IFNγ-inducible proteasome components in immune responses

The research described in this thesis was performed at the Division of Immunology, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht Univerity, The Netherlands.

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IFNγ-induceerbare proteasoom componenten in immuunresponsen

(met een samenvatting in het Nederlands)

Proefschrift

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CHAPTER 1

General introduction

Immune system

The body is constantly exposed to infectious pathogens like viruses, bacteria, fungi and parasites. In order to protect itself against these disease causing agents, the body is equipped with a highly complicated but well developed immune system. The first mode of action of the immune system is to recognize the presence of pathogens or foreign material followed by a reaction to eliminate it. The immune response can roughly be divided into two categories: the innate and the adaptive immune response. The innate immune response involves mainly macrophages, dendritic cells (DCs), granulocytes, natural killer cells (NK cells) and the complement system and is regarded as a non-specific, quick responding part of the immune system. The adaptive immune response is characterized by B- and T lymphocytes which are highly specific in recognition and elimination of a particular pathogen. Furthermore, the adaptive system forms memory which prevents a pathogen to cause disease upon a next encounter.

Ubiquitin proteasome system

CD8 T cells are an important part of the adaptive immune system. They recognize peptides presented in the context of MHC class I molecules on the cell surface. MHC class I molecules present peptides of all proteins that are degraded. The ubiquitin proteasome system (UPS) plays an important role in protein degradation.

The UPS was originally identified as the second intracellular proteolytical pathway next to the lysosome (1). In contrast to the lysosomal pathway, protein degradation by the UPS is a highly specific process that is tightly regulated. By removing damaged or misfolded proteins the UPS controls the protein level of the cell thereby maintaining the cell's quality control system. In order to recognize proteins that require degradation, cells mark these proteins with ubiquitin, a protein of 76 amino acids that is highly conserved in eukaryotes. Conjugation of multiple ubiquitin molecules involves three biochemical steps mediated by E1, E2 and E3 enzymes (2). Subsequently, the ubiquitinated proteins are recognized and degraded by the 26S proteasome into small peptides that mostly end up in single amino acids because of further degradation in the cytosol by proteases. However, a small fraction of peptides generated by the ubiquitin proteasome pathway escapes further degradation by translocation to the ER, where they encounter MHC class I molecules to form a complex that can be transported to the cell surface for presentation to CD8 T cells.

Although ubiquitination seems to be critical in proteasome-mediated protein degradation, there is some evidence that proteasomes can degrade certain proteins in an ubiquitin-independent way. One of the first and best studied examples is the enzyme

ornithine decarboxylase (ODC) that is degraded by the 26S proteasome upon binding of antizyme, which seems to form an alternative degradation signal (3-5). Also recent studies have identified proteins that do not require ubiquitination for proteasomal degradation (6, 7).

The proteasome and its activators 19S and PA28

As described above, the proteasome is an important part of the intracellular degradation pathway and generates most of the peptides presented in MHC class I molecules. Proteasomes are complex intracellular proteases that are ubiquitously expressed both in the cytosol as well as the nucleus of all eukaryotic cells. The complex is highly abundant, representing up to 2% of total cell proteins (8). The major functions of the proteasome are degradation of intracellular proteins and regulation of several cellular processes like the stress response, cell cycle control, transcription activation, apoptosis and metabolic adaptation (9, 10). The 26S proteasome complex is formed by the 20S core particle together with two 19S regulatory complexes that are attached at both sites of the 20S core proteasome. The 20S core proteasome has a cylindrical structure that is composed of four stacked rings of which the outer α-rings consist of seven different α-subunits (α1-7) and the inner β-rings contain seven β-subunits (β1-7). Three of the β-subunits (β1, β2 and β5) are proteolytically active and are thus responsible for the cleavage of proteins that have entered the proteasomal channel (11, 12). To protect substrates in the cytosol from unwanted degradation, the 20S channel is usually closed by the N-termini of the α-subunits, that form a gate that blocks substrate entry to the 20S. Upon binding of 19S or PA28, both proteasome activators, the gate is opened and 20S proteasomes are activated. The 19S complex (or PA700) was identified as one of the proteasome activators that can bind to both α-rings of the 20S core complex in an ATP-dependent manner, forming the 26S proteasome. The 19S regulator is formed by two subcomplexes, the base and a lid, the former containing six ATPases that probably function in unfolding captured substrates to facilitate substrate entry into the catalytic 20S core (13, 14). The lid of the 19S complex consists of non-ATPase subunits and is assumed to be responsible for recognition of ubiquitin-linked substrates and their deubiquitination (10, 13).

The proteasome activator 28 (PA28 or 11S) is another regulatory complex that forms a heptameric ring composed of α- and β-subunits Like the 19S complex, PA28 forms a cap that is able to bind to either one or both ends of the 20S complex (15, 16), but unlike binding of 19S to the 20S proteasome, association of PA28 is not dependent on ATP and is much weaker. PA28 is expressed in all eukaryotic cell types and was therefore thought to exert a basal cellular function (17). This assumption became less likely when PA28 gene-deficient mice were shown to be viable (18). PA28 stimu-

lates the catalytic activities of the 20S proteasome by assisting in substrate entry or exit without influencing the active sites (16, 19). In *in vitro* digestion experiments where epitope containing peptides were digested by 20S proteasomes in the presence of PA28, generation of MHC class I ligands was accelerated and proteasomal double cleavages were enhanced (20, 21).
The subunits of PA28 are inducible by IFNγ (22) and high levels of expression were shown in pAPC (23-25) suggesting a role for PA28 in MHC class I antigen processing (26). To examine a potential role for PA28 in MHC class I antigen processing, transfectant cell lines overexpressing the PA28 α- and β-subunits were generated. Although efficient processing and presentation of some viral epitopes was enhanced by PA28, presentation of other epitopes was not affected by PA28 (27-31). So far, generation of the $TRP2_{181-188}$ and $TRP2_{360-368}$ epitopes are the only known examples of epitopes that are dependent on PA28 (18, 29). Surprisingly, the PA28 dependence of the $TRP2_{360-368}$ epitope was demonstrated to be due to its flanking sequences, of which the N-terminal flanking sequence of the $TRP2_{360-368}$ epitope was sufficient for PA28 dependence (32). Although only the above mentioned TRP2 epitopes are PA28-dependent, PA28 aids in the generation of quite a lot of other epitopes, indicating that PA28 plays a role in antigen processing. However, the exact function of PA28 still remains to be resolved.

The proteasome generates the correct C-terminus of CTL epitopes
MHC class I molecules acquire small protein fragments (peptides) for display to CD8 T cells. The heavy chain of the MHC class I molecule contains the groove for antigen binding, which is closed at both ends allowing only binding of peptides with a defined length of 8-11 amino acids (33). Only a small percentage of peptides generated by the proteasome is of the correct size for binding to MHC class I molecules or longer (usually at its N-terminus) (34). The proteasome is considered to be responsible for the generation of the correct C-terminus of CTL epitopes to enable binding to class I molecules. Peptides that are extended at the N-terminus can be cleaved by other (cytosolic) proteases to acquire the correct size for binding to MHC class I molecules. Of all the proteases present in the cytosol or ER, the proteasome has long been the only protease detected with C-terminal trimming activity. Upon proteasome inhibition, the correct C-terminus of different CTL epitopes was no longer generated (35-37). Together these results suggested that only the proteasome generates the C-terminus of epitopes (38). However, generation of the HIV-derived Nef_{73-82} epitope was shown to depend on tripeptidyl peptidase II (TPPII) (39) and insulin-degrading enzyme has been reported to produce an epitope of the human melanoma antigen MAGE-A3 (40). Furthermore, a study by Kessler et al. showed that two cytosolic endopeptidases, TOP and nardilysin, were required (both of them or only one) for

the generation of three defined CTL epitopes (41). Together these results indicate that the proteasome is the major but not the only protease involved in generating the C-termini of epitopes.

