Antigen processing and immune regulation in the response to tumours

Short title: Antigen processing in tumours

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1. Introduction

The host immune system plays a fundamental role in the elimination as well as the progression and maintenance of cancer. Control and prevention of tumour growth relies on immunosurveillance mechanisms by innate and adaptive immune responses, involving macrophages, NK cells, IFN-γ secretion and CD8+ cytotoxic T lymphocytes (CTL), to eliminate transformed cells. However, the selective pressure of the evolving tumour microenvironment (TME) may alter the immune response, allowing transformed cells to survive undetected despite the presence of immune cells, supporting progression into solid tumours (1). Targeted immune evasion strategies occur through multiple mechanisms and are not exclusive to individual cells or tumour type. One of the main characteristics utilised by tumour cells is a reduction in immune recognition through targeting antigen processing and presentation (APP). This review will focus on the adaptation of antigen processing to support tumour growth and immune evasion, and the role of the tumour microenvironment in response to this altered antigen presentation.

2. MHC class I antigen processing and presentation in tumour immune escape

Endogenous cellular antigens are displayed to CTL through presentation as part of the major histocompatibility class I (MHC I) complex on the surface of all nucleated cells, including malignant cells. This presentation of peptide:MHC I (pMHC I) complexes provides a snapshot of the intracellular

environment to circulating CTL. Activation of CTL occurs in response to the detection of ‘foreign’ antigens, such as those derived from virus or bacteria, and results in the destruction of the presenting cell. Therefore, the effective immune targeting of tumour cells relies on a tightly regulated, effective APP pathway to display tumour antigens (tAgs) allowing the identification of malignant cells. Indeed, defects and/or alterations in expression levels of components of this pathway are common mechanisms utilised by transformed cells to reduce visibility to CTL, a strategy that proves highly detrimental to the host (2).

The expression of cell surface pMHC I involves processing and presentation of antigens. Many studies have reported the loss or downregulation of cell surface pMHC I in a vast array of tumours of different origins, revealing associations with progression of disease, level of tumour infiltrating lymphocytes (TIL), and overall survival (3-11). The events that lead to reduced pMHC I may be genetic (mutations and gene deletions) or regulatory (loss or reduction in transcription). These effects can be i) directly on MHC I genes, ii) as a result of defects in peptide generation, targeting immunoproteasomal components LMP2, LMP7 and LMP10 and the endoplasmic reticulum aminopeptidases (ERAP1/2), or iii) in peptide transport or loading of MHC I molecules, involving the transporter associated with antigen processing (TAP), chaperones calnexin (CNX), calreticulin (CRT) and ERp57, and the peptide editor tapasin (Tpn, figure 1) (2). These alterations have been most commonly documented in melanoma, cervical, colorectal, gastric, head and neck squamous cell carcinoma (HNSCC), renal cell

carcinoma (RCC), breast, prostate and ovarian cancers (4, 5, 7, 12-17). The precise alterations and their consequence are discussed below.

2.1. Antigen processing

MHC I molecules present a diverse array of peptides at the cell surface. These peptides originate from both functional proteins involved in a number of cellular processes and defective ribosomal products (DRiPs) that arise from defective protein synthesis and are rapidly degraded within the cytosol. The peptide binding groove formed within the α1 and α2 domains of MHC I heavy chain (HC) is highly polymorphic in order to accommodate a vast number of peptides. To ensure high affinity binding into the peptide binding groove of MHC I, peptides are typically cleaved to 8-11 amino acids in length and contain specific amino acid properties at anchor residues (position2/5 and C-terminal amino acids). In order to generate optimal peptide antigens, two key processing events exist within the APP pathway. The initial peptide proteolysis occurs within the cytosol of the cell and degrades larger protein fragments into smaller peptides by the proteasome/immunoproteasome (figure 1). This processing event is often responsible for generating the final C-terminal residue of peptides that bind to MHC I. Upon entering the ER, the majority of peptides require further ‘fine-tuning’ through processing of the N-terminal region by ERAP1/2 prior to its association with MHC I (figure 1). Whilst these two processing events occur independently, defects in either one or both results in a significant alteration in the ability to produce stable pMHC I complexes that reach the cell surface. In turn, the orchestration of a sufficient

immune response, where necessary, becomes impaired and therefore indicates a successful immune escape mechanism for tumour growth.

