques in regions rich in macrophages and foam cells (134). In fact, TL1A has been shown to promote foam cell formation from human macrophages by regulating expression of several genes involved in low density lipoproteins uptake and

cholesterol efflux (156). TL1A is involved in this disease also via induction of pro-inflammatory cytokines and extracellular matrix degrading enzymes, such as matrix metalloproteinase-9 (134). Administration of monoclonal α-TL1A antibodies protects mice from antigen-induced arthritis (AIA), a mouse model of RA (102) while administration of TL1A increased penetration and clinical score of AIA in mice (155). $DR3^{-/-}$ mice show reduction in histopathological hallmarks of AIA (102) and in clinical score of Th17 and Th1-mediated experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis (123). TL1A/DR3 interactions play a role also in the pathogenesis of Th2-mediated lung inflammation (123, 125).

## 1.10. Aims of the project

Members of the TNF and the TNFR superfamilies are important modulators of T cell-mediated responses. The results published by several groups within the last four years showed that the interaction between TL1A and its receptor, DR3, enhances some aspects of T cell activation but full understanding of the role of TL1A in the immune system is currently incomplete.

The major aim of this project is to define the role of TL1A in T cell-mediated immune responses. More specifically, this study aims to examine the expression pattern of TL1A on dendritic cells and T cells, to investigate the influence exerted by TL1A on proliferation and effector functions of $CD4^+$ and $CD8^+$ T cells, to determine whether TL1A acts predominantly during the primary or the secondary immune response and finally to answer the question whether TL1A skews the immune response towards Th1, Th2 or Th17 type. Some molecules belonging to the TNF or TNFR superfamily have been shown to inhibit the suppressive activity of regulatory T cells. Therefore, other aspect of this study is investigation whether TL1A exerts any effect on Tregs. Finally, another aim of this project was to examine the role of TL1A in inflammatory diseases, such as bowel inflammation, and in anti-tumour immunity.

In this study, the role of TL1A was examined carefully both *in vitro* and *in vivo* using soluble recombinant TL1A and transgenic mice overexpressing TL1A under the control of either CD11c or CD2 promoter.

Detailed investigation of the role of TL1A in the immune system will have impact on understanding the processes of immune pathology in inflammatory diseases and determine whether TL1A can be used as a safe and efficient adjuvant for vaccines.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Reagents, cells and antibodies

### *2.1.1. Reagents*

Ovalbumin peptide 257-264 ($OVA_{257-264}$; SIINFEKL) was purchased from Peptide Protein Research Ltd. The lyophilised peptide was (>95% purity) was dissolved in phosphate buffered saline (PBS; 120 nM NaCl / 24 mM $Na_2HPO_4$ / 5.8 mM $KH_2PO_4$) and stored at -20$^{o}$C. LPS from *Salmonella minnesota* was purchased from Sigma; it was dissolved in water (1 mg/ml), aliquoted and stored at -20$^{o}$C. PE-labelled H-2K$^{b}$ SIINFEKL tetramer was obtained from the University of Southampton, Cancer Sciences Division protein expression facility. DR3.Fc and TL1A.Fc constructs were produced in-house. Polyinosinic-polycytidylic acid (poly(I:C); Sigma) was dissolved in water (10 mg/ml) and stored at -20$^{o}$C. α-Galactosylceramide (α-GalCer; gift from Dr. Bruno Linclau, School of Chemistry, University of Southampton, UK) was dissolved in DMSO (1 mg/ml), aliquoted and stored at -20$^{o}$C. Unmethylated CpG DNA (Invitrogen) was dissolved in water and stored at -20$^{o}$C. Synthetic bacterial lipoprotein, Pam3CSK4 (InvivoGen), was dissolved in water (1 mg/ml) and stored at +4$^{o}$C. TAPI-0 (Calbiochem) was dissolved in DMSO (20 mM) and stored at -20$^{o}$C. Allophycocyanin (APC)-conjugated streptavidin was purchased from BD Pharmingen. Phycoerythrin (PE)-conjugated streptavidin and horseradish peroxidise (HRP)-conjugated streptavidin were purchased from Sigma-Aldrich.

### *2.1.2 Antibodies*

The following monoclonal antibodies were produced and purified in-house from hybridoma lines: anti-rat CD4 domains 3 and 4 (OX68), anti-IFN-γ (HB170), anti-CD86 (GL-1), anti-Bcl1 idiotype (Mc39-16), anti-CD3 (145.2C11), anti-TL1A (TAN2-2), anti-CD4 (YTA3.2.1.), anti-CD8 (YTS169), anti-MHC class II (N22), anti-CD25 (PC61), anti-IL-2 (S4B6), anti-CD80 (106A1), anti-FcRII and III (2.4G2). Monoclonal antibodies listed above, used for flow cytometry, were fluorescein (FITC), PE or APC-conjugated in-house. APC-conjugated anti-CD11c (HL3), FITC-conjugated anti-CD49b (DX5) , FITC- and

APC-conjugated anti-CD25 (7D4), PE-conjugated anti-Vα2 chain (B20.1), FITC-conjugated anti-Vβ5 chain (MR9-4), APC-conjugated anti-KLRG-1 (2F1), FITC- and PE-conjugated anti-CD62L (MEL14), PE-conjugated anti-CD44 (IM7), anti-IL-4 and biotinylated anti-IL-4 (11B11) monoclonal antibodies were purchased from BD Pharmingen. APC-conjugated anti-CD4 (GK1.5), APC-conjugated anti-CD8a (53-6.7), PE-conjugated anti-IFN-γ (AN-18) and APC- and PE-conjugated anti-FoxP3 (FJK-16s) monoclonal antibodies were purchased from eBiosciences. Polyclonal biotin-conjugated anti-DR3 antibody was purchased from R&D Systems. Anti-asialo GM1 antibody was purchased from Wako. Biotinylated anti-IFN-γ (R4-6A2) monoclonal antibody was purchased from AbD Serotec. Polyclonal PE-conjugated anti-human IgG and anti-rat IgG antibodies were purchased from Sigma-Aldrich. Human IgG and biotin-conjugated goat IgG were purchased from Sigma-Aldrich.

### *2.1.3. Cell lines and cell culture*

HEK293T (a human embryonic kidney cell line) and J558L (a mouse plasmacytoma cell line) cells were cultured *in vitro* in DMEM (Invitrogen) medium supplemented with 10% (v/v) foetal calf serum (FCS; Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 2 mM glutamine (Invitrogen) and 1 mM pyruvate (Invitrogen). J558L cells stably transfected with BALB/c mouse TL1A cDNA which had been cloned into pEF1/V5-HisA vector (Invitrogen) containing also the neomycin resistance gene (J558L-TL1A) and J558L cells stably transfected with empty pEF1/V5-HisA vector (J558L-PEF) were cultured *in vitro* in DMEM medium containing the supplements listed above and 800 μg/ml of geneticin (Invitrogen). EG.7 (a mouse thymoma cell line) and mouse spleen cells were cultured *in vitro* in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) FCS (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 2 mM glutamine (Invitrogen), 1 mM pyruvate (Invitrogen) and 50 μM 2-mercaptoethanol. Untransfected CHO.K1 (a Chinese hamster ovary cell line) cells were cultured in RPMI 1640 medium containing all of the supplements listed above, except for 2-mercaptoethanol. All cell lines were re-cultured every 2-3 days and maintained at 37$^{o}$C in a 5% $CO_2$ humidified incubator. Adherent cells (HEK293T and CHO.K1) were detached from culture flasks by incubation with Trypsin-EDTA (Invitrogen) for 5 min in the tissue culture incubator.

### *2.1.4. Cell quantitation*

Cells were counted using a Coulter Industrial D Cell Counter (Coulter Electronics) or by manual counting using a haemocytometer by dilution 1:1 with trypan blue dye (Sigma) and observation of dye exclusion from viable cells.

### *2.1.5. LPS testing*

Antibodies and sTL1A construct used for *in vivo* experiments were tested for endotoxin contamination using the Endosafe®-PTS$^{TM}$ system (Charles River) according to the manufacturer's instructions. Typically, the contamination with endotoxin has never been higher than 0.9 ng of LPS / 1 mg of protein.

### *2.1.6. Biotinylation of antibodies*

Biotinylation of antibodies was conducted using EZ-Link Sulfo-NHS-Biotinylation Kit (ThermoScientific) according to the manufacturer's instructions. Briefly, 2-5 mg of an antibody was incubated with 20-fold molar excess of Sulfo-NHS-Biotin in 1 ml of PBS at room temperature for 30 min. The antibodies were then dialysed overnight against 5 L of PBS and their concentration was measured using Nanodrop ND-1000 spectrophotometer (ThermoScientific).

## 2.2. Molecular biology

### *2.2.1 Bacterial transformation and amplification*

2 ng of DNA were mixed with DH5α or TOP10 competent cells (Invitrogen) and incubated on ice for 30 min before heat shocking at 42$^{o}$C for 1 min. The cells were then incubated on ice for 2 min before addition of 300 µl of SOC medium (10% (w/v) bacto tryptone, 2.5% (w/v) yeast extract, 0.29% (w/v) NaCl, 0.093% (w/v) KCl; Invitrogen) and incubation for 1h in an orbital incubator at 37$^{o}$C. 150 µl of the bacterial culture was spread on agarose plates (1.5% (w/v) Bacto-Agar (Difco), 10% (w/v) tryptone (Sigma), 5% (w/v) NaCl) containing the appropriate selection agent (100 µg/ml of ampicilin or 50 µg/ml of kanamycin) and incubated overnight at 37$^{o}$C. Individual colonies were amplified by

overnight culture in 10 ml Luria Broth (10% (w/v) tryptone (Sigma), 5% (w/v) yeast extract (Sigma), 5% (w/v) NaCl) containing the appropriate selection agent. Plasmid DNA was subsequently isolated and analysed by sequencing and/or restriction digest analysis. 300 µl of the culture were then added to 150 ml of Luria Broth and again grown overnight to amplify the plasmid DNA.

### *2.2.2. Isolation of plasmid DNA from bacterial cultures*

Plasmid DNA was isolated from bacterial cultures using QIAprep Spin Miniprep Kit (10 ml culture; Qiagen) or HiSpeed Maxi Plasmid Kit (150 ml culture; Qiagen) according to the manufacturer's instructions. Briefly, the bacteria were lysed under alkaline conditions, the lysate was subsequently neutralized and adjusted to high-salt binding conditions. Following the clearing of the lysate, the plasmid DNA was bound to the silica membrane and eluted with water.

### *2.2.3. Isolation of mouse genomic DNA*

Mouse ear or tail tip was added to 100 µl of complete isolation buffer (50 mM tris, 12.5 mM $MgCl_2$, 0.5% (v/v) Tween-20, 0.5 mg/ml proteinase K, pH=8.9; proteinase K was added just before the isolation of DNA) and incubated overnight at 55$^{o}$C in a thermocycler with a heated lid (PTC-100; MJ-Research, Inc.). The mixture was centrifuged for 5 min at 13000 rpm. The supernatant containing the genomic DNA was collected and stored at -20$^{o}$C.

### *2.2.4. Restriction enzyme digests*

Restriction enzyme digests were performed by incubation of DNA for 2 h at 37$^{o}$C with the required restriction enzyme (Promega) at 3 U/µg of DNA in the presence of the relevant restriction enzyme buffer (Promega) at 1x final concentration.

### *2.2.5. DNA ligation*

Vector and insert DNA fragments were ligated at 1:1 or 1:3 molar ratios in the presence of 1.5 U of T4 DNA Ligase (Promega) and 1x T4 Ligase Buffer (Promega) for 3 h at room temperature. The total volume of the reaction mixture was 10 µl.

### *2.2.6. Agarose electrophoresis of DNA fragments*

DNA electrophoresis was performed in 0.7 – 1% agarose gels made in TBE (89 mM tris, 89 mM boric acid, 2 mM EDTA) containing 20 µg/ml of ethidium bromide. DNA samples were mixed 10:1 with loading buffer (20% (w/v) Ficoll 400, 0.1 M EDTA, 0.25% (w/v) bromophenol blue) and loaded on the gel. Gels were run in TBE buffer at 70 mA in a BioRad electrophoresis tanks and visualized on a UV transilluminator (BioRad).

### *2.2.7. DNA sequencing*

DNA sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). 5 µl of template DNA at the concentration of 0.1 µg/µl was added to the reaction mixture containing 2 µl of 5x sequencing buffer, 2 µl of BigDye reaction mix and 1 µl of the desired primer at the concentration of 1.6 pmol/µl. The reaction was run in GeneAmp 9700 thermocycler (Applied Biosystems) for 25 cycles of: 96$^{o}$C 10 s, 50$^{o}$C 5s, 60$^{o}$C 2 min. Afetr the PCR reaction, DNA was precipitated with ethanol and sodium acetate. 10 µl of the PCR reaction mixture was added to 67 µl of ethanol/sodium acetate stock solution (550 µl of 100% ethanol, 40 µl of 3 M sodium acetate) in an Eppendorf tube. The mixture was incubated for 15 min at room temperature to allow precipitation of DNA. Subsequently, it was spun for 20 min at 13000 rpm in a bench-top centrifuge. The supernatant was removed with a pipette and the DNA pellet was washed in 150 µl of 75% ethanol. Following 15 min spin at 13000 rpm, the supernatant was removed and the DNA pellet was resuspended in 10 µl of formamide. The samples were run on a polyacrylamide gel on the ABI-PRISM 300 sequencer (Applied Biosystems) and the data were analysed using DNAStar software.

### *2.2.8. Isolation of RNA*

Total RNA was isolated from the cells using RNeasy Mini Kit (Qiagen) or RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Briefly, $1x10^7$ of bone marrow-derived dendritic cells (RNeasy Mini Kit) or $5x10^5$ of cells isolated from the mesenteric lymph nodes (RNeasy Micro Kit) were disrupted in a buffer containing guanidine isothiocyanite and homogenized using QIAShredder Spin Column (Qiagen). Alternatively, 30 mg of the spleen or the gut tissue (RNeasy Mini Kit) was homogenized in the same buffer using TissueRuptor homogenizer (Qiagen). Ethanol was added to the cell lysate to create conditions that promote binding of RNA to silica-gel membrane. The sample was applied to RNeasy spin column and total RNA was bound to the membrane. Contaminants were washed away and RNA was eluted into 40 µl (RNeasy Mini Kit) or 14 µl (RNeasy Micro Kit) of RNase-free water. When it was required, genomic DNA was digested using DNA-free kit (Ambion) according to the manufacturer's instructions. Briefly, 1 µl of DNase I and 5 µl of 10x DNase I Buffer were added to 50 µl of RNA solution in water (0.2 µg/µl). The reaction mixture was incubated at 37$^o$C for 30 min. Following that time, DNase was inactivated by addition of 5 µl of DNase Inactivation Reagent and incubation at room temperature for 2 min. Subsequently, the reaction mixture was spun at 10000 rpm and the supernatant containing RNA free from genomic DNA contamination was collected and stored at -80$^o$C. RNA concentration was measured using Nanodrop ND-1000 spectrophotometer (ThermoScientific).

### *2.2.9. Reverse transcription of RNA*

First-strand cDNA was reverse transcribed from RNA templates using SuperScript III First-Strand Synthesis Super Mix for qRT-PCR (Invitrogen) according to the manufaturer's instructions. Briefly, 1-2 µg of total RNA were added to 10 µl of 2x Reaction Mix (containing 2.5 ng/µl of random hexamers, 10 mM $MgCl_2$ and dNTPs), 2 µl of Reverse Transcriptase Enzyme Mix and DEPC-treated water to 20 µl. The reaction mixture was incubated at room temperature for 10 min and transferred to 50$^o$C for 30 min. The reaction was stopped by incubation at 85$^o$C for 5 min. The reaction mixture was chilled on ice, 2 U (1 µl) of RNase H were added and the mixture was incubated for 20 min at 37$^o$C to digest the remaining RNA.

*2.2.10. Quantitative real-time PCR (qPCR)*

Quantitative real-time PCR was used to determine the level of expression of TL1A, IL-17A, IL-13, IL-4, IL-2 and IFN-γ. The reactions were run in iCycler thermocycler (BioRad) using cDNA-specific TaqMan gene expression assays (TL1A – Mm00770031_m1, IL-17A – Mm00439619_m1, IL-13 – Mm99999190_m1, IL-4 – Mm00445260_m1, IL-2 – Mm00439861_m1, IFN-γ – Mm00801778_m1; Applied Biosystems). Fold expression of TL1A mRNA was calculated using $\Delta\Delta C_T$ method and HPRT (TaqMan assay Mm00446869_m1; Applied Biosystems) as a reference gene. For relative expression of cytokine genes (Figures 5.10. and 5.11.), the measurement was first normalized to expression of the reference gene, HPRT, ($\Delta C_T$) and then $2^{-\Delta C}{}_T$ was used to obtain relative gene expression levels. Gene expression levels were then normalized to the average gene expression on control mice.

*2.2.11. Generation of pcDNA3.1/Zeo(-).TL1A plasmid*

A C57BL/6 mouse was injected *i.v.* with 40 μg of LPS. 4 hours later, total RNA was isolated from the spleen cells of the mouse and reverse transcribed to obtain cDNA. TL1A cDNA was amplified from the total cDNA by nested PCR. The PCR consisted of two separate reactions – 1st with external primers (forward – 5'-AGAAGGGATCAGAAGTCTCTC-3' and reverse – 5'-GGAAATTGGAAAGTAGGAGGCAG-3') and 2nd with internal primers (forward – 5'-GAAGGATGGCAGAGGAGCTG-3' and reverse – 5'-CCTCCTTATAGCAAGAAAGCT-3'). The PCR mixture after the first reaction was used as a template in the second reaction. Each reaction contained 1.25 μl of 10 mM dNTPs (Promega), 5 μl of the respective 5 μM forward and reverse primer (Invitrogen), 2 μl of the template, 5 μl of 10x Pfu polymerase reaction buffer (Promega), 30.75 μl of water and 3 U (1 μl) of Pfu polymerase (Promega) which was added after the initial denaturing step in order to reduce non-specific amplification. The reactions were run in PTC-100 thermocycler (MJ-Research, Inc.) under the following conditions: 95°C for 7 min, 25 cycles of 94°C for 4 min, 58°C for 1 min, 72°C for 2 min followed by the final extension step at 72°C for 20 min. The PCR product (742 bp) was isolated from the agarose gel and ligated with pCR-Blunt II-TOPO vector (Invitrogen) using Zero Blunt TOPO Cloning Kit (Invitrogen) according to the manufacturer's instructions. Briefly, 4 μl of the PCR product

were mixed with 1 µl of salt solution (1.2 M NaCl / 0.06 M $MgCl_2$), 1 µl (10 ng) of pCR-Blunt II-TOPO vector and incubated for 5 min at room temperature. The obtained construct was sequenced to verify the orientation and sequence of the cloned TL1A cDNA. The sequencing was conducted in two separate reactions, using two different primers: (1) 5'-GAAGGATGGCAGAGGAGCTG-3' and (2) 5'-CCTCCTTATAGCAAGAAAGCT-3'. The insert was subsequently excised from pCR-Blunt II-TOPO vector using *BamHI* and *XbaI* restriction enzymes and cloned into pcDNA3.1/Zeo(-) vector (Invitrogen) using the same restriction sites.

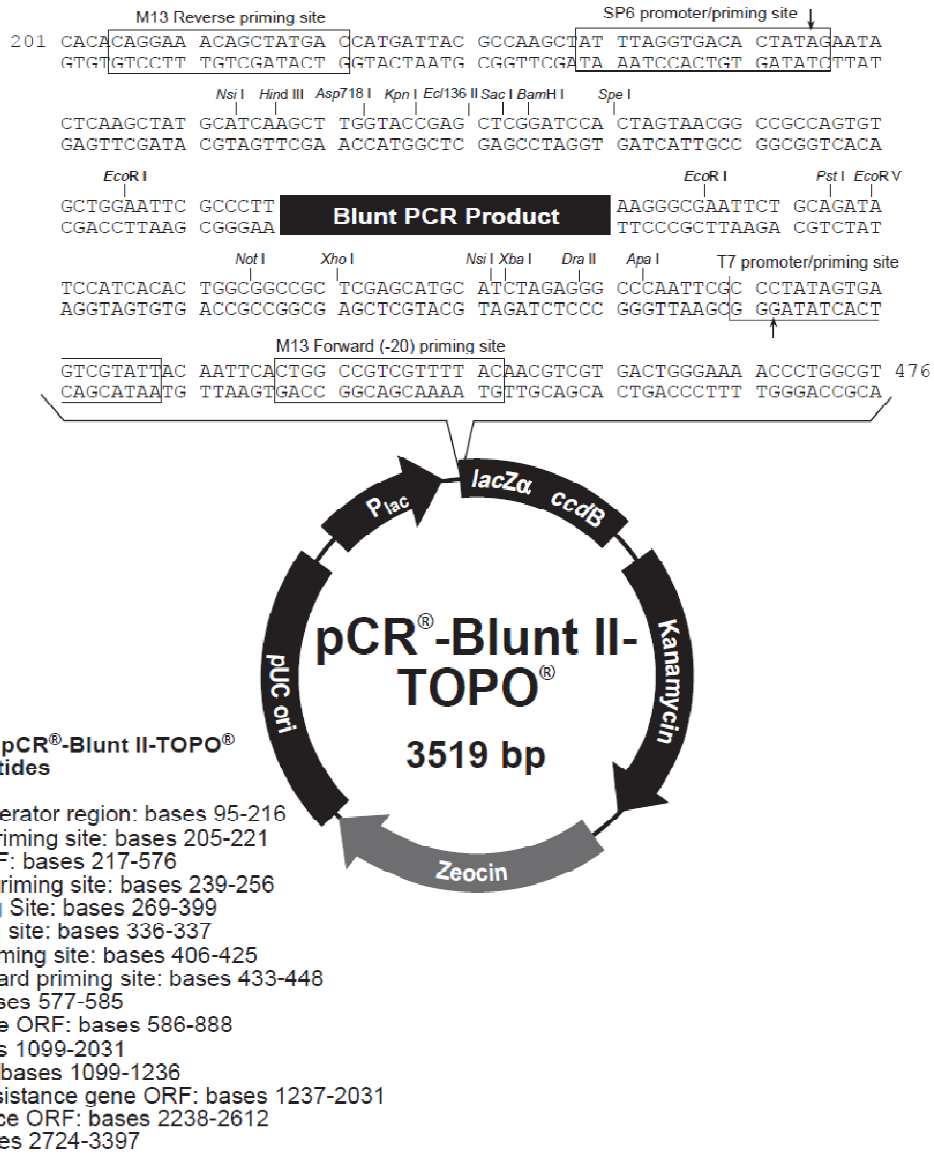
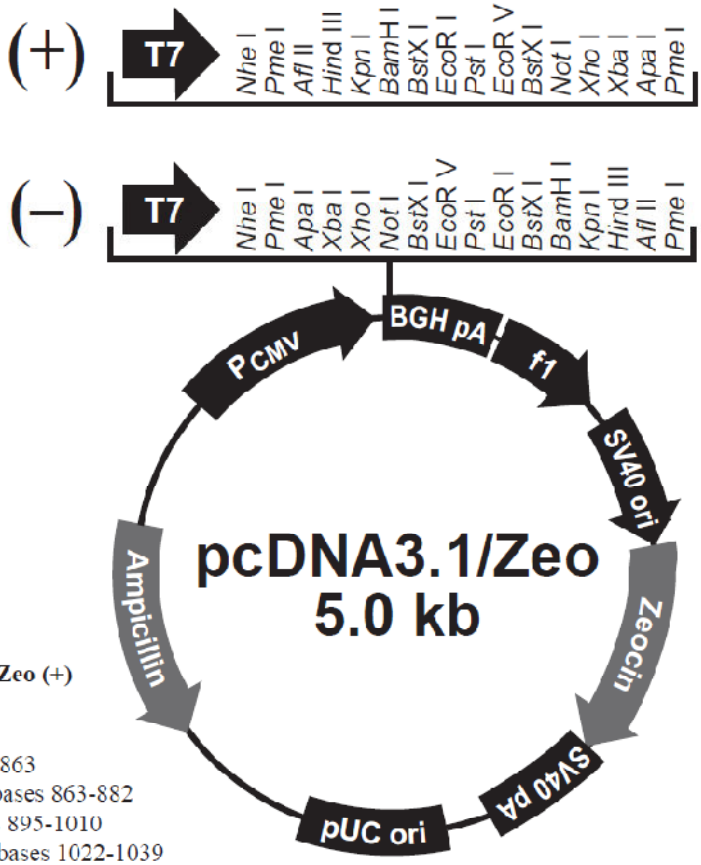



**Figure 2.1.** Map of pCR-Blunt II-TOPO vector (Invitrogen; http://tools.invitrogen.com/content/sfs/vectors/pcrbluntiitopo_map.pdf




**Comments for pcDNA3.1/Zeo (+)**
**5015 nucleotides**

CMV promoter: bases 209-863
T7 promoter priming site: bases 863-882
Multiple cloning site: bases 895-1010
BGH reverse priming site: bases 1022-1039
BGH polyadenylation signal: bases 1021-1235
f1 origin: bases 1298-1711
SV40 promoter and origin: bases 1776-2101
EM7 promoter: bases 2117-2183
Zeocin™ resistance gene: bases 2184-2558
SV40 polyadenylation: bases 2688-2817
pUC origin: bases 3201-3874 (C)
*bla* promoter: bases 4880-4978 (C)
Ampicillin (*bla*) resistance gene: bases 4019-4879 (C)

**Figure 2.2.** Map of pcDNA3.1/Zeo(-) vector (Invitrogen; http://www.synthesisgene.com/vector/pcDNA3.1%20Zeo%28-%29.pdf).

*2.2.12. Generation of soluble recombinant TL1A*

The cDNA sequence encoding the extracellular part of TL1A, with added *EcoRI* restriction site at the 5' end, was amplified by PCR from pcDNA3.1/Zeo(-) vector (Invitrogen) containing TL1A cDNA cloned between *BamHI* and *XbaI* restriction sites (see section 2.2.11.), using the following primers – forward: 5'-C**GAATTC**CACAGAAGAGAGATCTGAGCCT-3' (*EcoRI* site is shown in bold) and reverse: 5'-CCTCCTTATAGCAAGAAAGCT-3'. The PCR reaction mixture contained 1.25 µl of 10 mM dNTPs (Promega), 5 µl of 5µM forward and reverse primer, 5 µl of 10x Pfu polymerase reaction buffer (Promega), 30.75 µl of water and 3 U (1 µl) of Pfu polymerase (Promega) which was added after the initial denaturing step in order to reduce non-specific amplification. The reaction was run in PTC-100 thermocycler (MJ-Research, Inc.) under the following conditions: 95$^{o}$C for 5 min, 25 cycles of 94$^{o}$C for 1 min, 57$^{o}$C for 1 min, 72$^{o}$C for 2 min followed by an extension step at 72$^{o}$C for 10 min. The obtained fragment (540 bp) was isolated from the agarose gel and ligated with pCR-Blunt II-TOPO vector (Invitrogen) using Zero Blunt TOPO Cloning Kit (Invitrogen) according to the manufacturer's instructions as described in the section 2.2.11. The construct was sequenced to verify the orientation and the sequence of the cloned insert, using the following primer 5'-CCTCCTTATAGCAAGAAAGCT-3'. Subsequently, the insert was excised from pCR-Blunt II-TOPO vector using *EcoRI* restriction enzyme and cloned into pEE14.CD4(d3+4) vector (containing the sequence of rat CD4 domains 3 and 4, cloned between the restriction sites *XbaI* → *EcoRI*) using the same restriction site. The sequence and the orientation of the obtained construct were verified by DNA sequencing, using the following primer 5'-CCTCCTTATAGCAAGAAAGCT-3'. As a result, cDNA encoding domains 3 and 4 of rat CD4 (rCD4 d3+4) was added to the 5' end of the cDNA encoding the extracellular part of TL1A. Rat CD4(d3+4) tag is recognized by mouse monoclonal OX68 antibody which allowed to purify the recombinant protein. The obtained vector was transfected into CHO.K1 cells using calcium phosphate precipitate method. The cells were grown in DMEM medium (Safc) supplemented with 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), 5% dialysed foetal calf serum (First Link, Ltd.), GS supplements (containing alanine, asparagine, aspartic acid, glutamic acid, proline, serine, adenosine, thymidine, uridine and cytidine; Sigma) and 20 µM methionine sulfoximine (MSX; Sigma). The clone secreting the highest amount of the recombinant

TL1A.rCD4(d3+4) protein was selected by ELISA screening, expanded and the recombinant protein was purified on an OX68 antibody column. TL1A.CD4(d3+4) recombinant protein has molecular weight of 43.3kDa (full amino acid sequence – **MCRGFSFRHLLPLLLLQLSKLLVVTQGSTSITAYKSEGESAEFSFPLNLGEESL QGELRWKAEKAPSSQSWITFSLKNQKVSVQKSTSNPKFQLSETLPLTLQIPQV SLQFAGSGNLTLTLDRGILYQEVNLVVMKVTQPDSNTLTCEVMGPTSPKMRL ILKQENQEARVSRQEKVIQVQAPEAGVWQCLLSEGEEVKMDSKIQVLSKG**LN STEERSEPSPQQVYSPPRGKPRAHLTIKKQTPAPHLKNQLSALHWEHDLGMAFTK NGMKYINKSLVIPESGDYFIYSQITFRGTTSVCGDISRGRRPNKPDSITMVITKVADS YPEPARLLTGSKSVCEISNNWFQSLYLGATFSLEEGDRLMVNVSDISLVDYTKEDK TFFGAFLL; rat CD4(d3+4) sequence is shown in bold). Extinction coefficient is 0.95, as determined by ProtParam online database (www.expasy.ch/cgi-bin/protparam). Each batch of sTL1A was tested for LPS contamination which varied between 0.5 – 0.7 ng of LPS per 1 mg of sTL1A.

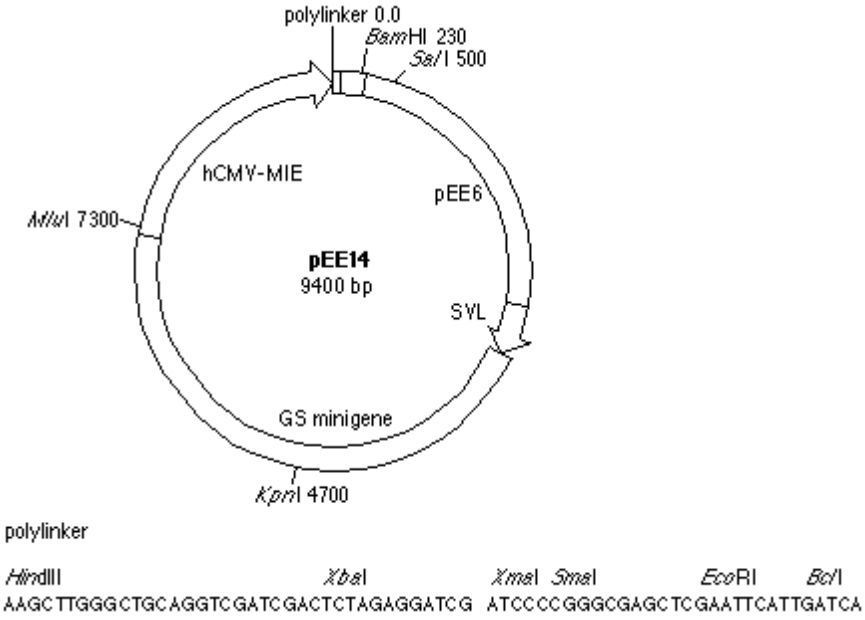



**Figure 2.3.** Map of pEE14 vector (Celltech).

## 2.3. Cellular assays, cell transfection and flow cytometry

### *2.3.1. Isolation of murine spleen cells*

Spleens were aseptically removed from mice and a single cell suspension was prepared by passing the spleens through a cell strainer. The cells were centrifuged (5 min, 400 g) and resuspended in 5 ml of red cell lysis buffer (0.83% (w/v) $NH_4Cl$, 0.1% $KHCO_3$ in water) and incubated for 5 min at room temperature. Subsequently, the cells were washed twice in PBS and resuspended in growth medium (see section 2.1.3.).

### *2.3.2. Enrichment of splenic dendritic cells*

3 ml of RPMI 1640 medium (Invitrogen) containing collagenase (1 mg/ml) and DNase (50 μg/ml) were added to the appropriate number of wells in a 6-well plate containing a spleen isolated from a C57BL/6 mouse. The medium was injected into the spleens which were then cut into small pieces and incubated for 45 min at 37$^{o}$C to allow digestion of collagen and the release of dendritic cells. After that time, EDTA was added to each well to the final concentration of 10 mM and incubated for 10 min at room temperature. Subsequently, a single cell suspension was prepared from the cells using a cell strainer. The cell suspension was transferred into 15 ml tubes and centrifuged (5 min, 400 g). The cell pellet was resuspended in 5 ml of red cell lysis buffer (0.83% (w/v) $NH_4Cl$, 0.1% $KHCO_3$ in water) and incubated for 10 min at room temperature. The cells were washed with RPMI 1640 (Invitrogen) supplemented with 10% (v/v) Foetal Calf Serum (FCS; Invitrogen), 10 mM EDTA and 20 mM Hepes (Sigma) and resuspended in 2 ml of this medium. The cell suspension was loaded onto 2 ml of 14.5% Nycodenz (Axis Shield) gradient in RPMI 1640 supplemented with 10% FCS, 10 mM EDTA and 20 mM Hepes and centrifuged for 20 min at 530 g at room temperature. The interface containing the enriched dendritic cells was collected and washed in RPMI 1640 supplemented with 10% (v/v) FCS, 10 mM EDTA and 20 mM Hepes. The percentage of $CD11c^+$ $CD11b^+$ cells was measured by flow cytometry. Before the centrifugation of Nycodenz gradient, $CD11c^+$ $CD11b^+$ represented typically 2.0-2.5% of total cell population; after the enrichment they represented typically 23-25% of total cells.

### *2.3.3. Generation of bone marrow-derived dendritic cells*

Bone marrow was isolated from mouse femurs and tibias. The cell suspension was filtered through a cell strainer to remove particles and obtain a single cell suspension. The cells were centrifuged for 10 min at 280 g and resuspended in 5 ml of red cell lysis buffer (0.83% (w/v) $NH_4Cl$, 0.1% (w/v) $KHCO_3$ in water). Following 5 min of incubation at room temperature, the cells were centrifuged as before and washed twice with RPMI 1640 (Invitrogen), each time by centrifuging for 10 min at 280 g. The cell concentration was adjusted to $1x10^6$ cells/ml in RPMI 1640 (Invitrogen) supplemented with 5% (v/v) FCS (Invitrogen), 100 U/ml of penicillin (Invitrogen), 100 μg/ml of streptomycin (Invitrogen), 1 mM pyruvate (Invitrogen) and 20 ng/ml of GM-CSF (PeproTec). The cell suspension was plated out at 1 ml/well in a 24-well plate. The cells were washed and fed every two days, each time by removing the old medium, gently washing with plain RPMI 1640 and replacing the old medium with 1 ml of RPMI 1640 supplemented with 5% (v/v) FCS (Invitrogen), 100 U/ml of penicillin (Invitrogen), 100 μg/ml of streptomycin (Invitrogen), 1 mM pyruvate (Invitrogen) and 20 ng/ml of GM-CSF (PeproTec). The cells were used for experiments after 7 days of culture which allowed optimal differentiation of bone marrow-derived dendritic cells. The level of $CD11c^+$ after that time has always been >85%, as determined by flow cytometry analysis.

### *2.3.4. Isolation of cells from the peritoneal cavity*

Mice were sacrificed, their skin was cut and pulled back to expose the inner skin lining of the peritoneal cavity. 5 ml of ice-cold PBS was injected into the peritoneal cavity using a 27 G needle. The peritoneal cavity was gently massaged to dislodge any attached cells into PBS. PBS containing the cells was removed from the peritoneal cavity using the same syringe and needle.

### *2.3.5. $^3$H-thymidine incorporation assay*

Methyl-$^3$H-thymidine (0.5 μCi/well in a 96-well plate; Amersham) was added to the cell cultures for the last 16 h of a 72 h culture. After that time, the cells were harvested onto glass fibres (Unifilter GF/B; Perkin Elmer) using an automated harvester (Filtermate;

Packard). The glass fibres were dried for 1 h at 37°C and the incorporation of $^{3}$H-thymidine was measured via liquid scintillation counting.

### *2.3.6. Flow cytometry*

The cells were stained for 30 min at 4°C in darkness with fluorophore-conjugated (direct) or unlabelled (indirect) antibodies or protein constructs (unless stated otherwise, the final concentration was 10 μg/ml) in PBS/BSA buffer (0.2% (w/v) Bovine Serum Albumin fraction V (BSA; First Link, Ltd.) in PBS) in transparent FACS tubes (Becton Dickinson). The cells were washed twice in PBS/BSA buffer and resuspended. For indirect immunofluorescence, the cells were further incubated for 30 min at 4°C in darkness with fluorophore-conjugated secondary antibodies directed against the primary antibodies or protein constructs, washed twice and resuspended in PBS/BSA buffer and subsequently analysed on FACSCalibur (Becton Dickinson) or FACS Canto II (Becton Dickinson) flow cytometers. Forward scatter versus side scatter analysis was used to gate viable cells or lymphocyte population. Ten thousand viable cells were typically collected per sample. Bone marrow-derived dendritic cells, blood cells, splenic dendritic cells and spleen cells were pre-incubated for 15 min at 4°C with 10 μg/ml of anti-FcR II and III antibody (2.4G2) prior to staining with primary antibodies to prevent their binding to Fc receptors. Blood samples (~100 μl) were directly stained in FACS tubes with antibodies or protein constructs, washed once with red cell lysis buffer and once with PBS/BSA buffer, resuspended and analysed on a flow cytometer. The data were analysed using CellQuest or WinMDI software.

### *2.3.7. Intracellular staining for the presence of IFN-γ and FoxP3*

Intracellular staining for the presence of IFN-γ was performed using Cytofix/Cytoperm Kit (BD Biosciences) according to the manufacturer's instructions. 1 μl/ml of GolgiPlug (containing brefeldin A; BD Biosciences) was added to the cell culture for the last 6 h of culture. The cells were pre-incubated with 2.4G2 antibody in the staining buffer (PBS/BSA containing 1 μl/ml of GolgiPlug) as described above and surface antigens were stained with fluorophore-conjugated antibodies in the staining buffer as described above. The cells were washed twice with staining buffer and fixed in Cytofix/Cytoperm buffer (1 ml/tube) for 20 min at 4°C. The cells were vortexed, resuspended in Fixation/Permeabilization

solution (250 µl/tube) and incubated for 20 min at 4°C. The cells were washed twice in 1x Perm/Wash buffer (containing foetal bovine serum and saponin; 1 ml/tube). Subsequently, the cells were resuspended in 50 µl of 1x Perm/Wash buffer containing fluorophore-labelled anti-IFN-γ antibody (10 µg/ml). Finally, the cells were again washed twice in 1x Perm/Wash buffer, resuspended in the staining buffer and analysed by flow cytometry. Intracellular staining for the presence of FoxP3 was performed using FoxP3 Staining Buffer Set (eBiosciences) in a similar way as described above.

