Dissection of spontaneous and therapy-induced T cell immunity in mice
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Chapter 1

General introduction
General introduction

The immune system protects organisms from microbes like bacteria, viruses, fungi and parasites. The primary defense machinery that is referred to as the innate immune response is based on the recognition of non-self. It is immediately active upon infection, and mediates inflammatory reactions to fight the pathogen. This protection mechanism is very conserved in evolution, considering that plants and animals apply the same molecular modules (1). The cell types that belong to the innate immune system in vertebrates include macrophages, polymorphonuclear leukocytes, dendritic cells and mast cells. These cells recognize conserved molecular patterns that are shared by large groups of microorganisms such as lipopolysaccharides in the cell walls of gram-negative bacteria, and bacterial unmethylated cytidine-phosphate-guanosine (CpG)-rich DNA. Upon recognition of these pathogen-associated molecular patterns (PAMPs), the immune cells bind and engulf the pathogen for processing and destruction.

Vertebrates have developed an additional immune system, the adaptive immune system that comprises B cell and T cell responses. In contrast to the innate immune system that relies on germ-line encoded receptors, B and T cells recognize pathogens by somatically generated, antigen-specific receptors, which renders these cells specific for a broad range of pathogens. During receptor rearrangement, auto-aggressive B and T cells are generated as well, but these self-specific cells are generally depleted from the B and T cell repertoire. The adaptive immune responses also differ from the innate immune system in the capacity to provide immunological memory of infection.

When B cells encounter pathogens, they produce antigen-specific antibodies that bind to the pathogen, resulting in neutralization of i.e. fungi or bacteria. T cells recognize pathogenic structures that are displayed at the cell surface by Major Histocompatibility complex (MHC) molecules of an infected cell or of an Antigen Presenting cell (APC). T cells can be divided in many subtypes, the major subtypes being CD4+ and CD8+ T cells. CD4+ T cells can be subdivided into T helper and T regulatory cells. Naïve T helper cells get activated by antigen presenting cells (APCs), and through direct cell-cell interaction and through secretion of cytokines, they provide help to (amongst others) B cells and to the other major class of the T cell population, the CD8+ T cells. Conversely, T regulatory cells suppress the activity of (self-specific) T cells, thereby reducing the risk of developing auto-immunity. The other class of T cells, the cytotoxic CD8+ T cells have the capacity to kill cells that are infected with viruses and intracellular bacteria, thereby clearing the body from infected cells and preventing further spread of a pathogen throughout the body.

In addition to the battle against microbial pathogens, the adaptive immune system may also play a role in clearing the body from malignant cells. In the 1950s, it has already been proposed by Burnet and Thomas that the immune system plays an important role in preventing outgrowth of spontaneous tumors (2,3). Recently, convincing support for the immunosurveillance hypothesis was provided, by showing that mice that were devoid of T and B cells have a higher prevalence of spontaneous tumors than their immunocompetent littersmets (4).

Evidence for a role of the immune system in preventing outgrowth of tumors has emerged from epidemiological studies in humans. Immuno-compromised individuals like HIV-infected patients, or patients that receive
immunosuppressive medication suffer more often from virally induced tumors than healthy individuals. This includes Epstein Barr virus (EBV)-induced leukemias and Human Herpes Virus-8 (HHV-8)-induced Kaposi’s sarcomas (5-7). T cell responses have also been observed towards non-virally induced tumors. For instance, spontaneous CD8$^+$ T cell responses have been detected to melanoma specific antigens like MART-1, gp100, and TRP-2 (8-10), to the MAGE-1 and NY-ESO-1 antigens that are widely expressed in epithelial tumors (11,12), and to the WT1 and proteinase 3 antigens in myeloid leukemia (AML) patients (13). The spontaneous induction of T cell responses towards these self-antigens is quite remarkable, because T cells undergo a tight selection program during maturation in the thymus and in the periphery to prevent self-reactivity. Furthermore, self-specific T cells that escape this selection procedure are generally of low affinity and non-reactive. Alternatively, self-specific T cells survive because they did not meet the antigen during selection, which is referred to as T cell ignorance. To understand how T cell responses towards tumors can occur, this thesis addresses the following questions:

I. What are the underlying mechanisms that lead to the spontaneous induction of tumor-specific CD8$^+$ T cell responses?

II. How can tumor-specific T cell responses be efficiently induced for therapeutic purposes?

III. When the self-specific T cell population is absent, can we apply adoptive T cell transfer as a tool for therapy?

Antigen recognition by CD8$^+$ T cells through the T cell receptor

Upon antigen recognition, cytotoxic CD8$^+$ T cells can kill virally infected cells or tumor cells. CD8$^+$ T cells recognize small peptides with a length of 8-11 amino acids that are presented at the cell surface of virtually all nucleated cells by Major Histocompatibility Complex (MHC) class I molecules. The peptide recognition is specific and unique for every single T cell and is solely defined by the T cell receptor (TCR). The TCR is an immunoglobulin-like surface protein that consists of a $\alpha$ chain and a $\beta$ chain, or in some cases of a TCR $\gamma\delta$ heterodimer.