2.1.1. Proteasome and immunoproteasome

The proteasome, a multicatalytic enzyme residing within the cytosol, is responsible for the turnover of the majority of cellular proteins and has two major roles; i) to regulate cellular protein homeostasis through the ubiquitin-proteasome system, preventing the aggregation of misfolded proteins and ii) to generate peptide antigens for MHC I presentation at the cell surface (18-20). Three catalytic subunits, β1, β2 and β5, are responsible for the proteolytic activity of the 20S subunit core of the proteasome. However, in non-malignant cells, inflammatory cytokines, such as IFN-γ, promotes an upregulation of specific catalytic subunits; LMP2, LMP7, and LMP10, incorporated into the 20S proteasome in place of β1, β2 and β5 subunits (21-23). These alterations result in the increased generation of antigenic peptides, favouring cleavage after hydrophobic residues, providing the optimal C-terminal anchor residue for stable binding to MHC I. The deletion or reduction of these immunoproteasome subunits significantly impacts the quantity of suitable peptides available, reducing pMHC I expression by up to 50%, and is exploited by a large number of tumours to evade the immune response (24, 25). If observed at an early stage, immunoproteasome downregulation is associated with an increase in both metastasis and disease recurrence (26). Furthermore, single nucleotide polymorphisms (SNPs) in LMP2 and LMP7 are

associated with a worse overall survival in cervical carcinoma, most likely as a consequence of altered protein function (17, 27),

These associations suggest that restoring immunoproteasome expression by IFN-γ would be therapeutically beneficial. Modulation of proteasomal function is being used in the clinic, however, these drugs inhibit proteasome function to prevent destruction of pro-apoptotic proteins and therefore may serve to promote immune evasion in any surviving tumour cells (28).

2.1.2. Endoplasmic reticulum aminopeptidases

Peptides generated in the cytosol are transported through TAP into the ER. As TAP preferentially transports peptides of 11-14 amino acids, peptides are often too long for stable binding to MHC I and require further processing. ERAP1 and ERAP2 serve as editors of the peptide repertoire by trimming N-terminal extensions of antigenic peptides, creating a pool of peptides with high affinity for MHC I binding (29-32). Interestingly, loss of ERAP1 results in qualitative and quantitative changes in pMHC I reducing expression by up to 50% dependent on the allele (33-35). With its role in editing the presented peptide repertoire, tumours target ERAP1 by altering expression at both the transcriptional and post-transcriptional level (36, 37). Interestingly, the ERAP1 expression is highly variable in tumours being both increased and decreased, however, total loss of expression is not common (37). Why expression varies so much in tumours is not known, but we have shown that ERAP1 destroys the tAg GSW11 in the murine tumour model CT26 and reducing ERAP1

expression increased GSW11 generation leading to CTL-mediated tumour protection (38). An increase in ERAP1 expression may therefore allow immune evasion in some tumours through the destruction of antigenic peptides. Consistent with this, increased ERAP1 expression was observed in colorectal adenocarcinoma (39). By contrast, a loss of ERAP1 expression, associated with a poorer prognosis and overall survival in cervical carcinoma, may restrict the ability to generate tAgs and reduce pMHC I expression (7, 40).