Different types of proteasomes and their role in epitope generation

The proteasome containing the catalytic subunits β1, β2 and β5 is known as the "standard" or "constitutive" proteasome. Each subunit has its preference to cleave substrates after certain residues, β1 cleaving after acidic residues (caspase-like activity), β2 cleaves after basic amino acids (trypsin-like activity) and β5 prefers cleavage after hydrophobic residues (chymotrypsin-like activity) (42). Based on this, the proteasome should be able to cleave after almost all amino acid residues and generate all possible epitopes. However, the majority of peptides generated are too short for presentation in MHC class I molecules (< 8 amino acids). Furthermore, the proteasome has a strong preference for particular cleavage sites and these dominant sites are more frequently used than the less preferred cleavage sites. Epitope generation is therefore strongly influenced by the presence of preferred cleavage sites. Epitopes may even be destroyed or fail to be generated when the preferred cleavage site resides in the middle of an epitope or in the immediate flanking regions of the epitope (43-45).

Constitutive proteasomes are present in all cells, but lymphoid cells and cells that reside in an inflammatory environment and are exposed to cytokines such as type I interferons (46), TNFα (47) or IFNγ (48, 49), express three different homologous subunits, LMP2, MECL-1 and LMP7, replacing the β1, β2 and β5 subunits, respectively. This type of proteasome is referred to as the immunoproteasome and is shown to be much more efficient in generating certain antigenic peptides than constitutive proteasomes (30, 42, 50, 51). These data were supported by studies performed with mice deficient for one or more immunoproteasome subunits (52, 53). Deficiency for LMP7 resulted in reduced expression of MHC class I molecules on the cell surface, due to a reduced peptide supply (53). In contrast, Morel et al. showed that immunoproteasomes are not capable of processing the antigenic peptide $RU1_{34-42}$ and the melanoma differentiation antigens Melan-A_{26-35} and $gp100_{209-217}$ that are efficiently produced by constitutive proteasomes (54). In addition, some tumor antigenic epitopes are not only produced more efficiently by constitutive proteasomes, but they are rather destroyed by immunoproteasomes due to their cleavage site preferences (55). These data indicate that the peptide pool that is presented on the cell surface is largely dependent on the type of proteasome that is expressed inside the cell, which can determine the outcome of CTL responses.

Recently, a third type of proteasome was discovered by Murata et al.: the thymic proteasome. The subunit β5t is expressed in cortical thymic epithelial cells (cTECs)

and prefers to incorporate into proteasomes containing LMP2 and MECL-1 (56). It was shown that this thymic subunit altered the cleavage site usage in proteasomes which directly influences the peptide pool that these cells present. Mice deficient for β5t showed decreased levels of CD8 T cells and were unable to positively select T cells specific for several known viral CD8 T-cell epitopes (56, 57). Expression of β5t and immunoproteasomes is therefore crucial in the generation of MHC class I presented peptides involved in positive selection and shaping the T-cell repertoire.

Functions of immunoproteasomes unrelated to antigen processing

Although the main function of immunoproteasomes is still believed to be related to antigen processing, several recent findings suggest that immunoproteasomes fulfill functions unrelated to antigen processing. In a study with LMP2$^{-/-}$ mice, the number of B- and T cells was severely reduced and defects were found in function of B cells and DCs (58). The defects in LMP2$^{-/-}$ mice were explained by the formation of mixed proteasomes, a combination of constitutive subunits (β1) and immunosubunits (MECL-1 and LMP7) that is normally not generated when LMP2 is present. In absence of LMP2, signaling pathways like the NF-κB pathway would be deregulated leading to immunologic problems. Furthermore, it was suggested that immunoproteasomes are important for T-cell survival or expansion. Studies performed with cells from different immunoproteasome-deficient mice (LMP2$^{-/-}$, MECL-1$^{-/-}$ or MECL-1+LMP7$^{-/-}$) showed that these cells did not expand upon transfer into wildtype (wt) mice followed by infection (59, 60). The hypothesis of immunoproteasome-dependent survival of T cells was recently confirmed in an extensive study (60). Although a clear explanation for these results was not present, a deregulated cytokine profile in immunoproteasome-deficient mice was suggested as inhibition of LMP7 or deletion of LMP2 showed a reduced production of (pro-inflammatory) cytokines (58, 61). An alternative role of immunoproteasomes in antigen processing was shown in a study by Seifert et al. (62). They showed that interferons induce the formation of oxidation-damaged proteins by induction of reactive oxygen species. Processing by 26S proteasomes is required to degrade the increasing pool of oxidant-damaged DRiPs (Defective Ribosomal Products). However, the amount of DRiPs that are generated exceeds the capacity of degrading proteasomes, leading to accumulation of these proteins and formation of ALIS, aggresome-like-induced structures. By induction of immunoproteasomes, the degradative capacity for oxidant-damaged proteins is enhanced which leads to rapid clearance of accumulated oxidized proteins (62). Mice lacking LMP7 were unable to degrade the enhanced amount of DRiPs, resulting in ALIS formation and these cells were more susceptible to apoptosis. Due to their role in the maintenance of protein homeostasis, the enhanced degradation automatically leads to the increased production of peptides available for MHC class

I presentation. So this suggests that peptide generation by immunoproteasomes in part is a result of its role in the maintenance of protein homeostasis under conditions of cellular stress induced by cytokines.

Effect of immunoproteasome expression on TCR repertoire selection

As previously mentioned, thymoproteasomes are involved in selection of T cells. However, some recent studies suggested also a role for immunoproteasomes in generating the TCR repertoire (59, 63). In a study of Chen et al. it was found that in LMP2$^{-/-}$ mice, responses to two dominant Influenza epitopes decreased significantly (63). Adoptive transfer experiments with CD8 T cells from either wt or LMP2$^{-/-}$ mice showed that reduced responses to $PA_{224-233}$ were the result of decreased processing and generation of the epitope, whereas reduced responses to $NP_{366-374}$ could be attributed to alterations in the CD8 T-cell repertoire. In addition, Basler et al. performed studies with LCMV infection in MECL-1$^{-/-}$ mice and found reduced CD8 T-cell responses to the LCMV $GP_{276-286}$ epitope. Presentation of the epitope in MECL-1-deficient mice was comparable to wt mice, thus the authors concluded that the T-cell precursor repertoire for the $GP_{276-286}$ epitope was altered in MECL-1$^{-/-}$ mice (59). These findings, together with the notice that immunoproteasome subunits are expressed in the thymus (64, 65) as well as the thymus specific β5t subunit strongly suggest that these subunits can influence T-cell selection in the thymus thereby shaping the T-cell repertoire.