In order to protect the body from as many pathogens as possible, the T cell repertoire has a broad range of antigen specificities. To create a T cell population with such a high variety, both the variable regions of the TCR$\alpha$ and TCR$\beta$ chain are somatically generated. The variable region of the murine TCR$\alpha$ chain is encoded by 100 V segment genes, and 50 J (joining) segment genes, whereas the variable part of the $\beta$ chain is encoded by about 30 V segment genes in addition to 2 D (diversity) and 12 J segments (14). During T cell development in the thymus, these segments are randomly recombined, resulting in a diverse T cell population with each cell expressing one specific TCR. In addition, the diversity of both TCR chains is further increased by insertion or deletion of a few base pairs at the junction of the V (D) J regions to generate the Complementarity-Determining Region 3 (CDR 3 region) (15). The CDR3 regions of the TCR $\alpha$ chain and TCR $\beta$ chain form the main determinants of antigen specificity of a T cell receptor since they interact with the peptide bound to the MHC class I molecule as demonstrated by structural analysis (16).

Although a high variability of the TCR repertoire is required to provide protection from as many pathogens as possible, the development of self-specific T cells must be blocked in order to prevent autoimmunity. To ensure
that CD8+ T cells ignore self-antigens, the T cell repertoire is shaped during T cell development by a tightly regulated process. When immature T cells recognize self-antigens that are presented by MHC class I molecules within the thymus, they undergo apoptosis and are deleted (17,18). The first direct evidence for this negative selection process was provided in a study with transgenic mice expressing a TCR specific for a male antigen. In male mice, these transgenic cells were negatively selected, whereas T cell maturation could be observed in female littermates (18). Recently, it has been shown that not only ubiquitously expressed self-antigens are presented to immature T cells in the thymus, but also tissue-specific antigens can be ectopically expressed under regulation of the transcription factor Aire (19). Due to this ectopic gene expression, negative selection of T cells specific for peripherally expressed antigens can take place as well within the thymus (20).

When a peripherally expressed self-antigen is not ectopically expressed within the thymus, or when the TCR is of low affinity or avidity, autoreactive T cells can escape clonal deletion and enter the periphery. These self-specific T cells can be controlled by peripheral tolerance mechanisms, including T cell deletion (21,22) and induction of T cell anergy when the antigen-presenting APC does not provide costimulatory signals (reviewed in (23)). In other cases, self-specific T cells may simply ignore antigens when expressed in peripheral tissues (24).

**Origin of peptides presented by MHC class I molecules**

Mature CD8+ T cells leave the thymus and enter the periphery to survey the body for cells with unusual protein expression, either due to an intracellular infection, or due to mutations within a cell. In order to understand which antigens form good candidates for antigen recognition, it is important to know which peptides are presented by MHC class I molecules.

MHC class I molecules present peptides that are derived from protein degradation products generated within a cell, be it from self-proteins, or proteins that are derived from viruses or intracellular bacteria. The degradation of most cellular proteins begins with the proteasome. Proteins are tagged with small molecules called ubiquitin within the cytosol, which singles them out for proteosomal destruction. The proteasome chops up the proteins into peptide fragments, generally to a length of between 3-22 residues (25). The proteasome is responsible to generate the precise carboxy termini of many presented peptides, and appears to be the major activity in cells that can make this cleavage (26). Several cytosolic endopeptidases and aminopeptidases such as thimet oligopeptidase (TOP), tripeptidylpeptidase II (TPPII), puromycin-sensitive aminopeptidase (PSA) and bleomycin hydrolase (BH) can trim the amino terminus of extended peptides to their proper size (27-29). The cytosolic cleavage by these peptidases can be pivotal for epitope generation as has been shown for the trimming of the HIV Nef epitope by TPP II (30). When the peptides have reached a size of preferentially 9-12 amino acids, the Transporter associated with Antigen Processing (TAP) transports the fragments from the cytosol into the lumen of the endoplasmic reticulum (ER) in an ATP-dependent manner. Within the ER, further aminoterminal processing can take place by a recently identified aminopeptidase, the Aminopeptidase associated with Antigen Processing in the ER (ERAAP) (31).
In the ER, MHC class I molecules await the peptide in association with the peptide-loading complex (PLC) that comprises TAP, calreticulin, Erp57, and the class I-specific accessory molecule tapasin (for a review see (32)). The emerging function of this complex is to retain MHC class I molecules within the ER until they become loaded with high affinity peptides. Upon peptide binding, MHC class I molecules dissociate from TAP, and travel to the cell membrane where they present the antigen to the CD8+ T cells.