SNPs in ERAP1 have been associated with poorer prognosis and greater disease progression in HPV+ cervical carcinoma (40). A combination of SNPs in ERAP1, TAP2 and LMP7 is associated with an increased risk of developing cervical carcinoma (40, 41). In addition, the presence of a homozygous ERAP1 haplotype (ERAP1-56 major and ERAP-127 minor) correlates with a significantly worse overall survival, highlighting ERAP1 as an independent predictor of survival (40). The functional consequences of these SNPs are currently unknown, however, other ERAP1 SNPs that exist in multiple combinations as discrete allotypes significantly alter function and, since they are co-dominantly expressed, the combination of allotypes also affects overall function (35, 42). These differences in ERAP1 function may alter the ability to generate tAgs and reduce anti-tumour CTL responses, suggesting a significant role of ERAP1 in the control of tumours. Furthermore, reduced function may serve to reduce pMHC I expression as observed in cervical carcinoma and head and neck squamous cell carcinoma cases (3, 7).

Therefore modulating ERAP1 activity through inhibitors may provide a unique and novel tool to induce protective anti-tumour CTL responses (43).

2.2. Antigen presentation

The presentation of optimal high affinity peptide antigens depends on a sequence of crucial steps involving peptide transport, folding and loading onto MHC I. The events that result in successful antigen presentation require transport of peptides from the cytosol into the ER through the TAP heterodimer. This transport provides a critical supply of peptides into the ER for further processing and loading onto MHC I. Within the ER lumen, the association of immature HC and β_2m is orchestrated by the chaperones CNX, BiP and CRT and is required for stable folding of HC before association and formation of the macromolecular peptide loading complex (PLC) comprised of; TAP, Tpn, ERp57 and MHC I (figure 1). The formation of the PLC is a fundamental final step in loading peptides onto the MHC I. Optimal peptides are selected and edited by Tpn to ensure the best available peptides are loaded and most stable pMHC I complex is formed, resulting in dissociation from the PLC and transit to the cell surface via the golgi apparatus.
These events involved in antigen presentation act together to allow stable expression of pMHC I at the cell surface. Alterations in any part of this process, either by mutation or altered expression, results in defective protein presentation and often results in a reduction of cell surface pMHC I complexes, abrogating any immune response required. Similarly to alterations

in antigen processing, tumours utilise the tightly regulated nature of antigen presentation to avoid immune targeting.

2.2.1 Transporter associated with antigen processing

TAP, consisting of two ATP-hydrolysing subunits (TAP1 and TAP2), efficiently transports peptides into the ER and facilitates peptide loading onto MHC I as part of the PLC (44). Loss or downregulation of TAP1 and/or TAP2 has been documented in a variety of cell lines and primary tumours and correlates with a reduction in pMHC I expression (45, 46). Interestingly, in colorectal carcinoma the loss of TAP expression could be classified into three phenotypes; i) synchronous loss of expression of TAP, β_2m and MHC I, ii) loss of MHC I and β_2m but expression of TAP1 and iii) loss of TAP only, however this resulted in the loss of detectable pMHC I complex, suggesting TAP is essential for the formation of stable pMHC I complexes (47). Therefore, variations in TAP expression or function can impact on pMHC I expression by significantly altering the quantity of peptides available for MHC I loading in the ER (48, 49).

2.2.2 Chaperones

ER-resident chaperones aid the folding and association of immature HC and β_2m before association with the PLC. CRT and CNX are involved in a vast number of cellular processes including APP. Altered expression of CRT and CNX has been linked with a number of tumour types; bladder, prostate, hepatocellular carcinoma, oesophageal, colon, cervical, breast, melanoma

and leukaemia, although, most of these associations are due to other cellular functions; cell cycle regulation (down), cell migration and adhesion (up), and ER stress (up) (2, 50, 51). CRT exposure at the cell surface promotes phagocytosis and engulfment by macrophages, resulting in destruction of tumour cells by immunogenic cell death (52). Interestingly, like CRT, the additional cellular role of CNX in ER stress has been implicated in cancer, with an increase in CNX expression linked with poor prognosis in colorectal cancer (53), and shown to be a diagnostic marker in lung cancer (54). The reduced expression of the thiol oxidoreductase, ERp57, that aids the formation of disulphide bonds to form the PLC, was shown to be an independent predictor of overall survival in cervical carcinoma (55) and both the reduction and loss of ERp57 are correlated with progression of gastric cancer (56).