CD8 T-cell responses

CD8 T cells play an important role in clearing viral and other intracellular pathogenic infections and in eradication of tumors. They recognize (foreign) antigenic peptides associated with MHC class I molecules with their T-cell receptor (TCR). Naïve CD8 T cells are activated when recognition of an antigenic peptide on professional antigen presenting cells (pAPCs; signal 1) is followed by costimulatory signals (signal 2) provided by the pAPC in the lymph node. Under the influence of cytokines (signal 3) this activation phase (priming) is followed by expansion of antigen specific CD8 T cells that exert their function at the site of infection. After expansion the antigen specific CD8 T-cell response contracts dramatically, leaving a relatively small number of memory CD8 T cells that can respond quickly upon reinfection. Development of T cells occurs in the thymus, where they undergo a selection process resulting in positive selection (thus survival) when the TCR (weakly) recognizes self-MHC and negative selection (thus elimination) upon strong interaction with MHC molecules complexed with self-antigens. Although every pathogen contains lots of potential CD8 T-cell epitopes, CD8 T-cell responses are usually directed to only a small number of peptides. This is known as immunodominance and is determined by multiple fac-

tors that act on the antigen processing or T-cell level (66). At the antigen processing level, a candidate epitope must first be cleaved out of its protein surroundings, then be transported via the transporter associated with antigen processing (TAP) to the endoplasmic reticulum (ER) and subsequently bind to MHC class I molecules with sufficiently high affinity. The dominant T-cell determinants are defined as peptides that elicit strong CD8 T-cell responses after immunization, whereas subdominant determinants induce only weak responses (67). Deol et al. (68) showed that also the early processing kinetics determine whether peptides are able to elicit vigorous CD8 T-cell responses. These peptides that are the first to be presented on priming pAPCs have a higher chance to bind to naïve T cells and elicit CD8 T-cell responses than peptides that are generated later.
The (immuno)proteasome generates the majority of MHC class I presented peptides and is therefore important for shaping the CD8 T-cell responses against pathogens and tumors.

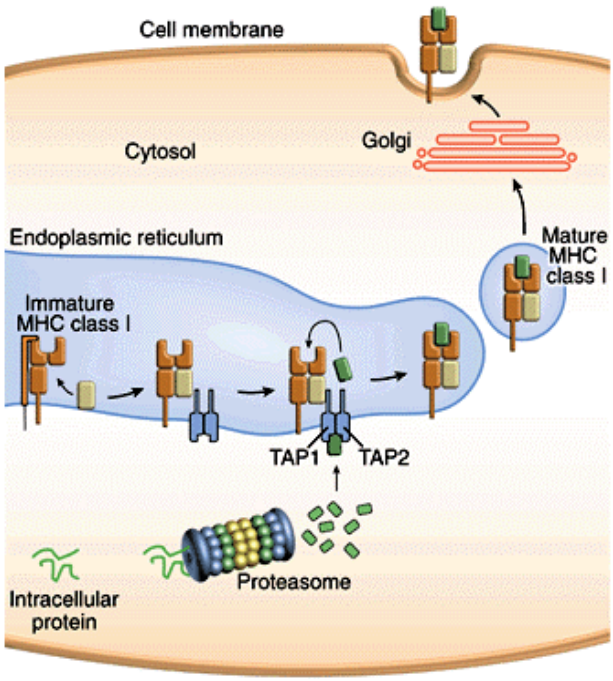

Figure 1. Overview of the MHC class I antigen processing pathway. Basic representation of the MHC class I antigen presentation pathway. Endogenous proteins are degraded into peptides by the proteasome. Peptides are translocated into the ER via TAP, where they associate with newly synthesized MHC class I molecules. Upon peptide binding, the MHC class I-peptide complex is transported via the Golgi system to the cell surface, where the complex is presented to CD8 T cells.

Cross-presentation

It has long been suggested that peptides presented in MHC class I molecules were derived from endogenous proteins degraded by the Ubiquitin Proteasome System (UPS), whereas MHC class II presented peptides were the result of degradation of exogenous proteins in the endocytic pathway. However, this classical view where endogenous and exogenous protein degradation pathways are strictly separated has been revised. One of the best known examples that shows connection between the different protein degradation pathways is cross-presentation. pAPCs, such as DCs, are essential in starting an immune response (69). They can activate naïve CD8 T cells by providing costimulatory signals upon recognition of a MHC class I-peptide complex presented at the cell surface of DCs. However, DCs are often not infected by pathogens, which prevents direct presentation of intracellular proteins in MHC class I molecules to CD8 T cells. Therefore, DCs have the capacity to take up antigens from the periphery and in addition to presenting this exogenous material in the MHC class II pathway, the antigens are processed and presented in the MHC class I pathway. This process is termed cross-presentation. The ($CD8^+$)DC is the main cell type known to be involved in cross-presentation (70, 71), but B cells (72), endothelial cells (73, 74) and macrophages (75, 76) have been reported to be able to cross-present as well. How do endocytosed proteins end up as peptides in MHC class I molecules? At least two different pathways have been well described: the phagosome-to-cytosol pathway and the vacuolar pathway of cross-presentation. For the first pathway, the finding that both proteasomes and the transporter associated with antigen processing (TAP) are required for cross-presentation of several antigens was very important (8, 77). This indicated that the captured exogenous antigen must be transferred from the phagosome to the cytosol. Although this mechanism is not completely understood, a role for Sec61 has been suggested. Once in the cytosol, the antigen is cleaved by the proteasome into peptides that are either transported by TAP followed by loading on MHC class I molecules in the endoplasmic reticulum (ER) or the peptides are transported back into phagosomes by TAP and loaded on MHC class I molecules. In the latter case, the phagosome probably obtained these ER-proteins (TAP, MHC class I) (78-80) by fusion with the ER membrane during phagocytosis (81, 82). The vacuolar pathway did not require TAP and was also proteasome-independent, indicating that the peptides that were cross-presented were not generated in the cytosol. It was shown that the cysteine protease Cathepsin S (Cat S) is important in the generation of cross-presented peptides in the vacuolar pathway, since DCs from Cat S-deficient mice were unable to cross-present via the vacuolar pathway whereas there was no defect in cross-presentation via the phagosome-to-cytosol pathway (83). So in the vacuolar pathway, antigens are degraded into peptides in the phagosome, which involves Cat S, where loading onto MHC

class I molecules occurs. Whether these MHC class I molecules are internalized, transported from the ER or obtained by ER-phagosome fusion remains unclear. It is clear however, that cross-presentation provides an important mechanism for the immune system to respond to infections that occur in the periphery.

Infection models: Listeria and Influenza

Listeria

In this thesis, a recombinant Listeria strain containing an immunoproteasome-dependent ($E1B_{192-200}$) and –independent epitope ($E1A_{234-243}$) was used as a model system for studies on proteasome mediated antigen processing and CD8 T-cell responses. The intracellular Gram-positive bacterium *Listeria monocytogenes* is well characterized, relatively safe, easy to use and therefore forms the perfect model pathogen to study the immune system in mice (84). Upon infection, Listeria enters the spleen and liver where bacteria are taken up by macrophages. By secretion of listeriolysin O (LLO), the phagosomal membrane is destroyed providing a route to escape the phagosome and to enter the cytosol (85). Using LLO for membrane perforation, Listeria can travel to other cells without leaving the cell. CD8 T cells have been shown to limit the spread of this intracellular pathogen (86). However, also innate effector mechanisms such as IFNγ produced by natural killer cells (NK) and inflammatory DCs (TiPDCs) are an important control of infection (87). CD8 T-cell responses are mainly directed to proteins that are secreted into the cytosol such as LLO and the hydrolase p60 and are processed via the MHC class I pathway (88, 89). It has been reported that marginal zone macrophages in the spleen are the main cells to take up antigen from the bloodstream after intravenous inoculation (90) and to spread the infection to the spleen. Recently, the $CD8\alpha^+$ DCs were shown to be the first cell type to become infected, as hardly any Listeria were found in the spleen after depletion of $CD11c^+$ cells (91).