Originally, it was assumed that mainly aged proteins that have reached the end of their working life were targeted for proteasomal degradation. Recently, however, a short cut to this degradation pathway has been discovered. During protein synthesis, about 25-30% of the newly synthesized material is not able to achieve a functional state either due to incomplete synthesis or to improper folding and is therefore rapidly degraded by the proteasomal machinery (33). It has been suggested that antigens presented by MHC class I molecules are mainly derived from these newly synthesized, rapidly degraded proteins rather than from mature proteins (33,34). From an immunological point of view, presentation of this antigen source may be beneficial because it allows rapid T cell recognition of virally infected cells and thereby may prevent further spreading of the pathogen.

Most of the epitopes contained in proteins that are targeted for degradation do not reach the MHC class I molecules as their final destination. First, the proteasome does not always provide the accurate carboxy terminus that leads in many cases to useless fragments. Second, the processed peptide must escape complete degradation within the cytosol to reach the ER for MHC class I-peptide binding, and only a small fraction of peptides appears to succeed (35). Third, the aminopeptidase ERAAP that awaits peptides in the ER for aminoterminal trimming has a broad substrate specificity and might therefore also destroy a certain amount of peptides before binding to the MHC class I complex has occurred. Calculations that are partially based on experimental results have suggested that 1000 to 10,000 proteins need to be degraded in order to have one peptide-MHC complex at the cell surface (36-38). Despite this poor yield upon antigen processing, the amount of peptides that reaches the antigen presentation pathway often is sufficient for CD8+ T cell recognition.

Activation of naïve CD8+ T cells

In order to kill an infected cell or a tumor cell, cytotoxic CD8+ T cells only need to recognize a few antigen-containing MHC class-I complexes on the target cell. However, before a T cell can perform this effector function it has to become activated within lymphoid organs where naïve T cells reside exclusively. T cell activation is generally carried out by professional antigen presenting cells (APCs).

APCs like macrophages and dendritic cells (DCs) are widely dispersed throughout the body and constitutively take up self and non-self antigens. Their primary function is to clear effete host cells and microorganisms. The recognition of danger signals like pathogen-associated molecular patterns (PAMPs) engages Toll like receptors (TLRs), which results in activation of APCs. Upon activation, APCs increase antigen uptake and intracellular transport as well as degradation of the engulfed material. This increased protein turnover enhances peptide loading, and the expression of at least MHC class II molecules. Maturated APCs also migrate out of peripheral
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tissue to the secondary lymphoid organs where they present the processed pathogenic material in MHC class II and class I molecules to naive CD4+ and CD8+ T cells, respectively.

For effective T cell activation, antigen presentation alone is not sufficient. Maturation of APCs by PAMP recognition is manifested by increased expression of costimulatory molecules such as B7.1 (CD80) and B7.2 (CD86), CD70, and 4-1BBL, and the secretion of cytokines such as IL-12 and IL-18 that provide maturation signals to T cells (reviewed in (39)).

Several costimulatory molecules are involved in providing proliferation and survival signals to activated T cells (reviewed in (40)). For instance, the engagement of CD28 by interaction with B7 molecules increases transcription of the T cell growth factor IL-2 (41,42) and provides survival signals for activated T cells by increased expression of the anti-apoptotic protein Bcl-XL (43,44). CD28 costimulation also decreases the threshold of antigen required for T cell activation (45).

APCs take up and present antigens irrespective of their origin, including self-antigens. Induction of self-specific T cell responses in general is prevented, because APC maturation is impaired in the absence of PAMP recognition. In this case, a naïve antigen-specific T cell sees the antigen without costimulatory signals, and may still undergo activation and proliferation. However, instead of differentiating, the cells either get functionally inactivated (anergic) or die upon antigen encounter due to the lack of costimulatory signals during the priming phase (23,46).

APCs receive an additional maturation signal by the interaction of CD40 with CD40L that is provided by T helper cells (47,48). T cell help is generally required for effective CD8+ T cell activation (49-51), although in some model systems efficient induction of primary CD8+ T cell responses could be achieved without CD4 T cell help ((52,53) and chapter 4).

Upon antigen-specific activation, T cells proliferate. Activated CD8+ T cells enter the circulation and migrate to the site of inflammation where they can perform their cytolytic function. The factors that regulate cell exit from lymphoid organs are unclear, but decreased expression of lymphoid homing receptors such as CD62L, and CCR7 may play a role in combination with differential expressions of other molecules such as integrins and other chemokine receptors.

In the past years, much effort has been put in the development of models to understand the underlying mechanisms of T cell activation. The recent development of MHC class I tetramer technology (54) gave important new insights in the kinetics of antigen-specific T cell immune responses, together with functional assays such as ex vivo IFNγ and cytotoxicity assays. These newly developed assays allow the analysis of antigen-specific T cell responses directly ex vivo, and therefore provide a better understanding of the actual antigen-specific T cell response than the classical in vitro restimulation assays.