### *2.3.8. CFSE dilution assay*

$5x10^7$ cells/ml were incubated for 10 min at 37°C with 5 µM or indicated concentrations of carboxyfluoresceine diacetate succinimidyl ester (CFSE; Molecular Probes) in PBS containing 0.1% (w/v) bovine serum albumin. Labelled cells were then washed twice with RPMI 1640 containing 10% FCS. The level of labelling was checked by flow cytometry analysis.

### *2.3.9. Depletion of $CD4^+$ T cells for in vitro cellular assays*

Depletion of $CD4^+$ T cells was performed using CD4 (L3T4) MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. First, $CD4^+$ T cells were stained with magnetically labelled anti-CD4 (L3T4) antibodies (Mitenyi Biotec) and the cell suspension ($8x10^7$ cells) was loaded on MACS LD column (Miltenyi Biotec) which was then placed in the magnetic field. The magnetically labelled $CD4^+$ T cells were retained on the column while the unlabelled cells run through it and were used for cellular assays. The depletion of $CD4^+$ T cells was confirmed by flow cytometry. Usually ~90% of $CD4^+$ T cells were removed from the total population of spleen cells.

### *2.3.10. Isolation of $CD49b^+$ cells for in vitro cellular assays*

$CD49b^+$ cells were isolated from spleens of SCID BALB/c mice using DC49b (DX5) MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, $DX5^+$ cells were stained with magnetically labelled anti-CD49b antibodies and the cell suspension ($15x10^7$ cells) was loaded onto a MACS LS column (Miltenyi Biotec) which was then placed in a magnetic field. The magnetically labelled $CD49b^+$ cells were retained

on the column, while the non-labelled cells run through the column. $CD49b^+$ cells were eluted from the column after it had been removed from the magnetic field.

*2.3.11. Stable transfection of CHO.K1 cells by calcium phosphate precipitate*

Mammalian expression vector pEE14.rCD4(d3+4).TL1A vector, containing cDNA encoding the extracellular part of TL1A fused to rat CD4 domains 3 and 4 was stably transfected into CHO.K1 cells via calcium phosphate precipitate method. Cell clones that stably expressed soluble TL1A were selected using glutamate synthetase system. Glutamine is a metabolite synthesised from glutamate and ammonia in a reaction catalysed by glutamine synthetase. Inhibition of this enzyme using methionine sulfoximine (MSX), in the absence of exogenous glutamine, will cause death of the cell. However, pEE14 vector contains a glutamine synthetase minigene, therefore cells transfected with this vector become resistant to sub-optimal concentrations of MSX because of elevated expression of the enzyme.

The day before the transfection, CHO.K1 cells were seeded at $1x10^6$ cells per 80 $cm^2$ flask in DMEM media (Safc) supplemented with10% (v/v) FCS (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), 1 mM pyruvate (Invitrogen), 2 mM L-glutamine (Invitrogen) and GS supplements (containing alanine, asparagines, aspartic acid, glutamic acid, proline, serine, adenosine, guanosine, cytidine, uridine and thymidine; Sigma). A sterile solution containing 15 µg of pEE14.rCD4(d3+4).TL1A vector and 186 µl of 1M $CaCl_2$ in a total volume of 750 µl was added dropwise with agitation to 750 µl of 2x HBS, pH=7.05 (1.636% (w/v) NaCl, 1.188% (w/v) Hepes, 0.04% (w/v) $Na_2HPO_4$) and incubated for 10 min at room temperature to allow the DNA-calcium phosphate to form. The precipitate was added to the CHO.K1 cells in 20 ml of the media and the cells were incubated for 5 h in a tissue culture incubator. After that time, the cells were washed twice with plain DMEM (Safc) media and incubate with 2 ml of 15% (v/v) glycerol in 1x HBS for 2 min. The cells were subsequently washed twice with plain DMEM media (Safc) and cultured overnight in DMEM (Safc) supplemented with 10% (v/v) FCS (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen) and GS supplements (Sigma). The transfected CHO.K1 cells were plated over 96-well flat-bottomed plates ($5x10^4$ cells/ml) at 200 µl/well. The plates were demi-fed after 7 daysand screened by ELISA. After the expansion into 24-well plates, the clones were re-

screened by ELISA to confirm stable expression of sTL1A. Clones that expressed the highest amounts of sTL1A were further expanded and used for the production of sTL1A.

*2.3.12. Transient transfection of HEK293T cells with pcDNA3.1/Zeo(-).TL1A vector*

Transient transfection of HEK293T cells with pcDNA3.1/Zeo(-).TL1A vector was performed using Effectene Transfection Reagent (Qiagen) according to the manufacturer's instructions. Briefly, the day before the transfection, the $8x10^5$ cells were seeded on a 60 mm dish in DMEM (Invitrogen) medium supplemented with 10% (v/v) foetal calf serum (FCS; Invitrogen), 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen) and 1 mM pyruvate (Invitrogen). The dishes were 80% confluent on the day of the transfection. 1 µg of the DNA construct in 10 µl of TE buffer (10 mM tris, 1 mM EDTA, pH=7.5) was added to 140 µl of DNA-condesation buffer (EC buffer). Then, 8 µl of Enhancer buffer was added; the mixture was briefly vortexed and incubated for 5 min at room temperature. After that time, 25 µl of Effectene Transfection Reagent (containing liposomes) was added to the mixture which was then incubated for another 10 min at room temperature. Next, 1 ml of growth medium was added to the DNA/liposomes solution and was added dropwise to the cells. The transfected cells were used for experiments 48 h after the transfection.

## 2.4. Protein analysis and purification

*2.4.1. SDS polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS-PAGE was performed using a mini-gel system (BioRad). The stacking gel consisted of 4% (v/v) acrylamide/bisacrylamide (National Diagnostics), 0.4% (w/v) sodium dodecyl sulphate (SDS), 0.005% (w/v) N N N' N'-tetramethylethylene (TEMED) in 0.125 M tris, pH=6.8. The resolving gel consisted of 12% (v/v) acrylamide/bisacrylamide, 0.4% (w/v) SDS, 0.01% (w/v) TEMED in 0.375 M tris, pH=8.8. Polymerization of the gels was initiated with a fresh 10% (w/v in water) solution of ammonium persulfate, added at the final concentration of 1% in stacking gels and 0.5% in resolving gels. 10 µg of protein per lane was analysed. Non-reduced samples were diluted 1:1 in 2x loading buffer (40% (w/v) urea, 1.6% (v/v) SDS, 0.16 M tris-HCl, 0.08% (w/v) bromophenol blue, pH=8.0). Reduced samples were diluted 1:1 in 2x loading buffer containing also 50 mM DTT. Samples were

denatured by heating at 95°C for 5 min and immediately loaded on the gel. Gels were run at 150 V in running buffer (0.0125 M tris-HCl, 0.096 M glycine, 0.1% (w/v) SDS). Gels were fixed by submerging in 25% isopropanol, 10% acetic acid for 30 min and protein was detected using coomassie brilliant blue stain (0.006% (w/v) coomassie brilliant blue in 5% (v/v) acetic acid). Gels were destained to the desired extent with 10% (v/v) acetic acid.

### *2.4.2. TL1A, IFN-γ and IL-4 ELISA*

96-well microtiter plates were coated overnight at 4°C with 10 μg/ml of anti-TL1A (TAN2-2), 4 μg/ml of anti-IFN-γ (HB170) or 1.25 μg/ml of anti-IL-4 monoclonal antibodies in 50 mM bicarbonate buffer (1.59 g $Na_2CO_3$, 2.93 g $NaHCO_3$ in 1 L of water). The plates were blocked with PBS/1% (w/v) bovine serum albumin for 2 h at room temperature and washed 3 times with PBS / 0.05% (v/v) Tween-20. Diluted standards of mouse soluble recombinant TL1A (TL1A.Fc), IFN-γ or IL-4 and supernatants from the cell cultures were added to the plates which were then incubated for 2 h at 37°C. The plates were washed 5 times with PBS/0.05% (v/v) Tween-20. Biotinylated anti-TL1A (TAN2-2; 5 μg/ml), anti-IFN-γ (0.5 μg/ml) or anti-IL-4 (0.125 μg/ml) were added to the wells and incubated for 1.5 h at 37°C. The plates were washed 5 times with PBS/0.05% (v/v) Tween-20 and 1:1000 dilution of streptavidin-HRP in PBS/1% (w/v) bovine serum albumin. The plates were incubated for 30 min at room temperature, washed 5 times with PBS/0.05% (v/v) Tween-20 and OPD (o-phenylene dihydrochloride; Sigma) substrate for colorimetric detection of HRP was added. The plates were incubated for 10 min at room temperature in darkness. After that time, 50 μl/ml of 2.5M $H_2SO_4$ was added. The plate was read at 460 nm on Dynatech MR4000 plate reader.

### *2.4.3. Purification of soluble recombinant TL1A (sTL1A)*

The clone of CHO.K1 cells stably transfected with pEE14.rCD4(d3+4).TL1A construct, showing the highest level of expression of sTL1A (extracellular region of TL1A fused to rat CD4 domains 3 and 4) was cultured in 175 $cm^2$ triple layer culture flasks (Nunc) in 150 ml of DMEM medium (Safc) supplemented with 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 5% dialysed foetal calf serum (First Link, Ltd.), GS supplements (containing alanine, asparagine, aspartic acid, glutamic acid, proline, serine, adenosine, thymidine, uridine and cytidine; Sigma) and 20 μM methionine sulfoximine

(MSX; Sigma). 5 L of supernatant collected from the cell culture was filterded through Whatman paper and concentrated to 1 L using Dicea 150 Dialyzer (Baxter). A 15 ml column containing anti-rat CD4 (OX68) antibody-conjugated sepharose beads was washed 10 times with 200 ml of low tris buffer (1:7 dilution of stock tris buffer (200 mM tris, 1 M NaCl, 10 mM EDTA, pH=8.0 in water) and 10 times with 200 ml of high tris buffer (1:2 dilution of stock tris buffer in water). 1 L of the concentrated CHO.K1 cell culture supernatant was run through the column (~200 µl/sec). The column was washed 10 times with 200 ml of low tris buffer and the protein was eluted with 0.1 M glycine-HCl, pH=2.5. The whole process of elution was then repeated, so that 2 batches of protein were collected from 1 L of the concentrated supernatant. Usually, 5 mg of sTL1A were collected from 5 L of non-concentrated cell culture supernatant.

### *2.4.4. Conjugation of anti-rat CD4 (OX68) antibody to sepharose beads*

30 mg of antibody was dialysed against 5 L of citrate buffer (0.2 M citric acid, 0.58 M NaOH, pH=6.5). The antibody was then diluted to a final concentration of 2 mg/ml. 200 ml of 10 mM HCl was added to 1.2 g of CNBr-activated sepharose 4B beads (Amersham Biosciences) and left at room temperature for 20 min. The beads were poured over a sintered glass filter and washed with 500 ml of 10 mM HCl, followed by 500 ml of the citrate buffer. The beads were placed into a universal tube and 26 mg of OX68 antibody was added. The beads together with antibody were left for 4 h at room temperature, rotating. Antibody binding to the beads was confirmed by spectrophotometry at 280 nm of the residual citrate buffer. The beads were then incubated with 20 ml of 1 M ethanolamine-HCl, pH=9.5 for 1 h, rotating. The beads were again poured onto a sintered glass filter and washed with 1 L of 0.1M tris, 0.5 M NaCl, 5 mM EDTA, followed by ammonium thiocyanate (1 M KSCn, 0.7 M ammonium), followed by a second wash with 0.1 M tris, 0.5 M NaCl, 5 mM EDTA. Conjugated beads were then packed over glass wool in a 30 ml syringe (the volume of the beads was approximately 15 ml).

## 2.5. Mice and *in vivo* experiments

### *2.5.1. OT-I, OT-II transgenic mice, wild-type C57BL/6 and BALB/c mice*

OT-I and OT-II C57BL/6 mice were bred in the in-house animal facility (Tenovus Research Laboratory). Wild-type C57BL/6 and BALB/c mice were purchased from Harlan and bred in the in-house animal facility (Tenovus Research Laboratory). C3H/HeN and C3H/HeJ mice were purchased from Jackson Laboratory. The mice were used at 8-12 weeks of age. All animal experiments described in this thesis were carried out according to the UK Home Office licence guidelines and were approved by the University of Southampton's ethical committee.

### *2.5.2. CD11c-TL1A transgenic mice*

CD11c-TL1A transgenic mice were generated in-house by injection of a DNA construct containing the coding sequence of TL1A under the control of CD11c promoter into the male pronuclei of FVB/N zygotes. The transgenic progeny was then backcrossed to the C57BL/6 strain for 5-6 generations. Transgenic mice and age-matched control littermates were used at 6-24 weeks of age. CD11c-TL1A transgenic mice were identified by PCR using the following primers: forward – 5'-CTCACCTCCCAGAGGCAAG-3' and reverse – 5'-GGAGTCTGGCTTGTTTGGTCG-3'. The following reaction mixture was used: 0.5 µl of 10 mM dNTPs, 1 µl of the forward and reverse primer at the concentration of 100 ng/ml, 5 µl of 5x Green GoTaq Polymerase Buffer (Promega), 1 µl of mouse genomic DNA, 0.5 µl (0.5 U) of GoTaq Polymerase (Promega) and 16 µl of water. The reaction was run under the following conditions: 95$^{o}$C 5 min, 25 cycles of 95$^{o}$C 1 min, 60.5$^{o}$C 1 min, 72$^{o}$C 2 min, followed by the final extension step 72$^{o}$C 10 min. The reaction was run in PTC-100 thermocycler (MJ-Research, Inc.). The primers used for this PCR can amplify both cDNA and the whole genomic sequence of TL1A, giving products of 720 bp and 5500 bp, respectively. However, the extension step used in this reaction is too short to amplify the larger fragment. The products of the PCR were analysed by agarose gel DNA electrophoresis.

### *2.5.3. CD2-TL1A transgenic mice*

CD2-TL1A transgenic mice were generated at Cancer Research UK using the prepared in-house DNA construct containing the coding sequence of TL1A under the control of CD2 promoter. The DNA construct was injected into male pronuclei of CBN zygotes. The transgenic progeny was then backcrossed to the C57BL/6 strain for 4-5 generations. The transgenic mice and age-matched control littermates were used at 8-20 weeks of age. CD2-TL1A transgenic mice were identified by PCR, as described in the previous section.

### *2.5.4. Adoptive transfer of OT-I and OT-II T cells*

For adoptive transfer of OT-I and OT-II to naive C57BL/6 recipients, a single cell suspension of lymph node (inguinal, brachial and mesenteric) and spleen cells was prepared from OT-I or OT-II transgenic C57BL/6 mice. The proportion of OT-I T cells was determined by H-2K$^b$ SIINFEKL tetramer and anti-CD8 antibody staining and flow cytometry analysis. The proportion of OT-II T cells was determined by staining the cells with anti-CD4, anti-Vα2 and anti-Vβ5 antibodies and analysis by flow cytometry. The cells were then injected *i.v.* into sex-matched C57BL/6 recipients. The number of transferred cells is indicated in the figure legends.

### *2.5.5. In vivo depletion of CD4$^+$, CD8$^+$ T cells and NK cells*

CD4$^+$ and CD8$^+$ T cell depletion was carried out by *i.p.* injection of anti-CD4 (YTA3.1.2.; 1 mg) or anti-CD8 (YTS169; 0.5 mg) monoclonal antibodies, respectively on days -3, -1 and 3. NK cell depletion was carried out by *i.v.* injection of 25 μg of anti-asialo GM1 antibody on days -3, -2 and -1, where day 0 was the day of tumour challenge.

## 2.6. Histology

Tissue was fixed in 10% neutral buffered formalin (Sigma). Samples were embedded, sectioned and stained with H&E. The histology analysis was conducted by University of Southampton, School of Medicine core facility.

## 2.7. Statistical analysis

Statistical analyses were performed with GraphPad Prism 5 software and involved the use of two-tailed Student t test, except for comparing survival curves where the log-rank (Mantel-Cox) test was used. $P \leq 0.05$ was considered statistically significant.

## CHAPTER 3

## EXPRESSION OF TL1A ON DENDRITIC CELLS AND T CELLS

### 3.1. Introduction

There are several reports showing that TL1A acts as a co-stimulatory molecule for T cells (116, 123, 145, 146). Expression of co-stimulatory molecules is usually localized to antigen presenting cells and T cells and is up-regulated following their activation (59). This chapter shows that expression of transmembrane TL1A is transiently up-regulated on activated T cells, bone marrow-derived dendritic cells and splenocytes stimulated with ligands for Toll-like receptor (TLR) 3 and TLR4.

Dendritic cells undergo activation and maturation following the interaction with pathogen-associated molecular patterns (PAMPs) or ligation of certain receptors present on their surface (e.g. CD40). Therefore, expression of TL1A was investigated in T cells, bone marrow-derived dendritic cells and total population of splenocytes activated both *in vitro* and *in vivo* with several PAMPs recognized by TLRs and dectin-1 and other factors that activate antigen presenting cells or T cells either directly or indirectly.

PAMPs are recognized by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) which are the best characterized class of PRRs. There are 10 to 15 different TLRs in mammals. They are key components of the innate immune system that detect microbial infection and trigger antimicrobial host defence responses. TLRs are expressed on epithelial cells and on a variety of cells of the immune system, including dendritic cells, and detect multiple PAMPs including lipopolysaccharide (LPS), flagellin, unmethylated CpG DNA and double-stranded viral RNA (157). TLRs use four adaptor proteins (MyD88, TIRAP, TRIF and TRAM) to transduce signals leading to activation of several transcription factors – NF-κB, AP-1, IRF-3, IRF-5 and IRF-7. IRFs induce expression of type I interferons which, in turn, activate transcription of interferon-induced genes (158).

Another PRR is dectin-1. It is a C-type lectin receptor recognizing β-1,3-glucan which is a component of yeast cell wall. Dectin-1 expressed in high levels on dendritic cells, monocytes, macrophages and neutrophiles and its stimulation results in activation of these cells. Dectin-1 signalling, via Src and Syk tyrosine kinases and an adaptor protein Card9, leads to activation of NF-κB. It also synergizes with TLR2 to augument secretion of

pro-inflammatory cytokines. It is known that dectin-1/Syk and Dectin-1/TLR2 pathways act independently (159, 160).

The other issue described in this chapter is the specificity of binding of TAN2-2 (α-TL1A) antibody and DR3.Fc construct to transmembrane TL1A expressed on cells transfected with TL1A cDNA and cells naturally expressing TL1A. This part the research led to a hypothesis that TL1A exists in two forms – homo- and heterotrimeric.

## 3.2. Results

### *3.2.1. Expression of TL1A on activated splenocytes, bone marrow-derived dendritic cells and T lymphocytes*

Expression of TL1A mRNA in bone marrow-derived dendritic cells (BMDCs) stimulated *in vitro* with 100 ng/ml of lipopolysaccharide (LPS; ligand for TLR4) or 50 μg/ml of polyinosinic-polycytidylic acid (poly(I:C); ligand for TLR3) for 4 h was determined by quantitative PCR (qPCR). LPS induced an average 442-fold increase of TL1A mRNA level compared to non-stimulated cells, while poly(I:C) induced an average 177-fold increase of TL1A mRNA level compared to non-stimulated cells (Figure 3.1.).

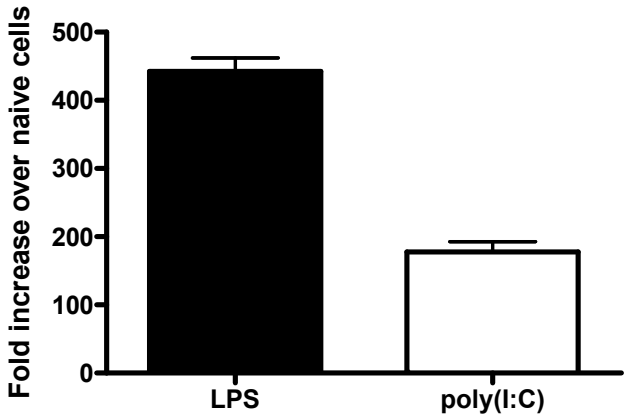



**Figure 3.1.** Expression of TL1A mRNA in bone marrow-derived dendritic cells stimulated with LPS or poly(I:C). The cells were stimulated *in vitro* with 100 ng/ml of LPS or 50 μg/ml of poly(I:C). After 4 h of stimulation, total RNA was extracted from the cells, genomic DNA in the RNA samples was digested, RNA was reverse transcribed and TL1A transcript was quantified by qPCR. The graph represents mean values from three separate experiments ±SE. In each experiment the fold change was calculated using triplicate qPCR samples.

Expression of the transmembrane form of TL1A was detected on $CD11c^{high}$ fraction of BMDCs by flow cytometry after 4 h and 24 h of *in vitro* stimulation with 100 ng/ml of LPS. The level of activation of BMDCs was estimated by the expression of the CD86 molecule which increased following stimulation with LPS (Figure 3.2.). The cells were grown also in the presence of both LPS and 20 μM TAPI-0 which is an inhibitor of the TNF convertase (TACE), belonging to the family of matrix metalloproteinases (MMPs), responsible for shedding of TNF from the cell surface. Taking into account the broad substrate specificity of MMPs and high level of homology between the TNFSF

members, it is possible that the same MMPs which induce the release of soluble TNF are also involved in shedding of TL1A. However, BMDCs grown in the presence of TAPI-0 did not show higher expression of TL1A than the control cells (Figure 3.2.A). This experiment indicates that TL1A is not shed from the surface of BMDCs in these conditions or enzymes other than TACE are responsible for shedding of this molecule, since others have reported the release of soluble TL1A from human dendritic cells and monocytes *in vitro* (119).

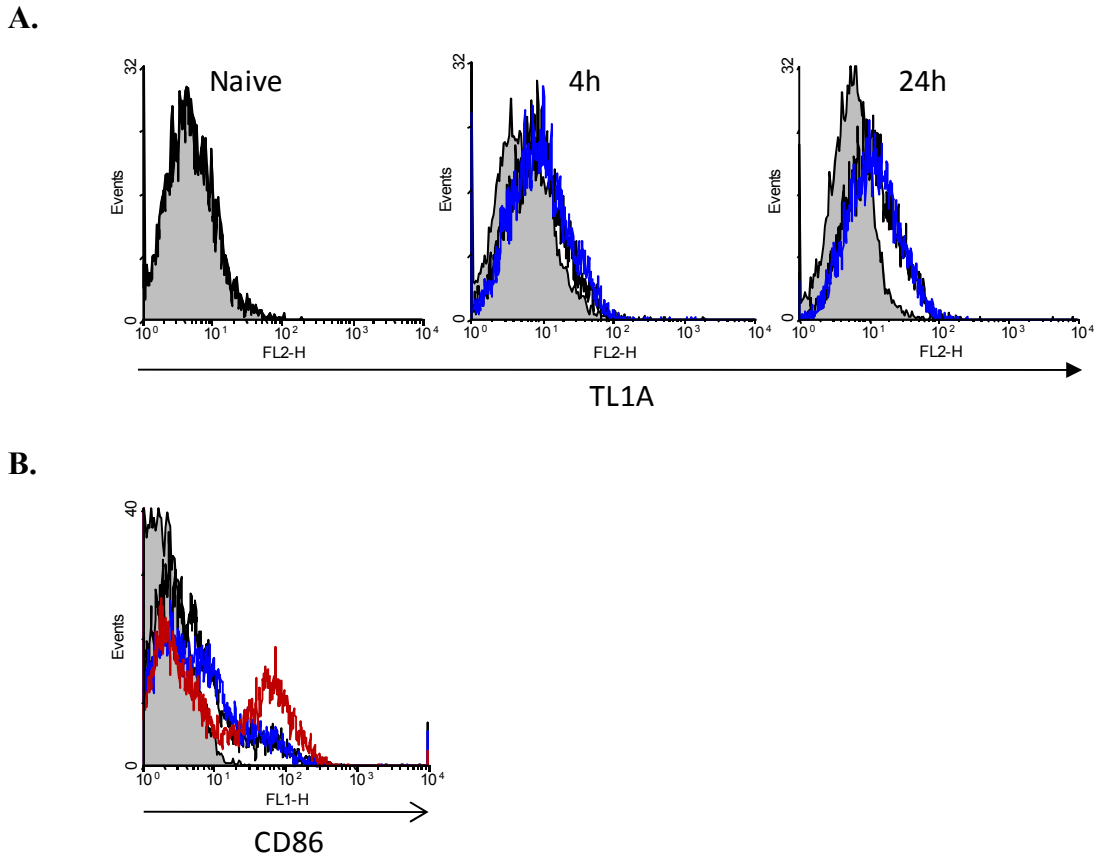



**Figure 3.2.** Expression of TL1A and CD86 on bone marrow-derived dendritic cells (BMDCs) stimulated with LPS. (A) BMDCs from a WT C57BL/6 mouse were stimulated *in vitro* with 100 ng/ml of LPS in the presence (blue line) or absence (black line) of 20 μM TAPI-0 and stained for the presence of TL1A with DR3.Fc construct and PE-conjugated goat anti-human IgG antibody. Grey histograms represent staining with the isotype control for DR3.Fc (human IgG). (B) BMDCs were stimulated as above and stained for the presence of CD86 with FITC-conjugated GL1 antibody. Black line – naive cells, blue line – 4h of stimulation, red line – 24h of stimulation, grey histogram – staining with the isotype control antibody for GL1 (FITC-conjugated Mc39-16 antibody). The cells presented on the histograms were gated on both $CD11c^{high}$ and viable cells. Presented results are representative of three separate experiments.

TL1A expression was examined by flow cytometry also on mouse T cells activated with 10 μg/ml of anti-CD3 antibody. CD3 takes part in signal transduction from T cell receptor, therefore stimulation of this co-receptor results in activation of T cells, but not other cells, present in the population of splenocytes. Activated T cells transiently up-regulate the expression of transmembrane TL1A after 24 h of stimulation with anti-CD3 antibody (Figure 3.3.).

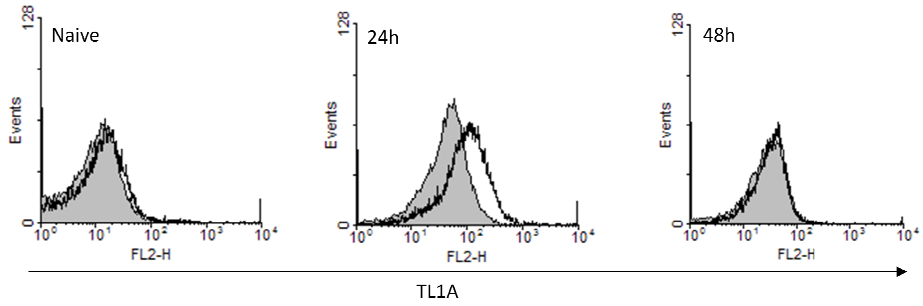



**Figure 3.3.** Expression of TL1A on activated T cells. Splenocytes isolated from a WT C57BL/6 mouse were stimulated *in vitro* with 10 μg/ml of anti-CD3 antibody for 24 h and 48 h. The cells were stained for the presence of TL1A with DR3.Fc construct and PE-conjugated goat anti-human IgG antibody (black line). Grey histograms represent staining with the isotype control for DR3.Fc (human IgG). The cells presented on histograms were electronically gated on viable population. The presented results are representative of five separate experiments.

Expression of TL1A mRNA was investigated also in splenocytes of C57BL/6 mice injected *i.v.* with 40 μg of LPS (ligand for TLR4), 40 μg of Lipid A (lipid component of LPS; ligand for TLR4), 50 μg of poly(I:C) mimicking double-stranded viral RNA (ligand for TLR3), 50 μg of unmethylated CpG sequences (CpG; ligand for TLR9), 50 μg of synthetic lipopeptide (S)-(2,3-bis(polmitoyloxy)-(2RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-$Lys_4$-OH trihydrochloride (Pam3CSK4; ligand for TLR2), 2 μg of α-galactosylceramide (α-GalCer), or *i.p.* with 5 mg of curdlan (β-1,3-glucan; ligand for dectin-1), or 500 μg of α-CD40. TL1A transcript was quantified by qPCR following 4 h, 6 h and 24 h of stimulation. The most potent inducers of TL1A mRNA were poly(I:C) which induced an average 18-fold increase of TL1A mRNA expression in comparison to naive animals, Lipid A (average 19-fold increase) and LPS (average 12-fold increase) (Figure 3.4.). TL1A mRNA level peaked after 4h of stimulation with LPS and Lipid A, and after 6h of stimulation with poly(I:C). It returned to the level of naive cells after 24 h. The other tested factors did not significantly change the level of TL1A mRNA expression.

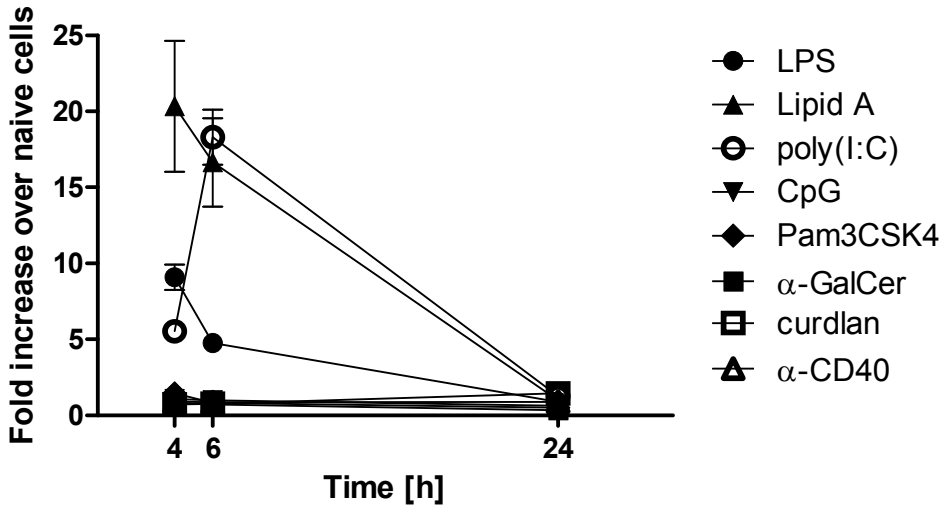



**Figure 3.4.** Expression of TL1A mRNA in mouse splenocytes. WT C57BL/6 mice (n=4 mice per time point) were injected *i.v.* with 40 μg of LPS, 40 μg of Lipid A, 50 μg of poly(I:C), 50 μg of CpG, 50 μg of Pam3CSK4, 2 μg of α-GalCer, or *i.p.* with 5 mg of curdlan or 500 μg of α-CD40 antibody. Following 4 h, 6 h or 24 h of stimulation, total RNA was isolated from splenocytes, genomic DNA in the RNA samples was digested, RNA was reverse transcribed and the amount of TL1A transcript was quantified by qPCR. In each experiment the fold change was calculated using triplicate qPCR samples.

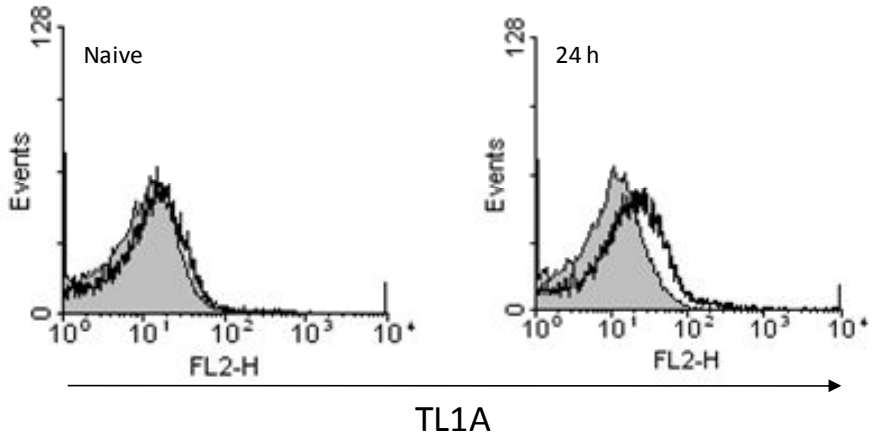



**Figure 3.5.** Expression of TL1A on splenocytes stimulated with LPS *in vivo*. A C57BL/6 mouse was injected *i.v.* with 40 μg of LPS. After 24 h of stimulation, splenocytes were stained for the presence of TL1A with DR3.Fc construct and PE-conjugated goat anti-human IgG antibody (black line). Grey histograms represent staining with the isotype control for DR3.Fc (human IgG). The cells presented on the histograms were gated on viable population. Presented results are representative of two separate experiments.

Expression of the transmembrane form of T1A was detected on splenocytes of C57BL/6 mice 24h after the *i.v.* injection of 40 μg of LPS (Figure 3.5.), although it was much smaller than the level of up-regulation of TL1A mRNA following stimulation with LPS. No expression of transmembrane TL1A was detected at the earlier time point (12 h).

To determine whether TLR4 is the receptor responsible for up-regulation of TL1A in splenocytes, C3H/HeJ mice (bearing an inactivating mutation in TLR4 (161)) and wild-type C3H/HeN control mice were injected *i.v.* with 40 μg of LPS and the amount of TL1A transcript in their splenocytes was quantified after 4h of stimulation. C3H/HeN mice showed 8-fold higher expression of TL1A mRNA than non-stimulated mice while C3H/HeJ mice showed only 1.5-fold increase in the expression of TL1A transcript compared to non-stimulated C3H/HeN mice (Figure 3.6.). This clearly shows that up-regulation of TL1A is mediated by TLR4.

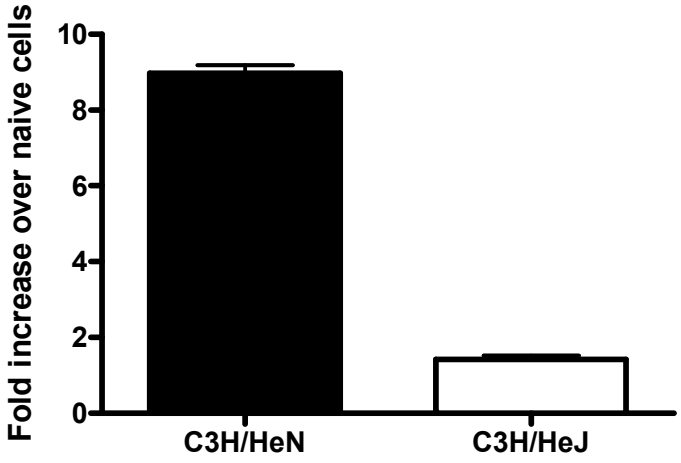



**Figure 3.6.** Expression of TL1A mRNA in splenocytes of C3H/HeN and C3H/HeJ mice (n=3 mice of each strain) stimulated with LPS. The mice were injected *i.v.* with 40 μg of LPS. 4 h later, total RNA was isolated from splenocytes, genomic DNA in the RNA samples was digested, RNA was reverse transcribed and the amount of TL1A transcript was quantified by qPCR.

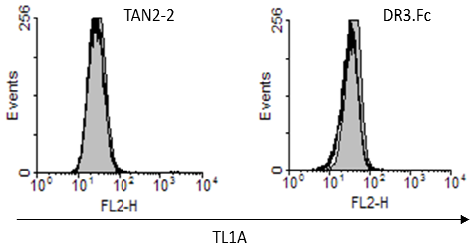
### *3.2.2. Specificity of TAN2-2 and DR3.Fc binding*

Two reagents were used for detection of TL1A by flow cytometry – a monoclonal antibody (TAN2-2) and a soluble receptor (DR3.Fc). TAN2-2 antibody was raised in rats against the mouse homotrimeric form of TL1A. DR3.Fc is a fusion protein consisting of the mouse extracellular domain of the DR3 receptor and the Fc fragment of human IgG.

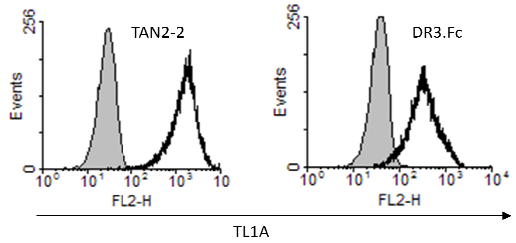
To check the binding specificity of these reagents, J558L cells stably transfected with a plasmid vector carrying TL1A cDNA (J558L-TL1A) and J558L cells stably transfected with an empty control vector (J558L-PEF) were stained with TAN2-2 and DR3.Fc. Both TAN2-2 and DR3.Fc showed binding to J558L-TL1A cells but none of the reagents bound to J558L-PEF cells (Figure 3.7.A and B). Both reagents showed binding also to EG.7 cells - mouse thymoma cells, naturally expressing transmembrane TL1A (Figure 3.7.C). Interestingly, TAN2-2 bound more strongly to J558L-TL1A cells than to EG.7 cells, whereas DR3.Fc appeared to bind equally well to both J558L-TL1A and EG.7 cells (Figure 3.7.B and C). Unlike DR3.Fc, TAN2-2 did not show any binding to T cells activated *in vitro* with 10 μg/ml of anti-CD3 antibody (Figure 3.7.E), whereas neither TAN2-2 nor DR3.Fc bound to non-activated T cells (Figure 3.7.D).

TAN2-2 and DR3.Fc were used also for staining of HEK293T-TL1A cells which were transiently transfected with pcDNA3.1/Zeo(-).TL1A vector carrying TL1A cDNA and non-transfected HEK293T cells. Both TAN2-2 and DR3.Fc showed higher binding to the transfected cells, although there was also some low level binding of DR3.Fc to the non-transfected cells (Figure 3.8.). Titration of DR3.Fc binding to HEK293T cells showed that above a certain point ($10^2$ on logarithmic scale) this binding is saturable and specific on HEK293T-TL1A cells (Figure 3.9.). Also, pre-incubation of HEK293T-TL1A cells with TAN2-2 but not with isotype control Mc39-16 antibody reduced the binding of DR3.Fc to the cells (Figure 3.10.A). Pre-incubation with TAN2-2, however, did not block binding of DR3.Fc to activated T cells (Figure 3.10.B).