Influenza

Influenza A virus (IAV) belongs to the family of orthomyxoviruses and is an enveloped, single stranded RNA virus. The surface proteins hemagglutinin (HA) and neuraminidase (NA) are important for virus attachment. The HA protein interacts with sialic acid on the host cell and the virus is subsequently internalized. Infection with IAV is mainly restricted to the lungs (92) and this respiratory virus is widely used as a model pathogen to investigate CD8 T-cell responses. Infection of C57BL/6 mice with Influenza causes pneumonia and the virus is usually cleared at day ten after infection (92). Many of the epitopes of the IAV recognized by CD8 T cells are derived from the internal proteins like nucleoprotein (NP) and the acidic polymerase (PA) or from the surface protein haemagglutinin (HA). Therefore, reïnfection with a second

Influenza virus (PR8 H1N1 or HKx31, H3N2) that have the same internal proteins of the virus but differ in their expression of surface proteins is possible without the presence of neutralizing antibodies. This is used as a model to analyse the protective capacity of CD8 T cells of previously immunized mice. Different CD8 T-cell epitopes have been characterized of which the H2-D^b epitopes $NP_{366-374}$ and $PA_{224-233}$ are the best studied (93). As processing and presentation of the IAV $PA_{224-233}$ epitope requires presence of immunoproteasomes (94), in contrast to the $NP_{366-374}$ epitope, IAV infection was used as a control system next to the processing of epitopes derived from recombinant Lm strains, to investigate the role of different cell types involved in antigen processing and presentation to CD8 T cells (see chapter 6 of this thesis).

Scope of the thesis

It is becoming increasingly more evident that the IFNγ-inducible components of the proteasome system play a central role not only in MHC class I antigen processing, but also in many other aspects of immune responses. To further unravel the role of these proteasome components, several questions were addressed in this thesis. Immunoproteasomes and PA28 can dramatically enhance the generation of several MHC class I presented antigenic peptides, so we questioned whether the IFNγ-inducible proteasome components (PA28 and the immunosubunits) are responsible for processing of the immunodominant epitopes targeted by pathogen-specific, protective CD8 T-cell responses. In **chapter 2** we describe the effects of both the immunosubunits and PA28 on MHC class I antigen processing and CD8 T-cell responses by quantifying production of MHC class I ligands and CD8 T cell responses in mice deficient for either or both PA28 and LMP7+MECL-1.
MECL-1-deficient mice maintain different CD4/CD8 T-cell ratios in the periphery than wt or also LMP7-deficient controls. To determine whether the effects of immunoproteasomes on CD4/CD8 T-cell ratios are caused by differences in antigen processing, in cytokine exposure or by T-cell intrinsic mechanisms, we investigated how immunoproteasomes influence T-cell regulation in **chapter 3**, by analyzing T-cell compartments of mice that differed in immunosubunit composition and were developed in the same environment.
NK cell education requires interactions of inhibitory receptors on NK cells with MHC class I molecules, and peripheral NK cells in mice with severe deficits in MHC class I antigen processing are hyporesponsive. Do immunosubunits, by decreasing MHC class I expression, affect the functional phenotype of NK cells? This question was addressed in **chapter 4**, where we investigated the presence of educated NK cells in immunoproteasome-deficient mice by comparing the phenotype and effector func-

tions of NK cells in wt and immunosubunit-deficient mice. Furthermore, NK cell mediated rejection of cells lacking immunoproteasomes was studied after transfer into wt mice in the presence or absence of an infection.
Chapter 5 describes the kinetics of NK cell proliferation, contraction and the survival after infection with a respiratory virus.
Priming of CD8 T cells is believed to be performed by non-infected pAPCs like DCs. Where antigen processing of the presented peptides that prime CD8 T cells occurs remains unknown. The site where antigen processing occurs and the mechanism by which antigens are delivered to DCs was studied in **chapter 6**. For this, different bone marrow-chimeric mice were generated in order to control the expression of immunoproteasomes in specific cell types and a recombinant *Listeria monocytogenes* infection model was used that harbors an immunoproteasome-dependent epitope. Finally, in **chapter 7** the function of the immunoproteasome is discussed based on current knowledge together with the data described in this thesis.

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CHAPTER 2

PA28 and the proteasome immunosubunits play a central and independent role in the production of MHC class I-binding peptides in vivo

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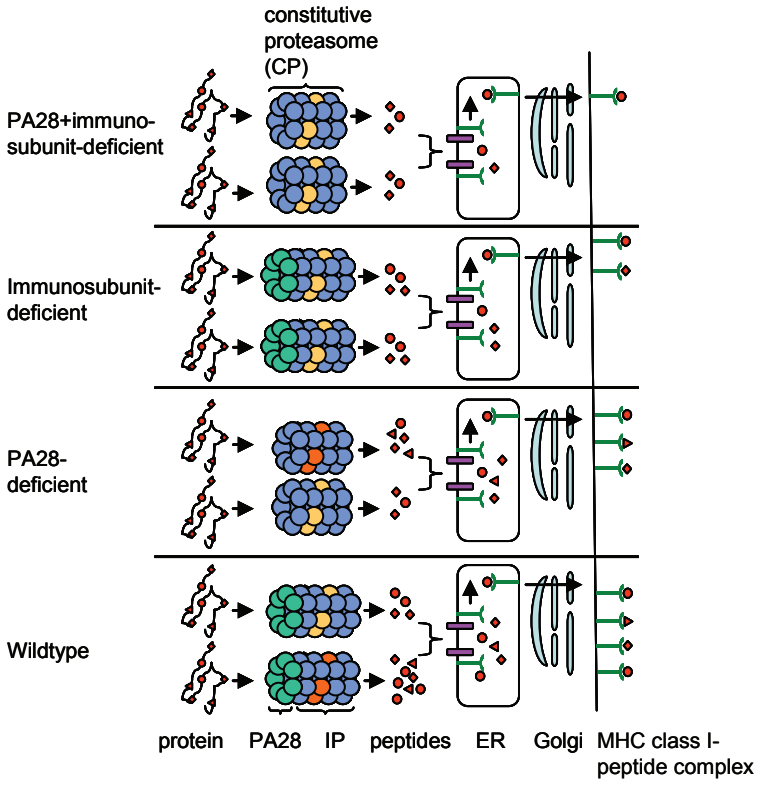
In this issue

Additive role for PA28 and proteasome immunosubunits in MHC class I ligand production

CD8+ T cells recognize peptides that are presented on the cell surface by MHC class I molecules. These peptides are generated upon degradation of intracellular proteins by the proteasome. Antigen presenting and cytokine-exposed cells express three inducible catalytic proteasome subunits, the immunosubunits, and the proteasome regulator PA28. In this issue, De Graaf et al. investigate whether these inducible proteasome components play a compounded role in antigen processing, using mice lacking either the immunosubunits, PA28, or both. The authors report that PA28-deficiency reduces the production of MHC class I-binding peptides, both in cells with and without immunosubunits. In the latter cells, PA28-deficiency further decreases the already diminished production of MHC ligands seen in the absence of immuno-proteasomes. These findings suggest that the immunosubunits and PA28 effects are additive, indicating that the immunosubunits and PA28 must use fundamentally different mechanisms to enhance the supply of MHC class I-binding peptides.



Abstract

Proteasomes play a fundamental role in the processing of intracellular antigens into peptides that bind to MHC class I molecules for the presentation to $CD8^+$ T cells. Three IFNγ-inducible catalytic proteasome (immuno)subunits as well as the IFN-γ-inducible proteasome activator PA28 dramatically accelerate the generation of a subset of MHC class I-presented antigenic peptides. To determine whether these IFNγ-inducible proteasome components play a compounded role in antigen processing, we generated mice lacking both PA28 and the immunosubunits β5i/LMP7 and β2i/MECL-1. Analyses of MHC class I cell surface levels *ex vivo* demonstrated that PA28-deficiency reduced the production of MHC class I-binding peptides both in cells with and without immunosubunits, in the latter cells further decreasing an already diminished production of MHC ligands in the absence of immunoproteasomes. In contrast, the immunosubunits but not PA28 appeared to be of critical importance for the induction of $CD8^+$ T-cell responses to multiple dominant Influenza and Listeria-derived epitopes. Taken together, our data demonstrate that PA28 and the proteasome immunosubunits use fundamentally different mechanisms to enhance the supply of MHC class I-binding peptides; however, only the immunosubunit-imposed effects on proteolytic epitope processing appear to have substantial influence on the specificity of pathogen-specific $CD8^+$ T-cell responses.