2.2.3. Tapasin

Tpn serves as a bridge between MHC I, TAP and CRT and is essential for ERp57 association to form a stable PLC. Tpn, as part of the PLC, has a unique role in peptide editing of MHC I bound peptides, by facilitating the release of sub-optimal fast off-rate peptides to enable optimal slow-off rate peptides to bind and form a stable pMHC I molecule (57). This quality control feature of Tpn is essential and influences the peptide repertoire; more stable peptides with a longer half-life are favoured for presentation (58). As such, Tpn deficiency has severe effects on most MHC I alleles, resulting in fewer stable pMHC I expressed at the cell surface (57, 59)

Heterogenous expression of Tpn levels, but an overall downregulation, is observed in comparison to normal controls in RCC, colon carcinoma, small cell lung carcinoma, HNSCC, and pancreatic carcinoma cells (2). Levels of Tpn directly correlate with pMHC I expression, suggesting Tpn may contribute to the immune escape phenotype of these tumours (60). More recently, a reduction in Tpn (down-regulated in 48% cases) was correlated not only with prognosis but also levels of CTL responses in colorectal carcinoma; high levels Tpn expression correlated with high CD8+ infiltrate, independent of pMHC I levels (61). The association of loss of Tpn function with clinical progression of disease is observed in melanoma, with advanced stages correlating with a significant downregulation (62). Interestingly, restoring Tpn in murine lung carcinoma (CMT.64) restored MHC and increased the CD8+, CD4+ and CD11C+ infiltrate to the tumour site (63). In addition, when both Tpn and TAP function were restored, a significantly greater protective response was generated, suggesting that multiple aspects of the APP pathway need to function in order to generate a maximal response (63). Expression of Tpn is not always associated with a positive outcome as increased Tpn expression in the murine pancreatic tumour cell line, Panc02, reduced the presentation of an immunodominant tAg resulting in lack of immune recognition (64). Therefore, the activity of Tpn in tumours is able to carefully modulate both the quality and quantity of tAgs displayed by MHC I.

There is a vast array of evidence that demonstrates alterations within the APP pathway among most tumour types. These encompass almost all the

components of the APP and whilst inducing down regulation of MHC I they also alter the repertoire of antigenic peptides presented to CTL. Interestingly, IFN-γ stimulation is able to restore expression of APM in cancer cell lines, suggesting that targeting these components and modulating their expression may provide immunotherapeutic strategies to increase the visibility of cancerous cells and allow recruitment of specialised immune cells to target and eliminate cancer (65).

3. MHC II antigen processing and presentation in tumour immune escape

In normal cellular environments, classical major histocompatibility complex class II (MHC II; HLA-DR, -DQ, -DP) are only expressed on ‘professional’ antigen presenting cells (APCs) such as dendritic cells (DC) or macrophages. Exogenous antigens that are internalised by phagocytosis are primarily presented on MHC II to CD4+ T cells, however a small subset of cytosolic antigens are expressed on MHC II as a result of autophagy (66). MHC II are synthesised in the ER, where they bind invariant chain (Ii) that occupies the peptide binding groove to prevent aggregation of proteins and aberrant peptide binding (67). The Ii facilitates MHC II transportation to endosomes where it is degraded by proteases such as cathepsins; leaving class II associated invariant chain (CLIP) bound (68, 69). The non-classical HLA-DM molecule mediates the exchange of CLIP with optimal peptides, where function is attenuated by HLA-DO, before the pMHC II is expressed at the cell surface. Many tumour cells do not express pMHC II and therefore the

involvement and activation of CD4+ T cells relies on infiltrating APCs that engulf tumour cells or internalise tAgs.