**A.**




**B.**

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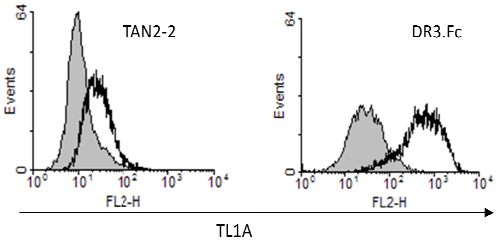
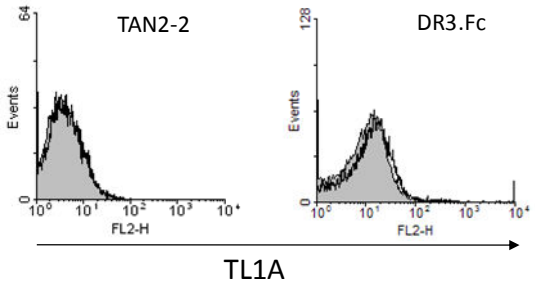



**D.**




**E.**

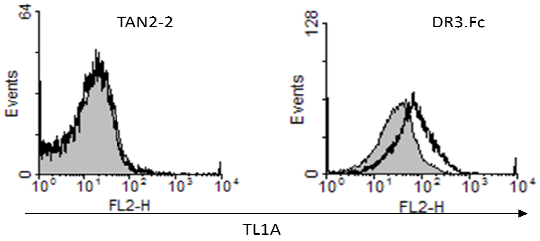
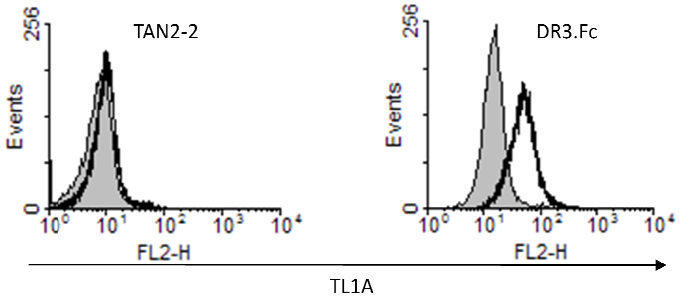



**Figure 3.7.** Binding of TAN2-2 and DR3.Fc to J558L-PEF (A), J558L-TL1A (B), EG.7 (C) non-activated (D) and activated T cells (E). The cells were stained for the presence of TL1A with TAN2-2 antibody and PE-conjugated goat anti-rat IgG antibody or DR3.Fc construct and PE-conjugated goat anti-human IgG antibody (black lines). Grey histograms represent staining with isotype controls for TAN2-2 or DR3.Fc (MC39-16 and human IgG, respectively). The cells were electronically gated on viable population. The presented results are representative of three separate experiments.

**A.**




**B.**

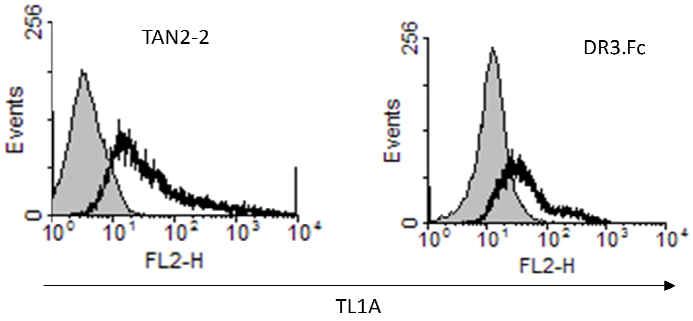



**Figure 3.8.** Binding of TAN2-2 and DR3.Fc to non-transfected HEK293T cells (A) and HEK 293T cells transfected with a plasmid encoding TL1A. The cells were stained with 10 µg/ml of TAN2-2 and PE-conjugated goat anti-rat IgG antibodies or 10 µg/ml of DR3.Fc construct and PE-conjugated goat anti-human IgG-PE antibody (black lines). Grey histograms represent staining with the isotype controls for TAN2-2 and DR3.Fc (Mc39-16 and human IgG, respectively). The cells were electronically gated on viable population. The presented results are representative of four separate experiments.

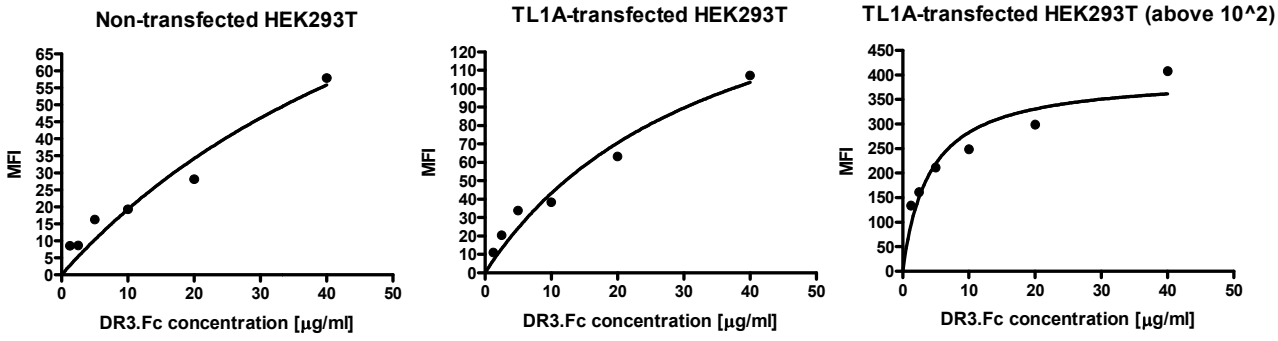
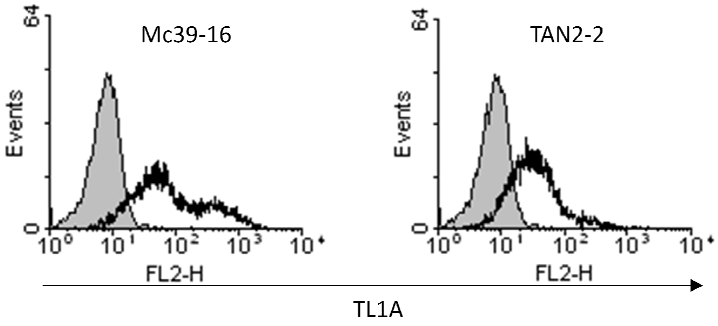



**Figure 3.9.** Titration of DR3.Fc binding to HEK293T cells transfected with a plasmid vector encoding TL1A or non-transfected cells.

**A.**




**B.**

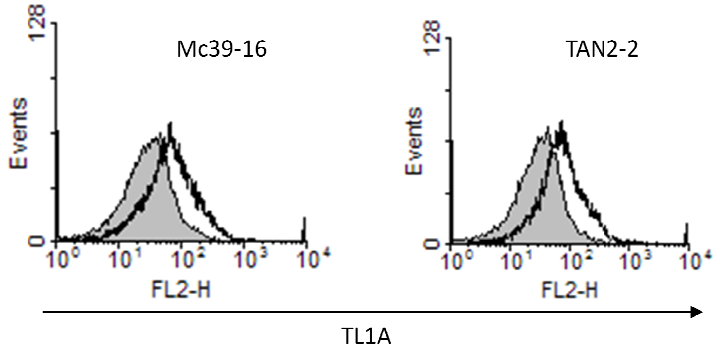



**Figure 3.10.** Binding of DR3.Fc to HEK293T cells transfected with a plasmid vector encoding TL1A (A) and T cells activated *in vitro* with 10 μg/ml of anti-CD3 antibody (B). The cells were pre-incubated on ice with 20 μg/ml of TAN2-2 antibody or its isotype control (Mc39-16) and stained for the presence of TL1A with DR3.Fc and PE-conjugated goat anti-human IgG (black lines). Grey histograms represent staining with the negative control for DR3.Fc (human IgG). The cells were electronically gated on viable population. The presented results are representative of three separate experiments.

## 3.3. Discussion

The most potent inducers of TL1A expression were LPS, Lipid A (ligands for TLR4) and poly(I:C) (ligand for TLR3) which very rapidly up-regulated the expression of TL1A transcript. It is consistent with the results obtained by other research groups (121, 123, 124) which were published after the completion of this part of research. In contrast to the data published by Shih *et al.* (121), no up-regulation of TL1A expression was detected following stimulation of TLR2 and TLR9. In contrast to the research presented in this thesis, Shih *et al.* (121) investigated the expression of TL1A in human monocytes where TL1A expression may be regulated in a different way than in murine cells. Rapid (4–6 h) up-regulation of TL1A transcript in activated bone marrow-derived dendritic cells and splenocytes and lack of its expression on naive cells of the immune system indicates that this molecule is involved in co-stimulation of T cells. Furthermore, up-regulation of TL1A in response to stimulation with pathogen-associated molecular patterns, such as LPS or poly(I:C), suggests that TL1A is involved in immune response directed against pathogen infection, although there are no published reports concerning the role of TL1A in host defence.

The increase of the expression of the transmembrane form of TL1A on bone marrow-derived dendritic cells and splenocytes stimulated *in vitro* or *in vivo* with LPS is much smaller than the increase of the expression of TL1A mRNA in these cells. However, the expression of transmembrane TL1A was much higher on activated T cells than on bone marrow-derived dendritic cells which is consistent with the finding of Meylan *et al.* (123) who found that TL1A can be cleaved off the plasma membrane in endothelial and dendritic cells but not in T cells. Other report shows that TL1A is released from monocytes and dendritic cells stimulated with immune complexes *in vitro* and the level of soluble TL1A detected in the culture supernatants correlates with peak TL1A mRNA levels. However, high expression of TL1A was detected only on the surface of monocytes, whereas dendritic cells show only a modest induction of transmembrane TL1A on their surface following stimulation with immune complexes. Furthermore, supernatant of dendritic cell culture contained higher concentration of soluble TL1A than the monocyte supernatant which suggests that there is a difference in the protease activity in dendritic cells and monocytes, although the enzyme involved in shedding of TL1A remains unidentified (119). Therefore it is highly possible that TL1A produced by bone marrow-derived dendritic cells exists

primarily in a soluble form, although the relatively low expression of the transmembrane form of TL1A may also be biologically significant.

Transient up-regulation of TL1A on activated T cells suggests that it plays an important role during clonal expansion phase of the immune response where T cells can be co-stimulated by other T cells expressing TL1A in an autocrine or paracrine manner. Similar occurrence was observed in an *in vitro* system where expansion and cytolytic function of cytotoxic T cells was enhanced by $CD4^+$ T cells which provided co-stimulatory signals mediated by cell-surface molecules (162).

The data presented in this chapter show that the only receptors which stimulation induces expression of TL1A are TLR3 and TLR4, although they up-regulate TL1A with different kinetics. IRF-3 is the only transcription factor activated by both TLR3 and TLR4 (via adaptor proteins TRIF and TRAM) but not other TLRs or dectin-1 (158, 163). Furthermore, triggering of TLR4 by LipidA induces only the TRIF-mediated signalling pathway, but not the MyD88 pathway, which results in activation of IRF3 (164). IRF3 is constitutively expressed in cells as a monomer and undergoes dimerization and nuclear translocation following the phosphorylation which leads to its activation. IRF3 binds to target DNA sequences but does not have an intrinsic transcriptional activity; it activates transcription of target genes by recruiting co-activators p300 and cAMP-responsive-element-binding protein (CREB)-binding protein (CBP) (158, 165). Some reports suggest that IRF3 is activated by two different signalling pathways – one is triggered as a result of stimulation of TLR3 and the other as a result of stimulation of TLR4 (166). On the other hand, others speculate that TLR3- and TLR4-mediated induction of IRF-3 differs quantitatively rather than qualitatively (158). Finally, TLR3 and TLR4 may have different expression patterns on the investigated cells. These are possible explanations as to why stimulation of TLR3 up-regulates TL1A expression with slightly delayed kinetics in comparison to TLR4-mediated up-regulation of TL1A. An interesting question is whether IRF3 activated the transcription of TL1A gene directly or via other proteins. IRF3 is known to induce expression of type I interferons, which in turn activate the expression of interferon-inducible genes via JAK/STAT signalling pathway (158), therefore it is possible that expression of TL1A is induced indirectly via type I interferon.

Very little is known about the quaternary structure of TL1A. Members of the TNF superfamily are trimeric proteins, therefore the difference in binding specificity of TAN2-2 and DR3.Fc led to the hypothesis that TL1A naturally exists in two biologically active forms – homo- and heterotrimeric, although it remains unknown which molecule forms the

heterotrimer together with a monomer (or two monomers) of TL1A. We hypothesize that DR3.Fc binds to both homo- and heterotrimeric form of TL1A while TAN2-2 (raised against the homotrimeric form of TL1A) binds only to the homotrimeric form. TAN2-2 antibody as well as DR3.Fc construct showed binding to both J558L-TL1A and HEK293T-TL1A cells transfected with TL1A cDNA, therefore these cells probably express only the homotrimeric form of TL1A. In contrast to DR3.Fc, TAN2-2 does not bind to activated T cells which are expected to express the heterotrimeric form of TL1A. EG.7 cells probably express express both forms of TL1A, although the heterotrimeric form predominates, as DR3.Fc shows higher binding to these cells than TAN2-2. The epitope recognised by TAN2-2 is most probably located on the border between two monomeric subunits of homotrimeric TL1A (Figure 3.11.). If it was located on the monomeric subunit of TL1A, TAN2-2 would bind to both forms of TL1A. There is evidence that injection of TAN2-2 reduces clinical score in mice with collagen-induced arthritis (102) which suggests that the homotrimeric form of TL1A has a biological activity. Furthermore, homotrimeric recombinant TL1A and homotrimeric TL1A expressed by J558L-TL1A cells show biological activity (Chapter 4). Domains responsible for ligand binding and trimerization of TNFSF members are 150 amino acid long, C-terminal, TNF homology domains (THDs) (78). Since all of the three isoforms of TL1A – VEGI-174, VEGI-192 and the full-length TL1A – share the same 151 amino acid long C-terminal domain (113), it is possible that the binding partner for TL1A is one of its truncated isoforms – VEGI-174 or VEGI-192. Possible differences in the secondary or tertiary structure of the THD domain in the three isoforms of TL1A may result from the fact that homotrimers of the isoforms bind to different receptors (the main receptor for VEGI-174 and VEGI-192 has not been identified yet, although it is different than DR3 (111)) but still may allow the formation of heterotrimers. The experiments presented in this thesis, that led to the hypothesis that TL1A exists in both homo- and heterotrimeric form were conducted on mice, on mice cells or using mice cDNA of TL1A. VEGI-174 and VEGI-192 were detected only in humans (111-113), however it is possible that other isoforms of TL1A exist in mice.

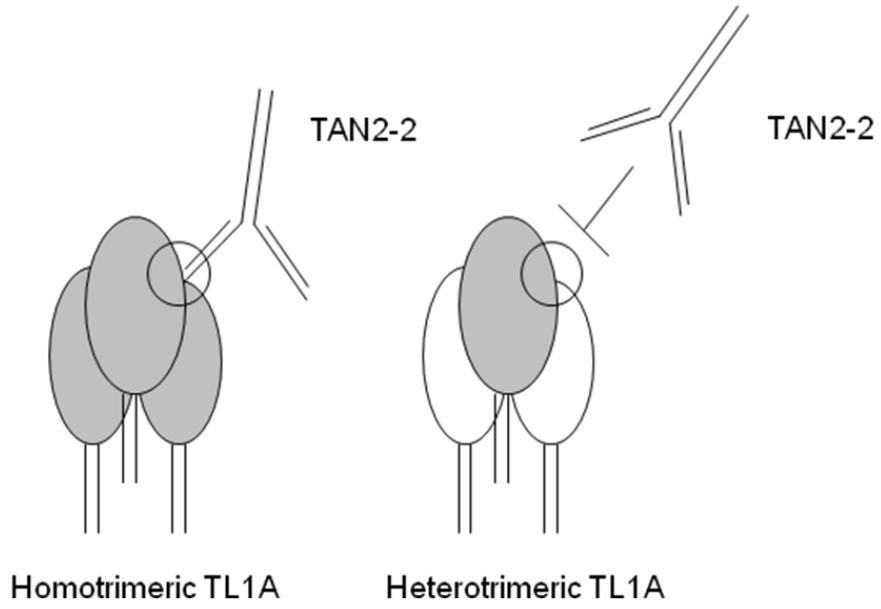



**Figure 3.11.** Epitope recognised by TAN2-2 is present only on the homotrimeric form of TL1A.

# CHAPTER 4

# THE ROLE OF TL1A/DR3 INTERACTIONS IN COSTIMULATION OF T CELLS

## 4.1. Introduction

The research presented in this chapter concentrates mostly on analysis of how TL1A/DR3 interactions affect proliferation and effector functions of $CD8^+$ T cells. A study by Tan *et al.* (135) shows that DR3 mRNA is strongly up-regulated in activated $CD8^+$ T cells but a role for DR3 in $CD8^+$ T cell responses has not been described so far. $CD8^+$ cytotoxic T cells play a critical role in tumour rejection *in vivo*. They kill tumour cells through secretion of granzymes, perforin or molecules belonging to the tumour necrosis factor (e.g. FasL) (29, 30). Treatment of melanoma patients with $CD8^+$-enriched lymphocytes results in the regression of the tumour in most of the patients (167, 168). Also, the transfer of antigen-specific $CD8^+$ T cells into tumour-bearing mice results in the eradication of tumour (169). Furthermore, $CD4^+$ T cells can develop cytotoxic activity and mediate rejection of melanoma tumours based on MHC class II-restricted recognition of tumours (170, 171) which indicates that cytotoxic T cells play a key role in anti-tumour immunity.

The role of TL1A in $CD8^+$ T cell-mediated antitumour immunity was examined in BALB/c mice injected subcutaneously with J558L tumour cells expressing transmembrane TL1A (J558L-TL1A) or control J558L cells transfected with an empty control vector (J558L-PEF). J558L are plasmacytoma cells, derived from BALB/c mice, which form solid tumours following a subcutaneous injection. To determine whether $CD8^+$ T cells take part in TL1A-mediated rejection of tumour, the mice were depleted of $CD4^+$ and/or $CD8^+$ T cells using the appropriate depleting antibodies. The influence of TL1A/DR3 interactions on the activity of $CD8^+$ T cells was also investigated *in vitro* by measuring their proliferation, secretion of effector cytokines and expression of activation/memory markers in response to TL1A stimulation.

There are reports showing that TL1A enhances proliferation (136) of NK cells and their cytotoxicity (150); furthermore, the initial tumour experiments presented in this chapter suggest that NK cells may play a role in TL1A-mediated rejection of J558L tumour, therefore the influence of TL1A on the activity of NK cells was also investigated.

NK (natural killer) cells are cytotoxic lymphocytes that constitute a major part of the innate immune system and represent 5-15% of circulating blood lymphocytes, although they make-up greater proportion in some tissues (e.g. liver) (172). NK cells play an important role in rejection of tumours and in response against viral and parasitic infections and act during the first 4-6 hours of the infection. They kill target cells by releasing perforin and granzymes (2) in a way similar to cytotoxic T cells (section 1.3.). NK cells do not express T cell receptor or CD3 molecules. So far, no universal marker of NK cells has been identified but they usually express CD16 and CD56 molecules in humans and NK1.1 in C57BL/6, FVB/N and NZB mice. CD49b and asialo-GM1 are used as markers for identification of NK cells in mice strains that do not express NK1.1 (e.g. BALB/c) (173), although the expression of asialo-GM1 was detected also on a subpopulation of central memory $CD44^{high}$ $CD8^{+}$ T cells (174). Their activity is strictly regulated to prevent autoreactivity against the host. It is believed that the net balance of signals from activating and inhibitory receptors enables NK cells to provide effective killing of target cells, while maintaining self-tolerance. Key regulators of NK cell activity are MHC class I molecules that provide NK cells with a means of identifying host cells. Down-regulation of MHC class I molecules on tumour or virus-infected cells permits activation of NK cells. MHC class I molecules are recognized by several inhibitory receptors expressed by NK cells – the most important are killer inhibitory receptors (KIRs) and CD94/NKG2A. The ability of NK cells to lyse target cells requires both the lack of MHC class I expression and expression of appropriate ligands on target cells that are recognised by activating receptors of NK cells. These receptors include natural cytotoxicity receptors (NKp30, NKp44 or NKp46), immunoglobulin G receptor CD16 but also some receptors belonging to the KIR superfamily (172). Activating receptors recognise ligands or cytokines secreted by on accessory cells that help in activation of T cells, such accessory cells are monocytes, macrophages and dendritic cells. It is also possible that NK cells directly bind pathogen-associated molecular patterns (175).

## 4.2. Results

### *4.2.1. TL1A-mediated rejection of tumours*

To investigate whether TL1A enhances anti-tumour response, wild-type BALB/c mice were injected subcutaneously with $5x10^6$ J558L cells transfected with a plasmid carrying TL1A cDNA (J558L-TL1A cells) or $5x10^6$ control J558L cells transfected with an empty vector (J558L-PEF cells). J558L-TL1A cells used for tumour experiments were a mixture of equal amounts of three different clones of J558L-TL1A cells – J558L-TL1A #1.1, J558L-TL1A #7.5 and J558L-TL1A #19.2. All of the J558L-TL1A clones showed equal, high expression of transmembrane TL1A, while J558L-PEF cells did not express it (Fig. 4.1.A). The growth rate of all three clones of J558L-TL1A cells and J558L-PEF cells was measured by *in vitro* $^3$H-thymidine incorporation assay. In this method, $^3$H-thymidine is incorporated into DNA of proliferating cells and its accumulation inside the cells can be determined by measuring its β decay by liquid scintillation counting. Even though the uptake of $^3$H-thymidine was low, the three clones of J558L-TL1A and J558L-PEF cells showed a similar growth rate (Fig. 4.1.B). Furthermore, J558L-TL1A and J558L-PEF cells grew at the very similar rate in BALB/c SCID mice (Fig. 4.3.). In wild-type BALB/c mice J558L-PEF tumour cells grew progressively and all of the mice had to be sacrificed 30 days after the inoculation of tumour (median survival 25 days). In contrast, J558L-TL1A cells were rejected in most wild-type mice (Fig. 4.2.). In many cases, J558L-TL1A tumour cells grew initially following the injection into BALB/c mice, but the tumours regressed later.

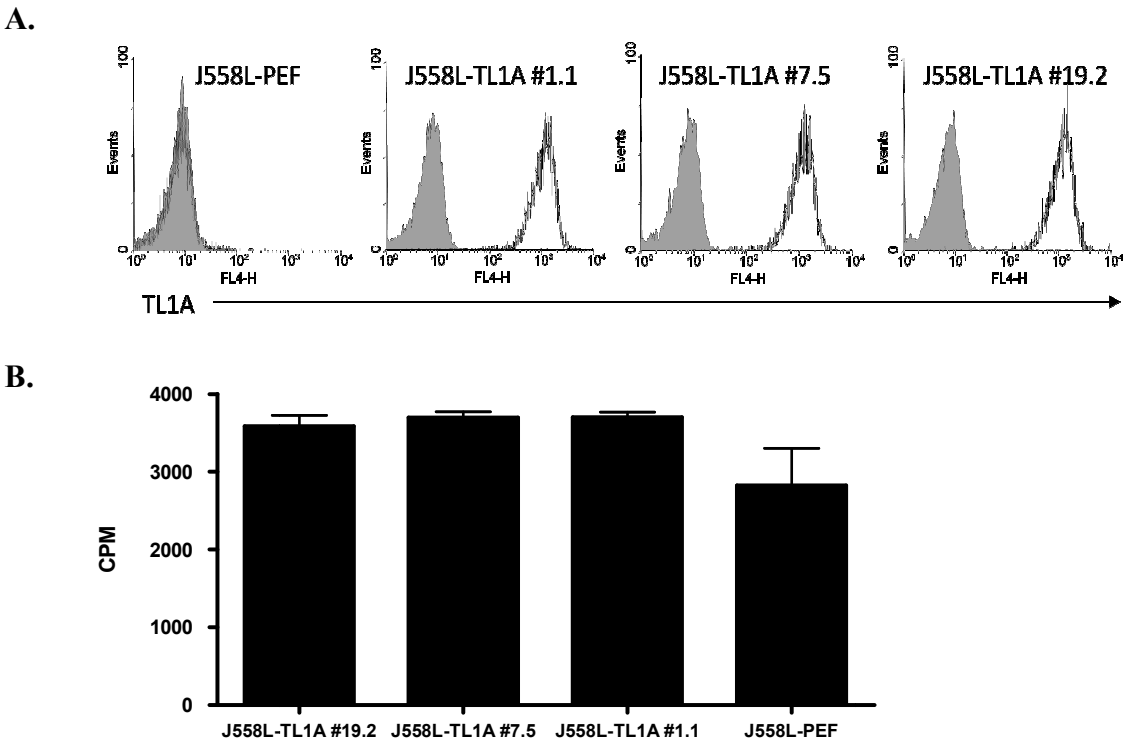



**Figure 4.1.** Expression of TL1A and proliferation of J558L-TL1A #1.1, J558L-TL1A #7.5, J558L-TL1A #19.2 and J558L-PEF cells. (A) Expression of TL1A. The cells were stained with biotin-conjugated TAN2-2 antibody and APC-conjugated streptavidin. The presented cells were gated on a viable population. The presented results are representative of three separate experiments. (B) *In vitro* proliferation assay. The growth rate was measured by incorporation of $^{3}$H-thymidine assay. The experiment was conducted in triplicates, the presented results are mean values ±SE.

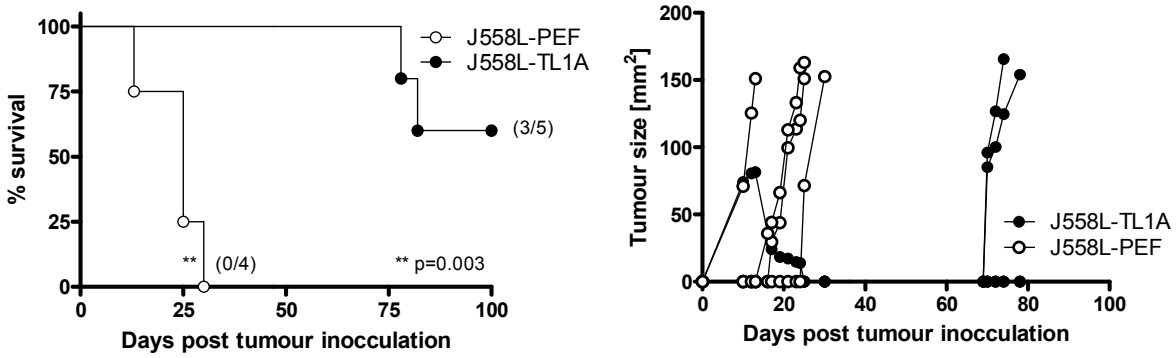



**Figure 4.2.** Survival and tumour growth in wild-type BALB/c mice injected *s.c.* with 5x10$^{6}$ J558L-PEF or J558L-TL1A cells (4 or 5 animals per group, respectively). Animals were sacrificed when the tumour size reached 150 mm$^{2}$. The presented results are representative of three separate experiments. P value was calculated using log-rank (Mantel-Cox) test.

Similar experiment was conducted in T and B cell-deficient BALB/c SCID mice. In this case, both J558L-PEF and J558L-TL1A cells grew progressively following the subcutaneous injection of tumour cells, although the survival of mice injected with J558L-TL1A cells was slightly, but statistically insignificantly longer – median survival for the mice injected with J558L-PEF cells was 12 days, while for the mice injected with J558L-TL1A cells was 18 days (Fig 4.3.). These results show that TL1A-mediated tumour rejection requires adaptive immune response.

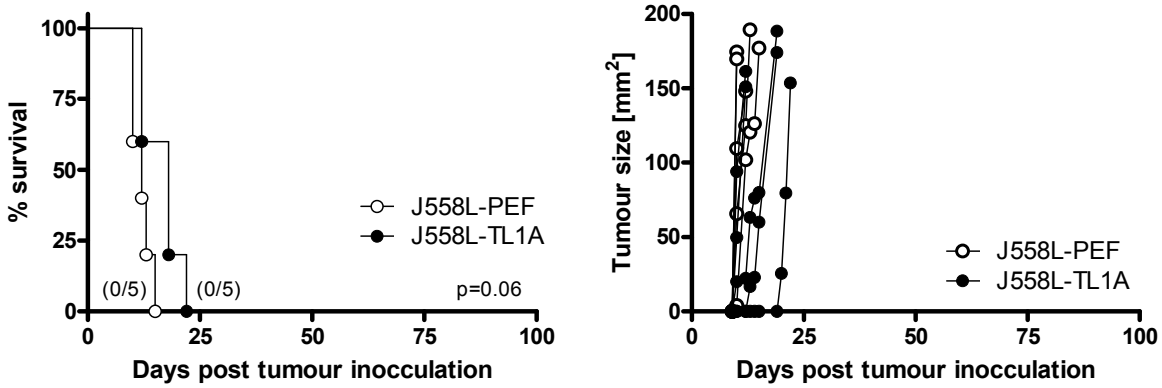



**Figure 4.3.** Survival and tumour growth in BALB/c SCID mice injected *s.c.* with $5x10^6$ J558L-PEF or J558L-TL1A cells (5 animals per group). Animals were sacrificed when the tumour size reached 150 $mm^2$. The presented results are representative of two separate experiments. P value was calculated using log-rank (Mantel-Cox) test.

To assess the role of T cell subsets in TL1A-mediated anti-tumour immunity, wild-type BALB/c mice were depleted of $CD4^+$, $CD8^+$ cells or both of these populations and inoculated *s.c.* with J558L-TL1A cells. There was also a control group of wild-type BALB/c mice inoculated with J558L-PEF cells. Depletion of $CD4^+$ and/or $CD8^+$ cells was confirmed in one randomly chosen mouse from each group by flow cytometry analysis of blood samples 10 days following the administration of depleting antibodies. The percentage of $CD4^-$ T cells in mice depleted of $CD8^+$ cells decreased from 11.55% in non-depleted mice to 1.56% and in mice depleted of both $CD4^+$ and $CD8^+$ cells to 1.09% while the percentage of $CD8^-$ T cells decreased from 31.59% in non-depleted mice to 0.69% in mice depleted of $CD4^+$ cells and to 0.92% in mice depleted of both $CD4^+$ and $CD8^+$ cells (Figure 4.4.A), showing that the depletion had worked according to the expectations. Only depletion of $CD8^+$ cells and both $CD4^+$ and $CD8^+$ cells resulted in prevention of tumour

rejection, clearly showing that $CD8^+$ cells are crucial for TL1A-mediated anti-tumour immunity (Figure 4.4.B).

Slightly prolonged survival of BALB/c SCID mice inoculated with J558L-TL1A tumour cells compared to the same mice inoculated with J558L-PEF cells (Figure 4.3.) and longer survival of mice depleted of $CD8^+$ and/or $CD4^+$ cells and inoculated with J558L-TL1A cells compared to wild-type BALB/c mice inoculated with J558L-PEF cells (Figure 4.4.B) suggests that there may be also other cells of the immune system that take part in TL1A-mediated tumour rejection. Since BALB/c SCID mice are devoid of T and B cells but have NK cells, these cells were potential candidates. To investigate this problem in more detail, NK cells were isolated from spleens of BALB/c SCID mice, which have higher percentage of NK cells compared to wild-type BALB/c mice, and stained for the presence of DR3 to check whether these cells can respond to TL1A stimulation. The isolation of the cells was based on the expression of CD49b which is known to be a marker of NK cells. The obtained population of cells contained 86.74% of $CD49b^+$ cells and 15.73% of them expressed DR3 (Figure 4.5.). To investigate whether TL1A-mediated tumour rejection depends also on NK cells, wild-type BALB/c mice were injected with anti-asialo GM1 antibody and inoculated with J558L-TL1A. In contrast to the control group (non-depleted wild-type BALB/c mice), NK-depleted animals did not reject TL1A-transfected tumours and showed relatively short survival rate – median survival for NK-depleted mice was 16 days compared to undefined median survival time for the animals from the control group (Figure 4.6.). Because NK cells are the main effector cells that take part in the rapid anti-tumour response mediated by the innate immune system, an *in vivo* killing assay was performed to determine whether TL1A-transfected tumour cells are preferentially eliminated during the first hours after the injection into mice. BALB/c SCID mice were injected *i.p.* with a mixture of $5x10^6$ J558L-PEF and $5x10^6$ J558L-TL1A cells labelled with different concentrations of carboxyfluoresceine succinimidyl ester (CFSE; 0.5 μM and 10 μM, respectively). CFSE is a fluorescent cell-staining dye used for monitoring of cell division which binds to the cell surface and therefore cell divisions cause progressive halving of CFSE fluorescence on daughter cells. The cells were isolated from the peritoneal cavity of mice and the amount of $CFSE^{low}$ (J558L-PEF) and $CFSE^{high}$ (J558L-TL1A) cells together with the number of total population of tumour cells were assessed after 1h and 7h. Even though the overall number of tumour cells decreased with time, there was no difference between the amounts of J558L-PEF and J558L-TL1A cells (Figure 4.7.), which clearly shows that TL1A expression on tumour cells does not induce

their selective killing by the cells of the innate immune system. The decrease in the total numbers of tumour cells can be explained by either non-selective killing or migration of the tumour cells from the peritoneal cavity, while the decrease in the percentage of total population of the tumour cells can be additionally explained by the migration of the cells of the innate immune system into the peritoneal cavity.

To check whether TL1A can induce long-term memory, mice that had been inoculated with J558L-TL1A cells and had rejected tumours were re-challenged with wild-type J558L cells 110 day after the initial inoculation. In contrast to the control group (wild-type mice inoculated with J558L cells), the re-challenged mice showed delayed tumour growth and significantly prolonged survival – median survival for the re-challenged group was 48 day, while for the control group only 16 days (Figure 4.8.), suggesting that the initial injection of TL1A-transfected tumour cells led to formation of memory cells and consequently more effective immune response to the secondary challenge with tumour.

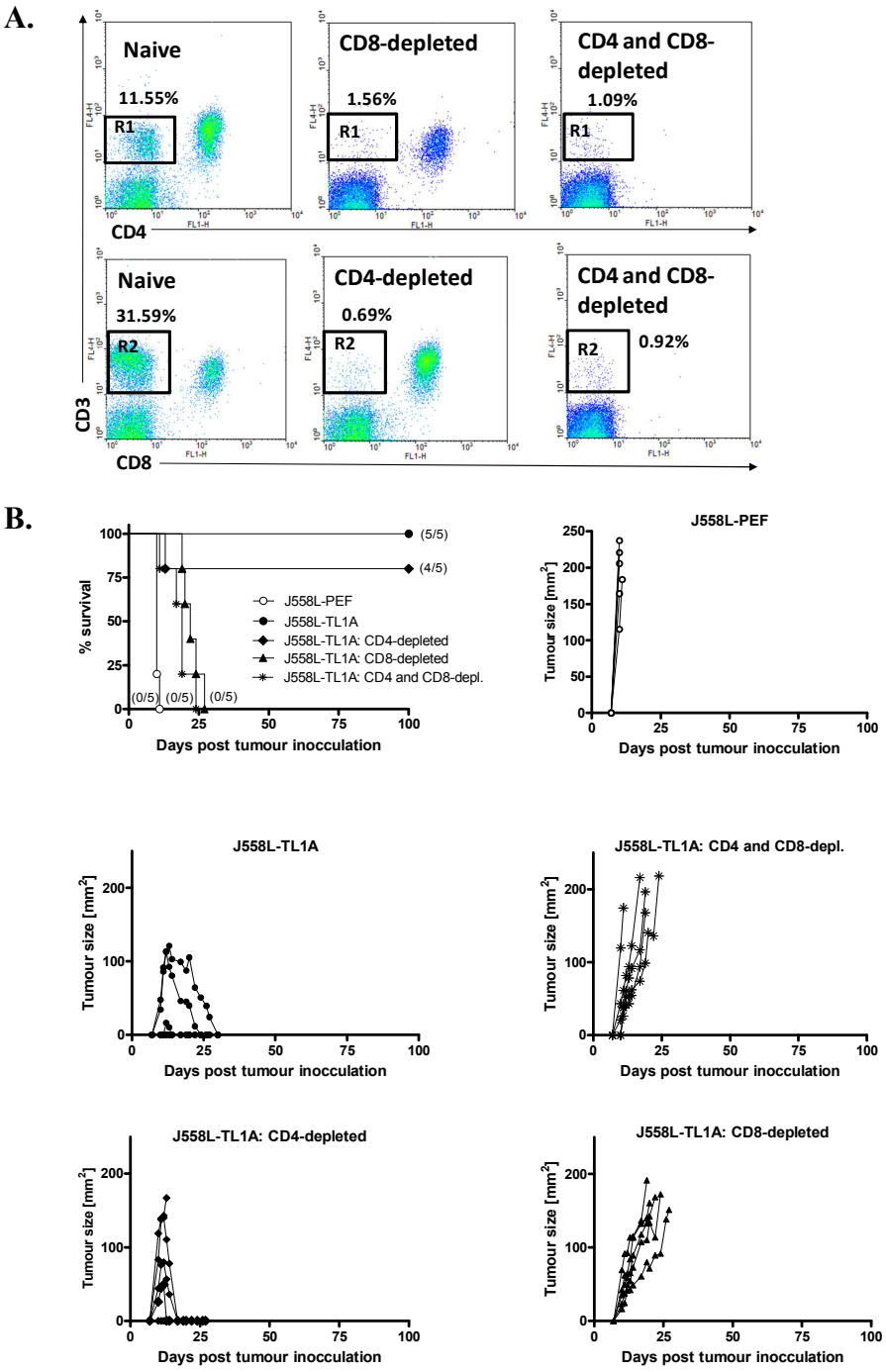



**Figure 4.4.** (A) Staining of blood samples from naive, CD4-depleted, CD8-depleted or both CD4 and CD8-depleted wild-type BALB/c mice. The cells were stained with APC-conjugated anti-CD3 antibody and FITC-conjugated anti-CD4 or FITC-conjugated anti-CD8 antibody and analysed by flow cytometry. The cells presented on the histograms were electronically gated on viable cells. The presented results are representative of two separate experiments. (B) Survival and tumour growth in wild-type BALB/c mice or wild-type BALB/c mice depleted of $CD4^+$, $CD8^+$ or both $CD4^+$ and $CD8^+$ cells (5mice per group) and injected *s.c.* with $5x10^6$ J558L-PEF or J558L-TL1A cells. The mice were sacrificed when the tumour size reached 150 $mm^2$.