Introduction

Cells continuously turn over their intracellular proteins, leaving short peptides of which a small percentage is translocated into the ER and binds to MHC class I molecules for display on the cell surface. Here, they can be recognized by $CD8^+$ T cells, allowing these cells to react to intracellular pathogens. The previous studies have shown that the kinetics and efficiency of epitope processing from pathogen-derived antigens play an important role in both $CD8^+$ T-cell activation (1-4) and their ability to recognize infected cells (5-7). The production of most MHC class I-presented peptides starts with the proteolysis of mature proteins or defective ribosomal products by the proteasome, an abundant cellular protease. Proteasomes consist of a catalytic core particle (20S) and one or more regulatory complexes. In most cells, the enzymatic activity of the 20S particle is exerted by three constitutively expressed subunits, β1, β2 and β5 (8). In contrast, lymphoid cells and cells exposed to cytokines such as type I interferons (9), TNFα (10) and IFNγ (11, 12) express three facultative, homologous subunits, induced (i) β1i/LMP2 (LMP, low-molecular-mass polypeptide), β5i/LMP7 and β2i/MECL-1 (MECL-1, multicatalytic endopeptidase complex-like-1), that replace the constitutive subunits in newly assembled so-called immunoproteasome complexes. Cytokine exposure further upregulates the expression of the proteasome regulator PA28αβ (PA28, proteasome acticator 28 kDa) (13), which is also found at enhanced quantities in professional APC (pAPC) (14-16), as well as that of many other proteins involved in antigen processing.

Both immunosubunit and PA28 expression are IFNγ-inducible, suggesting that these components of the proteasome system play an important role in immune recognition of infected host cells. A variety of studies have shown that incorporation of the immunosubunits β1i/LMP2, β5i/LMP7 and β2i/MECL-1 changes the 20S' cleavage preferences and enhances the proteasome-mediated generation of a significant number of antigenic peptides (17). This is most clear in cells lacking β5i/LMP7, where a defect in peptide supply leads to reduced MHC class I cell-surface levels (18). The immunosubunit-induced changes of proteasome-mediated epitope production have clear effects on the fine specificity of $CD8^+$ T-cell responses to intracellular pathogens. Thus, mice lacking β1i/LMP2, β5i/LMP7 and/or β2i/MECL-1 fail to mount $CD8^+$ T-cell responses to epitopes that are inefficiently generated by the constitutive proteasome subunits, probably because epitopes that are presented at relatively late time points after infection fail to prime $CD8^+$ T-cell responses (1). Nevertheless, the absence of immunosubunits does not impair the responses against other epitopes that are made by constitutive proteasomes and mice lacking either β1i/LMP2, β5i/LMP7 or β2i/MECL-1 or both β5i/LMP7 and β2i/MECL-1, despite partially impaired $CD8^+$ T-

cell responses, are capable of resolving infections with intracellular pathogens (19, 20). Thus, the inducible proteasome subunits do not seem to play an essential role in immune protection. Instead, a recent study suggested that immunoproteasomes may serve primarily to remove protein aggregates, accumulating due to IFN-induced oxidative stress (21).

The effects of PA28 on antigen processing and $CD8^+$ T-cell responses are less clear. The PA28 α- and β-subunits form a heptamer (22) that attaches to the 20S particle and activates this *in vitro*, leading to an enhanced turnover of short peptide substrates and an increase in double cleavages in polypeptide substrates (23, 24). *In vitro* studies, in which polypeptide substrates were digested with 20S proteasome/PA28 complexes, PA28$\alpha\beta$-gene-deficient cells or cells transfected with PA28α and or PA28β-encoding vectors were used, indicated that PA28 enhanced MHC class I presentation of several antigenic peptides (17, 25), but that the effects of PA28 were confined to specific MHC class I alleles (26). Mice lacking PA28 did not show clear defects in $CD8^+$ T-cell responses to Influenza virus or viral resistance (25); however, they were not analyzed for $CD8^+$ T-cell responses or resistance to any other pathogen. Notably, peritoneal macrophages of these mice failed to upregulate MHC class I cell-surface expression following IFNγ treatment (27). Such defects in MHC class I upregulation were not observed in mouse embryonic cells of these gene-deficient mice (28). Taken together, these studies suggest that the effects of PA28 on antigen processing and immune reponses are rather mild.

Although cytokine-exposed cells in infected tissues as well as professional APCs usually express both immunosubunits and PA28, the effects of PA28 or immunosubunits on MHC class I antigen processing and $CD8^+$ T-cell responses have been studied separately, so far. Thus, it is unknown whether these two types of IFNγ-inducible proteasome components have synergistic, additive or perhaps largely overlapping roles in antigen processing. To dissect the effects of PA28 on peptide production by immuno- versus constitutive proteasomes, we quantified 20S/PA28-mediated peptide liberation from a model substrate *in vitro*, and crossed PA28 gene-deficient (25) with β5i/LMP7+β2i/MECL-1 gene-deficient mice (29) and quantified proteasome-mediated production of MHC class I ligands and $CD8^+$ T-cell responses in the different proteasome component-deficient strains.

Materials and methods

Cells

MEC-PA28 and T2 cells were cultured as described previously (6, 30). LPS blasts were expanded by the stimulation of splenocytes with 30 μg/mL LPS in RPMI with

10% FCS, glutamax, antibiotics and 30 μM β-mercaptoethanol.

Mice

C57BL/6 (B6) mice were purchased from Charles River. B6 β5i/LMP7+β2i/MECL-1 gene-deficient (29) and PA28αβ gene-deficient mice (25) were maintained by in-house breeding. PA28+β5i/LMP7+β2i/MECL-1 gene-deficient mice were generated by crossing $PA28^{-/-}$ with β5i/LMP7+β2i/MECL-$1^{-/-}$ mice. All animal experiments were performed with age-matched mice and approved by the Committee of Animal Experiments of the University of Utrecht.

Infection and immunization of mice

Infections with rLM and immunizations with peptide-pulsed DCs were performed as described previously (1). For infection with Influenza strain HKx31, mice were anesthetized with isofluorane and then infected *i.n.* with 5 x 10^4 hemagglutination units in 30 μL PBS.

Western blot analysis

The presence of PA28, β5i/LMP7 or β2i/MECL-1 was analyzed by Western blotting as described previously (6, 31).

Abs and flow cytometry

mAbs used in this study included fluorochrome- or biotin-conjugated anti-mouse H2-K^b (clone AF6-88.5) and anti-mouse H-$2D^b$ (clone 28-14-8), fluorochrome-conjugated anti-mouse CD8α (clone 53-6.7), anti-mouse CD19 (clone MB19-1), anti-mouse CD4 (clone RM 4-5), anti-mouse IFNγ (clone XMG1.2) and anti-mouse B220 (clone RA3-6B2). Immunofluorescence staining and FACS analyses of specific cell subsets were performed as described previously (31).

Intracellular cytokine staining

Mice were sacrificed at the time points as specified in the figure legends and spleens or BAL was collected. Percentages of $E1B_{192-200}$-, $NP_{366-374}$ - or $PA_{224-233}$ -specific $CD8^+$ T cells and $LLO_{189-201}$-specific $CD4^+$ T cells were determined by intracellular cytokine staining and FACS analysis as described previously (1).