In MHC II positive tumours, expression usually correlates with Ii expression, however in a number of cancers (breast, B-CLL and colorectal) discordant expression has been observed (70-72). Specifically in breast cancer, HLA-DR and Ii are more strongly upregulated than HLA-DP or DQ; the prognostic significance of this remains unclear however the particular expression phenotype HLA-DR+/Ii+/HLA-DM+ resulted in better overall survival (72, 73). In leukaemia, a reduction in pMHC II cell surface expression correlated with genomic instability at the class II transactivator, a gene involved in regulation of MHC II APM components (74). In addition, modifications of endosomes and downregulation of cellular processes such as autophagy has a significant impact on the generation and presentation of tAgs (75). Similar to MHC I, defects of the MHC II APP pathway is likely to significantly alter the peptide repertoire and the expression of pMHC II at the cell surface, affecting the anti-tumour response and serving as an effective immune evasion strategy.

4. Tumour microenvironment

The TME is a complex and dynamic interaction between tumour cells and cells of the surrounding stromal tissue. The stroma is formed from a heterogeneous population of cells including immune cells, fibroblasts and endothelial cells, and evolves to support the establishment and progression of the tumour. Cancer associated fibroblasts (CAF) are one of the most abundant populations of cells surrounding the tumour and promote tumour

invasion and metastasis through matrix remodelling and angiogenesis (76). CAFs also have the potential to impact on immune cell recruitment and maturation, contributing to the immunosuppressive nature of the TME.

Immune cells are an essential component of the TME and the positive effect of immune surveillance is demonstrated by the correlation between level of TILs at the site of the tumour and overall prognosis. The composition of TILs can vary depending on the site and origin of the tumour; high levels of CD8+ CTL in TILs is often a predictor for better prognosis and has been demonstrated in a number of different tumour types; breast, colorectal, ovarian, oral squamous cells carcinoma, oesophageal and melanoma (3, 61, 77, 78) In addition, infiltration of NK cells to the tumour site has positively correlated with survival in colorectal carcinoma (79). However, as highlighted in this review, the presentation of tAgs by MHC I on tumour cells is a clear and effective target for immune evasion (figure 2).

CTL are also involved in antigen presentation, through trogocytosis, where CTL extract pMHC I from the plasma membrane of APCs and present them to other CTL, initiating a cascade of activation. Interestingly, this activation may result in CTL elimination through fratricide (killing) and as such, may play a role in autoimmunity (80). Although little is known about the role of trogocytosis and fratricide in the tumour environment, tumour cells have been shown to donate membrane fragments containing tAgs to CTL, with the level of trogocytosis correlating with the anti-tumour reactivity generated by CTL clones (81). In melanoma, trogocytosis led to melanoma derived tAgs being presented on melanoma specific CTL to both fraternal CTL and those with different TCR specificities. The resulting effect was activation of effector CTL,

but also CTL fratricide (82). These findings suggest an alternative route of tAg presentation in addition to the direct (tumour cells) and indirect (professional APCs) antigen presentation in the TME. However, this route of antigen presentation may prove detrimental to the host, as tAg presenting CTL become a target for fratricide by other reactive CTL. This was demonstrated when HLA-specific fratricide occurred in CTL expressing a transgenic T cell receptor for survivin, an apoptosis inhibitor protein expressed on many tumour cells (83). This HLA-specific mediated CTL fratricide suggests caution must be observed when using cancer vaccines generated towards specific proteins that can be expressed on both tumour cells and CTL. Nonetheless, this route of antigen presentation may amplify the efficacy of CTL activation cascade; providing another target for antigen specific immune therapy.