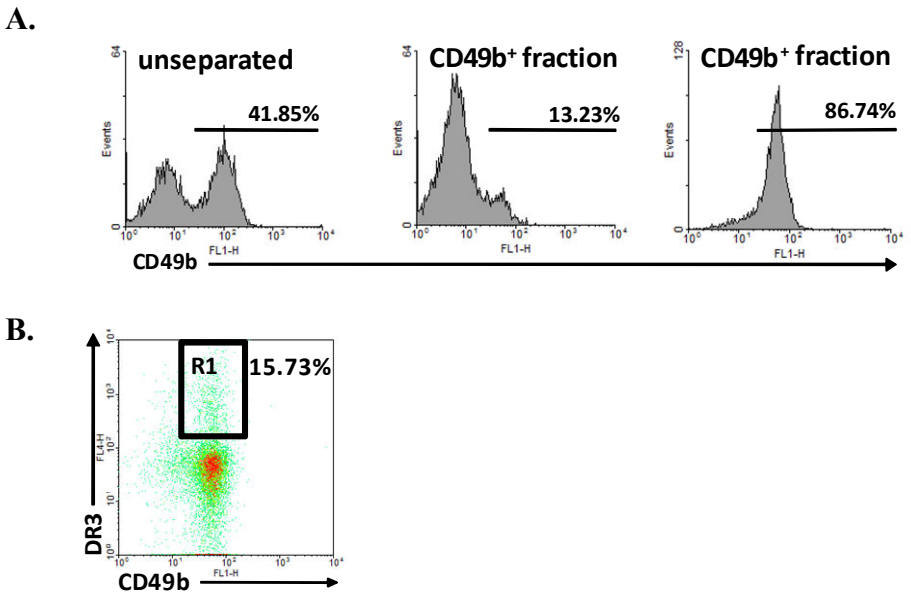



**Figure 4.5.** (A) Isolation of NK cells ($CD49b^+$) from two spleens of BALB/c SCID mice. The cells were stained with FITC-conjugated anti-CD49b antibody and analysed by flow cytometry before or after the isolation on CD49b-specific magnetic beads ($CD49b^-$ and $CD49b^+$ fractions). The gates on histograms show the percentage of $CD49b^{high}$ population in each fraction of cells. The presented cells were electronically gated on viable cells. (B) Expression of DR3 on NK cells ($CD49b^+$). The cells from the $CD49b^+$ fraction were stained also with both FITC-conjugated anti-CD49b, biotin-conjugated anti-DR3 antibody and APC-conjugated streptavidin, and analysed by flow cytometry. The gate R1 represents the percentage of $CD49b^+DR3^+$ cells in the total population of the cells in the $CD49b^+$ fraction. The presented cells were electronically gated on viable cells.

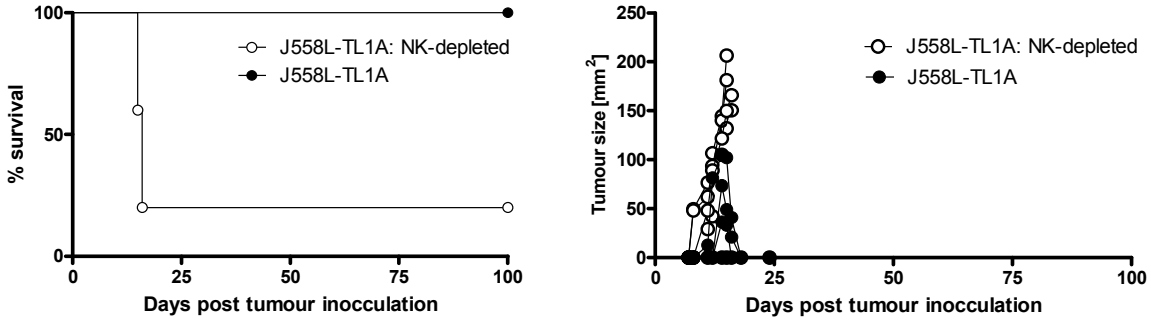



**Figure 4.6.** Survival and tumour growth in wild-type BALB/c mice either depleted or non-depleted of NK cells and injected *s.c.* with $5x10^6$ J558L-PEF or J558L-TL1A cells (5 animals per group). Animals were sacrificed when the tumour size reached 150 $mm^2$.

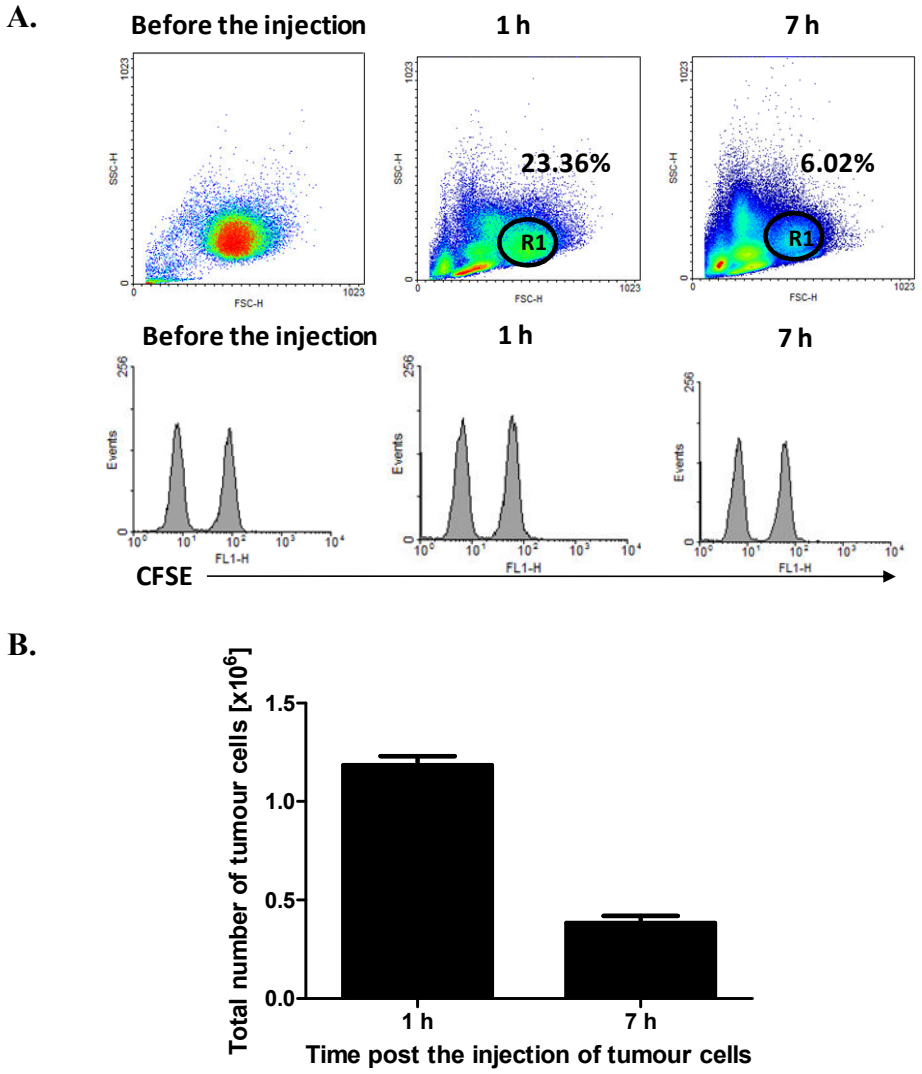



**Figure 4.7.** *In vivo* killing assay of J558L-PEF and J558L-TL1A cells. A mixture of $5x10^6$ J558L-PEF cells (labelled with 0.5 µM CFSE) and $5x10^6$ J558L-TL1A cells (labelled with 10 µM CFSE) was injected *i.p.* into BALB/c SCID mice. The cells were counted and analysed by flow cytometry for the presence of CFSE before the injection and after the indicated time-points. (A) The gate R1 represents the percentage of tumour cells in the whole population of the cells isolated from the peritoneal cavity. The cells present on the '1h' and '7h' histograms were electronically gated on the gate R1. The histogram representing the cells before the injection was gated on the whole population of cells. The presented results are representative of two experiments. (B) Total numbers of tumour cells isolated from the peritoneal cavity at the indicated time points. The presented results are average values from two experiments ± SE.

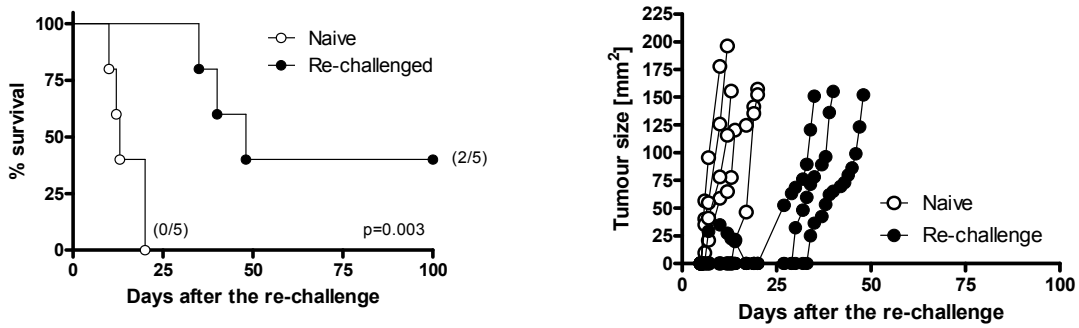



**Figure 4.8.** Survival and tumour growth in wild-type BALB/c mice re-challenged with $5x10^6$ J558L cells 110 days after the initial challenge with J558L-TL1A cells and in wild-type naive BALB/c mice inoculated with $5x10^6$ J558L cells for the first time (5mice per group). The mice were sacrificed when the tumour size reached 150 $mm^2$.

### *4.2.2. Characteristics of soluble recombinant TL1A (TL1A.CD4(d3+4))*

Soluble, recombinant fusion TL1A protein (sTL1A) was used as an alternative approach to investigate the role of DR3 in $CD8^+$ T cells. sTL1A, consisting of the extracellular part of TL1A (amino acids 72-251) fused to rat CD4 domains 3 and 4, was generated to investigate the function of TL1A in details both *in vivo* and *in vitro*. The rat CD4(d3+4) was used as a tag necessary for purification of the recombinant protein. Furthermore, it does not form multimeric quaternary structures, therefore the recombinant protein should retain the quaternary structure of native TL1A. cDNA encoding the protein has the length of 1233 bp, while the protein is 388 amino acids-long. It has the mass of 43.3 kDa, but taking into account the presence of four glycosylation sites, the overall mass of sTL1A is approximately 55 kDa.

Following the purification, sTL1A was analysed by SDS-polyacrylamide gel electrophoresis under both reducing and non-reducing conditions. According to the expectations, the size of the protein was approximately 55 kDa (Figure 4.9.A). To check whether the protein has biological activity, splenocytes isolated from a C57BL/6 mouse were stimulated *in vitro* with anti-CD3 antibody with or without sTL1A for 72h. The recombinant TL1A significantly increased proliferation and secretion of IL-4 and IFN-γ from the cells (Figure 4.9.B). sTL1A was not contaminated with endotoxin, therefore this effect confirms the biological activity of the recombinant TL1A.

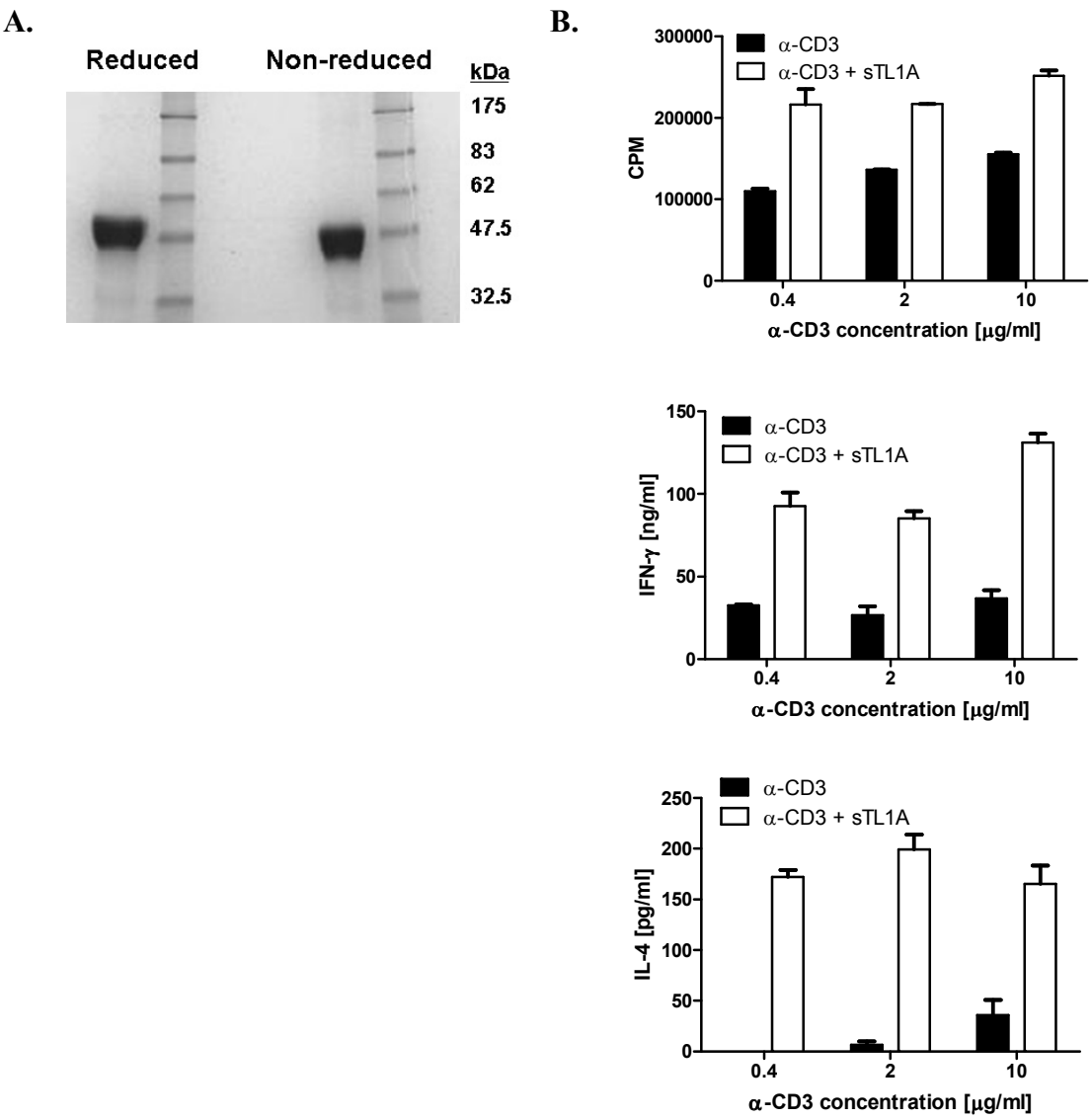



**Figure 4.9.** (A) Analysis of 10 μg of sTL1A by SDS-PAGE under reducing and non-reducing conditions. The size of sTL1A is approximately 55 kDa. (B) Evaluation of biological activity of sTL1A. Splenocytes isolated from a wild-type C57BL/6 mouse were stimulated *in vitro* on a 96-well plate ($1x10^5$ cells/well) with indicated concentrations of anti-CD3 antibody and 2 μg/ml of sTL1A. Proliferation of the cells was measured by $^3$H-thymidine assay, secretion of IL-4 and IFN-γ was measured by ELISA following 72 h of stimulation. The experiment was conducted in triplicates, the presented results represent mean values ± SE.

### *4.2.3. TL1A/DR3 interactions co-stimulate T cells in vitro*

Splenocytes isolated from OTII mice (which express TCR specific for $OVA_{323-339}$ peptide) were stimulated *in vitro* with $OVA_{323-339}$ peptide (ISQAVAAHAEINEAGR) with or without TL1A.CD4(d3+4) and TAN2-2 (anti-mouse TL1A) antibody, Mc39-16 antibody (anti-Bcl1 idiotype; isotype control for TAN2-2) or N22 (anti-murine MHC class II) antibody. $OVA_{323-339}$ peptide stimulates TCR expressed by OTII cells ($CD4^+$ T cells) present in the population. Even though the whole population of splenocytes from OTII mice was used in this experiment, the increase of $^3$H-thymide incorporation was due to increased proliferation of OTII cells only, which was confirmed by N22 antibody stimulation that completely blocked the response to $OVA_{323-339}$ peptide (Figure 4.10.). Stimulation with sTL1A increases proliferation of OTII cells 1.15- to 1.75-fold depending on the concentration of $OVA_{323-339}$ peptide (0.01 or 0.1 μM, respectively). This effect was not present when the cells were stimulated with 1 μM $OVA_{323-339}$ peptide because the increase of OTII cells proliferation induced by the high dose of the peptide exceeded the effect induced by sTL1A which was possibly overrun by other costimulatory molecules present in the environment. The effect induced by sTL1A was specific because it was blocked by TAN2-2 antibody; furthermore, when the cells were stimulated with sTL1A in the presence of the control monoclonal antibody Mc39-16 antibody, the increase in proliferation was similar to the one achieved when the cells were stimulated only with sTL1A and $OVA_{323-339}$ peptide (Figure 4.10.).

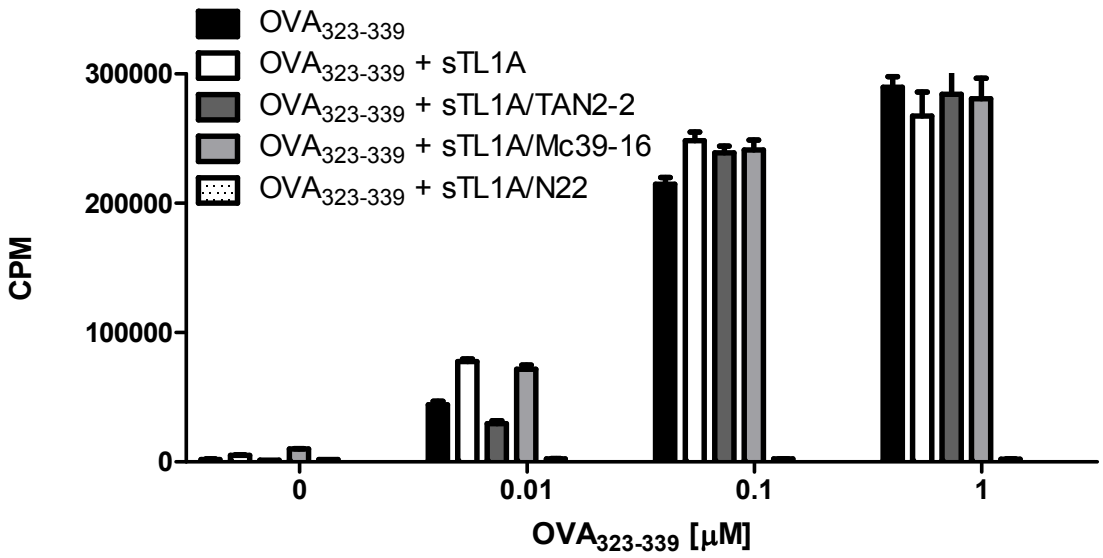



**Figure 4.10.** Proliferation of OTII cells *in vitro*. Splenocytes isolated from an OTII-transgenic mouse were stimulated on a 96-well plate ($1x10^5$ cells/well) in the presence or absence of indicated concentrations of $OVA_{323-339}$ peptide, 2 μg/ml of sTL1A, 50 μg/ml of TAN2-2, 50 μg/ml of Mc39-16 and/or 10 μg/ml of N22. Following 72 h of stimulation, proliferation of the cells was measured by $^3$H-thymidine incorporation assay. The experiment was conducted in triplicates; the presented results represent mean values ± SE.

Because $CD8^+$, but not $CD4^+$ T cells, play a key role in TL1A-mediated rejection of J558L tumour, their response to TL1A stimulation was examined more carefully. The role of TL1A/DR3 interaction in antigen-specific $CD8^+$ T cell responses was examined on OVA-specific TCR transgenic OTI T cells. Unless stated otherwise in the figure legends, the experiments on OTI T cells were performed on $CD4^+$ cell-depleted population of splenocytes isolated from an OTI-transgenic mouse. A representative result of $CD4^+$ cell depletion is presented in the Figure 4.11. Naive OTI T cells do not express DR3 on their surface, however its expression is up-regulated following stimulation with $OVA_{257-264}$ peptide (SIINFEKL) (Figure 4.12.), showing that activated $CD8^+$ T cells can directly respond to TL1A stimulation. Addition of sTL1A to $CD4^+$ cell-depleted OTI splenocytes enhanced proliferation of antigen-specific $CD8^+$ T cells 1.5- to 1.97-fold depending on the SIINFEKL concentration used. This effect was abolished by anti-TL1A (TAN2-2) monoclonal antibody, but not by the isotype control monoclonal antibody Mc39-16, showing that it is specific for sTL1A (Figure 4.13.A). sTL1A also increased the CFSE dilution on OTI T cells stimulated with antigen for 48 h or 72 h. This effect was also inhibited by TAN2-2 antibody (Figure 4.13.B). Furthermore, stimulation of the cells in the presence of anti-CD25 and anti-IL-2 antibodies (PC61 and S4B6, respectively) or anti-

CD80/anti-CD86 antibodies (1610A1 and GL-1, respectively) and with or without sTL1A showed that the increase of OTI T cell proliferation in response to TL1A stimulation partially depends on IL-2 and CD80/CD86 stimulation (Figure 4.13.A). Further investigation showed that sTL1A enhances production of IL-2 by OTI T cells and up-regulates expression of IL2Rα chain (CD25) on these cells (Figure 4.14.), indicating that IL-2 may be involved in mediating TL1A-induced increase in proliferation of $CD8^+$ T cells.

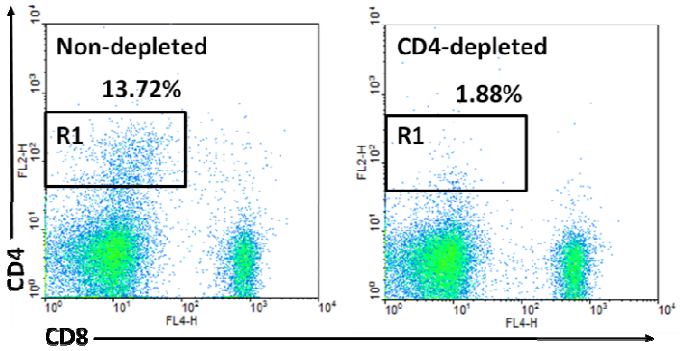



**Figure 4.11.** Example of depletion of $CD4^+$ cells from the whole population of splenocytes isolated from an OTI-transgenic mouse. The cells were stained with PE-conjugated anti-CD4 and APC-conjugated anti-CD8 antibodies and analysed by flow cytometry before and after the depletion. The presented cells were electronically gated on viable population.

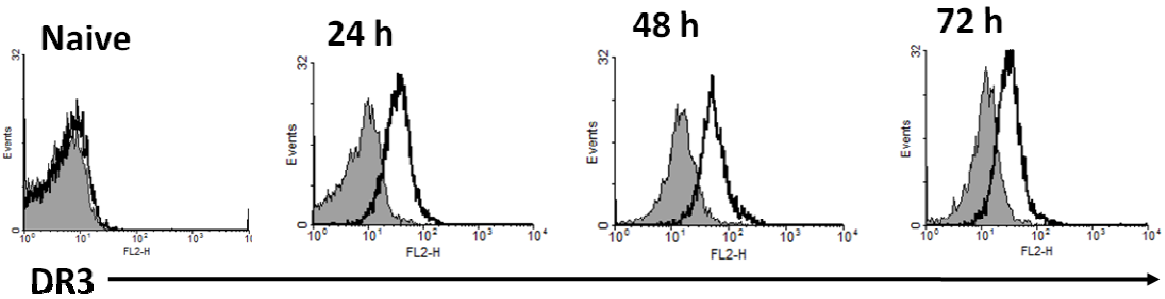



**Figure 4.12.** Expression of DR3 on OTI T cells stimulated with SIINFEKL. $CD4^+$ cell-depleted splenocytes isolated from an OTI-transgenic mouse were stimulated *in vitro* on a 96-well plate ($10^5$ cells/well) with 100 pM SIINFEKL for 24 h, 48 h or 72 h. The cells were stained with APC-conjugated anti-CD8 antibody, biotin-conjugated anti-DR3 antibody (black lines) or biotin-conjugated goat IgG (isotype control for biotin-conjugated anti-DR3 antibody; grey histograms) and PE-conjugated streptavidin before and after the stimulation. The presented cells were electronically gated on both $CD8^+$ and viable cells.

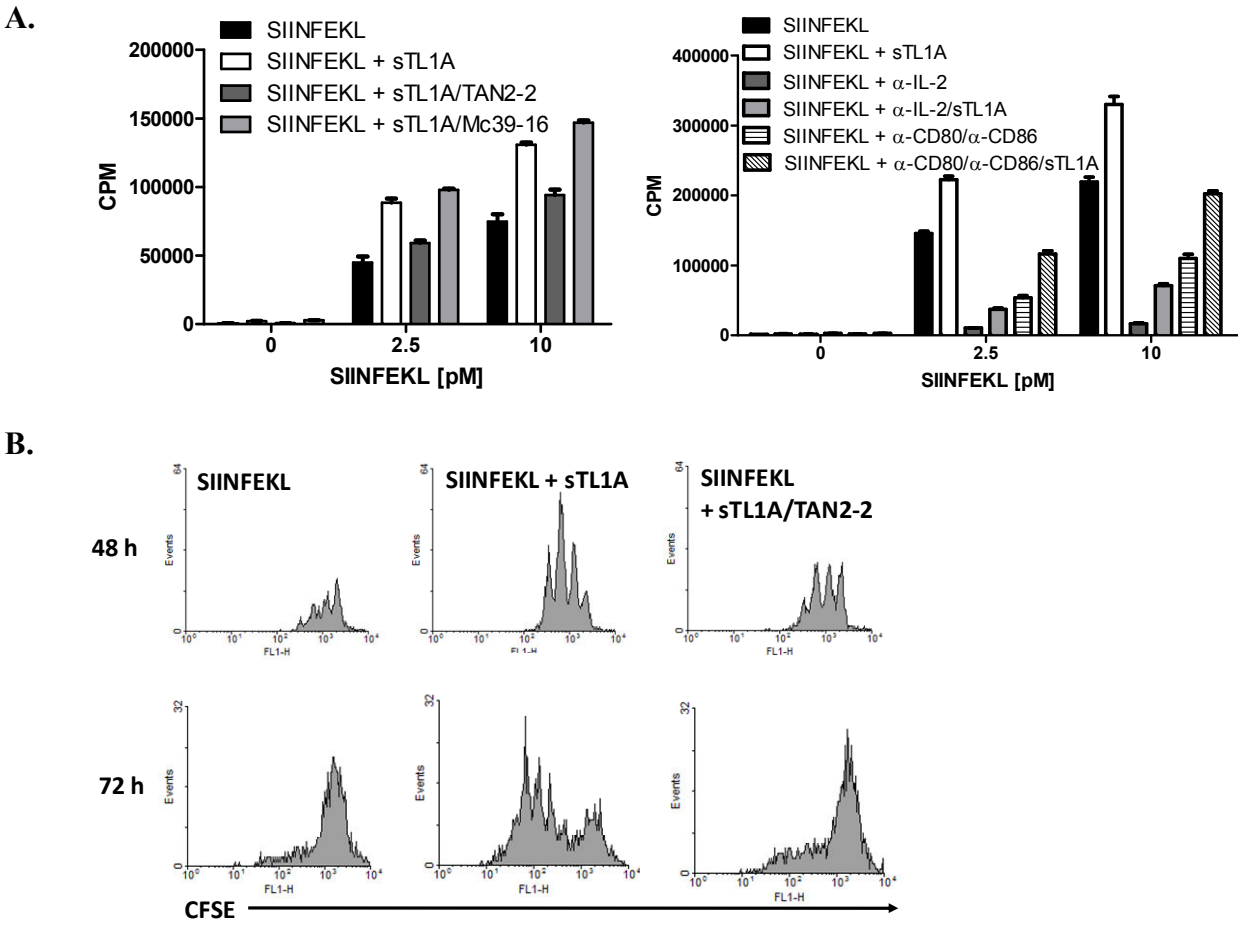
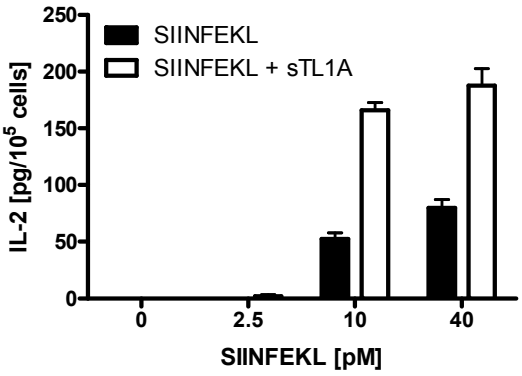



**Figure 4.13.** (A) Proliferation of OTI cells. $CD4^+$ cells-depleted splenocytes isolated from an OTI-transgenic mouse were stimulated *in vitro* on a 96-well plate ($10^5$ cells/well) with indicated concentrations of SIINFEKL and 2 μg/ml of sTL1A with or without 50 μg/ml of anti-TL1A (TAN2-2), 50 μg/ml of anti-Bcl1 idiotype (Mc39-16), anti-CD25 and anti-IL-2 antibodies (10 μg/ml of PC61 and 10 μg/ml of S4B6, respectively) or anti-CD80/anti-CD86 antibodies (10 μg/ml of 1610A1 and 10 μ/ml of GL-1). Proliferation of the cells was measured by $^3$H-thymidine incorporation assay after 72 h of stimulation. The experiments were conducted in triplicates, presented results are mean values ± SE. (B) CFSE dilution on OTI T cells. CFSE-labelled, $CD4^+$ cells-depleted splenocytes isolated from an OTI-transgenic mouse were stimulated *in vitro* on a 96-well plate ($10^5$ cells/well) with 2.5 pM SIINFEKL with or without 2 μg/ml of sTL1A and 50 μg/ml of anti-TL1A (TAN2-2). Following 48 h or 72 h of stimulation, the cells were stained with 1μg/ml of APC-conjugated anti-CD8 antibody and analysed by flow cytometry. The presented cells were electronically gated on both $CD8^+$ and viable cells. The presented results are representative of two separate experiments.

**A.**

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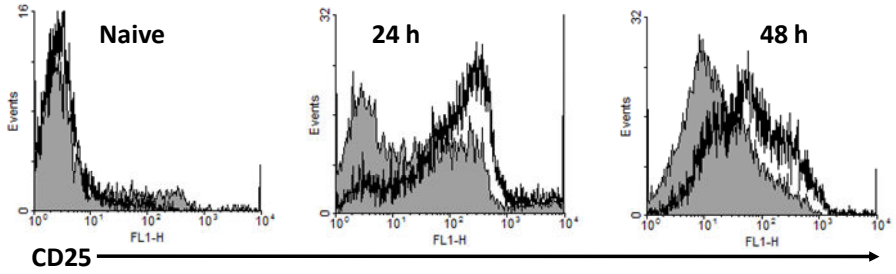



**Figure 4.14.** (A) Secretion of IL-2 by OTI T cells. $CD4^+$ cells-depleted splenocytes isolated from an OTI-transgenic mouse were stimulated *in vitro* on a 96-well plate ($10^5$ cells/well) were stimulated with indicated concentrations of SIINFEKL peptide with or without 2 μg/ml of sTL1A for 48 h. After that time, the cells were counted and the amount of IL-2 in the supernatant was measured by ELISA. The experiment was conducted in triplicates, the presented results represent mean values ±SE. (B) Expression of CD25 on OTI T cells stimulated with SIINFEKL peptide ± sTL1A. $CD4^+$ cells-depleted splenocytes isolated from an OTI-transgenic mouse were stimulated *in vitro* on a 96-well plate ($2x10^5$ cells/well) with 2.5 pM SIINFEKL peptide with (black line) or without (grey histogram) 2 μg/ml of sTL1A for 24 h or 48 h. The cells were stained with APC-conjugated anti-CD8 antibody and FITC-conjugated anti-CD25 antibody before or after the stimulation. The presented cells were electronically gated on both $CD8^+$ and viable cells.

### *4.2.4. TL1A/DR3 interactions co-stimulate T cells in vivo*

To determine how TL1A/DR3 interactions influence $CD4^+$ T cell responses *in vivo*, wild-type C57BL/6 mice received an adoptive transfer of $5x10^6$ OT-II T cells. Following the adoptive transfer, OT-II T cells represented ~3% of the total population of $CD4^+$ T cells. Administration of $OVA_{323-339}$ peptide resulted in 1.33-fold increase in the numbers of OT-II cells at the peak of the response (day 4), while administration of $OVA_{323-339}$ peptide together with sTL1A resulted in 2.66-fold increase in OT-II cells at the peak of the response (day 4), although this difference was not statistically significant (Figure 4.15.). The difference in the response between the mice that were injected with $OVA_{323-339}$ peptide alone and the ones which received $OVA_{323-339}$ peptide together with sTL1A was more significant during the contraction phase (days 6-10) (Figure 4.15.). This indicates that TL1A/DR3 interaction enhances survival rather than proliferation of $CD4^+$ T cells. On day 10, the mice from both groups received an injection of $OVA_{323-339}$ peptide in complete Freund's adjuvant to check whether previous stimulation with sTL1A can break tolerance to $OVA_{323-339}$ peptide induced after the first injection. However, there was not any striking difference in the secondary response between the mice from the two groups (Figure 4.15.).

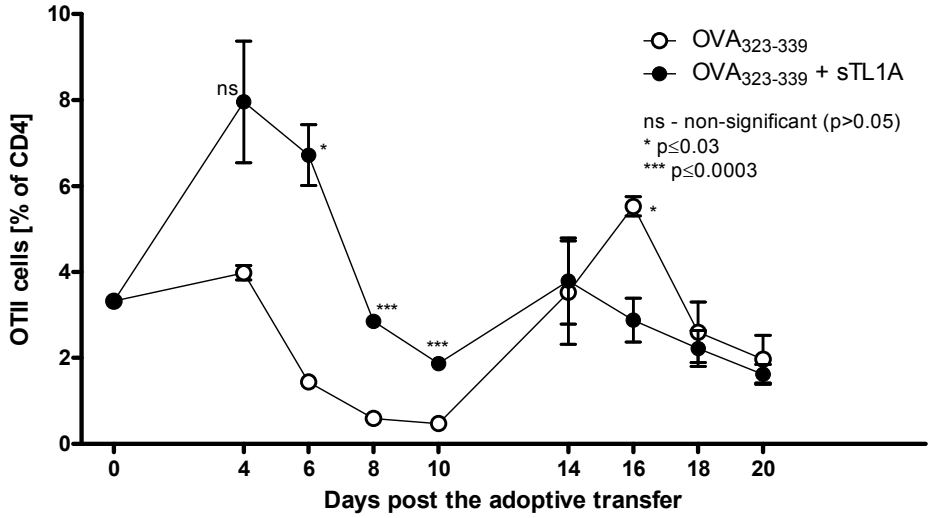



**Figure 4.15.** OT-II T cell response *in vivo*. 3 wild-type C57BL/6 mice adoptively transferred with OT-II cells were injected *i.v.* with 100 μg of $OVA_{323-339}$ peptide on day 1 (white dots), another 3 mice received also *i.v.* injections of 200 μg of sTL1A on days 1, 2 and 3 (black dots). Mice from both groups were injected *s.c.* at the base of the tail with 200 μg of $OVA_{323-339}$ peptide on day 10. Accumulation of OT-II cells was determined by flow cytometry analysis of peripheral blood samples stained with APC-conjugated anti-CD4, PE-

conjugated anti-Vα2 and FICT-conjugated anti-Vβ5 antibodies on indicated days. P values were calculated using two-tailed t test, n=3 mice per time point.

The effect of TL1A/DR3 interactions on $CD8^+$ T cell responses was determined *in vivo* in wild-type C57BL/6 mice that received an adoptive transfer of $1x10^6$ OT-I cells. Following the adoptive transfer, OT-I cells represented ~0.1% of the total peripheral blood lymphocytes (PBLs) and administration of SIINFEKL alone resulted in a 12-fold increase in their numbers at the peak of the response (day 3) while administration of SIINFEKL together with sTL1A resulted in 81-fold increase in the number of OT-I T cells at the peak of the response (Figure 4.16.A). A similar effect was observed also in the spleens of mice 3 days following the adoptive transfer and injection of SIINFEKL ± sTL1A; furthermore, the effect of sTL1A was abolished by concurrent injection of TAN2-2 (anti-TL1A) antibody (Figure 4.16.B). CFSE-labelled OT-I T cells adoptively transferred into wild-type C57BL/6 mice showed higher dilution of CFSE on day 3 after the adoptive transfer in mice injected with both SIINFEKL and sTL1A than in mice injected only with SIINFEKL. Again, this effect was not present in mice that were injected with SIINFEKL, sTL1A and TAN2-2 (Figure 4.16.C), showing that TL1A can directly enhance proliferation of $CD8^+$ T cells *in vivo*.

Experiments conducted *in vitro* showed that TL1A enhances production of IL-2 by $CD8^+$ T cells and that TL1A-induced proliferation of these cells is partially dependent on IL-2. However, *in vivo* the increased proliferation following DR3 stimulation is independent of IL-2, since administration of IL-2 neutralizing antibodies together with sTL1A did not change the accumulation of OT-I cells or the intensity of CFSE fluorescence on OT-I T cells which was checked at the peak of the response (day 3) following the adoptive transfer of OT-I cells into wild-type C57BL/6 mice and administration of antigen (Figure 4.17.). Expression of CD25 on $CD4^+$ T cells isolated from spleens of these mice was up-regulated following administration of sTL1A together with antigen but when IL-2 neutralizing antibodies were administered concurrently, it was similar to the level of the cells stimulated with the antigen alone (Figure 4.18.A). A similar effect was observed on OT-I T cells, although overall expression of CD25 was much lower than on $CD4^+$ T cells (Figure 4.18.B). This shows that IL-2 neutralizing antibodies worked properly and decreased the level of IL-2 which is known to up-regulate the expression of its own receptor (CD25) (176). The response was assessed also by measurement of the level of expression of killer lectin-like receptor G1 (KLRG1) and CD62L. KLRG1 is a C-

type lectin-like inhibitory receptor predominantly expressed on effector T cells. Its main role is raising the activation threshold for T cells and NK cells, thereby preventing autoreactivity (177). CD62L (L-selectin) is an adhesion molecule which acts as a homing receptor to enter secondary lymphoid organs, therefore its expression is down-regulated on activated T cells and effector memory T cells which are present in the periphery (2). *In vivo* stimulation with SIINFEKL and sTL1A with or without IL-2 neutralizing antibodies increased the percentage of $CD4^+$ $FoxP3^+$ regulatory T cells, but did not change the expression of KLRG1 when compared to the cells isolated from mice stimulated only with SIINFEKL (Figure 4.18.C and D). sTL1A significantly increased the frequency of $CD62L^{low}$ OT-I T cells *in vivo* and this effect was independent of IL-2 (Figure 4.18.E). Relative expression of granzyme B and perforin mRNA was measured in splenic cells of mice 3 days following an adoptive transfer of OT-I cells and administration of SIINFEKL with or without sTL1A and IL-2 neutralizing antibodies. Expression was normalized to that of CD3δ which takes into account differences in OT-I cell numbers between different groups of mice. sTL1A up-regulated expression of granzyme B and perforin beyond that induced by administration of SIINFEKL alone. Furthermore, sTL1A also induced expression of IL-2 mRNA and blockade of IL-2 signalling diminished the expression of granzyme B mRNA (Figure 4.19.).