Peptides

The synthetic polypeptide AYRPPNAPILSTLPETTVVRRRGRSPRRRTPS (HBV subtype ayw, cAg amino acids 131–162) was synthesized by Dr. P. Henklein of the Charité, Humboldt University, Berlin.

***In vitro* digestion assays**

PA28 was purified from 2 x 10^9 MEC-PA28 cells (6), grown in the absence of tetracycline, as described previously (32). Constitutive and immuno-20S proteasomes were purified from 6 to 8x10^8 T2 and T2 LMP2+7 cells, respectively, as described previously (30). The purity of the preparates was checked by Coomassie-stained SDS-PAGE and was >90%.

In total, 20 µg of HBV 32-mer polypeptide, 3 µg of purified proteasomes and 1.5 µg of purified PA28 were incubated in 300 µL assay buffer (20 mM Hepes/KOH, pH 7.8, 1 mM DTT) at 37°C for the time periods specified in the figure legends. Digestions in the absence of PA28 were performed in assay buffer containing 20 mM Hepes/KOH, pH 7.8, 2 mM $MgAc_2$, and 1 mM DTT. Samples of the digestion reactions were frozen at -20°C until the analysis by RP-HPLC and mass spectrometry, as described previously (32).

Results

PA28 and immunosubunits have synergistic effects on 20S-mediated peptide liberation in vitro

Previous studies have shown that PA28 enhances the proteasome-mediated production of different antigenic peptides (6, 32, 33), but neither directly compared the effects of PA28 versus immunosubunits on 20S-mediated fragmentation of substrates, nor determined the effects of PA28 on the cleavage profile of constitutive versus immunoproteasomes. To test how PA28 modulated the activity of constitutive versus immunoproteasomes, we digested a synthetic polypeptide derived from the hepatitis B virus core antigen (HBV cAg) (Figure 1A) with purified constitutive and immunoproteasome complexes, in the presence or absence of PA28. Peptide products were analyzed by RP-HPLC and online mass spectrometry. As shown previously, cleavage of the HBV polypeptide by constitutive proteasomes liberated the peptides Ser_{141}-Val_{149} and Ser_{141}-Arg_{152} (30). Even higher quantities of the same two fragments and, in addition, the fragments $Ser_{141\text{-}150}$ and Ser_{141}-Arg_{151} (30) were found in the digests of immunoproteasomes. Remarkably, the presence of PA28 in the digests dramatically enhanced the efficiency of liberation of the 141-149 fragment by both constitutive and immunoproteasomes, and of 141-150 by immunoproteasomes (Figure 1B) (note the difference in time scale between upper and lower panel). The presence of PA28, however, did not enhance the generation of fragments 141-151 and 141-152 by either constitutive or immunoproteasomes (Figure 1B). Thus, PA28 enhanced the generation of a subset of 20S double-cleavage products only and failed to confer new cleavage specificities to the 20S complex, which

was most evident from the finding that PA28 enhanced the liberation of 141-150 by immunoproteasomes but did not enable constitutive proteasomes to generate this fragment. Notably, as the set of peptides produced by immunoproteasomes seems more diverse (Figure 1) and better suited for MHC class I binding (18, 34) than that produced by constitutive proteasomes, it is possible that PA28 further enhances the immunoproteasome-mediated generation of MHC class I ligands.

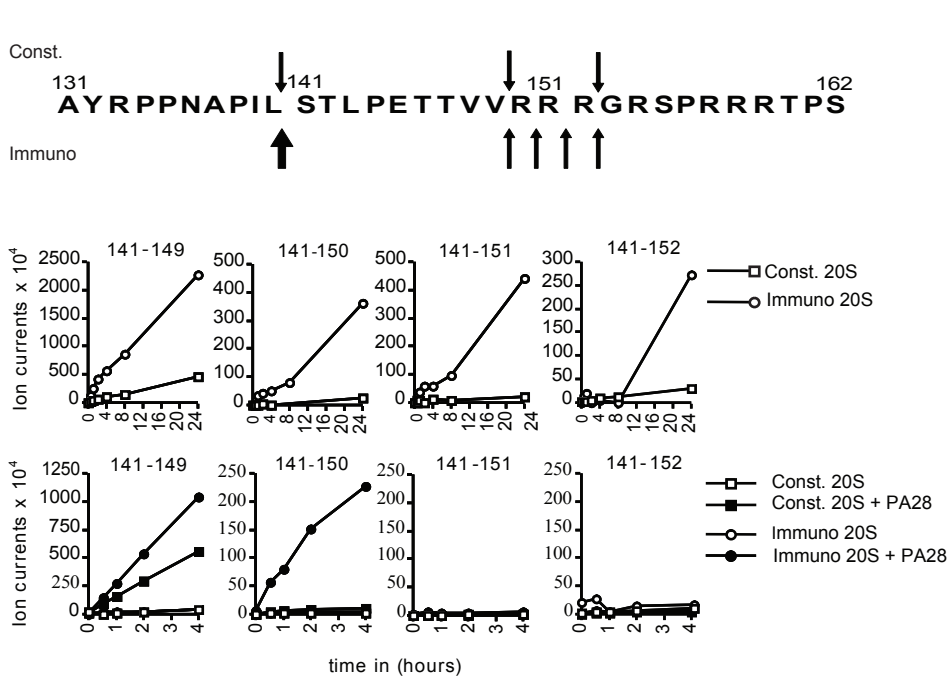


Figure 1. Kinetic analysis of the effects of PA28 and immunosubunits on the generation of HBV cAg131-162 cleavage products. (A) Amino acid sequence of the HBV cAg131-162 polypeptide substrate. Arrows indicate dominant cleavage sites of constitutive or immunoproteasomes (32). (B) HBV cAg131-162 was incubated with constitutive or immunoproteasomes of T2 or T2+β1i/LMP2+β5i/LMP7 cells, in the absence or presence of a four-molar excess of PA28, purified from induced MEC-PA28 cells (6), as indicated. Digestion products were separated by RP-HPLC and identified by mass spectrometry. Accumulation of different peptide products over time of digestion is shown. HBV cAg131-162 incubated with PA28 without proteasomes was not degraded. Data are representative of two independent experiments.

Generation of a gene-deficient mouse strain lacking PA28 and immunosubunit expression

To be able to determine the respective and potentially compounded roles of the IFNγ inducible catalytic subunits and PA28 in MHC class I antigen processing, β5i/

LMP7+β2i/MECL-1 double gene-deficient mice (29) were crossed with PA28αβ-double gene-deficient mice (25). As 20S complexes containing β1i/LMP2 assemble inefficiently in the absence of β5i/LMP7 (35), lymphoid- and cytokine-exposed cells of β5i/LMP7+β2i/MECL-1-deficient mice contain predominantly constitutive proteasomes. Homozygous mice lacking the genes coding for PA28α, PA28β, β5i/LMP7 and β2i/MECL-1 were identified by PCR (data not shown) and deficiency of these proteasome components was confirmed by immunoblot analyses (Figure 2). The PA28+β5i/LMP7+β2i/MECL-1 gene-deficient mice did not show any phenotypic characteristics or abnormalities in growth or fertility.

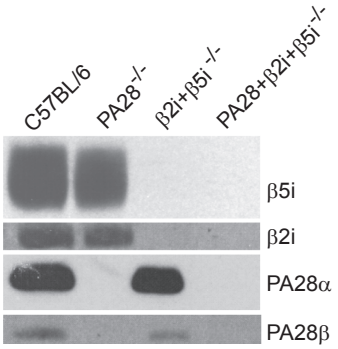


Figure 2. The absence of PA28 and immunosubunit expression in gene-deficient mouse strains. Splenocytes of indicated mouse strains were extracted, and the lysates were separated by SDS/PAGE, and β5i/LMP7, β2i/MECL-1, PA28α and PA28β were detected by Western blotting. Equal loading was confirmed by Ponceau S staining. Data are representative of one experiment.