Initiation of T cell responses by direct priming or by cross-priming?

CD8+ T cell activation occurs only to antigens that are present within secondary lymphoid organs. When infections or tumor growth take place systemically or within the lymphoid system, antigens are readily exposed
to naïve T cells. The process and the efficiency through which antigens can reach the T cell priming site from local infections or from tumors that reside outside the lymphoid organs is a matter of debate.

One possibility of T cell priming to peripheral antigens is that infected cells or tumor cells migrate to the lymphoid organs and activate naïve T cells by direct antigen presentation (direct priming). Alternatively, professional APCs may take up the antigen at the peripheral inflammatory site in form of cellular remnants or soluble antigens. Upon antigen uptake, APCs process the antigen and migrate to the lymphoid environment where they can activate naïve T cells upon antigen cross-presentation (cross-priming). Evidence for a role of direct priming and cross-priming in CD8+ T cell activation to peripheral antigens has been obtained in several studies.

Studies by Zinkernagel’s group have shown that tumor-specific T cell responses to the Lymphocytic choriomeningitis virus (LCMV)- derived GP33 antigen occurred only when the tumor cells migrated from the periphery to the lymphoid organs where they could directly prime antigen-specific T cells (55,56). Although the expression of costimulatory molecules by the tumor cells appeared not to be required for T cell priming (55), only continuous antigen exposure could maintain effective T cell immunity. In addition, the lack of T cell help could be detrimental for long term protection and functionality of antigen-specific CD8+ T cells in this setting (57-59).

Direct infection of professional APCs at the local infection site may also lead to T cell priming. In a recent study by Norbury and colleagues, it was shown that vaccinia virus infection in the footpad of mice resulted in migration of infected APCs within a few hours from the peripheral infection site to the lymph nodes (60). Within the lymphoid organ, infected APCs could directly prime naïve CD8+ T cells as shown in a setting where cross-priming was blocked due to a TAP deficiency. Because direct infection of APCs can engage TLRs by PAMP recognition, APC maturation can occur for efficient T cell induction. This study also pointed to DCs as inducers of viral-specific T cell response, because CD8+ T cell clusters were observed only around infected DCs and not around infected macrophages.

The first evidence for cross-priming was provided when female mice developed male-specific T cell responses upon injection of male cells, in spite of the fact that the antigen could not be directly presented by the male cells due to an MHC mismatch (61). Later, it has been demonstrated that bone marrow-derived APCs cross-present antigens for tumor- and viral-specific CD8+ T cell induction (62,63). Yet, many questions how antigen cross-presentation takes place remained unanswered. For instance, which antigens enter the cross-presentation pathway? What is the identity of the cross-presenting APC? And how does the antigen enter the antigen processing and presentation pathway?

Several studies have shown that cellular-associated material can efficiently induce T cell responses through cross-priming (61,62,64,65). Receptors like αvβ5 and CD36 are involved in uptake of apoptotic cells by APCs (66), which can result in efficient cross-presentation as shown in an in vitro model (67). In vivo models, however, do not uniformly point to apoptotic, necrotic, or non-dividing cells as the favored antigen source for cross-priming (68-70). Both DCs and macrophages can efficiently phagocytose cellular remnants and process and cross-present the antigenic material, but it appears that DCs are the major stimulators for adaptive immune responses (68,71), presumably because DCs can increase the expression of costimulatory molecules upon
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antigen uptake. The identity of the cross-presenting APC was first elucidated by Den Haan, showing that cell-associated OVA was cross-presented by CD11c⁺ CD8⁺ DCs (64). In addition, a recent study has elegantly demonstrated that short term in vivo depletion of CD11c⁺ cells impaired T cell responses to exogenous antigens from cell-associated OVA as well as several microbial infections (72).

An alternative source for cross-presentation is soluble material, but since very high antigen concentrations are required, cross-presentation of this antigen source is inefficient (65,73,74). Soluble antigens are mainly cross-presented by CD11c⁺CD8α⁺ DCs (75). APCs can also take up soluble antigens through Fc receptors when captured by immunoglobulins (76). Recently, it has been shown that these immune complexes are cross-presented not only by CD11c⁺ CD8⁺ DCs, as is the case for cellular associated material, but also by activated CD11c⁺ CD8⁻ DCs (77). The efficiency rate with which immune complexes get into the cross-presentation pathway is still undetermined, but recent in vitro data in our lab suggest that this pathway may be quite inefficient (Bakker et al., unpublished observations).