To investigate whether TL1A can generate *bona fide* memory $CD8^+$ T cells, wild-type C57BL/6 mice that had received an adoptive transfer of $1x10^6$ OT-I T cells and had been challenged with SIINFEKL with or without sTL1A were re-challenged with anti-CD40 antibody and SIINFEKL. The mice that had been initially injected with the antigen and sTL1A showed slightly higher response than the mice that had been injected with the antigen alone. The observed memory response in both groups was a true memory response, as it was much higher than the primary response in the mice from the control group (Figure 4.20.).

The effect of sTL1A on the expansion of memory T cells was also investigated. First, SIINFEKL-specific memory $CD8^+$ T cells were generated by priming naive C57BL/6 mice with OVA protein, anti-CD40 antibody and poly(I:C) as an adjuvant. Four weeks later, endogenous SIINFEKL-specific memory $CD8^+$ T cells represented ~0.3% of the total peripheral blood lymphocytes (PBLs). The mice were subsequently challenged with SIINFEKL with or without sTL1A. In contrast to SIINFEKL alone, administration of SIINFEKL together with sTL1A induced a robust secondary response. To confirm that that it was a true secondary response, the response of the pre-immunized mice was compared to

the response of naive animals in which no significant expansion of endogenous SIINFEKL-specific $CD8^+$ T cells could be detected (Figure 4.21.). These results indicate that TL1A/DR3 interactions can boost secondary expansion of memory $CD8^+$ T cells.

Accumulation of antigen-specific $CD8^+$ T cells was investigated also in peripheral blood of wild-type and $DR3^{-/-}$ C57BL/6 mice injected with OVA protein together with anti-CD40 antibody and poly(I:C) which is known to up-regulate TL1A expression (see Chapter 3). Wild-type mice showed slightly higher accumulation of SIINFEKL-specific $CD8^+$ T cells than $DR3^{-/-}$ mice (Figure 4.22.A) and there were more IFN-γ-producing $CD8^+$ T cells in the wild-type mice compared to $DR3^{-/-}$ after *in vitro* re-stimulation with a mitogen - phorbol 12-myristate 13-acetate (PMA) and ionomycin (Figure 4.22.B). This shows that TL1A not only up-regulates proliferation and/or survival of $CD8^+$ T cells but also enhances their effector functions.

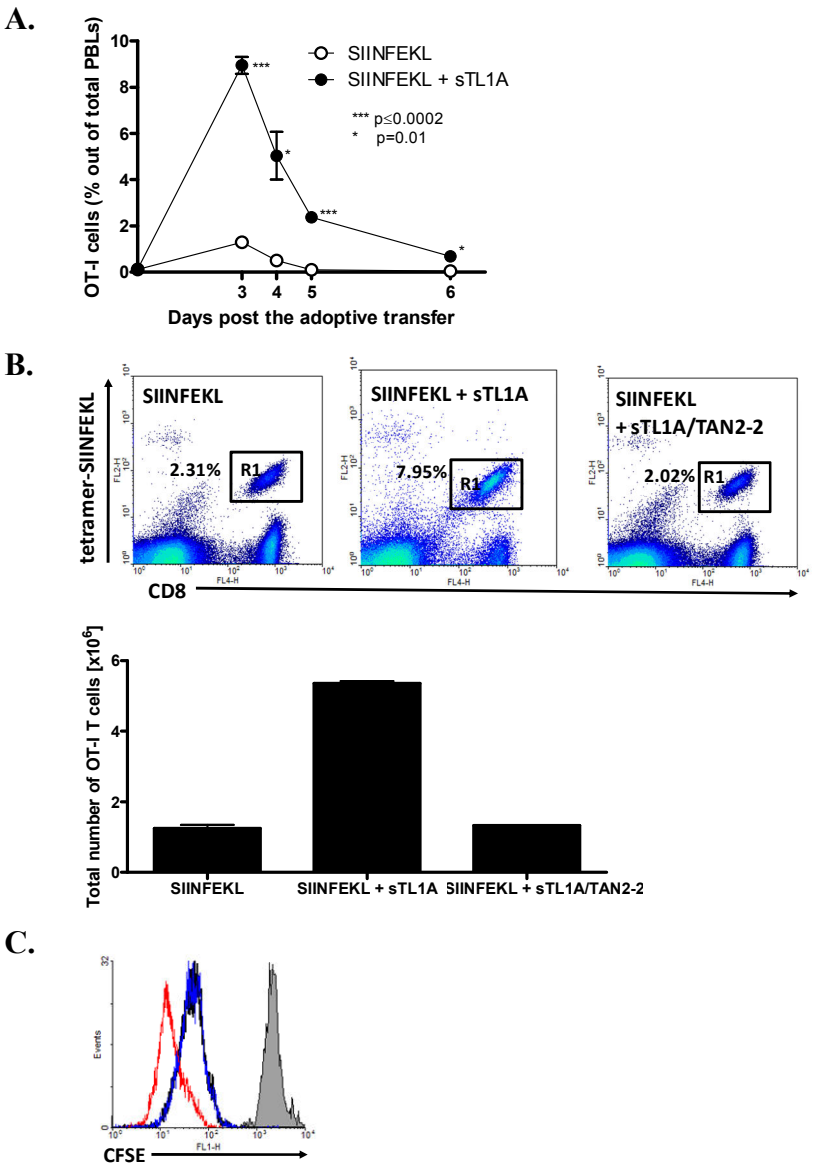



**Figure 4.16.** (A) OT-I T cell response *in vivo*. Wild-type C57BL/6 mice were adoptively transferred with 1x10$^6$ OT-I T cells and injected *i.v.* with 30 nM of SIINFEKL on day 0 with or without 150 µg of sTL1A on days 0, 1 and 2 (3 mice per group). Accumulation of OT-I T cells was measured by flow cytometry analysis of peripheral blood samples stained with APC-conjugated anti-CD8 antibody and 5 µl/tube of PE-conjugated H-2K$^b$ SIINFEKL tetramer on indicated days. P values were calculated using two-tailed t test. (B) Accumulation of OT-I T cells in the spleens on day 3 after the adoptive transfer and administration of SIINFEKL with or without sTL1A as described above (3 mice per group). Additionally, a group of 3 mice was injected also with 250 µg of TAN2-2 antibody on days 0, 1 and 2. Following the isolation from mice, splenocytes were counted and analysed by flow cytometry as described above. Dot-plots show representative results for each group, bar graph shows mean values ±SE for each group. (C) CFSE dilution of OT-I T cells from the spleens of mice adoptively transferred with CFSE-labelled OT-I T cells and injected with

SIINFEKL alone (black line), SIINFEKL + sTL1A (red line) or SIINFEKL + sTL1A/TAN2-2 (blue line) as described above. Grey histogram represents CFSE dilution on OT-I T cells before the adoptive transfer. The cells were stained with APC-conjugtaed anti-CD8 antibody and 5 µl/tube of PE-conjugated H-2K$^b$ SIINFEKL tetramer and analysed by flow cytometry. The cells presented on the histogram were electronically gated on viable OT-I T cells. Presented results are representative for each group (n=3).

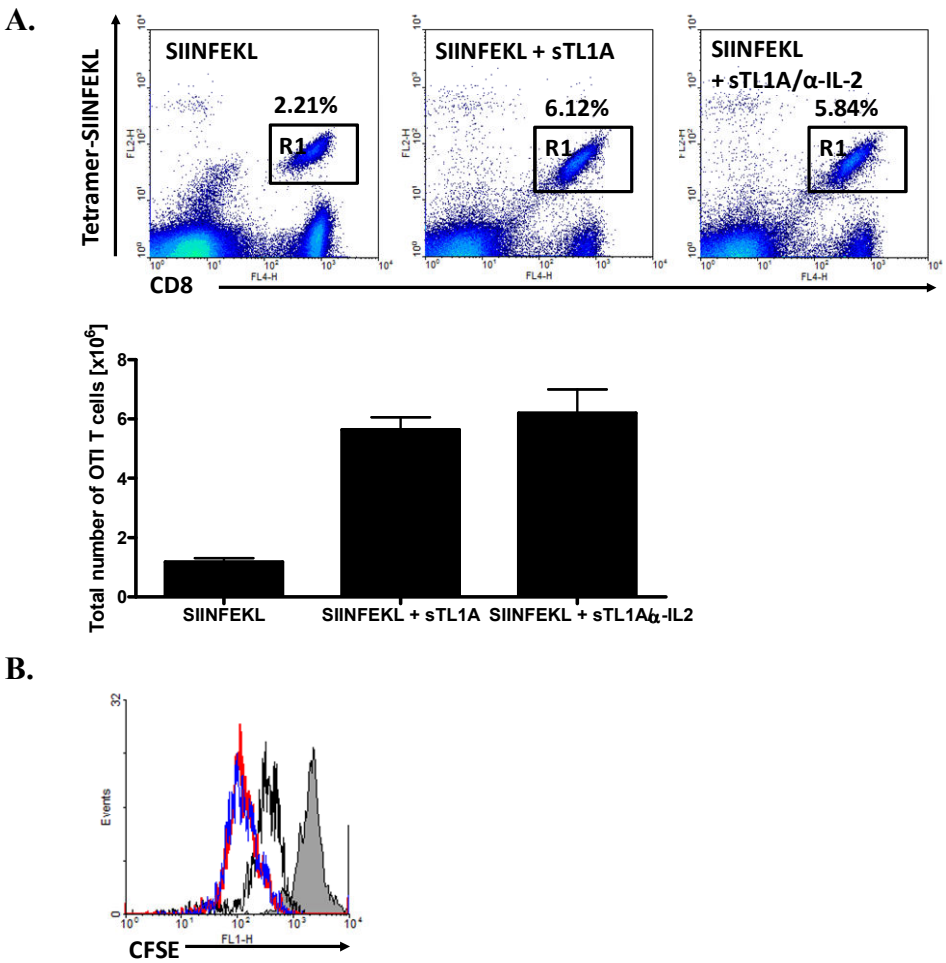



**Figure 4.17.** (A) Accumulation of OT-I T cells in the spleens on day 3 after the adoptive transfer and administration of SIINFEKL with or without sTL1A as described in the legend for Figure 4.16.A (3 mice per group). Additionally, a group of 3 mice was injected *i.p.* also with IL-2 neutralizing antibodies (75 µg of JES6-1A12 and 250 µg of S4B6) on days 0, 1 and 2. Following the isolation from mice, splenocytes were counted and analysed by flow cytometry as described in the legend for Figure 4.16.A. Dot-plots show representative results for each group, bar graph shows mean values ±SE for each group. (B) CFSE dilution of OT-I T cells from the spleens of mice adoptively transferred with CFSE-labelled OT-I T cells and injected with SIINFEKL alone (black line), SIINFEKL + sTL1A (red line) or SIINFEKL + sTL1A/anti-IL-2 (blue line) as described above. Grey histogram represents CFSE dilution on OT-I T cells before the adoptive transfer. The cells were stained with APC-conjugated anti-CD8 antibody and 5 µl/tube of PE-conjugated H-2K$^b$ SIINFEKL tetramer and analysed by flow cytmetry. The cells presented on the histogram were electronically gated on viable OT-I T cells. Presented results are representative for each group (n=3).

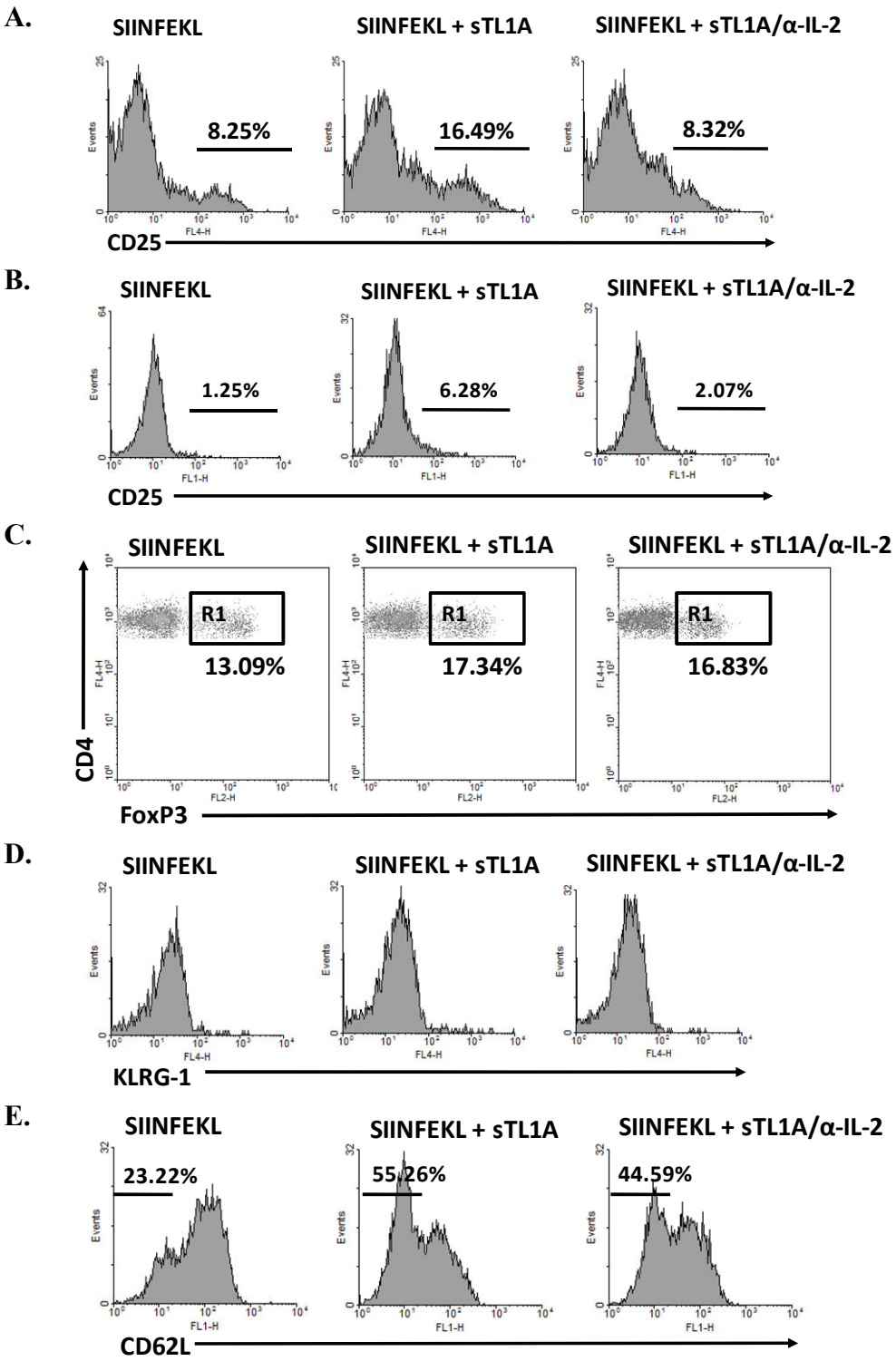



**Figure 4.18.** Expression of CD25 on $CD4^+$ T cells (A), OT-I T cells (B), percentage of Treg cells (C), expression of KLRG-1 on OT-I T cells (D) and CD62L on OT-I T cells (E) in the spleens of mice adoptively transferred with OT-I T cells and injected with SIIFEKL ± sTL1A or sTL1A and IL-2 neutralizing antibodies as described in the legend for Figure 4.17.A (3 mice per group). The cells presented on histograms were electronically gated on viable and $CD4^+$ (A, C), OT-I T cells (B, D and E). Presented results are representative for each group (n=3).

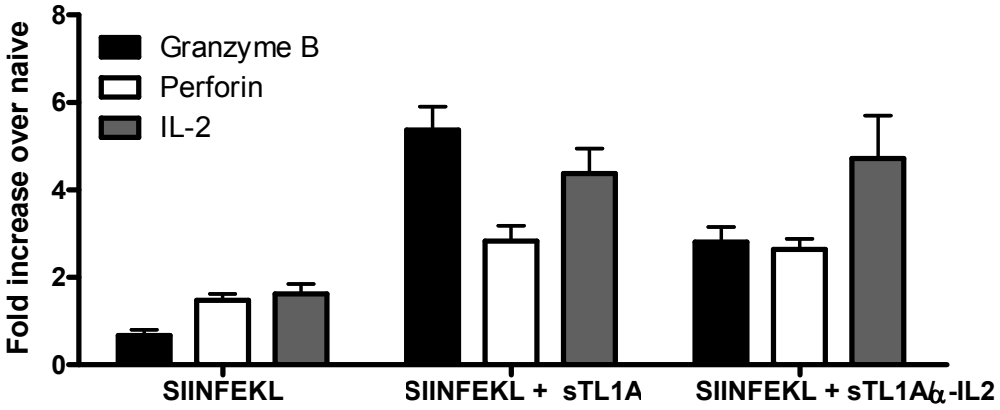



**Figure 4.19.** Relative expression levels of granzyme B, perforin and IL-2 mRNA in the spleens of wild-type C57BL/6 mice following adoptive transfer of OT-I T cells and administration of SIINFEKL, SIINFEKL with sTL1A or SIINFEKL with sTL1A and IL-2 neutralizing antibodies as described in the legend for Figure 4.17.A. Each bar represents mean fold change ±SE (n=6 mice per group for granzyme B and perforin, and 3 mice per group for IL-2).

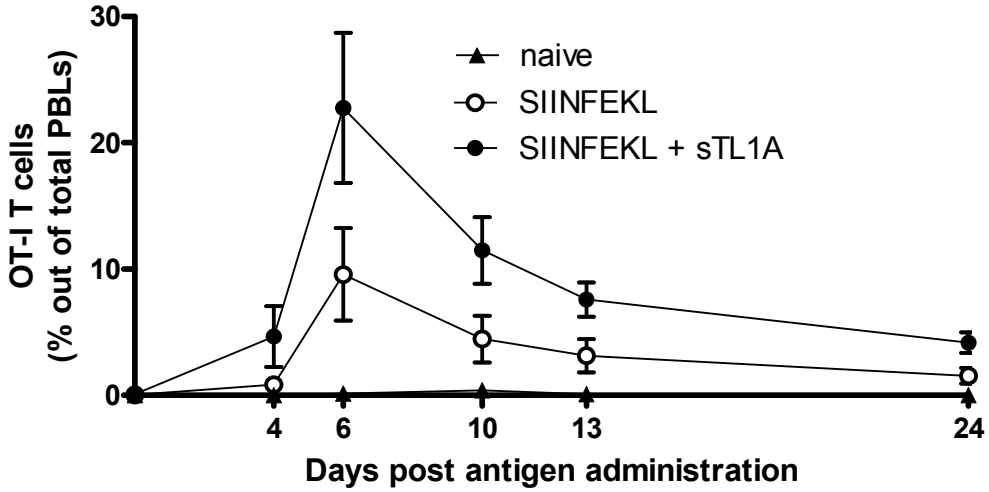



**Figure 4.20.** Secondary response of OT-I T cells in wild-type C57BL/6 mice that had been adoptively transferred with $1x10^6$ OT-I cells and injected with SIINFEKL ± sTL1A as described in the legend for Figure 4.16A (3 mice per group).87 days after the primary challenge, the mice were re-challenged with 30 nM of SIINFEKL and 75 μg of anti-CD40 antibody. The mice from the control group (black triangles) were injected *i.v.* with 30 nM of SIINFEKL and 75 μg of anti-CD40 antibody (3 mice). Accumulation of SIINFEKL-specific $CD8^+$ T cells was measured by flow cytometry analysis of peripheral blood samples stained with APC-conjugated anti-CD8 antibody and 5 μl/tube of PE-conjugated H-$2K^b$ SIINFEKL tetramer on indicated days.

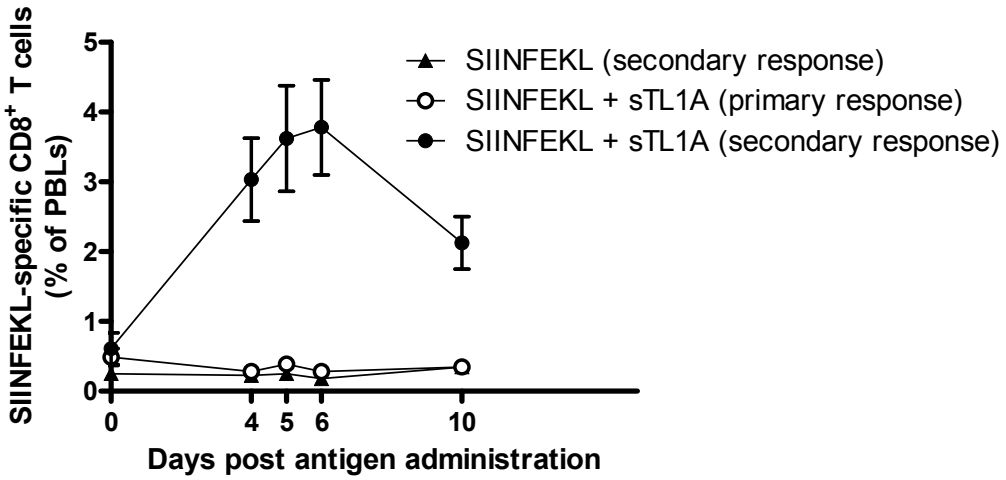



**Figure 4.21.** Memory response of endogenous SIINFEKL-specific $CD8^+$ T cells. Wild-type C57BL/6 mice had been injected *i.p.* with 100 μg of OVA protein, 100 μg of anti-CD40 antibody and 50 μg of poly(I:C). 28 days later, the mice were re-challenged with 30 nM of SIINFEKL with or without 150 μg of sTL1A. The mice from the control group (open circles) were challenged with 30 nM of SIINFEKL and 150 μg of sTL1A (n=3 mice per group). Accumulation of SIINFEKL-specific $CD8^+$ T cells was measured by flow cytometry analysis of peripheral blood samples stained with APC-conjugated anti-CD8 antibody and 5 μl/tube of PE-conjugated H-$2K^b$ SIINFEKL tetramer on indicated days.

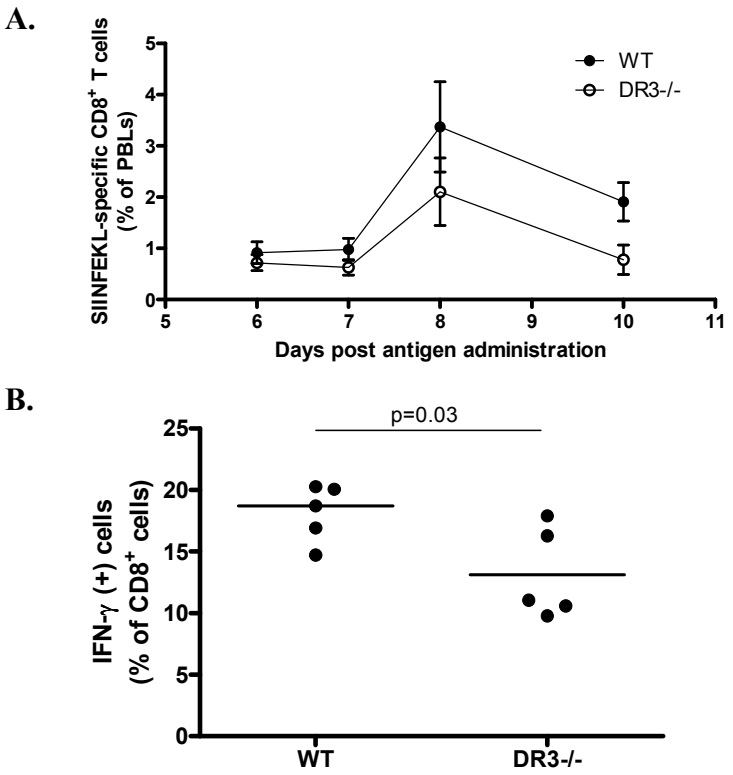



**Figure 4.22.** (A) Accumulation of SIINFEKL-specific $CD8^+$ T cells in wild-type and $DR3^{-/-}$ C57BL/6 mice injected *i.p.* with 100 μg of OVA protein, 100 μg of anti-CD40 antibody and 50 μg of poly(I:C) on day 0 (n=5 mice per group). Accumulation of SIINFEKL-specific $CD8^+$ T cells was measured by flow cytometry analysis of peripheral blood samples stained with APC-conjugated anti-CD8 antibody and 5 μl/tube of PE-conjugated H-$2K^b$ SIINFEKL tetramer on indicated days. (B) Percentage of IFN-γ-secreting $CD8^+$ T cells in spleens of wild-type and $DR3^{-/-}$ mice. The mice from the experiment described in point A were sacrificed on day 11, their splenocytes were re-stimulated *in vitro* with 2 μM PMA and 100 ng/ml of ionomycin for 4 h, stained intracellularly with APC-conjugated anti-CD8-APC antibody and PE-conjugated anti-IFN-γ antibody and analysed by flow cytometry. P value was calculated using two-tailed t test.

## 4.3. Discussion

The data presented in this chapter show that TL1A-expressing tumour cells are rejected by the adaptive immune system. Following the subcutaneous injection, they either grow transiently or do not show any growth. J558L-TL1A and J558L-PEF cells show similar proliferation rate, as measured *in vitro* by $^{3}$H-thymidine incorporation assay, therefore the delayed growth of J558L-TL1A cells *in vivo* was not the result of lower proliferation rate and was the consequence of enforced expression of TL1A on tumour cells. Further investigation showed that $CD8^+$ T cells are essential for TL1A-mediated rejection of J558L tumour.

Previous publications concerning the role of TL1A in co-stimulation of T cells concentrate mostly on its influence on $CD4^+$ T cells (see section 1.9.4.) and very little is known about its role in $CD8^+$ T cell-mediated immune response. This chapter shows that TL1A can act as a direct co-stimulatory molecule for both $CD4^+$ and $CD8^+$ T cells. The interaction between TL1A and DR3 enhances their proliferation and secretion of effector cytokines; the increase in proliferation following stimulation with sTL1A is more profound in $CD8^+$ than in $CD4^+$ T cells. In a more physiological setting, however, where wild-type and $DR3^{-/-}$ C57BL/6 mice were injected with OVA protein together with anti-CD40 antibody and poly(I:C), which is known to up-regulate TL1A expression, accumulation of SIINFEKL-specific $CD8^+$ T cells in wild-type mice was only slightly and statistically insignificantly higher than in $DR3^{-/-}$ mice, even though that $CD8^+$ T cells isolated from the wild-type mice produced higher amount of IFN-γ than $CD8^+$ T cells isolated from $DR3^{-/-}$ mice following *in vitro* restimulation with PMA and ionomycin, showing that the role of TL1A is much more evident in experiments involving the administration of soluble recombinant TL1A. This is the consequence of the presence of many costimulatory molecules in living organisms, where the inability to respond to one of them – TL1A – does not result in significant reduction of the accumulation of effector T cells. On the other hand, the use of experimental systems, where target cells are stimulated with soluble recombinant TL1A allow to investigate its role in the development of immune response more clearly; they also show that TL1A can potentially act as an adjuvant, boosting T cell-mediated immune response.

Down-regulation of expression of the lymph node-homing receptor CD62L on activated $CD8^+$ T cells stimulated with TL1A indicates that these cells acquire the phenotype of effector cells. Finally, DR3 triggering on activated $CD8^+$ T cells during the

primary response leads to formation of memory cells which contribute to the rejection of tumour during the secondary immune response.

Meylan *et al.* (123) show that TL1A enhances proliferation of $CD4^+$ T cells via up-regulation of IL-2 production. The data presented in this chapter show that *in vitro*, the increase in proliferation of $CD8^+$ T cells is partially dependent on IL-2. *In vivo*, this effect was not observed but both *in vitro* and *in vivo*, TL1A enhances the production of IL-2 on T cells and the expression of CD25 (IL-2Rα chain) on these cells which is the consequence positive feedback loop of IL-2 signalling (176) and is consistent with previous findings (111). IL-2 acts as a growth factor for T cells and also can enhance their effector functions. The results presented in this chapter are consistent with findings of other groups showing that even though IL-2 is essential for T cell responses *in vitro* (178) and expression of its receptor appears on T cells early (4-6 h) after the activation *in vitro* (179), it appears to be dispensable for T cell immunity *in vivo*, as effective immune responses occur in mice deficient for IL-2 (178, 180) or CD25 (181), although immune deficits are sometimes noticed. Furthermore, Obar *et al.* (182) show that CD25 signalling is not required for early effector $CD8^+$ T cell differentiation *in vivo* but has a negative impact on late accumulation of $CD8^+$ T cells and generation of memory cells. These findings stand in agreement with the data presented in this chapter showing that TL1A-induced early activation of $CD8^+$ T cells *in vivo* can occur in the absence of IL-2.

Quantitative PCR analysis of mRNA isolated from OT-I T cells isolated from mice stimulated with SIINFEKL, SIINFEKL + sTL1A or SIINFEKL + sTL1A and IL-2-neutralizing antibodies showed that IL-2 expression is required for optimal induction of granzyme B transcript by TL1A which is in agreement with previous studies showing that expression of granzyme B by activated T cells depends on IL-2R signalling (183, 184). There are reports showing that expression of perforin depends on two transcription factors – STAT-5 and eomesodermin which are activated by IL-2 (184, 185). The data presented in this chapter show that neutralization of IL-2 did not down-regulate the expression of perforin transcript induced by TL1A suggesting that, in contrast to induction of granzyme B expression, TL1A can by-pass the requirement of IL-2R signalling and can induce perforin expression in activated $CD8^+$ T cells without any other stimuli. However, the question whether TL1A signalling can lead to activation of STAT-5 and eomesodermin remains unanswered.

Slightly delayed growth of J558L-TL1A cells in BALB/c SCID mice and in wild-type BALB/c mice depleted of $CD8^+$ and both $CD4^+$ and $CD8^+$ T cells compared to the

growth of J558L-PEF cells suggests that NK cells may also respond to TL1A stimulation and take part in the rejection of the tumour. Approximately 15% of NK cells isolated from BALB/c mice expressed DR3, which is consistent with findings of other research groups (125, 136) and shows that these cells can respond to TL1A stimulation. Depletion of NK cells in wild-type BALB/c mice resulted in the lack of tumour rejection following a subcutaneous injection of J558L-TL1A cells. However, the depletion of NK cells in the mice was conducted using anti-asialo GM1 antibody, while Slifka *et al.* (173) showed that administration of this antibody results also in removal of most activated $CD8^+$ and $CD4^+$ T cells. In vivo depletion of NK cells is difficult because many naive T cells and most activated T cells express markers typical for NK cells (NK1.1, DX5 and asialo-GM1) (173); therefore the lack of tumour rejection in BALB/c mice injected with α-asialo GM1 antibody was probably the consequence of both T cell and NK cell depletion. An *in vivo* killing assay was performed to overcome this problem. NK cells act during the first 4-6 hours of the immune response, however no preferential killing of TL1A-expressing tumour cells was observed. Even though some research groups showed that TL1A enhances cytotoxicity and proliferation of NK cells (136, 150), the data presented in this chapter show that NK cells play a minor role in TL1A-mediated tumour rejection *in vivo*.

# CHAPTER 5.

# CHARACTERIZATION OF CD11c-TL1A AND CD2-TL1A TRANSGENIC MICE

## 5.1. Introduction

As shown in chapter 3, expression of TL1A on dendritic cells and T cells is transient and activation-dependent which makes it difficult to examine the function of endogenously expressed TL1A in detail *in vivo*. Therefore, constitutive overexpression of TL1A on dendritic cells (in CD11c-TL1A transgenic mice) and on T cells (in CD2-TL1A transgenic mice) was used as an alternative approach to reveal the role of TL1A in T cell-mediated immunity *in vivo*.

The data presented in chapter 4 show that sTL1A increases the frequency of regulatory T cells and enhances activation of T cells. The percentage of regulatory T cells and the expression of activation/memory markers were investigated in the transgenic mice to see how sustained expression of TL1A on dendritic cells and T cells affects the activation of T cells and accumulation of regulatory T cells.

The other aspect described in this chapter is the influence of the constitutive expression of TL1A on the development of bowel inflammation in mice. TL1A is thought to play a role in the pathogenesis of inflammatory bowel disease (IBD). Its elevated expression was detected on $CD11c^{high}$ dendritic cells isolated from lamina propria in two murine models of chronic ileitis (144); TL1A was elevated also on human mononuclear cells isolated form lamina propria in IBD (116). Increased expression of TL1A was detected also on lamina propria macrophages, monocytes, dendritic cells and T cells in patients with Crohn's disease (146, 149, 153) and on plasma cells in patients with ulcerative colitis (116). IBD patients show also a higher proportion of DR3-expressing intestinal lamina propria T cells than healthy subjects (116, 117). The level of TL1A expression correlates with the severity of the disease (116); furthermore, it has been shown that neutralization of TL1A *in vivo* reduces the symptoms of bowel inflammation in mice (134, 146). IBD is a group of inflammatory conditions of the gastrointestinal tract; its major forms are ulcerative colitis (affecting colon and rectum) and Crohn's disease (affecting any part of the gastrointestinal tract). IBD is an autoimmune disease which results from an inappropriate activation of the immune system driven by the presence of normal luminal flora. There are several genetic and environmental factors that contribute to

the susceptibility to IBD. It is not a uniform disease; for example, ulcerative colitis is mediated by Th2 cells, while in Crohn's disease the inflammation is driven by Th1 and Th17 cells (186, 187). There are several forms of mouse ileitis which are murine models of IBD and can be induced by administration of chemical agents (188, 189), transfer of autoreactive T cells (190) or spontaneously developed as a result of the genetic background (144). There are two haplotypes of TL1A gene which are associated with increased expression of TL1A protein and susceptibility to IBD (153).

For these reasons, CD11c-TL1A transgenic mice were investigated for any signs of ileitis and expression of cytokines associated with Th1 cells (IFN-γ), Th2 cells (IL-4 and IL-13) and Th17 cells (IL-17A) which was also investigated in CD2-TL1A transgenic mice.

## 5.2. Results

### *5.2.1. Expression of TL1A in CD11c-TL1A and CD2-TL1A transgenic mice*

CD11c-TL1A transgenic (CD11c-TL1A *tg*) mice constitutively express TL1A under the control of CD11c promoter, therefore in these animals sustained expression of TL1A is present on dendritic cells. The transgenic mice were fertile, healthy and born at the expected Mendelian frequencies; the total cellularity of the spleens and the lymph nodes of CD11c-TL1A transgenic mice was similar to that found in littermate control mice. Constitutive expression of TL1A mRNA in splenocytes of CD11c-TL1A transgenic mice was 10-fold higher than in control littermates and was comparable to that seen at the peak of the LPS stimulation response in wild-type mice (Figure 5.1. and Figure 3.4.), although the expression of transmembrane TL1A was only slightly higher on dendritic cells isolated from CD11c-TL1A transgenic mice when compared to control mice (Figure 5.2.).

CD2-TL1A transgenic (CD2-TL1A *tg*) mice constitutively express TL1A under the control of CD2 promoter which results in sustained expression of TL1A on T cells. Like CD11c-TL1A *tg*, CD2-TL1A *tg* mice were fertile, healthy and born at the expected Mendelian frequencies. Constitutive expression of TL1A mRNA in the spleens of the transgenic mice was 100-fold higher than in their control littermates (Figure 5.1.). Peripheral blood samples from 4 CD2-TL1A transgenic mice and their control littermates were stained for the presence of $CD3^+$ $TL1A^+$ T cells. 6.88% ±1.41% of total peripheral blood lymphocytes from the transgenic mice expressed transmembrane TL1A, compared to 0.46% ±0.13% in the control mice. When the cells were electronically gated on T cells ($CD3^+$ cells), the expression of transmembrane TL1A was detected on 22.61% ±2.98% of T cells from the transgenic mice, compared to 1.01% ±0.2% of T cell from the control littermates (Figure 5.3.A) and the average mean fluorescence intensity of the peak was 4.83±0.06 and 12.51±0.73 for the negative littermates and CD2-TL1A *tg* mice, respectively (Figure 5.3.B).

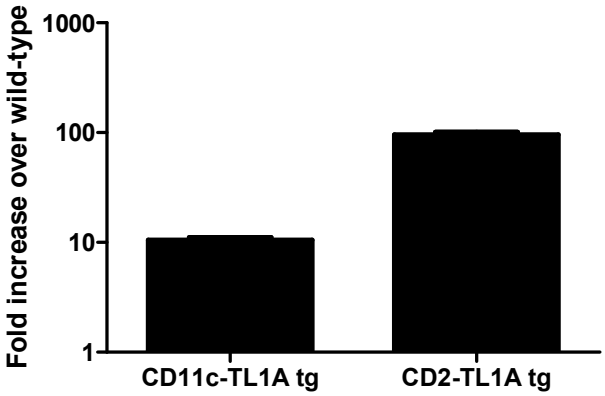



**Figure 5.1.** Expression of TL1A mRNA in the spleens of CD11c-TL1A and CD2-TL1A transgenic mice. Total RNA was isolated from the spleen cells, genomic DNA in the RNA samples was digested, RNA was reverse transcribed and the amount of TL1A mRNA was determined by qPCR. Bar graphs represent mean values ±SE, n=4 mice.

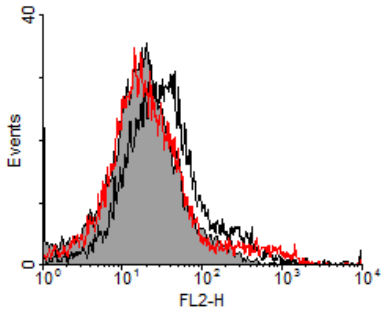



**Figure 5.2.** Expression of TL1A on dendritic cells from CD11c-TL1A mice (black line) or control littermates (red line). Dendritic cells were enriched on 14.5% Nycodenz gradient, stained with DR3.Fc construct or human IgG (isotype control for DR3.Fc; grey histogram) and PE-conjugated anti-human IgG antibody and analysed by flow cytometry. The cells presented on the histogram were electronically gated on a viable and $CD11c^+$ population. The presented results are representative of two separate experiments.