DCs and macrophages bridge the gap between innate and adaptive immune responses to the tumour and are found abundantly in the TME and surrounding lymph nodes (LN). DCs are highly specialised in antigen presentation, both classical MHC II and cross-presentation of internalised tAg on MHC I, and are required for activation of naïve T cells in the tumour-draining LN. Immature DCs (iDCs) infiltrating the tumour site express low levels of MHC I, MHC II and co-stimulatory molecules and are unable to efficiently active T cells. Upon tAg encounter, iDCs mature to up-regulate MHC I, MHC II and co-stimulatory molecules, and migrate to the LN where they present the tAgs to CD4+(classical) or CTL (cross-presentation). Although the role of DC in tumours is not well characterised, their presence has been well documented; DC infiltration in primary tumours has been

associated with better survival and reduced incidence of metastatic lesions in HNSCC, bladder, gastric and lung cancers (84) The immunosuppressive nature of the TME often supresses DC maturation, resulting in recruitment and infiltration of immature and functionally impaired DCs. These tumour associated DCs (tDCs) suppress endocytic activity and the reduction in the ability to internalise protein renders them as non-functional APCs, resulting in lack of activation of T cell response in LN and poor peripheral effector cell accumulation (85). Since most cancerous tissue do not express MHC II, stimulation of CD4+ cells at these sites depend on infiltrating DCs. The maturation of DCs to successful present tAgs is fundamental for the peripheral T cell responses. IFN-γ stimulation has been shown to upregulate MHC I expression at the cell surface as a direct effect of an increase in APM, described above. Although not widely reported, the increase in MHC I and MHC II in response to DC maturation is also likely to be as a result of increased APM components as well as MHC to enable the generation of a wider pool of tAgs for presentation, increasing the likelihood of sufficient anti-tumour responses.

One of the more abundant cells in the TME, macrophages, are tissue phagocytic cells with the ability to shape their phenotype in response to the surrounding microenvironment. Tumour associated macrophages (TAMs) differentiate from immature pre-cursors into two phenotypes; M1 ‘classical’ or M2 ‘alternative’. M1 TAMs express high levels of MHC I and MHC II and are able to efficiently present tAgs. In early tumourigenesis, interactions with Th1 and NK cells drive M1 TAMs to eliminate tumour cells, promoting an anti-

tumour phenotype. Conversely, M2 TAMs significantly reduce their expression of MHC I and II and are therefore unable to effectively present tAgs, displaying a pro-tumorigenic phenotype (86). Interestingly, during the course of tumour progression, TAMs may switch from M1 to M2, resulting in TAM-MHC II^{HIGH} to TAM-MHC II^{LOW} phenotypes (87). Although the tAg presenting capacity of both TAM phenotypes was lower than conventional DCs, the TAM-MHC II^{LOW} correlates with poorer APP capacity than TAM-MHC II^{HIGH} and are able to supress T cell activation (87). Whilst the effect of this altered M1 to M2 phenotype on APM components has not been studied, it is likely that components of both the MHC I and MHC II APP pathways are also downregulated in response to pro-tumourigenic activity, limiting the supply of peptides that are required for MHC presentation.

5. Conclusions

In this review we have discussed the regulation of the APP pathway in the promotion of tumour growth and survival. Successful antigen presentation on MHC I, and in particular the presentation of specific tAgs on tumour cells, is fundamental for immunological control of tumour growth, and requires a number of key APM components that work in concert to effectively express pMHC I.

It has become increasingly evident that defects within the APP pathways are associated with malignant transformation of cells. Reducing the expression of MHC I on tumour cells, through genetic or translational modifications of the APM components, is an effective immune escape strategy. Defects or altered antigen processing and presentation significantly alter both the peptide supply

and the presented peptide repertoire at the cell surface. Occurring either independently or cumulatively, the consequence of defects within the APP ultimately result in loss of pMHC I or change in peptide repertoire presented at the cell surface. Interestingly, in many malignancies, multiple parts of the APM are defective, which may indicate the evasion mechanisms employed by tumour cells at different stages of disease progression. In addition, altering APM components has significant implications for specific tAg, often resulting in a loss of expression. The frequency of abnormalities varies widely between different tumour types and as a direct consequence, the interaction between tumour cells and cells of the TME is significantly altered, impairing anti-tumour responses. During the progression of tumorigenesis, the TME components (both stromal and immune) can be co-opted into supporting tumour survival by promoting angiogenesis, tumour cell survival and supressing the anti-tumour immune responses.