Antigen presentation from exogenous sources may occur through several pathways. The antigens phagocytosed by professional APCs are transported into the cytosol as undigested protein, or are chopped up within the lysosome up to a size that they can exit the endocytic compartment. In the cytosol, the protein fragments can be processed by proteasomes and enter the classical MHC class I pathway as has been shown for immunoglobulin complexes (78). Alternatively, internalized antigens may already be processed into peptides within the endocytic compartment. These antigen-containing lysosomes can fuse with vesicles that contain recycling MHC class I molecules, resulting in antigen loading of MHC class I complexes and transport back to the cell surface (79). Third, upon antigen processing within the endocytic pathway, the antigen-containing vesicle may be transported back to the cell surface and exocytosed so that antigen loading on empty membrane-bound MHC class I complexes from the regurgitated material could occur (80). Although all three processes could occur in vivo, the cytosolic route of antigen cross-presentation might be most efficient for epitope generation because it provides all proteases and peptidases that are involved in epitope generation for the endogenous MHC class I pathway.

Albeit both direct priming and cross-priming have been shown to occur to peripheral antigens, it is still unclear how these two priming pathways contribute to CD8⁺ T cell induction, because different systems have been employed to study either of the priming pathways. In addition, antigen-specific T cell responses have been analyzed post in vitro restimulation, which may not represent the actual in vivo situation. Therefore, we developed a murine tumor model that allowed us to determine the contribution of both direct priming and cross-priming in one setting, and to study the induction of tumor-specific T cell responses directly ex vivo (chapter 4). Upon tumor cell inoculation at a peripheral site, we detected migration of tumor cells to the lymphoid organs. To determine whether these migrated tumor cells could directly prime tumor-specific T cells, we studied their priming capacity in a setting where the cross-priming pathway was blocked due to a TAP-deficiency. In addition to T cell proliferation, this antigen encounter resulted in IFNγ production (Wolkers et al., unpublished data), indicating that tumor cells that have migrated to the lymphoid organs can induce functional T cell responses through direct priming. Conversely, when the direct priming pathway was disrupted, tumor-specific T cell induction appeared to be as efficient as when both priming pathways were intact. Efficient T cell induction
required costimulation through CD28, but in contrast to the findings by Bennett and coworkers (81), this primary CD8+ T cell response did not depend on CD4+ T cell help. Recent data indicated that CD4+ T cell help is required for efficient induction of T cell memory rather than for primary T cell responses (57-59). Therefore, it would be of interest to examine if this also applies to T cell memory formation in this model. Our study indicated that both direct priming and cross-priming can occur and that they can be redundant for the induction of tumor-specific T cell responses. This finding has recently been confirmed in a viral model (82). Yet, in some cases, T cell induction through cross-priming was undetectable (55,56). Because these models study T cell responses towards a different antigen, this different outcome to be or not to be suitable for cross-presentation could be an intrinsic property of antigens.

Antigen bias in cross-priming

One factor that can determine the efficiency of antigen cross-presentation is the expression of immunoproteasomes by professional APCs. In the presence of IFN-γ, the three catalytic subunits of vertebrate proteasomes are replaced by their homologous subunits LMP2, MECL1, and LMP7 to form immunoproteasomes. Although the cleavage capacity of the immunoproteasome is unchanged, its cleavage preference differs from the standard proteasome, resulting in a modified spectrum of the produced antigenic peptides (83, 84). Indeed, whereas some epitopes are better processed by cells carrying immunoproteasomes, others are very poorly processed (reviewed in (85)). Immature DCs express normal proteasomes and immunoproteasome in equal amounts, but mature DCs contain only immunoproteasomes (86). Because DC maturation is required for efficient T cell induction, some antigens may be excluded from the cross-presentation pathway such as the melanoma-specific antigen MelanA\textsuperscript{MEL} (87), since melanocytes express only the normal proteasome at steady state.

We speculated that the location of an antigen within a protein could also affect the efficiency of cross-presentation. To address this issue, we introduced antigens either in a signal peptide or in the mature protein of the Green fluorescent protein (GFP; chapter 5). When these fusion genes were introduced into murine tumor cells, direct antigen presentation was unaffected by the antigen location. Nevertheless, the capacity of either epitope location to induce T cell responses through cross-priming was strongly influenced by the antigen location. Epitopes located within the mature protein induced massive antigen-specific CD8+ T cell responses, whereas the signal peptide-derived antigens failed to do so. Our data also suggest that the observed bias in T cell cross-priming is a result from antigen uptake of cell-associated material rather than from soluble material, which was also demonstrated to be the major antigen source in another cross-priming model (65).