PA28-deficiency does not alter the ratios of CD4:CD8 T-cell subsets

Previous studies have shown that, compared with wt B6 mice, mice lacking the immunosubunit β2i /MECL-1 display an enhanced relative ratio of $CD4^+$ to $CD8^+$ T cells in the spleen (29, 31, 36). Comparing the cellular composition of the spleens of the different gene-deficient mouse strains (Figure 3A), we found reduced frequencies of $CD8^+$ T cells in β5i/LMP7+β2i/MECL-1-deficient compared with wt mice, consistent with these previous studies. In addition, $PA28^{-/-}$ mice showed slightly diminished frequencies of $CD8^+$ T cells in comparison to wt B6 mice, but no clear differences were discernible between β5i/LMP7+β2i/MECL-$1^{-/-}$ and PA28+β5i/LMP7+β2i/MECL-$1^{-/-}$ mice (Figure 3A). Frequencies of splenic $CD4^+$ T cells were similar in wt, $PA28^{-/-}$ and PA28+β5i/LMP7+β2i/MECL-$1^{-/-}$ mice, whereas in β5i/LMP7+β2i/MECL-$1^{-/-}$ mice the numbers of $CD4^+$ T cells were slightly decreased (data not shown). Expressed as ratios, we detected increased CD4:CD8 ratios in β5i/LMP7+β2i/MECL-$1^{-/-}$ and PA28+β5i/LMP7+β2i/MECL-$1^{-/-}$ mice in comparison to wt or PA28-deficient mice, both in the spleen (Figure 3B) and in the lymph nodes (data not shown), whereas CD4:CD8 T-cell ratios in the thymus were similar in all groups (Figure 3B), consistent with Caudill et al. (29). Thus, the increased CD4:CD8 T-cell ratios in mice deficient for the immunosubunits (and PA28) are found only in the secondary lymphoid or-

gans. Probably, they are caused by the effects of β2i/MECL-1 on homeostatic expansion (31), and are not affected by the presence or absence of PA28.

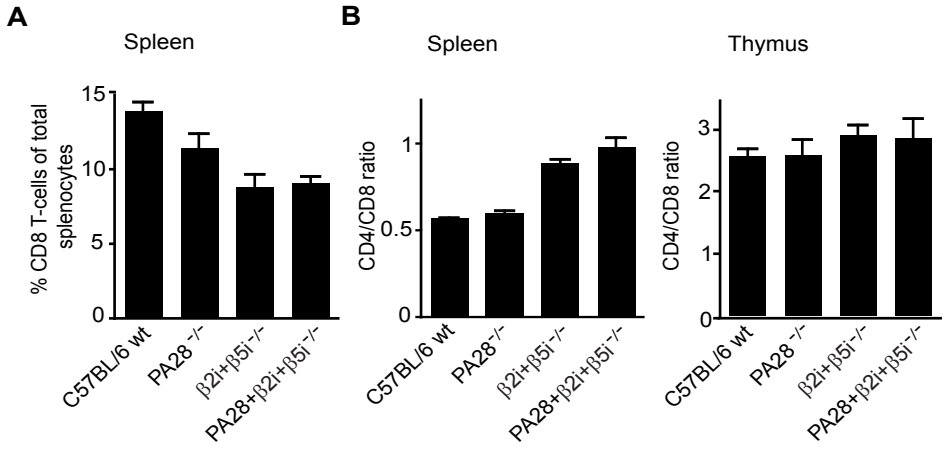


Figure 3. Effects of PA28 and immunosubunits on CD4:CD8 T-cell ratios in lymphoid tissues of uninfected mice. (A) $CD8^+$ T cells were detected in the spleens of B6 wt, $PA28^{-/-}$, β5i/LMP7+β2i/$MECL-1^{-/-}$ and PA28+β5i/LMP7+β2i/$MECL-1^{-/-}$ mice by staining with anti-CD8α mAb and FACS analysis. Frequencies of $CD8^+$ T cells as percentage of total lymphocytes are shown (data show mean + SEM, n = 3 mice per group, and are representative of two independent experiments). (B) CD4:CD8 T-cell ratios in the spleen and the thymus of B6 wt, $PA28^{-/-}$, β5i/LMP7+β2i/$MECL-1^{-/-}$ and PA28+β5i/LMP7+β2i/$MECL-1^{-/-}$ mice. Data show mean + SEM, n = 3 mice per group, and are representative of two independent experiments. FACS gating strategy for the detection of CD4 (or CD8)-positive cells is shown in Supporting Information Figure 2.

PA28 and immunosubunits play an additive role in the generation of MHC class I ligands

$H2^b$ cells that lack PA28 or β5i/LMP7 expression display diminished amounts of cell-surface MHC class I molecules, indicating that both these proteasome components play an important role in the production of MHC class I ligands (18, 26, 29, 31, 34, 37). As PA28 increases the production of only a subset of the peptides that are liberated by proteasomes (Figure 1) and immunosubunit incorporation changes the proteasomal cleavage profile to support the production of MHC class I ligands (18, 29, 31), it is possible that PA28 further enhances the positive effects of the immunosubunits on MHC class I antigen processing. To address this possibility, we compared the MHC class I cell-surface levels on splenocytes of wt and the different proteasome component-deficient mice. As expected, we found that defective immunosubunit expression resulted in reduced cell-surface expression of MHC class I H2-K^b and D^b molecules on splenocytes, such as B cells (Figure 4A) and T cells (data not shown), and also on BMDCs (data not shown). The absence of PA28 led to a reduction of MHC class I cell-surface expression on immunoproteasome-positive

splenic B cells, for H2-K^b to 14,3 % of that on wt cells and, surprisingly, reduced the already diminished MHC class I cell-surface expression on β5i/LMP7+β2i/MECL-1$^{-/-}$ cells even further (Figure 4), with 18,8 % (Table 1). These similar reductions in MHC class I cell-surface levels for PA28-deficient cells with and without immunoproteasomes argue against a compounded role of PA28 and immunosubunits in MHC class I antigen processing. Thus, PA28 enhances the generation of MHC class I-binding peptides by constitutive and immunoproteasomes equally.