Understanding the role of the host immune system and evasion strategies, in this instance the role of the altered APM and antigen presentation, will provide an essential insight and reveal targets for new immunotherapies based on the ability to generate tAgs and elicit an effective T cell response to presented target antigens. In addition, further investigation and understanding of the interplay between the different cells of the TME and their role in reducing anti-tumour responses will prove fundamental for development of better therapeutic agents to control and eliminate the tumour.

Competing Interests

The authors declare that there are no competing interests.

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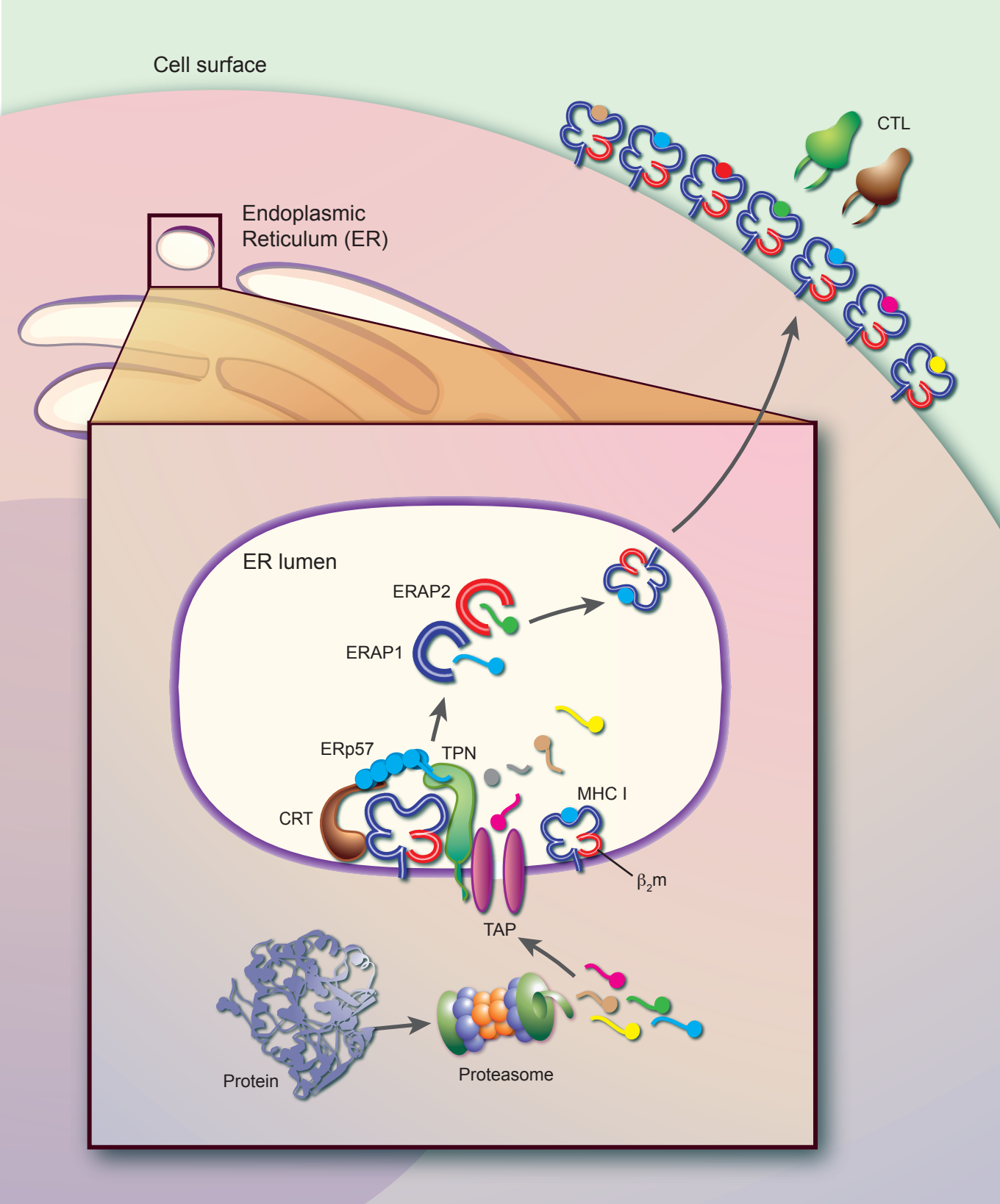
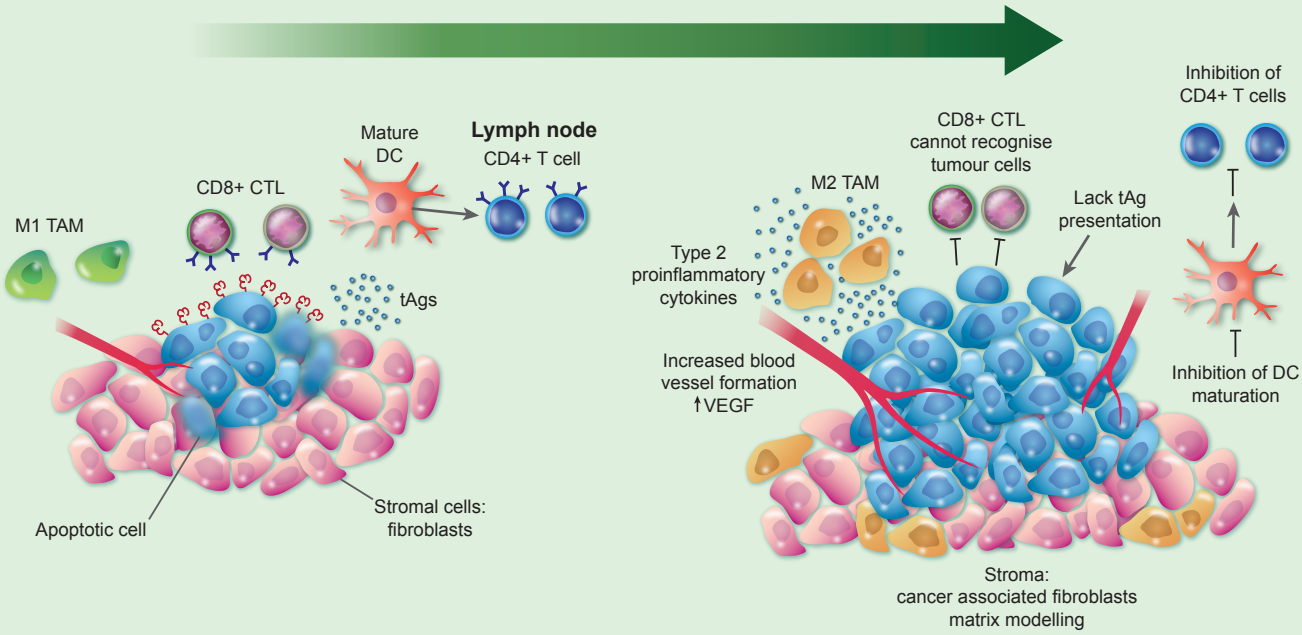

Cell surface
CTL
Endoplasmic
Reticulum (ER)
ER lumen
ERAP2
ERAP1
ERp57
TPN
CRT
MHC I
β_2m
TAP
Protein
Proteasome

Tumor progression - Immune evasion mechanism



Tumour Elimination
MHC I expression
APM intact
M1 TAM phenotype = anti-tumourigenic
Immune recognition and CTL mediated lysis

↓Antigen Presentation

Tumour Escape
Targeted defects in MHC I APM
Reduced MHC I presentation
Reduced CTL response
Inhibition of CD4+ T cell response
M2 TAM phenotype = pro-tumourigenic
Increased angiogenesis
Increase in cancer associated fibroblasts