Because direct antigen presentation was equally efficient for either antigen location, and cross-priming was biased towards antigens located within the mature protein, we were able to use this model to determine the relative contribution of these two priming pathways to the induction of tumor-specific T cell responses. We injected TAP-proficient tumor cells containing these constructs into wild type mice, thereby allowing both direct priming and cross-priming to occur (chapter 5). Strikingly, also in this system efficient antigen-specific T cell responses were induced only to the antigens located within the mature antigen and not to the signal-peptide
derived epitope, indicating that cross-priming is a very efficient pathway for T cell induction, albeit biased towards antigens that are derived from mature proteins. But how can we explain the dichotomy observed for direct presentation versus cross-presentation? Whereas at a steady state the efficiency of direct antigen presentation is independent of protein half-life (33,34), the cross-presenting APC may mainly deal with the left overs from cells. Consequently, the antigen composition of the cross-presented material may substantially differ from the one that is used for direct antigen presentation. Stable, mature proteins can accumulate within cells and thereby form a good antigen source for cross-presentation. In contrast, signal peptides are cotranslationally cleaved off the proteins, and are believed to be rapidly degraded (88). Hence, the observed bias in T cell priming towards the mature protein may be a reflection of a difference in antigen stability that determines the capacity to enter the cross-priming pathway. Recent studies by Yewdell and colleagues point as well to protein stability as a determining factor to enter the cross-presentation pathway (Yewdell, personal communication).

Our finding that antigen location can affect the capacity to enter the cross-presentation pathway may help to explain why the signal peptide-derived epitope GP\textsubscript{33} induces T cell responses through direct priming, but fails to do so through cross-priming (55,56).

If cross-priming is the dominant priming pathway for T cell induction, protein accumulation may affect the immunogenicity of otherwise ignored antigens. Anecdotally, increased expression of the self-antigen vinculin in apoptotic cells coincided with antigen-specific CD8\textsuperscript{+} T cell responses in HIV infected individuals (89). Similarly, p53 is generally ignored in healthy individuals, but when the protein degradation of the otherwise short lived p53 is impaired in tumor cells, spontaneous T cell responses can be detected (90).

Current investigations in our lab directly address the effect of protein stability on cross-priming by employing the N-end rule (91). Ubiquitin added to the 5' end of a gene is cotranslationally cleaved off, and allows the introduction of unconventional starting amino acids into proteins, which depending on their character decreases their stability. Comparison of T cell responses towards these model antigens should directly demonstrate if protein stability indeed affects the capacity to enter the cross-priming pathway.

Antigen cross-presentation is not only involved in the induction of T cell responses, but has also been shown to induce T cell tolerance to peripheral antigens (reviewed in (46)). If indeed protein accumulation is the determining factor to enter the cross-presentation pathway, one can predict that cross-tolerance may also mainly be induced towards stable antigens. When the Influenza-derived Haemagglutinin is exclusively expressed within the pancreas under the rat insulin promoter (RIP-HA), peripheral tolerance is induced by antigen cross-presentation (22). In contrast, expression of the LCMV-derived GP under the same promoter is simply ignored (24), presumably because the signal peptide-derived epitope GP\textsubscript{33} is excluded from cross-presentation pathway. Similarly, when the short-lived OVA-peptide is expressed under a Keratin-promoter, peripheral tolerance induction is impaired and the mice suffer from severe autoimmunity (92). Conversely, expression of the antigen in context of the whole protein either driven by the ubiquitously expressed actin promoter, or the pancreatic β cell-specific RIP promoter leads to tolerance induction (21,92).

In cases where peripheral antigens are ignored rather than tolerance induction has occurred, the antigen-specific T cell repertoire can still be present as shown for the GP\textsubscript{33} antigen (24). This T cell ignorance could be exploited.
for vaccination strategies. Importantly, recognition of target cells should be unaffected since direct antigen presentation of proteins is unaffected by their stability (see above).

Our findings may also have implications for the vaccine design. DNA vaccination, for instance, is generally administered in the periphery, and is considered to induce T cell immunity mainly via antigen cross-presentation. Therefore, the context in which the epitope is presented to the immune system could greatly influence the efficacy of DNA vaccines.

**Implications of T cell priming on epitope-directed vaccinations**

The induction of efficient CD8+ T cell responses relies on several parameters. We have shown that antigen location can influence the efficacy of T cell priming (chapter 5). Furthermore, the availability of the epitope for MHC class I loading upon antigen processing affects T cell priming, as well as the maturation status of APCs that is induced upon PAMP recognition by TLRs together with APC-CD4 T helper cell interaction. Likewise, T cell responses can solely be induced to non-self antigens, or to antigens that are ignored by the T cell repertoire. These conditions for efficient T cell induction are also indispensable for the development of potent epitope-directed vaccines.

One strategy to induce epitope-specific T cell responses is DNA vaccination. This vaccination strategy is based on vectors that encode a eukaryotic expression cassette. In addition to the antigen of interest, DNA vaccines contain CpG motifs that bind to TLR9 and thereby provide a 'danger signal' that is required for APC maturation (93). DNA vaccines are mainly administered intradermally or intramuscularly which results in transfection of host cells. Upon transcription and translation of DNA, the antigen is available for the immune system. Although it is still not fully understood through which priming pathway T cell responses are induced, it has been demonstrated that bone marrow-derived cells are responsible for the priming of immune responses after DNA vaccination (94). This indicates that T cell priming occurs either upon direct transfection of APCs by the naked DNA, or by cross-presentation upon uptake of transfected somatic cells. At least in some settings, T cell induction by DNA vaccination is mainly driven by antigen cross-presentation (95).