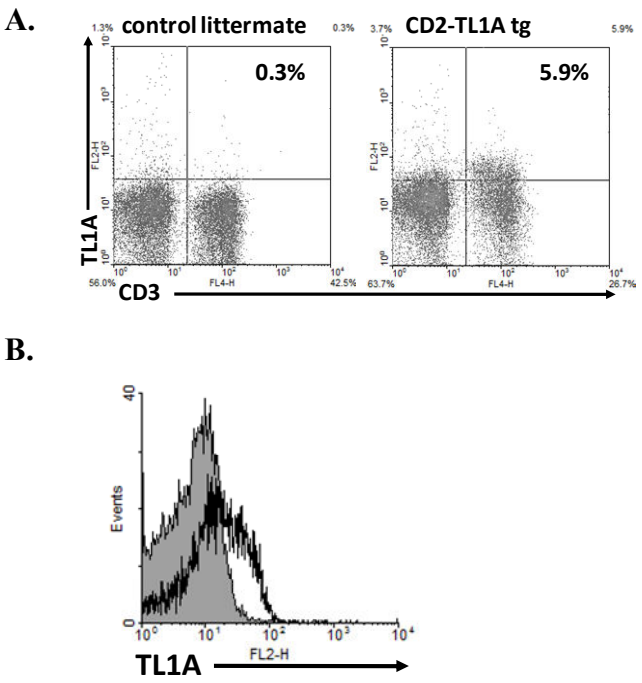



**Figure 5.3.** Expression of TL1A on T cells from CD2-TL1A transgenic mice and control littermates. (A) Expression of TL1A on total population of peripheral blood lymphocytes. The cells were electronically gated on viable population. (B) Expression of TL1A on T cells from CD2-TL1A transgenic mice (black line) and control littermates (grey histogram). The cells were gated on both viable and $CD3^+$ population. The cells were stained with APC-conjugated anti-CD3 antibody, biotin-conjugated anti-TL1A (TAN2-2) antibody and PE-conjugated streptavidin and analysed by flow cytometry. The presented results are representative of 4 experiments.

### *5.2.2. Analysis of T cell activation and accumulation of regulatory T cells in CD11c-TL1A and CD2-TL1A transgenic mice*

Expression of CD25, CD44 and CD62L on $CD4^+$ and $CD8^+$ T cells was investigated in both CD11c-TL1A *tg* and CD2-TL1A *tg* mice to examine the influence of sustained TL1A expression on T cells. CD25 (IL-2Rα chain) is an early activation marker of T cells (179). CD44 is commonly used as a marker for distinguishing activated and memory cells from their naive counterparts. Activated T cell up-regulate CD44 early during the clonal expansion phase and maintain its expression permanently. The functional role of CD44 remains unclear although a recent report shows that it regulates cell survival, therefore CD44 promotes effector cell survival (191). Lymph node-homing receptor CD62L (L-selectin) is used as a marker of effector memory T cells (2).

In CD11c-TL1A *tg* mice, the percentage of $CD62L^{low}$ $CD4^+$ T cells was only slightly higher than in wild-type mice and there was no difference in the percentage of $CD62L^{low}$ $CD8^+$ T cells (Figures 5.4. and 5.5.). However, the frequency of $CD44^+$ $CD4^+$ and $CD8^+$ cells was significantly elevated in CD11c-TL1A *tg* mice (Figures 5.4. and 5.5.). This demonstrates that sustained expression of TL1A results in enhanced activation of both $CD4^+$ and $CD8^+$ T cells that presumably recognize environmental antigens. There was no change in the percentage of $CD25^+$ $CD8^+$ T cells in the spleens of CD11c-TL1A *tg* mice (Figures 5.4. and 5.5.) but the frequency of $CD25^+$ $CD4^+$ T cells was higher in the transgenic mice than in wild-type mice (Figures 5.4. and 5.5.) which may reflect the elevated level of regulatory T cells. Surprisingly, this increase was not observed in the peripheral blood (Figures 5.4. and 5.5.).

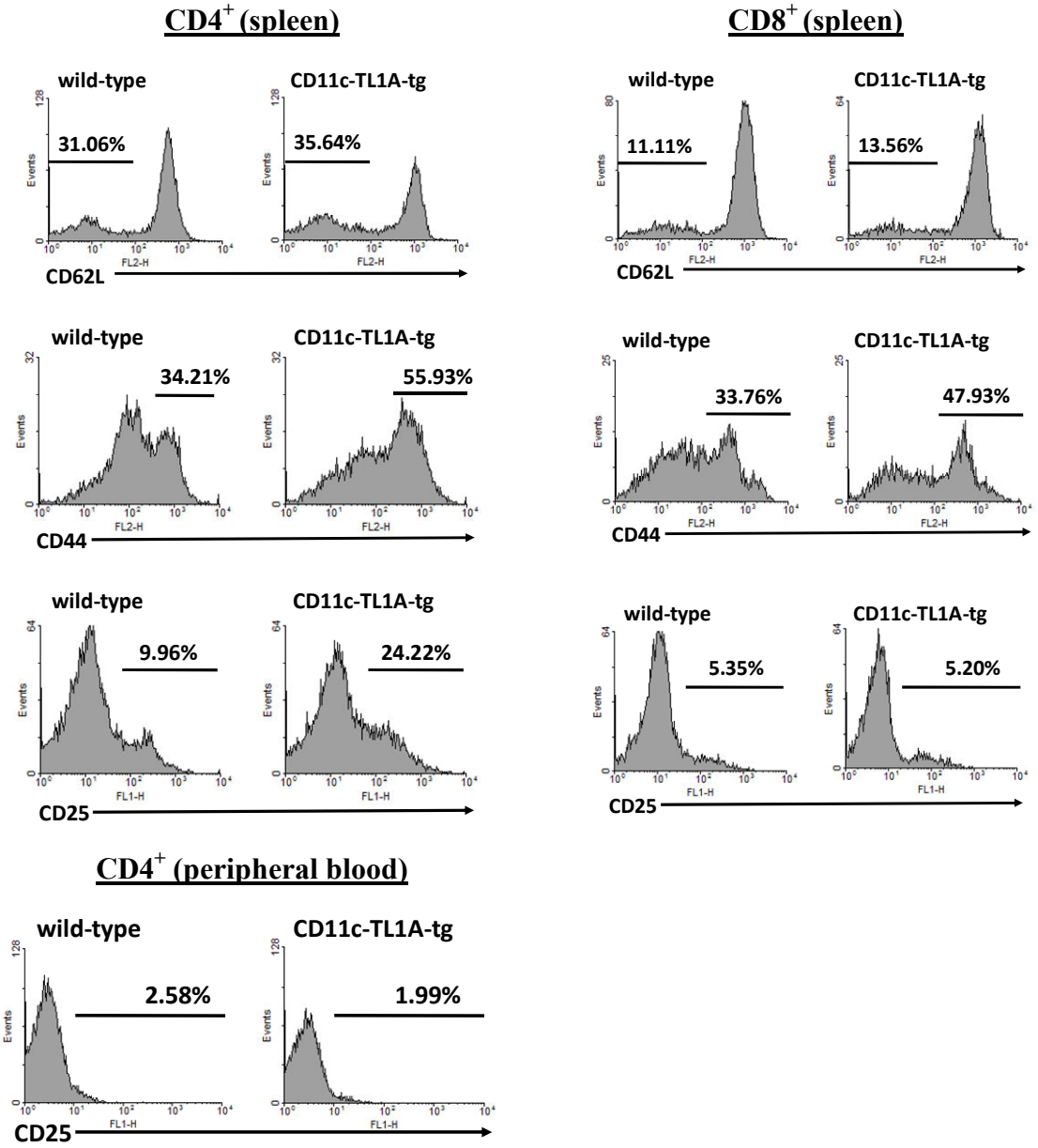



**Figure 5.4.** Expression of CD62L, CD44 and CD25 on $CD4^+$ or $CD8^+$ T cells from the spleens or peripheral blood from CD11c-TL1A *tg* mice or control wild-type mice. The cells were stained with APC-conjugated anti-CD4 or APC-conjugated anti-CD8 antibody and PE-conjugated anti-CD62L, PE-conjugated anti-CD44 or FITC-conjugated anti-CD25 antibody and analysed by flow cytometry. Presented cells were electronically gated on viable and $CD4^+$ or $CD8^+$ population. The presented results are representative of three separate experiments.

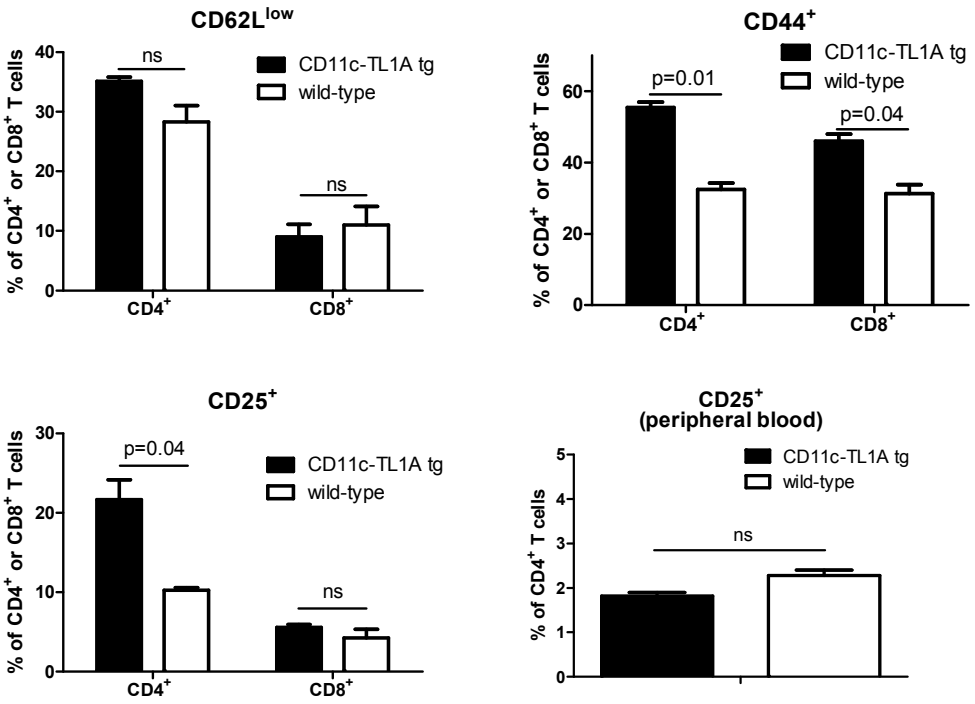



**Figure 5.5.** The frequency of $CD62L^{low}$, $CD44^+$ or $CD25^+$ $CD4^+$ or $CD8^+$ T cells in the spleens or peripheral blood from CD11c-TL1A *tg* mice or control wild-type mice. The presented results represent mean values ±SE, n=3 mice. P values were calculated using two-tailed t test; ns (not significant) indicates $p>0.05$.

The analysis of the expression of activation / memory markers on splenic T cells from CD2-TL1A *tg* mice was more detailed and based on the expression of regulatory T cell marker FoxP3 which allowed to distinguish conventional $CD4^+$ T cells from regulatory T cells.

Like in CD11c-TL1A *tg* mice, CD2-TL1A *tg* mice did not show any difference in the frequency of $CD62^{low}$ $CD8^+$ T cells when compared to control animals (Figures 5.6. and 5.8.). There was also no difference in the frequency of $CD62L^{low}$ $CD4^+$ T cells regardless of the expression of FoxP3 (Figures 5.7. and 5.8.). However, there was a higher percentage of $CD44^+$ $CD4^+$ $FoxP3^-$ T cells in CD2-TL1A *tg* mice than in the control mice (Figures 5.7. and 5.8.). The comparison of the percentage of $CD44^+$ $CD4^+$ $FoxP3^+$ T cells in CD2-TL1A *tg* mice and in the control animals was not possible, because no clear peak of $CD44^+$ cells could be observed on the histograms, however the comparison of mean fluorescence intensity (MFI) values revealed that regulatory T cells from the transgenic mice show higher expression of CD44 than the control cells (Figures 5.7. and 5.8.). In contrast to CD11c-TL1A *tg* mice, CD2-TL1A *tg* mice did not show an increase in the frequency of $CD44^+$ $CD8^+$ T cells when compared the wild-type mice (Figures 5.6. and 5.8.). Like CD11c-TL1A *tg* mice, CD2-TL1A *tg* mice had a higher proportion of $CD25^+$ $CD4^+$ T cells than the control mice (Figures 5.7. and 5.8.). However, when the cells were gated on $CD4^+$ $FoxP3^+$ or $CD4^+$ $FoxP3^-$ cells, no difference in the frequency of $CD25^+$ cells was observed which confirms that the elevated level of $CD25^+$ $CD4^+$ T cells is the consequence of the increased level of regulatory T cells (Figures 5.7. and 5.8.). CD2-TL1A *tg* mice did not show any difference in the percentage of $CD25^+$ $CD8^+$ T cells when compared to the control mice (Figures 5.6. and 5.8.).

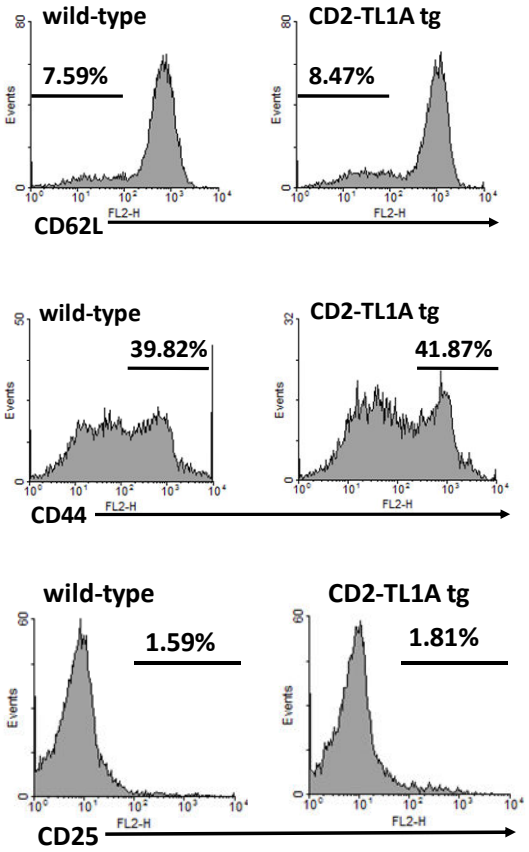



**Figure 5.6.** Expression of CD62L, CD25 and CD44 on $CD8^+$ T cells in the spleens of CD2-TL1A *tg* mice and control mice. The cells were stained with APC-conjugated anti-CD8 antibody and PE-conjugated anti-CD62L, anti-CD44 or anti-CD25 antibody. The cells presented on the histograms were electronically gated on both viable and $CD8^+$ population. The presented results are representative of two separate experiments.

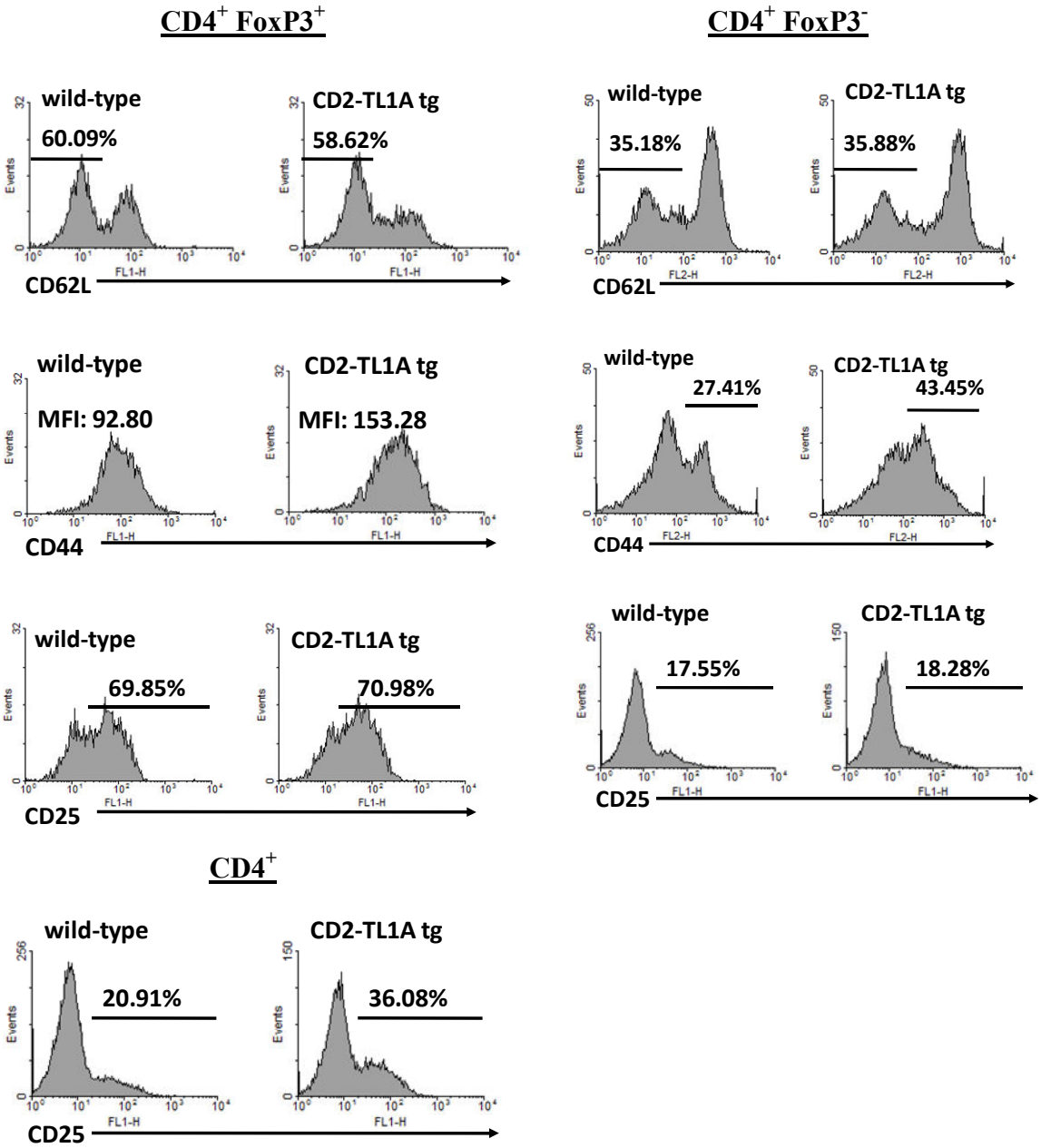



**Figure 5.7.** Expression of CD62L, CD44 and CD25 on $CD4^+$ $FoxP3^+$, $CD4^+$ $FoxP3^-$ or total population of $CD4^+$ T cells from the spleens of CD2-TL1A *tg* mice and control mice. The cells were stained with PE-conjugated anti-CD4 antibody, APC-conjugated anti-FoxP3 antibody and FITC-conjugated anti-CD62L, anti-CD44 or anti-CD25 antibody and analysed by flow cytometry. The cells presented on histograms were electronically gated on viable and $CD4^+$ $FoxP3^+$, $CD4^+$ $FoxP3^-$ or $CD4^+$ population. The presented results are representative of two separate experiments.

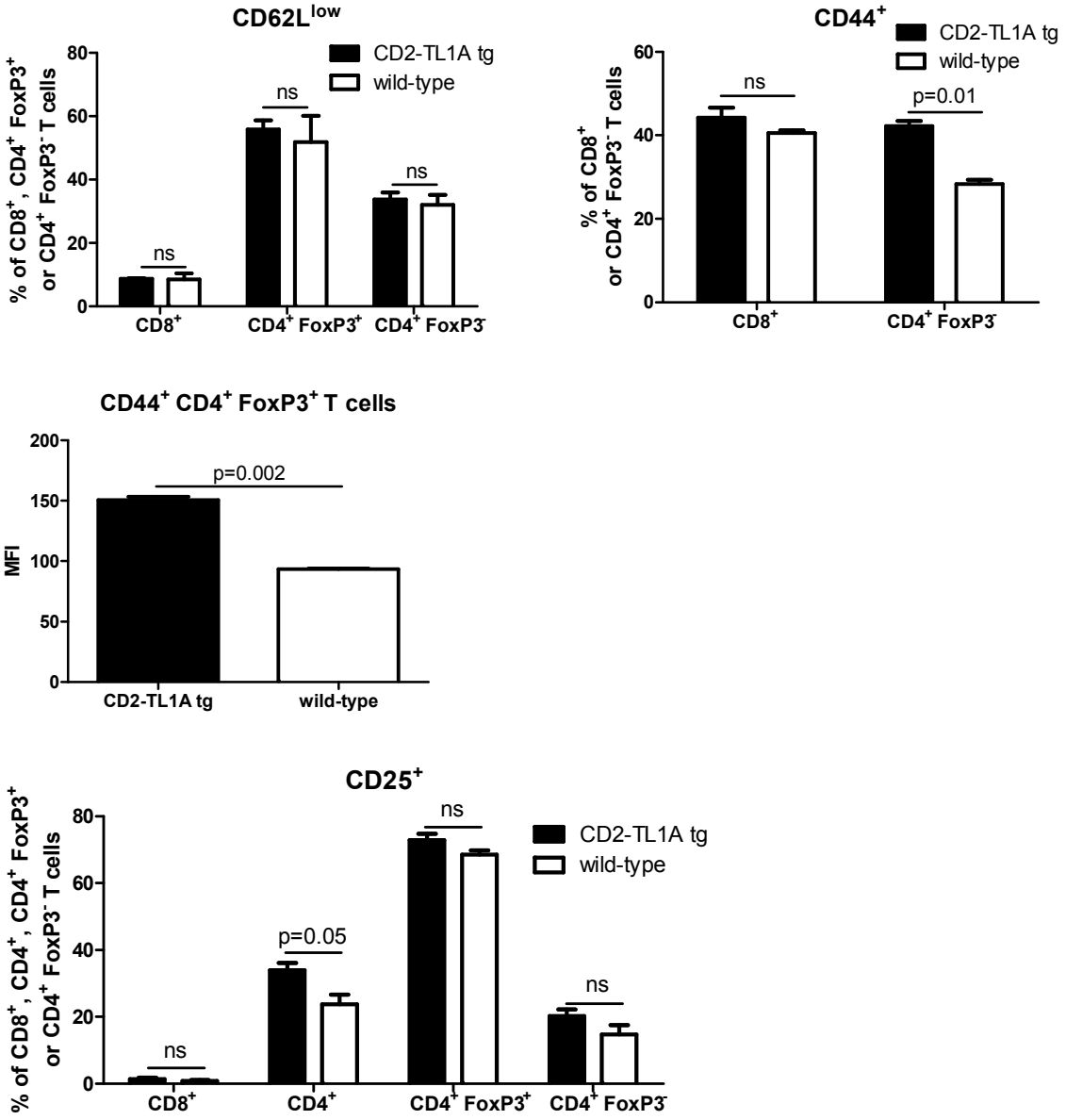



**Figure 5.8.** The frequency of $CD62L^{low}$, $CD44^+$ or $CD25^+$ $CD8^+$, $CD4^+$, $CD4^+$ $FoxP3^+$ or $CD4^+$ $FoxP3^-$ T cells and the mean fluorescence intensity (MFI) of the FITC-conjugated anti-CD25 antibody staining of $CD4^+$ $FoxP3^+$ T cells from CD2-TL1A *tg* mice and wild-type control mice. The presented results represent mean values ±SE, n=2 mice. P values were calculated using two-tailed t test; ns (not significant) indicates $p>0.05$.

*In vivo* stimulation with sTL1A increased the frequency of regulatory T cells in wild-type C57BL/6 mice that received an adoptive transfer of OT-I T cells and injection of SIINFEKL peptide (Figure 5.9.). To investigate how constitutive expression of TL1A affects the accumulation of regulatory T cells, spleen cells from CD11c-TL1A *tg*, CD2-TL1A *tg* mice and control mice were stained for the presence of $CD4^+$ $FoxP3^+$ cells. In both strains of the transgenic mice the percentage of $CD4^+$ $FoxP3^+$ T cells was almost 2 times higher than in the control animals (Figure 5.9.).

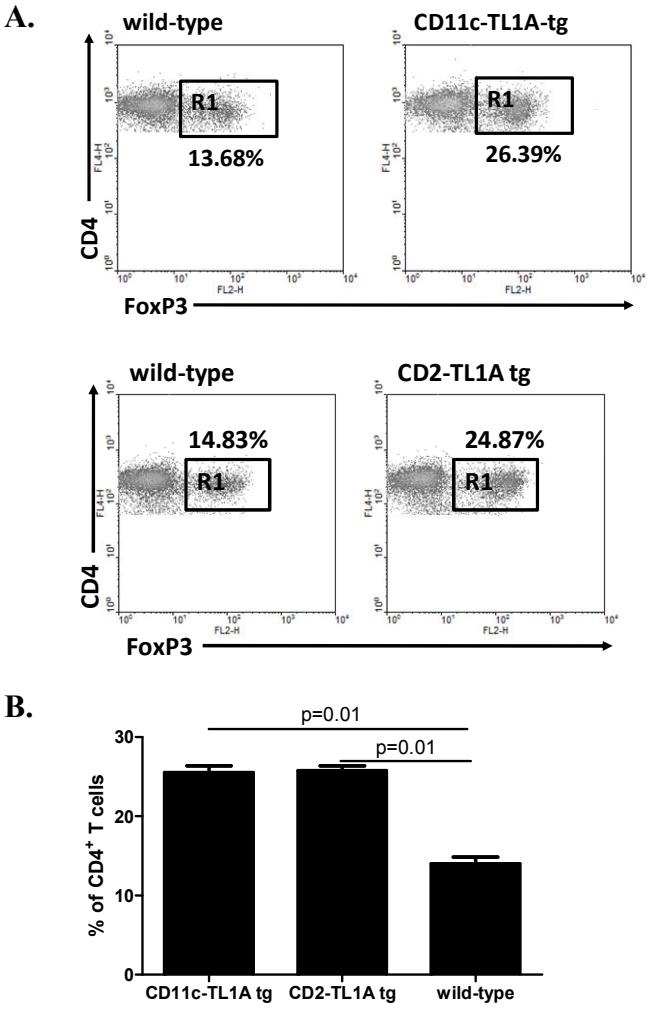



**Figure 5.9.** (A) Expression of FoxP3 on $CD4^+$ T cells from spleens of CD11c-TL1A *tg* and CD2-TL1A *tg* mice and control animals. The cells were stained with APC-conjugated anti-CD4 and PE-conjugated anti-FoxP3 antibodies. The cells were electronically gated on viable and $CD4^+$ population. The presented results are representative of 4 (for CD11c-TL1A *tg* mice) or 2 (for CD2-TL1A *tg* mice) separate experiments. (B) The frequency of $CD4^+$ $FoxP3^+$ regulatory T cells in CD11c-TL1A *tg* and CD2-TL1A *tg* mice. The data represent mean values ±SE, n=4 mice for CD11c-TL1A and 2 mice for CD2-TL1A *tg* mice. P values were calculated using two-tailed t test.

### *5.2.3. The influence of sustained TL1A expression on the immune homeostasis of the bowel in CD11c-TL1A tg and CD2-TL1A tg mice*

In order to investigate whether constitutive expression of TL1A affects the production of Th1, Th2 or Th17-associated cytokines and whether it can polarize the immune response to one of these types, the expression of IFN-γ (Th1 cytokine), IL-4, IL-13 (Th2 cytokines) and IL-17A (Th17 cytokine) transcripts was measured in the secondary lymphoid organs and the ilea of CD11c-TL1A and CD2-TL1A *tg* mice. CD2-TL1A *tg* mice were additionally examined for the expression of IL-2 transcript.

In CD11c-TL1A *tg* mice, the expression of IL-17A mRNA was elevated in the spleens (average 54.67-fold increase, compared to control littermates), mesenteric lymph nodes (average 2.56-fold increase) and ilea (average 3.39-fold increase) (Figure 5.10.). IL-13 mRNA showed a trend toward higher expression in the spleens (average 8.17-fold), mesenteric lymph nodes (average 5.08-fold) and ileum (average 1.74-fold), although the increase was statistically significant only in the spleen (Figure 5.10.). The levels of IFN-γ an IL-4 transcripts did not differ between CD11c-TL1A *tg* mice and the control animals (Figure 5.10.), although it was not possible to detect IL-4 transcript in the spleens and the ilea of either CD11c-TL1A *tg* or control mice, suggesting that the overall level of IL-4 in these organs is very low.

CD2-TL1A *tg* mice also show elevated level of IL-17A mRNA in the mesenteric lymph nodes (average 5.65-fold increase, compared to control littermates) and a trend toward higher expression of IL-13 mRNA in the mesenteric lymph nodes (average 3.83-fold increase), although the increase of the level of IL-13 transcript was not statistically significant (Figure 5.11.). The expression of IL-17A and IL-13 transcripts was also investigated in the ilea of CD2-TL1A *tg* mice, however the level of the cytokines' mRNA was too low to obtain reliable results. Furthermore, there was no difference in the expression of IL-2 and IFN-γ transcripts in the mesenteric lymph nodes of CD2-TL1A *tg* and control mice (Figure 5.11.).

IL-13 and IL-17A are known to increase goblet cell and mucus production (192, 193). Indeed, histological examination of the small intestine revealed an increase in the number of goblet cells in the ilea of CD11c-TL1A transgenic mice (Figure 5.12.A and C). The increase in the numbers of goblet cells was restricted to the ileum and was not

accompanied by inflammation. No signs of inflammation or goblet cell hyperplasia were observed also in the colons of the transgenic mice (Figure 5.12.B).

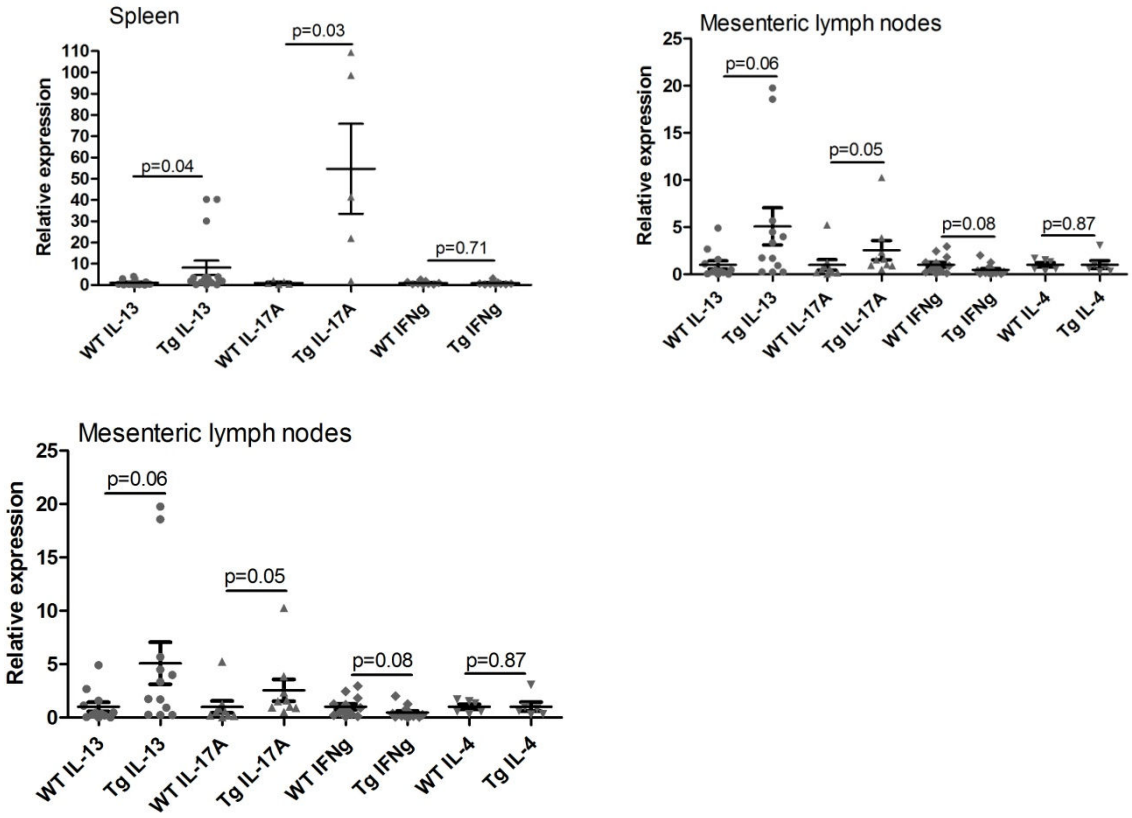



**Figure 5.10.** Relative expression of IL-13, IL-17A, IFN-γ and IL-4 mRNA in the spleens, mesenteric lymph nodes and ilea of CD11c-TL1A *tg* mice as determined by qPCR. Each data point represents the relative cytokine expression value of an individual mouse. Relative expression values were calculated from triplicate qPCR samples.

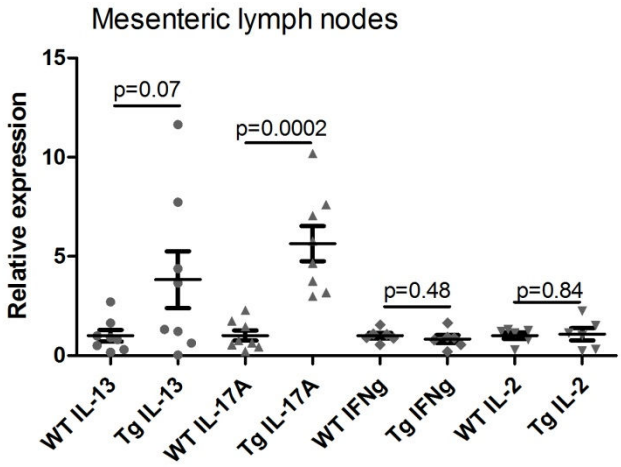
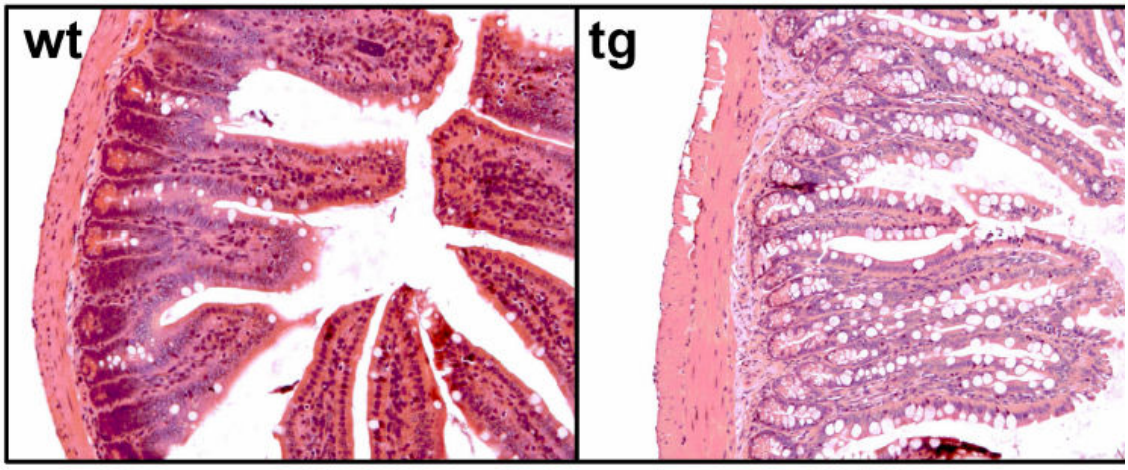
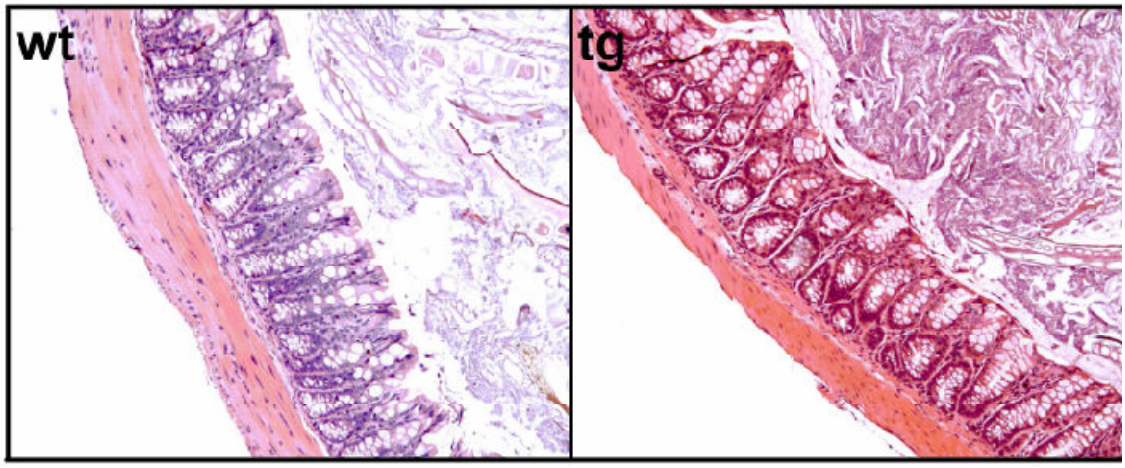



**Figure 5.11.** Relative expression of IL-13, IL-17A, IFN-γ and IL-2 mRNA in the mesenteric lymph nodes of CD2-TL1A *tg* mice as determined by qPCR. Each data point represents the relative cytokine expression value of an individual mouse. Relative expression values were calculated from triplicate qPCR samples.

A.




B.




C.

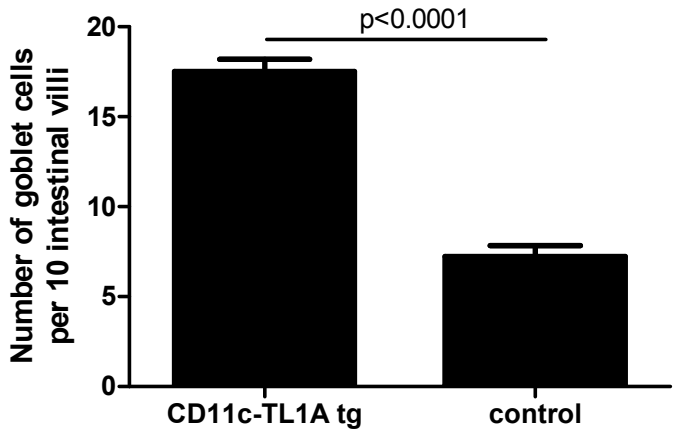



**Figure 5.12.** Goblet cell hyperplasia in the intestines of CD11c-TL1A transgenic mice. Representative (n=10) photographs (x 100 magnification) of sections from the ileum (A) and colon (B) of a 24 week old CD11c-TL1A transgenic mouse or a control littermate. Paraffin sections were stained with H&E. (C) Total number of goblet cell per 10 small intestinal villi of 24 week old CD11c-TL1A transgenic mice or control littermates. Goblet cells were counted in 10 randomly chosen small intestinal villi of 6 CD11c-TL1A transgenic mice or control littermates (60 villi in total). The bar graphs represent mean values ±SE.