In chapter 3 we determined several guidelines for potent epitope-directed T cell induction by DNA vaccination. Based on the finding that the parental gene of the Influenza-derived Nucleoprotein failed to efficiently induce T cell responses, we examined whether alterations in the context of the T cell epitope could lead to more potent T cell induction. We have shown that carboxy-terminal fusion of T cell epitopes to a carrier protein of foreign origin is most optimal. Fusion of the minimal epitope to a carrier protein reduces the required antigen trimming to the amino-terminus. In addition, fusion to a carrier protein may protect the antigen from rapid degradation by cytosolic proteases. The study also shows that efficient vaccination depends on non-self recognition of the carrier protein, presumably by providing a signal for CD4 T cell help. This is in line with previous findings that CD4 T cell help is required for efficient T cell induction by DNA vaccination (96). In addition to the carrier proteins that were employed in this study, recent work in our lab has demonstrated that tetanus-toxin fragment C (TTFC) is also a very potent carrier protein for the induction of CD8+ T cell responses (Bins et al., unpublished observations). Since the tetanus vaccine is included in childhood vaccination, it is important to establish whether
pre-existing T cell immunity to the carrier protein is detrimental to efficient T cell induction. Pre-existing T cell immunity does not appear to influence the efficacy of T cell induction by epitope-directed DNA vaccination (chapter 3). Yet, immunogenic CD8+ T cell epitopes should be depleted from the carrier protein (97), since competition for APC access may occur during T cell priming with the T cells specific for the antigen of interest (98).

Another factor that may increase the efficiency of T cell induction by epitope-directed DNA vaccination is the way of DNA delivery. Generally, DNA is administered intramuscularly or intradermally. The combination of both pathways significantly increased the efficiency (99). Moreover, it was recently shown that intralymphatic DNA vaccination could increase the efficiency of epitope-directed T cell induction by a 100-1000 fold (100). Recent studies in our lab indicate that skin tattooing can significantly increase the efficiency of intradermal DNA vaccination, possibly because the transfection efficiency is improved by this technique (Bins et al., personal communication).

Up to date, clinical trials that employed DNA vaccines have attained measurable but not extremely robust T cell responses. Several parameters can be improved to increase the efficacy of DNA vaccines such as optimizing the DNA construct and the way of delivery. Furthermore, DNA vaccination may be a very powerful therapy in combination with other vaccination regimens such as a modified vaccinia virus (101).

Cross-reactivity of memory T cells
Vaccination can serve two purposes, which is to induce effector cells for immediate action, and to induce T cell memory that provides long-term protection. Memory T cells differentiate more rapidly and efficiently to effector cells compared to naïve T cells (102), possibly because the gene expression profile has changed due to an altered chromatin structure (103). In addition, the precursor frequency of antigen-specific T cells in a memory pool has increased up to a 1000-fold (104-106), thereby enhancing the probability of rapid antigen encounter and of an augmented secondary T cell response.

The T cell memory pool generally comprises a polyclonal population with a highly variable TCR usage. Within this pool, different affinities can be found towards the specific epitope, and the form of interaction with the peptide differs. Therefore, cross-reactivity of TCRs could occur, which may contribute to cross-protection to minor mutations. To directly address the capacity of T cells to cross-react with antigenic variants, we determined the shape of the T cell population in an influenza model (chapter 2). Immunized mice received a secondary challenge with a variant influenza strain that contained a mutation within the immunodominant epitope. By direct ex vivo analysis of the T cell response with the simultaneous use of MHC class I tetramers specific for either epitope, we detected a selective expansion of cross-reactive memory T cells. The cross-reactive T cell population was oligoclonal and was also detectable upon rechallenge with peptides that were derived from other variant influenza strains, indicating that cross-reactivity was not restricted to one particular T cell clone, or to one single amino acid change. Furthermore, antigen encounter was sufficient for expansion of cross-reactive T cells, because rechallenge with the variant epitope in the context of tumor cells resulted in expansion of cross-reactive T cells and in reduction of the tumor burden.
The cross-reactive capacity of T cells can be explained by how the TCR interacts with the MHC class I-peptide complex. The TCR lays diagonally over the peptide groove of the MHC molecule (16,107). Depending on the peptide and the TCR, the contribution of the TCRα chain and the TCRβ chain on peptide recognition differs. Whereas the TCRα chain mainly contributes to the recognition of the amino-terminal part of the epitope, the TCRβ chain is involved in binding to the carboxy-terminal part. Alterations of amino acids that do not directly interact with the TCR may be tolerated, therefore this 'molecular mimicry' does not disturb the interaction of TCR with MHC peptide complex (108). Furthermore, recent studies have shown that the interaction of the CDR3 region of the TCR with the MHC class I-peptide complex is very flexible, which might lead to alternative recognition of epitopes by the TCR (109).

In some situations, altered peptide ligands can be detrimental for the protection towards the first virus as shown in a study with LCMV variants (110). Alterations of amino acids in peptides can lead to total or partial loss of functional recognition by cytotoxic T cells both in vitro and in vivo (110-113). This discrepancy with our findings remains unexplained. Nevertheless, subsequent studies confirmed that cross-reactive T cells can provide cross-protection, in some cases even to epitopes that bear no overt structural similarity (114,115). The cross-reactive nature of T cell responses is likely to benefit the host by providing protection against antigen-related variants. Furthermore, cross-reactivity may also be exploited to activate self-specific T cells. In a transgenic mouse model with ubiquitous expression of the influenza derived nucleoprotein (NP), activation of self-specific T cells of low affinity could be achieved with an antigenic variant (116). Similarly, vaccination of mice with the human variant of the melanoma-specific gp100 antigen induced self-specific T cell immunity (117).

**TCR gene transfer as anti-tumor therapy**

Vaccinations towards tumor antigens can be very efficient when the T cell repertoire is ignorant for the self-antigen, or when a cross-reactive population can be activated. When the T cell repertoire is tolerant towards the self-antigen, or when the patient is immunocompromised, adoptive T cell therapy is a very appealing strategy (for a review see chapter 7). The efficacy of adoptive therapy was established in spontaneous murine tumor models. In these systems, mice develop tumors due to transgenic oncoprotein expression, and the T cell repertoire of these mice is tolerant to the neo-self antigen. Adoptive transfer of T cells derived from non-transgenic mice (that recognize the tumor antigen as non-self) resulted in efficient anti-tumor reactivity (118,119).

In the early 1990s, adoptive therapy of TAA-specific T cells was introduced in cancer patients (120,121), but the in vivo persistence and the anti-tumor function of transferred cells remained limited (122,123). Two recent studies have presented more promising results in that tumor-specific T cell transfer efficiently induced regression of metastatic tumors (124,125). In addition, lymphodepletion prior to T cell transfer significantly increased the in vivo persistence of transferred cells (124). Because the antigen specificity of T cells is solely determined by the T cell receptor, an alternative approach for adoptive T cell therapy is the transfer of antigen-specific TCR genes into host cells (126). Upon short term in
in vitro culture, TCRs can be introduced by retroviral transduction into peripheral T cells (127-129). This strategy allows the generation of a large amount of antigen-specific T cells within a few days, and tumor-specific TCRs obtained from one individual can be employed for the treatment of a larger group of patients.

In chapter 6, the feasibility of TCR gene transfer is examined in a mouse model. This study provided the first evidence that upon antigen encounter, TCR-transduced T cells can strongly proliferate in vivo and home to the effector site. In addition, TCR gene transfer therapy resulted in tumor rejection in an immunodeficient setting. Redirected T cells persist for more than two months in immunodeficient mice post tumor clearance, and rechallenge with tumors led again to expansion of these T cells (chapter 6). In contrast, in immunocompetent mice redirected T cells failed to persist. A reason for this may be the reduced expression level of introduced TCRs compared to endogenous TCRs. Therefore, secondary responses may be dominated by endogenous T cells due to the reduced avidity of the redirected T cells (130). Alternatively, the poor persistence of redirected T cells in immunocompetent mice might be a result of graft rejection, because either the introduced retrovirus, or the exogenous TCR might render a T cell immunogenic. Current studies are addressing this issue by adoptive transfer of redirected T cells into mice that are tolerant towards this particular antigen. Since this setting precludes competition of transferred T cells with the endogenous T cell repertoire, it should elucidate why these cells persisted only for a short period.

Because T cells divide rapidly upon antigen encounter, the introduced TCR should be stably expressed. Most gene transfer studies reported thus far have employed a moloney murine leukaemia virus (MoMLV)-based vector system. However, stable integration of this retrovirus into the host genome can lead to cellular transformation. In a gene therapy trial carried out by Alain Fischer and colleagues (131), retroviral introduction of the IL-2γc chain into bone marrow-derived stem cells resulted in the development of leukemia in 2 out of 11 patients (132-134). Whether retroviral integration of TCRs into mature T cells with a limited life span harbors similar risks for the development of leukemia remains unclear to date. Nevertheless, episomal vectors that do not integrate into the host genome, but are maintained during cell division might be a good alternative for the retroviral system.

References

Chapter 1


Chapter 1


Chapter 1