## 5.3. Discussion

CD11c-TL1A transgenic and CD2-TL1A transgenic mice have higher proportion of $CD44^+$ $CD4^+$ and $CD8^+$ T cells than their control littermates. The percentage of $CD62L^{low}$ T cells was similar in the transgenic mice and the control littermates, although CD11c-TL1A *tg* mice showed a trend toward higher frequency of $CD62L^{low}$ $CD4^+$ T cells (Figure 5.5.). This shows that sustained expression of TL1A promotes enhanced activation of naive cells and accumulation of effector / memory T cells. Expression of CD25 is transient and present on conventional T cells only during the early stage of activation (176). CD62L is shed from T cells early (6-8 h) after the activation, however later it may be re-expressed on the cell surface of central memory T cells (194). CD44 is the only marker of activation that is permanently expressed on T cells which have encountered an antigen (191). This explains why CD44 was the most significantly altered activation marker in the TL1A transgenic mice. These results show that TL1A can co-stimulate T cells responding to environmental antigens not only when it is expressed on dendritic cells but also when it is expressed on T cells which can take part in co-stimulation of other T cells during the clonal expansion phase which confirms the findings described in the chapter 3.

Expression of the transmembrane form of TL1A on T cells from CD2-TL1A transgenic mice is higher than on dendritic cells from CD11c-TL1A transgenic mice which is in agreement with the findings of other research groups (119, 123) and the data presented in the chapter 3 which indicate that TL1A is cleaved off dendritic cells but not T cells. The other possible explanation of this expression pattern is the fact that expression of TL1A transcript is much higher in CD2-TL1A transgenic mice than in CD11c-TL1A transgenic mice which, in turn, may be the consequence of the copy number that integrated into the genome of CD2-TL1A *tg* mice or the strength of the CD2 promoter.

Both in CD11c-TL1A transgenic and CD2-TL1A transgenic mice, constitutive expression of TL1A results in elevated levels of IL-13 and IL-17A, without affecting the production of IFN-γ, IL-4 or IL-2. Potential sources of IL-13 and IL-17A in the transgenic mice are $CD4^+$ and $CD8^+$ T cells, as constitutive expression of TL1A results in increased frequencies of these cells with effector phenotype. Like IL-13, IL-4 is a Th2-type cytokine but recent studies showed that IL-13 plays a more important role than IL-4 in a Th2 immune response directed against intestinal parasites (192). Moreover, during Th2-type immune response, production of IL-13 is higher and more prolonged than IL-4 (195) which

explains the lack of IL-4 production in the mesenteric lymph nodes of CD11c-TL1A transgenic mice. Taken together, this suggests that in the presented transgenic models TL1A is preferentially amplifying Th2 and Th17-type responses and the observed effect is independent of the cell type (dendritic cells or T cells) that produces TL1A *in vivo*.

IL-13 and IL-17A are one of the cytokines involved in the regulation of intestinal immunity. Maintaining immune homeostasis of bowels is a crucial process, since intestines are exposed to a variety of environmental and food antigens which can lead to severe immune reactions. There are several reports showing that IL-13 is involved in the induction of goblet cell differentiation both in the airways and in the intestines. Receptor for IL-13 (termed "type II IL-4R") is a heterotrimer composed of IL-13Rα1 chain and IL-4Rα chain and binds both IL-13 and IL-4. IL-4α chain is present also in type I IL-4R which binds only IL-4 (196). Differentiation of goblet cells from intestinal epithelial cells in IL-4Rα$^{-/-}$ mice infected with worm *Nippostryngylus brasiliensis* was abrogated (197) and this effect was due to the inability of the mice to respond to IL-13, not to IL-4, stimulation because other report shows that IL-13$^{-/-}$ mice, but with normal expression of IL-4, fail to develop goblet cell hyperplasia that normally occurs coincident with *N. brasiliensis* expulsion (192). Other *in vivo* models using transgenic mice (198) or intratracheal injections of IL-13 (199) consistently show increased goblet cell hyperplasia in the airways of mice. Also the data presented in this thesis show that elevated expression of IL-13 is accompanied by increased numbers of goblet cells. IL-17A is also involved in the induction of goblet cell hyperplasia since it stimulates expression of mucin genes *MUC5B* and *MUC5AC* (193) which are considered to be markers of goblet cell metaplasia (200). Furthermore, neutralization of IL-17A in mice infected with respiratory syncytial virus resulted in significant decrease in the mucogenic response and goblet cell numbers (201).

Goblet cells are found in the intestinal and respiratory tracts and their main role is secretion of mucin which protects the intestinal barrier from infiltration by luminal contents and protects host organisms from parasitic worms (202, 203). In the intestines, goblet cells also produce resistin-like molecule β (RELMβ) which has a direct effector function against parasitic infections and also plays a pathogenic role in promoting colitis and ileitis probably by inducing secretion of pro-inflammatory cytokines such as TNF, IL-6 and RANTES (204-206). Production of RELMβ is directly induced by IL-13 but inhibited by IFN-γ. Other Th2-type cytokine, IL-4 does not play a role in induction of RELMβ expression (206, 207). Surprisingly, in humans, IBD is usually accompanied by the decreased level of goblet cells (208) and elevated production of antibodies directed

against goblet cells (209). On the other hand, in SAMP1/YitFc mice that spontaneously develop ileitis, goblet cell hyperplasia is one of the earliest symptoms of intestinal inflammation (206) showing that goblet cells may play a dual, not fully understood yet, role in the pathogenesis of bowel inflammation.

Apart from the participation in the induction of goblet cell formation, IL-17A also inhibits Th1-type immune response which is responsible for the development of intestinal inflammation in certain settings (210) and it limits infiltration of the intestine by $CD4^+$ T cells and granulocytes which increase the severity of the disease (189). In a trinitrobenzenesulfonic acid-induced model of colitis IL-17A appears to play a pro-inflammatory role (188) but, on the other hand, in a dextran sodium sulphate-induced model of this disease IL-17A plays a protective role (189). This shows that the role of IL-17A in pathomechanisms of bowel inflammation remains controversial and depends on the settings of the experiments, particularly on the type of the immune response that is involved in the development of the disease, as bowel inflammation can be mediated by Th1, Th2 or Th17-type immune response (186, 187).

In CD11c-TL1A transgenic mice used in the research presented in this thesis, no signs of bowel inflammation were observed, except for increased number of goblet cells which may be due to the elevated levels of IL-17A or increased accumulation of Tregs in these mice. CD2-TL1A transgenic mice were not investigated for the presence of bowel inflammation. On the other hand, a report published after the completion of the research presented in this thesis shows that transgenic mice constitutively expressing TL1A on lymphoid and myeloid cells develop mild inflammation in the small intestine which is accompanied by elevated levels of IL-13 and IL-17A mRNA in the mesenteric lymph nodes and in the spleen (211). This inconsistency with the data presented in this chapter can be explained by different gut flora in the tested animals or by expression of TL1A under the control of different promoters – in the above mentioned report TL1A expression on myeloid cells was controlled by *c-fms* promoter and was present not only on dendritic cells but also on macrophages and the total expression of TL1A protein was higher than in CD11c-TL1A transgenic mice used in the research presented here (211). A recent report from other research group shows that CD2-TL1A transgenic mice develop IL-13-dependent small bowel inflammation accompanied by goblet cell hyperplasia (212).

CD11c-TL1A and CD2-TL1A transgenic mice show also two times higher accumulation of Tregs when compared to negative littermates. Tregs were shown to be capable of suppressing several autoimmune diseases, including inflammatory bowel

disease (213). Sustained expression of TL1A both on dendritic cells and T cells contributes to elevated accumulation of regulatory T cells in the transgenic mice, although data from our laboratory (214), show that TL1A attenuates the suppression imposed by regulatory T cells on effector T cells *in vitro*, therefore it is not clear whether the lack of intestine inflammation in CD11c-TL1A transgenic mice is due to the elevated level of regulatory T cells or the activity of IL-17A.

In conclusion, this chapter demonstrates that TL1A promotes Th2 and Th17-type effector responses and activates T cells *in vivo*. It also contributes to the development of bowel inflammation most probably via induction of IL-13 and possibly IL-17A expression as well as activation of T cells.

## CHAPTER 6

## GENERAL DISCUSSION

The data presented in this thesis show that expression of TL1A is highly restricted and activation-dependent. TL1A is transiently expressed on anti-CD3-activated T cells, LPS-activated bone marrow-derived dendritic cells and total population of spleen cells isolated from mice injected with ligands for TLR3 (poly(I:C)) and TLR4 (LPS and Lipid A), while no expression of TL1A was detected on naive bone marrow-derived dendritic cells or T cells. Very little DR3 is expressed on non-activated $CD8^+$ T cells (125). The data presented here show that DR3 is up-regulated on $CD8^+$ T cells after their activation *in vitro* and is maintained at relatively high level even 72 h following the activation. In contrast to TNFR1, which is constitutively expressed in most tissues (83), expression of DR3 is up-regulated on activated immune cells, such as T cells, monocytes or NK cells (125, 134, 135), therefore its signalling does not have such a profound effect on the organism homeostasis as TNFR1 signalling. Other members of the TNF and TNFR superfamilies, which take part in co-stimulation of T cells, like CD70 and CD27, OX40L and OX40 or 4-1BBL and 4-1BB, are also expressed on activated antigen presenting cells and/or T cells (74). Function of CD70/CD27, 4-1BBL/4-1BB, OX40L/OX40, CD30L/CD30 and LIGHT/HVEM interactions during an immune response is dependent on the timing of their maximal expression. Therefore HVEM, expressed on naive T cells, plays an important role during initial activation of T cells, while CD70 promotes proliferation of activated T cells. By contrast, expression of OX40, 4-1BB, CD30 and their ligands is maintained longer during the immune response on proliferating T cells and activated antigen presenting cells; consequently they also act later (60, 215). Thus, the unique contribution of each TNF/TNFRSF member to regulation of T cell responses depends not only on their specialized function but, more importantly, on the timing of their action. Expression pattern of TL1A, similar to that of CD70 (60), indicates that its role in the development of T cell-mediated immune response is comparable to that of CD70. Transient expression of TL1A and the fact that it does not induce apoptosis of $CD8^+$ T cells (111) suggest that its interaction with DR3 does not play a role in quenching of the immune response; instead, its main function is co-stimulation of T cells during the expansion phase.

The differences in the binding specificity of TAN2-2 antibody and DR3.Fc construct to J558L-PEF, J558L-TL1A, HEK293T, HEK293T-TL1A, EG.7 cells and

activated T cells and the ability of TAN2-2 antibody to ameliorate the clinical score of collagen-induced arthritis *in vivo* (102), showing that it recognises biologically active form of TL1A, have led to a hypothesis that TL1A naturally exists in two different forms, homo- and heterotrimeric. All of the known TNFSF members exist as trimers (75). The only known heterotrimeric member of the TNFSF is lymphotoxin β (LTβ), the other members of the TNFSF are homotrimeric proteins (76). There are two forms of LTβ – LTα1β2, consisting of one α chain (monomer of lymphotoxin α; LTα) and two β chains, and LTα2β1, consisting of two α chains and one β chain (76). LTα1β2 binds only to LTβ receptor (LTβR) but LTα2β1 binds to TNFR1 and TNFR2 (216). This suggests that one of the forms of TL1A (homo- or heterotrimeric), or both of them may bind also to receptors other than DR3. This hypothesis is supported by the fact that truncated forms of TL1A, generated as the result of alternative splicing, VEGI-174 and VEGI-192 bind also to receptors other than DR3 (111). Furthermore, several receptors belonging to the TNFRSF can bind multiple ligands (75, 76). For example, LIGHT binds both LTβR and HVEM (216) and soluble decoy receptor 3 (DcR) binds Fas, LIGHT and TL1A (118, 138). Therefore there is a possibility that not only can TL1A bind to receptors other than DR3 but also DR3 can bind ligands other than TL1A. TNF homology domain (THD), present in the C-terminal part of the TNFSF members is responsible for their trimerization. Proteins showing high level of homology in the THD are more likely to form oligomers than the proteins with lower level of homology (75, 76, 78).

As described in chapter 1, death receptors can induce either apoptotic cell death or activation of pro-survival genes. The final outcome of their stimulation depends on the balance between pro- and anti-apoptotic factors inside the cell. Like TNFR1, DR3 recruits an adaptor protein TRADD which subsequently recruits TRAF2 and FADD (82, 132), therefore its triggering can also result either in apoptotic death of a cell or in activation of transcription of pro-survival genes. On the other hand, DR4, DR5 (receptors for TRAIL) and Fas do not bind TRADD; instead they bind FADD directly to their cytoplasmic domains (217) which results in their inability to activate NF-κB and therefore, in contrast to DR3, these receptors primarily induce apoptotic cell death. Apoptotic death of T cells induced by several death receptors, notably Fas, CD30 and TNFR1 (42-44), is crucial for contraction of the effector cell pool following the peak of the immune response. However, the data presented in this thesis shows that the primary role of TL1A/DR3 interactions is co-stimulation of T cells but not the participation in activation-induced cell death. Even

though DR3 is capable of inducing apoptotic cell death (129, 131, 132), triggering of this receptor on T cells results in their enhanced proliferation and effector function (111).

Previous publications concerning the role of TL1A in co-stimulation of T cells concentrate mostly on its influence on $CD4^+$ T cells and very little is known about its role in $CD8^+$ T cell-mediated immune response, therefore most of the research presented in this thesis is concentrated on the role of TL1A in co-stimulation of $CD8^+$ T cells. The other reason for that was the observation that $CD8^+$ T cells are responsible for TL1A-mediated rejection of J558L tumour in BALB/c mice. The data presented in this thesis show that TL1A acts as a co-stimulatory molecule for both $CD8^+$ T cells and can enhance their effector function. TL1A/DR3 signalling combined with TCR engagement leads to increased proliferation of both $CD4^+$ and $CD8^+$ T cells which has been shown both *in vitro* and *in vivo*. Constitutive expression of TL1A on T cells and dendritic cells (in CD2-TL1A and CD11c-TL1A transgenic mice, respectively) results in an increased frequency of activated, $CD44^+$ $CD4^+$ and $CD8^+$ T cells. Furthermore, wild-type C57BL/6 mice that received an adoptive transfer of OT-I T cells and injection of SIINFEKL together with sTL1A have higher percentage of activated / effector memory $CD62L^{low}$ $CD4^+$ and $CD8^+$ T cells than the control animals. *In vivo*, TL1A directly increases production of perforin and IL-2 by $CD8^+$ T cells. It also directly elevates the production of granzyme B from $CD8^+$ T cells, although IL-2 is required for optimal induction of granzyme B.

These results show that function of TL1A/DR3 interactions is similar to that of other pairs of TNFSF/TNFRSF members which act as co-stimulatory molecules for both $CD4^+$ and $CD8^+$ T cells – CD70/CD27, 4-1BBL/4-1BB, GITRL/GITR, OX40L/OX40 (89). *In vitro* data show that stimulation of OT-I T cells with sTL1A together with SIINFEKL can partially by-pass the requirement for CD28 signalling to enhance proliferation of $CD8^+$ T cells; also 4-1BB, GITR and OX-40 signalling can replace CD28 signalling, although in its presence the effect of co-stimulation is more profound (89, 90).

Very little is known about the influence of TL1A/DR3 signalling on memory T cell response. There is evidence that TL1A enhances proliferation of $CD45^{low}$ $CD4^+$ memory T cells *in vitro* (116) but there are no reports published to date discussing the role of TL1A in expansion of $CD8^+$ memory T cells. The data presented here show that TL1A enhances the generation of endogenous antigen-specific $CD8^+$ memory T cells and boosts the expansion of $CD8^+$ memory T cells in mice that received an adoptive transfer of OT-I T cells as well as in an endogenous setting *in vivo*.

Wild-type C57BL/6 mice that received an adoptive transfer of OT-I T cells and injection of SIINFEKL and sTL1A have higher percentage of regulatory T cells (Tregs) in the spleens than the control animals. Sustained, constitutive expression of TL1A on T cells and dendritic cells results in elevated frequency of Tregs in CD2-TL1A and CD11c-TL1A transgenic mice, respectively. Observations from our laboratory show that in CD11c-TL1A transgenic mice there is a clear increase in the proportion of splenic Tregs that express the proliferation antigen Ki-67 (214). *In vitro* experiments showed that TL1A directly enhances proliferation of Tregs which are stimulated also with anti-CD3 and anti-CD28 antibodies (214). Taken together, these data indicate that TL1A enhances proliferation of Tregs. Van Olffen *et al.* (218) show a similar occurrence in transgenic mice that constitutively express GITR under the control of CD19 promoter which also exhibit accumulation of Tregs as a result of their increased proliferation. However, it is possible that TL1A, like LIGHT (219), also induces differentiation of Tregs. Since there is no specific marker that would allow distinguishing inducible Tregs (differentiating in the periphery) from natural Tregs of thymic origin (9), it is not possible to determine whether these cells differentiate in the periphery from T cells or develop in the thymus. However, it is very likely that TL1A stimulates both ways of Treg development. Signals that influence the development of natural Tregs are not clear (4), but constitutive expression of TL1A in CD2-TL1A and CD11c-TL1A transgenic mice may contribute to enhanced development of Tregs in the thymus as well as in the periphery. On the other hand, short-term (3 days) *in vivo* stimulation of wild-type C57BL/6 mice with sTL1A might lead to differentiation of Tregs from naive, conventional $CD4^+$ T cells in the periphery. Stimulation of GITR, OX40 and 4-1BB present on Tregs results in loss of their suppressive activity and/or negatively regulates their differentiation (92, 99). The influence of TL1A/DR3 interactions on the biological activity of Tregs was not investigated here, although unpublished data from our laboratory show that TL1A attenuates the suppression of effector T cells by Tregs *in vitro* by direct stimulation of proliferation of both effector and regulatory T cells. However, because of transient nature of TL1A expression *in vivo*, Tregs are expected to regain their suppressive activity relatively quickly, therefore the net effect of TL1A on activity of Tregs in conditions of a normal immune response is probably positive due to their increased accumulation.

Wang et al. (219) describe a function of LIGHT comparable to that of TL1A. Its overexpression on T cells in transgenic mice results in development of colitis which is accompanied by enhanced expansion of Tregs. This is due to ability of LIGHT to promote

proliferation of Tregs as well as effector T cells even in a Treg-abundant environment. Transgenic mice, overexpressing GITR on B cells, also show enhanced proliferation of Tregs and conventional $CD4^+$ T cells (218). In a similar way, TL1A up-regulates proliferation of both conventional effector T cells ($CD4^+$ and $CD8^+$) as well as regulatory T cells.

CD11c-TL1A transgenic mice develop IL-13-induced goblet cell hyperplasia in the small intestine but do not show any other symptoms of bowel inflammation which may be the consequence of elevated expression of IL-17A which inhibits Th1-type immune response (210) and infiltration of the bowel by pro-inflammatory cells (189); the other possibility is enhanced proliferation of Tregs, as discussed in Chapter 5.

Proteins belonging to the TNF and TNFR superfamilies are involved in directing the immune response towards Th1, Th2 or Th17-type (60, 74, 92). Several research groups showed that TL1A skews the immune response towards Th1-type by inducing secretion of a Th-1-type cytokine, IFN-γ, from $CD4^+$ T cells (136, 144, 146, 147) and peripheral blood mononuclear cells *in vitro* (111, 117, 136, 142). Meylan et al. (123) show that TL1A induces secretion of both IFN-γ and IL-4 from activated $CD4^+$ T cells *in vitro*. On the other hand, other research groups demonstrate that TL1A induces secretion of Th2-type cytokines, IL-4 and/or IL-13, from peripheral blood monocytes (117) and NK cells (125) but has no effect on production of IFN-γ by these cells *in vitro*. Moreover, TL1A can also up-regulate secretion of IL-17 (Th17-type cytokine) from activated Th17 cells (145, 146, 149) and induce differentiation of Th17 cells from naive $CD4^+$ T cells (145). The data presented here show that TL1A up-regulates the production of both IL-4 and IFN-γ from activated T cells *in vitro*, furthermore splenocytes isolated from $DR3^{-/-}$ mice and restimulated *in vitro* with PMA and ionomycin secrete less IFN-γ than splenocytes isolated from wild-type mice. As described above, CD11c-TL1A transgenic mice and CD2-TL1A transgenic mice show an increase in expression of IL-17A mRNA accompanied by a modest increase of IL-13 mRNA expression in the secondary lymphoid organs and intestine but do not up-regulate the level of IFN-γ. This demonstrates that the observed effects of TL1A are independent of the cell type that produces TL1A *in vivo*. However, the observed phenotype of CD11c-TL1A and CD2-TL1A transgenic mice stands in contrast with the phenotype of transgenic mice overexpressing TNF or LIGHT where severe intestinal pathology is accompanied by elevated levels of IFN-γ and IL-12 (220, 221). Elevated levels of IFN-γ are present also in mice overexpressing CD70 and GITR (97,

218). On the other hand, transgenic mice overexpressing OX40 show bias towards Th2-type immune response and develop colon inflammation (222).

Taken together, these data suggest that TL1A does not skew the immune response to one, particular type. Instead, it can induce secretion of Th1, Th2 or Th17-type cytokines depending on the conditions of an experiment and differences in the cellular distribution and signalling capacity of DR3 in different types of an immune response. For example, Th17 cells show higher level of DR3 expression than Th1 and Th2 cells *in vitro* (145). Also, the lack of DR3 expression on $CD4^+$ T cells cultured *in vitro* under non-polarizing conditions negatively impacts the production of Th2-type cytokines but not IFN-γ (125). The observed impact of TL1A on secretion of Th1, Th2 and Th17-type cytokines is similar to the effect of OX40 which is known to induce differentiation of both Th1 and Th2 cells (92).

In conclusion, this thesis demonstrates that TL1A/DR3 interactions enhance T cell-mediated immunity. Further investigation revealed that $CD8^+$ T cells play a critical role in TL1A-mediated rejection of J558L tumour. TL1A acts as a direct co-stimulatory molecule for $CD4^+$ and $CD8^+$ T cells which enhances their activation. It also enhances generation of $CD8^+$ memory T cells and boosts their expansion. This suggests that in future TL1A might by used as molecular adjuvant to enhance T cell responses to vaccines or to boost immune response to tumours or infections with pathogens. Some members of the TNFSF are already used as molecular adjuvants to vaccines. GITRL and CD40L augument $CD8^+$ and/or $CD4^+$ responses to DNA vaccination (223). OX40L and CD40L boosts $CD8^+$ T cell responses to HIV-1 vaccine in mice (224). Furthermore, Kayamuro *et al.* (148) show that out of 16 TNFSF members tested as mucosal vaccine adjuvants, TL1A induced the strongest response to intranasal immunization with OVA, characterized by high serum levels of $IgG_1$ and elevated production of IL-4 and IL-5 from splenocytes. The ability of TL1A to act as a direct co-stimulatory molecule for T cells indicates that it may potentially be used as an adjuvant for vaccines or in anti-tumour therapy. However, considering application of TL1A in therapy it is important to pay attention that TL1A is involved in pathogenesis of several autoimmune diseases, such as inflammatory bowel disease (116, 134, 146, 149, 153, 225), rheumatoid arthritis (102, 154, 155), experimental autoimmune encephalomyelitis (123) and autoimmune lung inflammation (123, 125, 145).

Most of the experiments presented in this thesis involve stimulation of animals or cells with recombinant TL1A or is conducted on mice constitutively expressing high levels of TL1A. In physiological conditions, however, the expression of TL1A is tightly

regulated and transiently present on activated cells of the immune system, including T cells (123, 125), dendritic cells and monocytes (119). Furthermore, TL1A is one of many costimulatory molecules present in organisms of mammals, therefore it may seem that its role in the development of the immune response is not critical. On the other hand, neutralization of TL1A prevents the development of experimental bowel inflammation (134) and attenuates chronic colitis in mice (146); it also protects mice from antigen-induced arthritis (a mouse model of rheumatoid arthritis) (102). In addition, $DR3^{-/-}$ mice show reduced clinical score of antigen-induced arthritis (102) and experimental autoimmune encephalomyelitis (a mouse model of multiple sclerosis) (123). This shows that, in settings that do not involve overexpression of TL1A, it still is an important costimulatory molecule, required for triggering an immune response. Further investigation of the role of TL1A in physiological conditions would require the use of $TL1A^{-/-}$ and/or $DR3^{-/-}$ animals, however no such reports, except for the ones presented above, have been published to date.

## CHAPTER 7

## SUMMARY OF THE RESULTS

The data presented in this thesis show that expression of TL1A is tightly regulated and is transiently present on activated dendritic cells and T cells. TL1A increases accumulation and/or proliferation of antigen-stimulated $CD4^+$ and $CD8^+$ T cells both *in vitro* and *in vivo*. It also enhances functional activity of $CD8^+$ T cells and stimulation with high doses of TL1A results in acquisition of effector phenotype by $CD4^+$ and $CD8^+$ T cells. Furthermore, stimulation with high doses of TL1A *in vivo* results in elevated proportion of regulatory T cells; it also leads to elevated expression of IL-17A and IL-13 in TL1A-transgenic mice which may contribute to the development of bowel inflammation. Below is the summary of the findings presented in this thesis.

**1)** Expression of TL1A mRNA is transiently up-regulated on splenocytes isolated from mice injected with LPS, Lipid A and poly(I:C), but not with CpG, Pam3CSK4, α-GalCer, curdlan or anti-CD40 antibody. The expression peaks after 4 hours of stimulation with LPS or Lipid A (8 and 20-fold increase compared to non-stimulated mice, respectively) and after 6 hours of stimulation with poly(I:C) (18-fold increase).

**2)** Expression of TL1A mRNA is up-regulated on bone marrow-derived dendritic cells stimulated *in vitro* with LPS or poly(I:C) for 4 hours (442 and 177-fold increase compared to non-stimulated cells, respectively).

**3)** Transmembrane TL1A is detected on bone marrow-derived cells after 4 h and 24 h of *in vitro* stimulation with LPS.

**4)** Transmembrane TL1A is transiently expressed on T cells after 24 h of *in vitro* stimulation with anti-CD3 antibody.

**5)** Transmembrane TL1A is expressed on splenocytes isolated from 24 h following the *i.v.* injection with LPS.

**6)** Differences in the binding specificity of anti-TL1A antibody (TAN2-2) and DR3.Fc construct to TL1A-transfected cells and cells naturally expressing TL1A suggest that TL1A may exist as a homo- and heterotrimer.

**7)** Rejection of TL1A-transfected J558L tumour cells in BALB/c mice is mediated by $CD8^+$ T cells, but not by $CD4^+$ T cells or NK cells.

**8)** Initial *s.c.* injection of TL1A-transfected J558L tumour cells into BALB/c mice results in long-term protection from wild-type J558L tumour.

**9)** Expression of DR3 is present on antigen-stimulated OTI cells but not on naive OTI cells.

**10)** Soluble recombinant TL1A increases proliferation of antigen-stimulated OTII and OTI cells *in vitro* (average 1.5-fold increase compared to cells not stimulated with TL1A). The increase of proliferation of OTI cells stimulated with soluble recombinant TL1A partially depends on IL-2 and CD80/CD86 stimulation.

**11)** Soluble recombinant TL1A enhances the production of IL-2 by antigen-stimulated OTI cells and up-regulates the expression of IL-2Rα chain (CD25) on these cells *in vitro*.

**12)** Administration of soluble recombinant TL1A increases the accumulation of antigen-stimulated OTII and proliferation of antigen-stimulated OTI cells *in vivo*. The effect of increased proliferation of OTI cells is independent of IL-2 *in vivo*.

**13)** Administration of soluble recombinant TL1A increases the frequency of regulatory T cells in peripheral blood of wild-type C57BL/6 mice (2-fold increase, compared to control mice).

**14)** Soluble recombinant TL1A significantly increases the frequency of antigen-stimulated $CD62L^{low}$ OTI cells *in vivo* but does not change the frequency of antigen-stimulated $KLRG1^+$ OTI cells. It also increases the expression of granzyme B, perforin and IL-2 mRNA in antigen-stimulated OTI cells *in vivo*; the increase of granzyme B mRNA expression depends also on IL-2.

**15)** Administration of soluble recombinant TL1A during the initial priming of C57BL/6 mice with an antigen does not result in a significant increase of memory $CD8^+$ T cell accumulation after a subsequent re-challenge with the antigen and anti-CD40 antibody but soluble recombinant TL1A significantly enhances the expansion of memory $CD8^+$ T cells when it is administered together with an antigen during the re-challenge in an endogenous setting.

**16)** CD11c-TL1A transgenic mice have significantly higher proportion of $CD44^+$ $CD4^+$ T cells and $CD44^+$ $CD8^+$ T cells in the spleen than control animals, although no significant change was observed in the frequencies of $CD62L^{low}$ $CD4^+$ and $CD62L^{low}$ $CD8^+$ T cells. CD11c-TL1A transgenic mice develop goblet cell hyperplasia in the small intestine and show elevated level of IL-17A transcript in the spleen, small intestine and mesenteric lymph nodes; they also show a trend towards higher expression of IL-13 mRNA the spleen, small intestine and mesenteric lymph nodes, although the increase is not statistically significant when compared to control animals. The mice did not show any change in the level of IL-4 mRNA in the mesenteric lymph nodes and IFN-γ mRNA in the spleen, mesenteric lymph nodes and small intestine when compared to control animals. CD11c-TL1A transgenic mice have two times higher proportion of regulatory T cells than control mice.

**17)** CD2-TL1A transgenic mice have significantly higher proportion of $CD44^+$ $FoxP3^-$ $CD4^+$ T cells and $CD44^+$ $FoxP3^+$ $CD4^+$ T cells in the spleens and $CD44^+$ $CD4^+$ T cells in peripheral blood than control animals, although no significant changes were observed in the frequencies of $CD44^+$ $CD8^+$ T cells or $CD62L^{low}$ T cells. CD2-TL1A transgenic mice have higher expression of IL-17A mRNA in the mesenteric lymph nodes, than control animals; they also show a trend towards higher expression of IL-13 mRNA in the mesenteric lymph nodes than control animals. The mice did not show any difference in the level of IFN-γ and IL-2 mRNA in the mesenteric lymph nodes when compared to control mice. CD2-TL1A transgenic mice have also two times higher proportion of regulatory T cells than control mice.

## REFERENCES


1. Laky K, Fleischacker C, Fowlkes BJ. 2006. TCR and Notch signaling in CD4 and CD8 T-cell development. *Immunol Rev* 209: 274-83
2. Gołąb J, Jakóbisiak M, Lasek W. 2002. *Immunologia*. Warsaw: Wydawnictwo Naukowe PWN
3. Khattar M, Chen W, Stepkowski SM. 2009. Expanding and converting regulatory T cells: a horizon for immunotherapy. *Arch Immunol Ther Exp (Warsz)* 57: 199-204
4. Workman CJ, Szymczak-Workman AL, Collison LW, Pillai MR, Vignali DA. 2009. The development and function of regulatory T cells. *Cell Mol Life Sci* 66: 2603-22
5. Bonneville M, O'Brien RL, Born WK. 2010. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol* 10: 467-78
6. Miosge L, Zamoyska R. 2007. Signalling in T-cell development: is it all location, location, location? *Curr Opin Immunol* 19: 194-9
7. Chen W. 2004. The late stage of T cell development within mouse thymus. *Cell Mol Immunol* 1: 3-11
8. Seder RA, Ahmed R. 2003. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. *Nat Immunol* 4: 835-42
9. Zhu J, Paul WE. 2010. Heterogeneity and plasticity of T helper cells. *Cell Res* 20: 4-12
10. Annunziato F, Romagnani S. 2009. Heterogeneity of human effector CD4+ T cells. *Arthritis Res Ther* 11: 257
11. Zhu J, Paul WE. 2008. CD4 T cells: fates, functions, and faults. *Blood* 112: 1557-69
12. Grogan JL, Mohrs M, Harmon B, Lacy DA, Sedat JW, Locksley RM. 2001. Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity* 14: 205-15
13. Gri G, Piconese S, Frossi B, Manfroi V, Merluzzi S, Tripodo C, Viola A, Odom S, Rivera J, Colombo MP, Pucillo CE. 2008. CD4+CD25+ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. *Immunity* 29: 771-81
14. Brusko TM, Putnam AL, Bluestone JA. 2008. Human regulatory T cells: role in autoimmune disease and therapeutic opportunities. *Immunol Rev* 223: 371-90
15. Zhao DM, Thornton AM, DiPaolo RJ, Shevach EM. 2006. Activated CD4+CD25+ T cells selectively kill B lymphocytes. *Blood* 107: 3925-32
16. Vignali DA, Collison LW, Workman CJ. 2008. How regulatory T cells work. *Nat Rev Immunol* 8: 523-32
17. von Boehmer H. 2005. Mechanisms of suppression by suppressor T cells. *Nat Immunol* 6: 338-44
18. Strauss L, Bergmann C, Whiteside TL. 2009. Human circulating CD4+CD25highFoxp3+ regulatory T cells kill autologous CD8+ but not CD4+ responder cells by Fas-mediated apoptosis. *J Immunol* 182: 1469-80
19. Knutson KL, Disis ML, Salazar LG. 2007. CD4 regulatory T cells in human cancer pathogenesis. *Cancer Immunol Immunother* 56: 271-85
20. Levings MK, Bacchetta R, Schulz U, Roncarolo MG. 2002. The role of IL-10 and TGF-beta in the differentiation and effector function of T regulatory cells. *Int Arch Allergy Immunol* 129: 263-76
21. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DA. 2007. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 450: 566-9

22. Niedbala W, Wei XQ, Cai B, Hueber AJ, Leung BP, McInnes IB, Liew FY. 2007. IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur J Immunol* 37: 3021-9
23. Tang Q, Bluestone JA. 2008. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* 9: 239-44
24. Trzonkowski P, Szarynska M, Mysliwska J, Mysliwski A. 2009. Ex vivo expansion of CD4(+)CD25(+) T regulatory cells for immunosuppressive therapy. *Cytometry A* 75: 175-88
25. Scott-Browne JP, Shafiani S, Tucker-Heard G, Ishida-Tsubota K, Fontenot JD, Rudensky AY, Bevan MJ, Urdahl KB. 2007. Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. *J Exp Med* 204: 2159-69
26. Haribhai D, Lin W, Relland LM, Truong N, Williams CB, Chatila TA. 2007. Regulatory T cells dynamically control the primary immune response to foreign antigen. *J Immunol* 178: 2961-72
27. Gavin MA, Clarke SR, Negrou E, Gallegos A, Rudensky A. 2002. Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells in vivo. *Nat Immunol* 3: 33-41
28. Littman DR, Rudensky AY. 2010. Th17 and regulatory T cells in mediating and restraining inflammation. *Cell* 140: 845-58
29. Bots M, Medema JP. 2006. Granzymes at a glance. *J Cell Sci* 119: 5011-4
30. Zuccato E, Blott EJ, Holt O, Sigismund S, Shaw M, Bossi G, Griffiths GM. 2007. Sorting of Fas ligand to secretory lysosomes is regulated by mono-ubiquitylation and phosphorylation. *J Cell Sci* 120: 191-9
31. Keefe D, Shi L, Feske S, Massol R, Navarro F, Kirchhausen T, Lieberman J. 2005. Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis. *Immunity* 23: 249-62
32. Zhang D, Beresford PJ, Greenberg AH, Lieberman J. 2001. Granzymes A and B directly cleave lamins and disrupt the nuclear lamina during granule-mediated cytolysis. *Proc Natl Acad Sci U S A* 98: 5746-51
33. Hayday AC. 2000. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol* 18: 975-1026
34. Berzins SP, Smyth MJ, Baxter AG. 2011. Presumed guilty: natural killer T cell defects and human disease. *Nat Rev Immunol* 11: 131-42
35. Smyth MJ, Crowe NY, Hayakawa Y, Takeda K, Yagita H, Godfrey DI. 2002. NKT cells - conductors of tumor immunity? *Curr Opin Immunol* 14: 165-71
36. Mempel TR, Henrickson SE, Von Andrian UH. 2004. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* 427: 154-9
37. van Kooyk Y, Geijtenbeek TB. 2002. A novel adhesion pathway that regulates dendritic cell trafficking and T cell interactions. *Immunol Rev* 186: 47-56
38. Germain RN. 2001. The T cell receptor for antigen: signaling and ligand discrimination. *J Biol Chem* 276: 35223-6
39. Janssen EM, Droin NM, Lemmens EE, Pinkoski MJ, Bensinger SJ, Ehst BD, Griffith TS, Green DR, Schoenberger SP. 2005. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature* 434: 88-93
40. Sun JC, Williams MA, Bevan MJ. 2004. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat Immunol* 5: 927-33
41. Kaech SM, Wherry EJ, Ahmed R. 2002. Effector and memory T-cell differentiation: implications for vaccine development. *Nat Rev Immunol* 2: 251-62
42. Zhang J, Xu X, Liu Y. 2004. Activation-induced cell death in T cells and autoimmunity. *Cell Mol Immunol* 1: 186-92
43. Hildeman DA, Zhu Y, Mitchell TC, Kappler J, Marrack P. 2002. Molecular mechanisms of activated T cell death in vivo. *Curr Opin Immunol* 14: 354-9

44. Janssen O, Sanzenbacher R, Kabelitz D. 2000. Regulation of activation-induced cell death of mature T-lymphocyte populations. *Cell Tissue Res* 301: 85-99
45. Esser MT, Marchese RD, Kierstead LS, Tussey LG, Wang F, Chirmule N, Washabaugh MW. 2003. Memory T cells and vaccines. *Vaccine* 21: 419-30
46. Veiga-Fernandes H, Walter U, Bourgeois C, McLean A, Rocha B. 2000. Response of naive and memory CD8+ T cells to antigen stimulation in vivo. *Nat Immunol* 1: 47-53
47. Woodland DL, Kohlmeier JE. 2009. Migration, maintenance and recall of memory T cells in peripheral tissues. *Nat Rev Immunol* 9: 153-61
48. Davis MM. 2002. A new trigger for T cells. *Cell* 110: 285-7
49. Dustin ML, Chan AC. 2000. Signaling takes shape in the immune system. *Cell* 103: 283-94
50. Schulze-Luehrmann J, Ghosh S. 2006. Antigen-receptor signaling to nuclear factor kappa B. *Immunity* 25: 701-15
51. Batista FD, Harwood NE. 2009. The who, how and where of antigen presentation to B cells. *Nat Rev Immunol* 9: 15-27
52. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. 2010. Development of monocytes, macrophages, and dendritic cells. *Science* 327: 656-61
53. Banchereau J, Steinman RM. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245-52
54. Coquerelle C, Moser M. 2010. DC subsets in positive and negative regulation of immunity. *Immunol Rev* 234: 317-34
55. Amigorena S, Savina A. 2010. Intracellular mechanisms of antigen cross presentation in dendritic cells. *Curr Opin Immunol* 22: 109-17
56. Watts C. 2004. The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules. *Nat Immunol* 5: 685-92
57. Appleman LJ, Boussiotis VA. 2003. T cell anergy and costimulation. *Immunol Rev* 192: 161-80
58. Schwartz RH. 2003. T cell anergy. *Annu Rev Immunol* 21: 305-34
59. Sharpe AH. 2009. Mechanisms of costimulation. *Immunol Rev* 229: 5-11
60. Nolte MA, van Olffen RW, van Gisbergen KP, van Lier RA. 2009. Timing and tuning of CD27-CD70 interactions: the impact of signal strength in setting the balance between adaptive responses and immunopathology. *Immunol Rev* 229: 216-31
61. Yong PF, Salzer U, Grimbacher B. 2009. The role of costimulation in antibody deficiencies: ICOS and common variable immunodeficiency. *Immunol Rev* 229: 101-13
62. Frauwirth KA, Thompson CB. 2002. Activation and inhibition of lymphocytes by costimulation. *J Clin Invest* 109: 295-9
63. Lenschow DJ, Walunas TL, Bluestone JA. 1996. CD28/B7 system of T cell costimulation. *Annu Rev Immunol* 14: 233-58
64. Beverly B, Kang SM, Lenardo MJ, Schwartz RH. 1992. Reversal of in vitro T cell clonal anergy by IL-2 stimulation. *Int Immunol* 4: 661-71
65. Rudd CE, Taylor A, Schneider H. 2009. CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunol Rev* 229: 12-26
66. Viola A, Lanzavecchia A. 1996. T cell activation determined by T cell receptor number and tunable thresholds. *Science* 273: 104-6
67. Wetzel SA, McKeithan TW, Parker DC. 2002. Live-cell dynamics and the role of costimulation in immunological synapse formation. *J Immunol* 169: 6092-101
68. Nurieva RI, Liu X, Dong C. 2009. Yin-Yang of costimulation: crucial controls of immune tolerance and function. *Immunol Rev* 229: 88-100
69. Dong H, Zhu G, Tamada K, Chen L. 1999. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med* 5: 1365-9
70. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, Iwai Y, Long AJ, Brown JA, Nunes R, Greenfield EA, Bourque K, Boussiotis VA, Carter LL, Carreno BM,

Malenkovich N, Nishimura H, Okazaki T, Honjo T, Sharpe AH, Freeman GJ. 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2: 261-8

71. Kanai T, Totsuka T, Uraushihara K, Makita S, Nakamura T, Koganei K, Fukushima T, Akiba H, Yagita H, Okumura K, Machida U, Iwai H, Azuma M, Chen L, Watanabe M. 2003. Blockade of B7-H1 suppresses the development of chronic intestinal inflammation. *J Immunol* 171: 4156-63
72. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, Lennon VA, Celis E, Chen L. 2002. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 8: 793-800
73. Watts TH, DeBenedette MA. 1999. T cell co-stimulatory molecules other than CD28. *Curr Opin Immunol* 11: 286-93
74. Croft M. 2009. The role of TNF superfamily members in T-cell function and diseases. *Nat Rev Immunol* 9: 271-85
75. Hehlgans T, Pfeffer K. 2005. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. *Immunology* 115: 1-20
76. Locksley RM, Killeen N, Lenardo MJ. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 104: 487-501
77. Alexis Biochemicals. http://www.sapphirebioscience.com/app/template/Wallchart.vm/a/z. Accessed on 08/06/2011
78. Bodmer JL, Schneider P, Tschopp J. 2002. The molecular architecture of the TNF superfamily. *Trends Biochem Sci* 27: 19-26
79. Zhang M, Ko KH, Lam QL, Lo CK, Xu DJ, Shen L, Zheng B, Srivastava G, Lu L. 2004. Novel function of TNF cytokines in regulating bone marrow B cell survival. *Cell Mol Immunol* 1: 447-53
80. Haswell LE, Glennie MJ, Al-Shamkhani A. 2001. Analysis of the oligomeric requirement for signaling by CD40 using soluble multimeric forms of its ligand, CD154. *Eur J Immunol* 31: 3094-100
81. Arch RH, Gedrich RW, Thompson CB. 1998. Tumor necrosis factor receptor-associated factors (TRAFs)--a family of adapter proteins that regulates life and death. *Genes Dev* 12: 2821-30
82. Aggarwal BB. 2003. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 3: 745-56
83. Wajant H, Pfizenmaier K, Scheurich P. 2003. Tumor necrosis factor signaling. *Cell Death Differ* 10: 45-65
84. Jin Z, El-Deiry WS. 2006. Distinct signaling pathways in TRAIL- versus tumor necrosis factor-induced apoptosis. *Mol Cell Biol* 26: 8136-48
85. Hengartner MO. 2000. The biochemistry of apoptosis. *Nature* 407: 770-6
86. Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV. 1996. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity* 4: 387-96
87. Lemmers B, Salmena L, Bidere N, Su H, Matysiak-Zablocki E, Murakami K, Ohashi PS, Jurisicova A, Lenardo M, Hakem R, Hakem A. 2007. Essential role for caspase-8 in Toll-like receptors and NFkappaB signaling. *J Biol Chem* 282: 7416-23
88. He L, Wu X, Siegel R, Lipsky PE. 2006. TRAF6 regulates cell fate decisions by inducing caspase 8-dependent apoptosis and the activation of NF-kappaB. *J Biol Chem* 281: 11235-49
89. Watts TH. 2005. TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol* 23: 23-68
90. Gramaglia I, Weinberg AD, Lemon M, Croft M. 1998. Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J Immunol* 161: 6510-7

91. Rowley TF, Al-Shamkhani A. 2004. Stimulation by soluble CD70 promotes strong primary and secondary CD8+ cytotoxic T cell responses in vivo. *J Immunol* 172: 6039-46
92. Croft M, So T, Duan W, Soroosh P. 2009. The significance of OX40 and OX40L to T-cell biology and immune disease. *Immunol Rev* 229: 173-91
93. Cayabyab M, Phillips JH, Lanier LL. 1994. CD40 preferentially costimulates activation of CD4+ T lymphocytes. *J Immunol* 152: 1523-31
94. Wang C, Lin GH, McPherson AJ, Watts TH. 2009. Immune regulation by 4-1BB and 4-1BBL: complexities and challenges. *Immunol Rev* 229: 192-215
95. Cai G, Freeman GJ. 2009. The CD160, BTLA, LIGHT/HVEM pathway: a bidirectional switch regulating T-cell activation. *Immunol Rev* 229: 244-58
96. Sabbagh L, Snell LM, Watts TH. 2007. TNF family ligands define niches for T cell memory. *Trends Immunol* 28: 333-9
97. Arens R, Tesselaar K, Baars PA, van Schijndel GM, Hendriks J, Pals ST, Krimpenfort P, Borst J, van Oers MH, van Lier RA. 2001. Constitutive CD27/CD70 interaction induces expansion of effector-type T cells and results in IFNgamma-mediated B cell depletion. *Immunity* 15: 801-12
98. Xie P, Kraus ZJ, Stunz LL, Liu Y, Bishop GA. 2011. TNF receptor-associated factor 3 is required for T cell-mediated immunity and TCR/CD28 signaling. *J Immunol* 186: 143-55
99. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. 2002. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 3: 135-42
100. Telford WG, Nam SY, Podack ER, Miller RA. 1997. CD30-regulated apoptosis in murine CD8 T cells after cessation of TCR signals. *Cell Immunol* 182: 125-36
101. Algeciras-Schimnich A, Griffith TS, Lynch DH, Paya CV. 1999. Cell cycle-dependent regulation of FLIP levels and susceptibility to Fas-mediated apoptosis. *J Immunol* 162: 5205-11
102. Bull MJ, Williams AS, Mecklenburgh Z, Calder CJ, Twohig JP, Elford C, Evans BA, Rowley TF, Slebioda TJ, Taraban VY, Al-Shamkhani A, Wang EC. 2008. The Death Receptor 3-TNF-like protein 1A pathway drives adverse bone pathology in inflammatory arthritis. *J Exp Med* 205: 2457-64
103. Tracey D, Klareskog L, Sasso EH, Salfeld JG, Tak PP. 2008. Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. *Pharmacol Ther* 117: 244-79
104. Chan AC, Carter PJ. 2010. Therapeutic antibodies for autoimmunity and inflammation. *Nat Rev Immunol* 10: 301-16
105. Oliveira JB, Gupta S. 2008. Disorders of apoptosis: mechanisms for autoimmunity in primary immunodeficiency diseases. *J Clin Immunol* 28 Suppl 1: S20-8
106. Turbyville JC, Rao VK. 2010. The autoimmune lymphoproliferative syndrome: A rare disorder providing clues about normal tolerance. *Autoimmun Rev* 9: 488-93
107. Worth A, Thrasher AJ, Gaspar HB. 2006. Autoimmune lymphoproliferative syndrome: molecular basis of disease and clinical phenotype. *Br J Haematol* 133: 124-40
108. Bleesing JJ. 2003. Autoimmune lymphoproliferative syndrome (ALPS). *Curr Pharm Des* 9: 265-78
109. Kimberley FC, Lobito AA, Siegel RM, Screaton GR. 2007. Falling into TRAPS--receptor misfolding in the TNF receptor 1-associated periodic fever syndrome. *Arthritis Res Ther* 9: 217
110. Rezaei N. 2006. TNF-receptor-associated periodic syndrome (TRAPS): an autosomal dominant multisystem disorder. *Clin Rheumatol* 25: 773-7
111. Migone TS, Zhang J, Luo X, Zhuang L, Chen C, Hu B, Hong JS, Perry JW, Chen SF, Zhou JX, Cho YH, Ullrich S, Kanakaraj P, Carrell J, Boyd E, Olsen HS, Hu G, Pukac L, Liu D, Ni J, Kim S, Gentz R, Feng P, Moore PA, Ruben SM, Wei P. 2002. TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator. *Immunity* 16: 479-92

112. Metheny-Barlow LJ, Li LY. 2006. Vascular endothelial growth inhibitor (VEGI), an endogenous negative regulator of angiogenesis. *Semin Ophthalmol* 21: 49-58
113. Chew LJ, Pan H, Yu J, Tian S, Huang WQ, Zhang JY, Pang S, Li LY. 2002. A novel secreted splice variant of vascular endothelial cell growth inhibitor. *FASEB J* 16: 742-4
114. Hou W, Medynski D, Wu S, Lin X, Li LY. 2005. VEGI-192, a new isoform of TNFSF15, specifically eliminates tumor vascular endothelial cells and suppresses tumor growth. *Clin Cancer Res* 11: 5595-602
115. Tian F, Liang PH, Li LY. 2009. Inhibition of endothelial progenitor cell differentiation by VEGI. *Blood* 113: 5352-60
116. Bamias G, Martin C, 3rd, Marini M, Hoang S, Mishina M, Ross WG, Sachedina MA, Friel CM, Mize J, Bickston SJ, Pizarro TT, Wei P, Cominelli F. 2003. Expression, localization, and functional activity of TL1A, a novel Th1-polarizing cytokine in inflammatory bowel disease. *J Immunol* 171: 4868-74
117. Prehn JL, Mehdizadeh S, Landers CJ, Luo X, Cha SC, Wei P, Targan SR. 2004. Potential role for TL1A, the new TNF-family member and potent costimulator of IFN-gamma, in mucosal inflammation. *Clin Immunol* 112: 66-77
118. Kim S, Zhang L. 2005. Identification of naturally secreted soluble form of TL1A, a TNF-like cytokine. *J Immunol Methods* 298: 1-8
119. Prehn JL, Thomas LS, Landers CJ, Yu QT, Michelsen KS, Targan SR. 2007. The T cell costimulator TL1A is induced by FcgammaR signaling in human monocytes and dendritic cells. *J Immunol* 178: 4033-8
120. Jin T, Guo F, Kim S, Howard A, Zhang YZ. 2007. X-ray crystal structure of TNF ligand family member TL1A at 2.1A. *Biochem Biophys Res Commun* 364: 1-6
121. Shih DQ, Kwan LY, Chavez V, Cohavy O, Gonsky R, Chang EY, Chang C, Elson CO, Targan SR. 2009. Microbial induction of inflammatory bowel disease associated gene TL1A (TNFSF15) in antigen presenting cells. *Eur J Immunol* 39: 3239-50
122. Endo K, Kinouchi Y, Kakuta Y, Ueki N, Takahashi S, Shimosegawa T. 2010. Involvement of NF-kappa B pathway in TL1A gene expression induced by lipopolysaccharide. *Cytokine* 49: 215-20
123. Meylan F, Davidson TS, Kahle E, Kinder M, Acharya K, Jankovic D, Bundoc V, Hodges M, Shevach EM, Keane-Myers A, Wang EC, Siegel RM. 2008. The TNF-family receptor DR3 is essential for diverse T cell-mediated inflammatory diseases. *Immunity* 29: 79-89
124. Takimoto T, Sato K, Akiba Y, Takahashi K. 2008. Role of chicken TL1A on inflammatory responses and partial characterization of its receptor. *J Immunol* 180: 8327-32
125. Fang L, Adkins B, Deyev V, Podack ER. 2008. Essential role of TNF receptor superfamily 25 (TNFRSF25) in the development of allergic lung inflammation. *J Exp Med* 205: 1037-48
126. Screaton GR, Xu XN, Olsen AL, Cowper AE, Tan R, McMichael AJ, Bell JI. 1997. LARD: a new lymphoid-specific death domain containing receptor regulated by alternative pre-mRNA splicing. *Proc Natl Acad Sci U S A* 94: 4615-9
127. Warzocha K, Ribeiro P, Charlot C, Renard N, Coiffier B, Salles G. 1998. A new death receptor 3 isoform: expression in human lymphoid cell lines and non-Hodgkin's lymphomas. *Biochem Biophys Res Commun* 242: 376-9
128. Wang EC, Kitson J, Thern A, Williamson J, Farrow SN, Owen MJ. 2001. Genomic structure, expression, and chromosome mapping of the mouse homologue for the WSL-1 (DR3, Apo3, TRAMP, LARD, TR3, TNFRSF12) gene. *Immunogenetics* 53: 59-63
129. Chinnaiyan AM, O'Rourke K, Yu GL, Lyons RH, Garg M, Duan DR, Xing L, Gentz R, Ni J, Dixit VM. 1996. Signal transduction by DR3, a death domain-containing receptor related to TNFR-1 and CD95. *Science* 274: 990-2
130. Al-Lamki RS, Wang J, Thiru S, Pritchard NR, Bradley JA, Pober JS, Bradley JR. 2003. Expression of silencer of death domains and death-receptor-3 in normal human kidney and in rejecting renal transplants. *Am J Pathol* 163: 401-11

131. Varfolomeev EE, Schuchmann M, Luria V, Chiannilkulchai N, Beckmann JS, Mett IL, Rebrikov D, Brodianski VM, Kemper OC, Kollet O, Lapidot T, Soffer D, Sobe T, Avraham KB, Goncharov T, Holtmann H, Lonai P, Wallach D. 1998. Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity* 9: 267-76
132. Wen L, Zhuang L, Luo X, Wei P. 2003. TL1A-induced NF-kappaB activation and c-IAP2 production prevent DR3-mediated apoptosis in TF-1 cells. *J Biol Chem* 278: 39251-8
133. Yeh WC, Pompa JL, McCurrach ME, Shu HB, Elia AJ, Shahinian A, Ng M, Wakeham A, Khoo W, Mitchell K, El-Deiry WS, Lowe SW, Goeddel DV, Mak TW. 1998. FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. *Science* 279: 1954-8
134. Kang YJ, Kim WJ, Bae HU, Kim DI, Park YB, Park JE, Kwon BS, Lee WH. 2005. Involvement of TL1A and DR3 in induction of pro-inflammatory cytokines and matrix metalloproteinase-9 in atherogenesis. *Cytokine* 29: 229-35
135. Tan KB, Harrop J, Reddy M, Young P, Terrett J, Emery J, Moore G, Truneh A. 1997. Characterization of a novel TNF-like ligand and recently described TNF ligand and TNF receptor superfamily genes and their constitutive and inducible expression in hematopoietic and non-hematopoietic cells. *Gene* 204: 35-46
136. Papadakis KA, Prehn JL, Landers C, Han Q, Luo X, Cha SC, Wei P, Targan SR. 2004. TL1A synergizes with IL-12 and IL-18 to enhance IFN-gamma production in human T cells and NK cells. *J Immunol* 172: 7002-7
137. Papadakis KA, Zhu D, Prehn JL, Landers C, Avanesyan A, Lafkas G, Targan SR. 2005. Dominant role for TL1A/DR3 pathway in IL-12 plus IL-18-induced IFN-gamma production by peripheral blood and mucosal CCR9+ T lymphocytes. *J Immunol* 174: 4985-90
138. Yang CR, Hsieh SL, Teng CM, Ho FM, Su WL, Lin WW. 2004. Soluble decoy receptor 3 induces angiogenesis by neutralization of TL1A, a cytokine belonging to tumor necrosis factor superfamily and exhibiting angiostatic action. *Cancer Res* 64: 1122-9
139. Shi G, Wu Y, Zhang J, Wu J. 2003. Death decoy receptor TR6/DcR3 inhibits T cell chemotaxis in vitro and in vivo. *J Immunol* 171: 3407-14
140. Wu Y, Han B, Sheng H, Lin M, Moore PA, Zhang J, Wu J. 2003. Clinical significance of detecting elevated serum DcR3/TR6/M68 in malignant tumor patients. *Int J Cancer* 105: 724-32
141. Ohshima K, Haraoka S, Sugihara M, Suzumiya J, Kawasaki C, Kanda M, Kikuchi M. 2000. Amplification and expression of a decoy receptor for fas ligand (DcR3) in virus (EBV or HTLV-I) associated lymphomas. *Cancer Lett* 160: 89-97
142. Hsu TL, Wu YY, Chang YC, Yang CY, Lai MZ, Su WB, Hsieh SL. 2005. Attenuation of Th1 response in decoy receptor 3 transgenic mice. *J Immunol* 175: 5135-45
143. Shi G, Mao J, Yu G, Zhang J, Wu J. 2005. Tumor vaccine based on cell surface expression of DcR3/TR6. *J Immunol* 174: 4727-35
144. Bamias G, Mishina M, Nyce M, Ross WG, Kollias G, Rivera-Nieves J, Pizarro TT, Cominelli F. 2006. Role of TL1A and its receptor DR3 in two models of chronic murine ileitis. *Proc Natl Acad Sci U S A* 103: 8441-6
145. Pappu BP, Borodovsky A, Zheng TS, Yang X, Wu P, Dong X, Weng S, Browning B, Scott ML, Ma L, Su L, Tian Q, Schneider P, Flavell RA, Dong C, Burkly LC. 2008. TL1A-DR3 interaction regulates Th17 cell function and Th17-mediated autoimmune disease. *J Exp Med* 205: 1049-62
146. Takedatsu H, Michelsen KS, Wei B, Landers CJ, Thomas LS, Dhall D, Braun J, Targan SR. 2008. TL1A (TNFSF15) regulates the development of chronic colitis by modulating both T-helper 1 and T-helper 17 activation. *Gastroenterology* 135: 552-67
147. Wu SF, Liu TM, Lin YC, Sytwu HK, Juan HF, Chen ST, Shen KL, Hsi SC, Hsieh SL. 2004. Immunomodulatory effect of decoy receptor 3 on the differentiation and function of bone

marrow-derived dendritic cells in nonobese diabetic mice: from regulatory mechanism to clinical implication. *J Leukoc Biol* 75: 293-306

148. Kayamuro H, Yoshioka Y, Abe Y, Katayama K, Yoshida T, Yamashita K, Yoshikawa T, Hiroi T, Itoh N, Kawai Y, Mayumi T, Kamada H, Tsunoda S, Tsutsumi Y. 2009. TNF superfamily member, TL1A, is a potential mucosal vaccine adjuvant. *Biochem Biophys Res Commun* 384: 296-300
149. Kamada N, Hisamatsu T, Honda H, Kobayashi T, Chinen H, Takayama T, Kitazume MT, Okamoto S, Koganei K, Sugita A, Kanai T, Hibi T. 2010. TL1A produced by lamina propria macrophages induces Th1 and Th17 immune responses in cooperation with IL-23 in patients with Crohn's disease. *Inflamm Bowel Dis* 16: 568-75
150. Heidemann SC, Chavez V, Landers CJ, Kucharzik T, Prehn JL, Targan SR. 2010. TL1A Selectively Enhances IL-12/IL-18-Induced NK Cell Cytotoxicity against NK-Resistant Tumor Targets. *J Clin Immunol*
151. Hsu TL, Chang YC, Chen SJ, Liu YJ, Chiu AW, Chio CC, Chen L, Hsieh SL. 2002. Modulation of dendritic cell differentiation and maturation by decoy receptor 3. *J Immunol* 168: 4846-53
152. Su WB, Chang YH, Lin WW, Hsieh SL. 2006. Differential regulation of interleukin-8 gene transcription by death receptor 3 (DR3) and type I TNF receptor (TNFRI). *Exp Cell Res* 312: 266-77
153. Michelsen KS, Thomas LS, Taylor KD, Yu QT, Mei L, Landers CJ, Derkowski C, McGovern DP, Rotter JI, Targan SR. 2009. IBD-associated TL1A gene (TNFSF15) haplotypes determine increased expression of TL1A protein. *PLoS One* 4: e4719
154. Bamias G, Siakavellas SI, Stamatelopoulos KS, Chryssochoou E, Papamichael C, Sfikakis PP. 2008. Circulating levels of TNF-like cytokine 1A (TL1A) and its decoy receptor 3 (DcR3) in rheumatoid arthritis. *Clin Immunol* 129: 249-55
155. Zhang J, Wang X, Fahmi H, Wojcik S, Fikes J, Yu Y, Wu J, Luo H. 2009. Role of TL1A in the pathogenesis of rheumatoid arthritis. *J Immunol* 183: 5350-7
156. McLaren JE, Calder CJ, McSharry BP, Sexton K, Salter RC, Singh NN, Wilkinson GW, Wang EC, Ramji DP. 2010. The TNF-like protein 1A-death receptor 3 pathway promotes macrophage foam cell formation in vitro. *J Immunol* 184: 5827-34
157. Iwasaki A, Medzhitov R. 2004. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 5: 987-95
158. Akira S, Takeda K. 2004. Toll-like receptor signalling. *Nat Rev Immunol* 4: 499-511
159. Gross O, Gewies A, Finger K, Schafer M, Sparwasser T, Peschel C, Forster I, Ruland J. 2006. Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature* 442: 651-6
160. Rogers NC, Slack EC, Edwards AD, Nolte MA, Schulz O, Schweighoffer E, Williams DL, Gordon S, Tybulewicz VL, Brown GD, Reis e Sousa C. 2005. Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity* 22: 507-17
161. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282: 2085-8
162. Giuntoli RL, 2nd, Lu J, Kobayashi H, Kennedy R, Celis E. 2002. Direct costimulation of tumor-reactive CTL by helper T cells potentiate their proliferation, survival, and effector function. *Clin Cancer Res* 8: 922-31
163. Beutler B. 2004. Toll-like receptors and their place in immunology. Where does the immune response to infection begin? *Nat Rev Immunol* 4: 498
164. Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. 2007. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. *Science* 316: 1628-32

165. Yoneyama M, Suhara W, Fukuhara Y, Fukuda M, Nishida E, Fujita T. 1998. Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J* 17: 1087-95
166. Servant MJ, Grandvaux N, tenOever BR, Duguay D, Lin R, Hiscott J. 2003. Identification of the minimal phosphoacceptor site required for in vivo activation of interferon regulatory factor 3 in response to virus and double-stranded RNA. *J Biol Chem* 278: 9441-7
167. Dudley ME, Gross CA, Langhan MM, Garcia MR, Sherry RM, Yang JC, Phan GQ, Kammula US, Hughes MS, Citrin DE, Restifo NP, Wunderlich J, Prieto PA, Hong JJ, Langan RC, Zlott DA, Morton KE, White DE, Laurencot C, Rosenberg SA. 2010. CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. *Clin Cancer Res*
168. Prieto PA, Durflinger KH, Wunderlich JR, Rosenberg SA, Dudley ME. 2010. Enrichment of CD8+ cells from melanoma tumor-infiltrating lymphocyte cultures reveals tumor reactivity for use in adoptive cell therapy. *J Immunother* 33: 547-56
169. Hanson HL, Donermeyer DL, Ikeda H, White JM, Shankaran V, Old LJ, Shiku H, Schreiber RD, Allen PM. 2000. Eradication of established tumors by CD8+ T cell adoptive immunotherapy. *Immunity* 13: 265-76
170. Quezada SA, Simpson TR, Peggs KS, Merghoub T, Vider J, Fan X, Blasberg R, Yagita H, Muranski P, Antony PA, Restifo NP, Allison JP. 2010. Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *J Exp Med* 207: 637-50
171. Xie Y, Akpinarli A, Maris C, Hipkiss EL, Lane M, Kwon EK, Muranski P, Restifo NP, Antony PA. 2010. Naive tumor-specific CD4(+) T cells differentiated in vivo eradicate established melanoma. *J Exp Med* 207: 651-67
172. Cheent K, Khakoo SI. 2009. Natural killer cells: integrating diversity with function. *Immunology* 126: 449-57
173. Slifka MK, Pagarigan RR, Whitton JL. 2000. NK markers are expressed on a high percentage of virus-specific CD8+ and CD4+ T cells. *J Immunol* 164: 2009-15
174. Kosaka A, Wakita D, Matsubara N, Togashi Y, Nishimura S, Kitamura H, Nishimura T. 2007. AsialoGM1+CD8+ central memory-type T cells in unimmunized mice as novel immunomodulator of IFN-gamma-dependent type 1 immunity. *Int Immunol* 19: 249-56
175. Newman KC, Riley EM. 2007. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. *Nat Rev Immunol* 7: 279-91
176. Malek TR. 2008. The biology of interleukin-2. *Annu Rev Immunol* 26: 453-79
177. Li Y, Hofmann M, Wang Q, Teng L, Chlewicki LK, Pircher H, Mariuzza RA. 2009. Structure of natural killer cell receptor KLRG1 bound to E-cadherin reveals basis for MHC-independent missing self recognition. *Immunity* 31: 35-46
178. Kundig TM, Schorle H, Bachmann MF, Hengartner H, Zinkernagel RM, Horak I. 1993. Immune responses in interleukin-2-deficient mice. *Science* 262: 1059-61
179. Liu K, Li Y, Prabhu V, Young L, Becker KG, Munson PJ, Weng N. 2001. Augmentation in expression of activation-induced genes differentiates memory from naive CD4+ T cells and is a molecular mechanism for enhanced cellular response of memory CD4+ T cells. *J Immunol* 166: 7335-44
180. Steiger J, Nickerson PW, Steurer W, Moscovitch-Lopatin M, Strom TB. 1995. IL-2 knockout recipient mice reject islet cell allografts. *J Immunol* 155: 489-98
181. Tsunobuchi H, Nishimura H, Goshima F, Daikoku T, Nishiyama Y, Yoshikai Y. 2000. Memory-type CD8+ T cells protect IL-2 receptor alpha-deficient mice from systemic infection with herpes simplex virus type 2. *J Immunol* 165: 4552-60
182. Obar JJ, Molloy MJ, Jellison ER, Stoklasek TA, Zhang W, Usherwood EJ, Lefrancois L. 2010. CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. *Proc Natl Acad Sci U S A* 107: 193-8

183. Malek TR, Porter BO, Codias EK, Scibelli P, Yu A. 2000. Normal lymphoid homeostasis and lack of lethal autoimmunity in mice containing mature T cells with severely impaired IL-2 receptors. *J Immunol* 164: 2905-14
184. Pipkin ME, Sacks JA, Cruz-Guilloty F, Lichtenheld MG, Bevan MJ, Rao A. 2010. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity* 32: 79-90
185. Zhang J, Scordi I, Smyth MJ, Lichtenheld MG. 1999. Interleukin 2 receptor signaling regulates the perforin gene through signal transducer and activator of transcription (Stat)5 activation of two enhancers. *J Exp Med* 190: 1297-308
186. Podolsky DK. 2002. Inflammatory bowel disease. *N Engl J Med* 347: 417-29
187. Brand S. 2009. Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease. *Gut* 58: 1152-67
188. Zhang Z, Zheng M, Bindas J, Schwarzenberger P, Kolls JK. 2006. Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. *Inflamm Bowel Dis* 12: 382-8
189. Ogawa A, Andoh A, Araki Y, Bamba T, Fujiyama Y. 2004. Neutralization of interleukin-17 aggravates dextran sulfate sodium-induced colitis in mice. *Clin Immunol* 110: 55-62
190. Manocha M, Rietdijk S, Laouar A, Liao G, Bhan A, Borst J, Terhorst C, Manjunath N. 2009. Blocking CD27-CD70 costimulatory pathway suppresses experimental colitis. *J Immunol* 183: 270-6
191. Baaten BJ, Li CR, Deiro MF, Lin MM, Linton PJ, Bradley LM. 2010. CD44 regulates survival and memory development in Th1 cells. *Immunity* 32: 104-15
192. McKenzie GJ, Bancroft A, Grencis RK, McKenzie AN. 1998. A distinct role for interleukin-13 in Th2-cell-mediated immune responses. *Curr Biol* 8: 339-42
193. Chen Y, Thai P, Zhao YH, Ho YS, DeSouza MM, Wu R. 2003. Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. *J Biol Chem* 278: 17036-43
194. Obar JJ, Lefrancois L. 2010. Memory CD8+ T cell differentiation. *Ann N Y Acad Sci* 1183: 251-66
195. Minty A, Asselin S, Bensussan A, Shire D, Vita N, Vyakarnam A, Wijdenes J, Ferrara P, Caput D. 1997. The related cytokines interleukin-13 and interleukin-4 are distinguished by differential production and differential effects on T lymphocytes. *Eur Cytokine Netw* 8: 203-13
196. Ramalingam TR, Pesce JT, Sheikh F, Cheever AW, Mentink-Kane MM, Wilson MS, Stevens S, Valenzuela DM, Murphy AJ, Yancopoulos GD, Urban JF, Jr., Donnelly RP, Wynn TA. 2008. Unique functions of the type II interleukin 4 receptor identified in mice lacking the interleukin 13 receptor alpha1 chain. *Nat Immunol* 9: 25-33
197. Herbert DR, Yang JQ, Hogan SP, Groschwitz K, Khodoun M, Munitz A, Orekov T, Perkins C, Wang Q, Brombacher F, Urban JF, Jr., Rothenberg ME, Finkelman FD. 2009. Intestinal epithelial cell secretion of RELM-beta protects against gastrointestinal worm infection. *J Exp Med* 206: 2947-57
198. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, Elias JA. 1999. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 103: 779-88
199. Singer M, Lefort J, Vargaftig BB. 2002. Granulocyte depletion and dexamethasone differentially modulate airways hyperreactivity, inflammation, mucus accumulation, and secretion induced by rmIL-13 or antigen. *Am J Respir Cell Mol Biol* 26: 74-84
200. Zuhdi Alimam M, Piazza FM, Selby DM, Letwin N, Huang L, Rose MC. 2000. Muc-5/5ac mucin messenger RNA and protein expression is a marker of goblet cell metaplasia in murine airways. *Am J Respir Cell Mol Biol* 22: 253-60

201. Lukacs NW, Smit JJ, Mukherjee S, Morris SB, Nunez G, Lindell DM. 2010. Respiratory virus-induced TLR7 activation controls IL-17-associated increased mucus via IL-23 regulation. *J Immunol* 185: 2231-9
202. Sawicki W. 2008. *Histologia*. Warsaw: Wydawnictwo Lekarskie PZWL
203. Hasnain SZ, Wang H, Ghia JE, Haq N, Deng Y, Velcich A, Grencis RK, Thornton DJ, Khan WI. 2010. Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection. *Gastroenterology* 138: 1763-71
204. Nair MG, Guild KJ, Du Y, Zaph C, Yancopoulos GD, Valenzuela DM, Murphy A, Stevens S, Karow M, Artis D. 2008. Goblet cell-derived resistin-like molecule beta augments CD4+ T cell production of IFN-gamma and infection-induced intestinal inflammation. *J Immunol* 181: 4709-15
205. Steppan CM, Brown EJ, Wright CM, Bhat S, Banerjee RR, Dai CY, Enders GH, Silberg DG, Wen X, Wu GD, Lazar MA. 2001. A family of tissue-specific resistin-like molecules. *Proc Natl Acad Sci U S A* 98: 502-6
206. Barnes SL, Vidrich A, Wang ML, Wu GD, Cominelli F, Rivera-Nieves J, Bamias G, Cohn SM. 2007. Resistin-like molecule beta (RELMbeta/FIZZ2) is highly expressed in the ileum of SAMP1/YitFc mice and is associated with initiation of ileitis. *J Immunol* 179: 7012-20
207. Artis D, Wang ML, Keilbaugh SA, He W, Brenes M, Swain GP, Knight PA, Donaldson DD, Lazar MA, Miller HR, Schad GA, Scott P, Wu GD. 2004. RELMbeta/FIZZ2 is a goblet cell-specific immune-effector molecule in the gastrointestinal tract. *Proc Natl Acad Sci U S A* 101: 13596-600
208. Gersemann M, Becker S, Kubler I, Koslowski M, Wang G, Herrlinger KR, Griger J, Fritz P, Fellermann K, Schwab M, Wehkamp J, Stange EF. 2009. Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis. *Differentiation* 77: 84-94
209. Ardesjo B, Portela-Gomes GM, Rorsman F, Gerdin E, Loof L, Grimelius L, Kampe O, Ekwall O. 2008. Immunoreactivity against Goblet cells in patients with inflammatory bowel disease. *Inflamm Bowel Dis* 14: 652-61
210. O'Connor W, Jr., Kamanaka M, Booth CJ, Town T, Nakae S, Iwakura Y, Kolls JK, Flavell RA. 2009. A protective function for interleukin 17A in T cell-mediated intestinal inflammation. *Nat Immunol* 10: 603-9
211. Shih DQ, Barrett R, Zhang X, Yeager N, Koon HW, Phaosawasdi P, Song Y, Ko B, Wong MH, Michelsen KS, Martins G, Pothoulakis C, Targan SR. 2011. Constitutive TL1A (TNFSF15) expression on lymphoid or myeloid cells leads to mild intestinal inflammation and fibrosis. *PLoS One* 6: e16090
212. Meylan F, Song YJ, Fuss I, Villarreal S, Kahle E, Malm IJ, Acharya K, Ramos HL, Lo L, Mentink-Kane MM, Wynn TA, Migone TS, Strober W, Siegel RM. 2011. The TNF-family cytokine TL1A drives IL-13-dependent small intestinal inflammation. *Mucosal Immunol* 4: 172-85
213. Kullberg MC, Hay V, Cheever AW, Mamura M, Sher A, Letterio JJ, Shevach EM, Piccirillo CA. 2005. TGF-beta1 production by CD4+ CD25+ regulatory T cells is not essential for suppression of intestinal inflammation. *Eur J Immunol* 35: 2886-95
214. Taraban VY, Slebioda TJ, Willoughby JE, Buchan SL, James S, Sheth B, Smyth NR, Thomas GJ, Wang EC, Al-Shamkhani A. 2011. Sustained TL1A expression modulates effector and regulatory T-cell responses and drives intestinal goblet cell hyperplasia. *Mucosal Immunol* 4: 186-96
215. Croft M. 2003. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat Rev Immunol* 3: 609-20
216. Mauri DN, Ebner R, Montgomery RI, Kochel KD, Cheung TC, Yu GL, Ruben S, Murphy M, Eisenberg RJ, Cohen GH, Spear PG, Ware CF. 1998. LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. *Immunity* 8: 21-30

217. Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, Ashkenazi A. 2000. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity* 12: 611-20
218. van Olffen RW, Koning N, van Gisbergen KP, Wensveen FM, Hoek RM, Boon L, Hamann J, van Lier RA, Nolte MA. 2009. GITR triggering induces expansion of both effector and regulatory CD4+ T cells in vivo. *J Immunol* 182: 7490-500
219. Wang Y, Zhu M, Yu P, Fu YX. 2010. Promoting immune responses by LIGHT in the face of abundant regulatory T cell inhibition. *J Immunol* 184: 1589-95
220. Kontoyiannis D, Boulougouris G, Manoloukos M, Armaka M, Apostolaki M, Pizarro T, Kotlyarov A, Forster I, Flavell R, Gaestel M, Tsichlis P, Cominelli F, Kollias G. 2002. Genetic dissection of the cellular pathways and signaling mechanisms in modeled tumor necrosis factor-induced Crohn's-like inflammatory bowel disease. *J Exp Med* 196: 1563-74
221. Shaikh RB, Santee S, Granger SW, Butrovich K, Cheung T, Kronenberg M, Cheroutre H, Ware CF. 2001. Constitutive expression of LIGHT on T cells leads to lymphocyte activation, inflammation, and tissue destruction. *J Immunol* 167: 6330-7
222. Murata K, Nose M, Ndhlovu LC, Sato T, Sugamura K, Ishii N. 2002. Constitutive OX40/OX40 ligand interaction induces autoimmune-like diseases. *J Immunol* 169: 4628-36
223. Stone GW, Barzee S, Snarsky V, Kee K, Spina CA, Yu XF, Kornbluth RS. 2006. Multimeric soluble CD40 ligand and GITR ligand as adjuvants for human immunodeficiency virus DNA vaccines. *J Virol* 80: 1762-72
224. Liu J, Ngai N, Stone GW, Yue FY, Ostrowski MA. 2009. The adjuvancy of OX40 ligand (CD252) on an HIV-1 canarypox vaccine. *Vaccine* 27: 5077-84
225. Gomez-Garcia M, Oliver J, Marquez A, Mendoza JL, Lopez-Nevot MA, Fernandez-Arquero M, Gonzalez-Escribano MF, Diaz-Rubio M, de la Concha EG, Urcelay E, Martin J, Martinez A. 2007. Strong protective effect of DR3 against ulcerative colitis in the Spanish population. *Am J Gastroenterol* 102: 2762-6