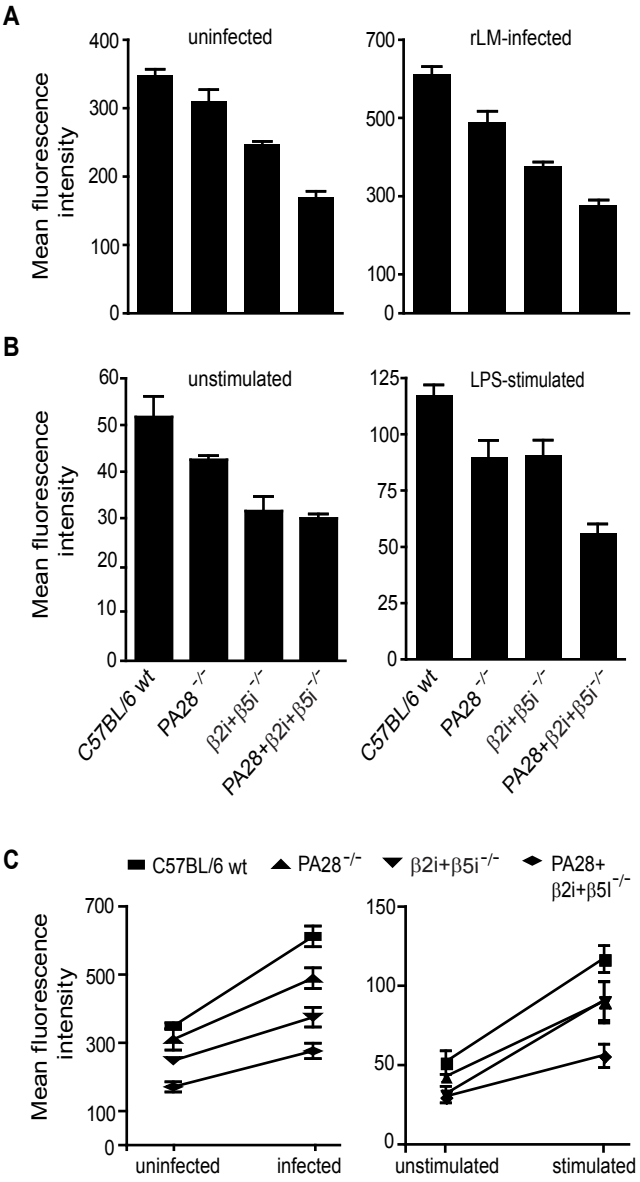


Figure 4. Role of immunosubunits and PA28 in the production of MHC class I ligands. (A) Splenocytes of uninfected mice and mice, infected 8 days earlier with rLM, were stained with anti-B220 and a conformation-dependent anti-H-2K^b mAb. Data show mean + SEM, n = 2 to 4 mice per group, and are representative of two to four independent experiments. FACS gating strategy is shown in Supporting Information Figure 1. (B) Unstimulated and LPS-stimulated splenocytes of uninfected mice were stained with anti-CD19 and anti-H-2K^b mAbs. MFIs of H-2K^b staining on B cells are shown (data show mean + SEM; n = 3 to 5 mice per group, and are representative of two to four independent experiments). (C) Upregulation of MHC class I H-2K^b cell surface levels on B220$^+$ splenocytes of rLM-infected mice compared to uninfected mice (left panel) or on LPS-stimulated compared to unstimulated B220$^+$ splenocytes (right panel). Data show mean ± SD, n = 2 to 4 mice per group and are representative of two independent experiments.

Listeria monocytogenes, administered *i.v.*, infects the mouse spleen and liver, leading to the recruitment of immune effector cells and cytokine secretion in these tissues. This, in turn, enhances the expression of the components of the MHC class I antigen processing pathway (38, 39) and therewith the demand for MHC class I ligands. To determine whether a potentially compounded role of PA28 and immunosubunits in antigenic peptide production is detectable under such conditions, we analyzed MHC class I cell-surface expression on splenic B cells of the different gene-deficient mice, 8 days after *i.v.* infection with recombinant Listeria. Indeed, the mean fluorescence channels detected for MHC class I (K^b) on splenocytes of recombinant *L. monocytogenes* (rLM)-infected mice were consistently higher than those on the cells of uninfected mice, measured in the same experiment (Figure 4A). Upregulation of MHC class I expression was detected for all mouse groups. Thus, despite the lack of expression of immunosubunits and/or PA28, cells are able to upregulate MHC class I cell-surface expression in response to external stimuli although wt B cells upregulated MHC class I markedly more efficiently than either $PA28^{-/-}$, β5i/LMP7+β2i/$MECL\text{-}1^{-/-}$ or PA28+β5i/LMP7+β2i/MECL-1-deficient cells (Figure 4C). Consistent with the observations on splenocytes of uninfected mice, PA28-deficiency diminished MHC class I cell surface expression on both immunoproteasome-positive and -deficient cells, again to approximately similar extents (Figure 4A and Table 1). To further verify these findings, MHC class I expression on splenocytes stimulated with LPS *in vitro* was compared with that on unstimulated B cells, analyzed *ex vivo*. As shown in Figure 4B and C, LPS treatment led to a similar upregulation of MHC class I K^b and D^b (data not shown) cell-surface expression as cytokine exposure, with the highest levels reached for cells of wt mice and the lowest upregulation observed on cells lacking both immunosubunits and PA28. Again, PA28-deficiency reduced the relative quantities of $H2K^b$ and D^b molecules on immunoproteasome-positive and immunoproteasome-deficient cells similarly (Figure 4B, Table 1). The cell surface levels of MHC class II and CD19 molecules were similar for gene-deficient and wt cells (data not shown), indicating that PA28 does not interfere with cell-surface trafficking of glycoproteins in general. Furthermore, $H2\text{-}K^b$ levels on immunosubunit and PA28-deficient LPS blasts were restored upon overnight incubation with synthetic $ova_{257\text{-}264}$ peptide (data not shown), indicating that overall generation of MHC class I molecules was not impaired, but that the reduced expression of K^b (and D^b) in mice deficient for one or more of the inducible components of the proteasome must be attributed to the defects in the supply of peptides that bind MHC class I with high affinity. Taken together, these data show that both the immunosubunits and PA28 play an important role in proteasome-mediated production of high-affine MHC class I ligands and that PA28 potentiates the production of MHC class I ligands by immunoproteasomes and constitutive proteasomes similarly. Moreover, the effects of

PA28 and the immunosubunits are additive, indicating that they use different, largely non-overlapping mechanisms to enhance proteasome-mediated generation of MHC class I ligands.

Table 1. Reduced MHC class I expression on lymphoid cells lacking PA28 [a].

	wt	PA28$^{-/-}$	β5i+β2i$^{-/-}$	PA28+ β5i+β2i$^{-/-}$
K^b (unstim)	100	85.7[a]	100	81.2
K^b (LPS)	100	74.4	100	72.0
K^b (inf)	100	80	100	74.5
D^b (unstim)	100	94.1	100	88.6
D^b (LPS)	100	78.4	100	69.8
D^b (inf)	100	91.6	100	82.5

a) Relative amounts of MHC class I molecules on PA28-deficient unstimulated (unstim) or LPS-stimulated B cells of uninfected or infected (inf) PA28$^{-/-}$ mice on B6 or B6 LMP7+MECL-1-deficient background, expressed as percentage of MHC class I expression on similarly treated B cells of wt or LMP7+MECL-1-deficient mice. MHC class I expression was measured by immunofluorescence staining and FACS analysis. Data are representative of two to four experiments with 3 to 5 mice per group.

PA28-deficiency has no dramatic effects on pathogen-specific CD8$^+$ T-cell responses

Although the proteasome immunosubunits are known to shape the immunodominance hierarchy of pathogen-specific CD8$^+$ T-cell responses (1, 4, 40, 41), the effects of PA28 on the specificity of CD8$^+$ T-cell responses are less clear. To determine whether PA28-deficiency diminishes the ability of wt or β5i/LMP7+β2i/MECL-1-deficient mice to mount T-cell responses, control B6, PA28$^{-/-}$, β5i/LMP7+β2i/MECL-1$^{-/-}$ and PA28+β5i/LMP7+β2i/MECL-1$^{-/-}$ mice were infected with a rLM strain secreting the p60E1 model antigen (rLM-E1) (1), with rLM-ova (42) or with Influenza virus strain HKx31. Quantification of CD8$^+$ T-cell responses to rLM epitopes in the spleen, 8 days after infection, showed that rLM-E1-infected mice lacking β5i/LMP7 and β2i/MECL-1 failed to respond to the immunoproteasome-dependent epitope E1B$_{192\text{-}200}$ (Figure 5A), consistent with our previous data (1). On the contrary, mice that lacked PA28 but expressed the immunosubunits mounted clearly detectable E1B$_{192\text{-}200}$-specific responses (Figure 5A) that did not significantly differ in magnitude from the responses detected in wt mice (Mann-Whitney, two-tailed test, 95% CI). Thus, PA28 does not play a critical role in the processing of the immunoproteasome-dependent epitope E1B$_{192\text{-}200}$.

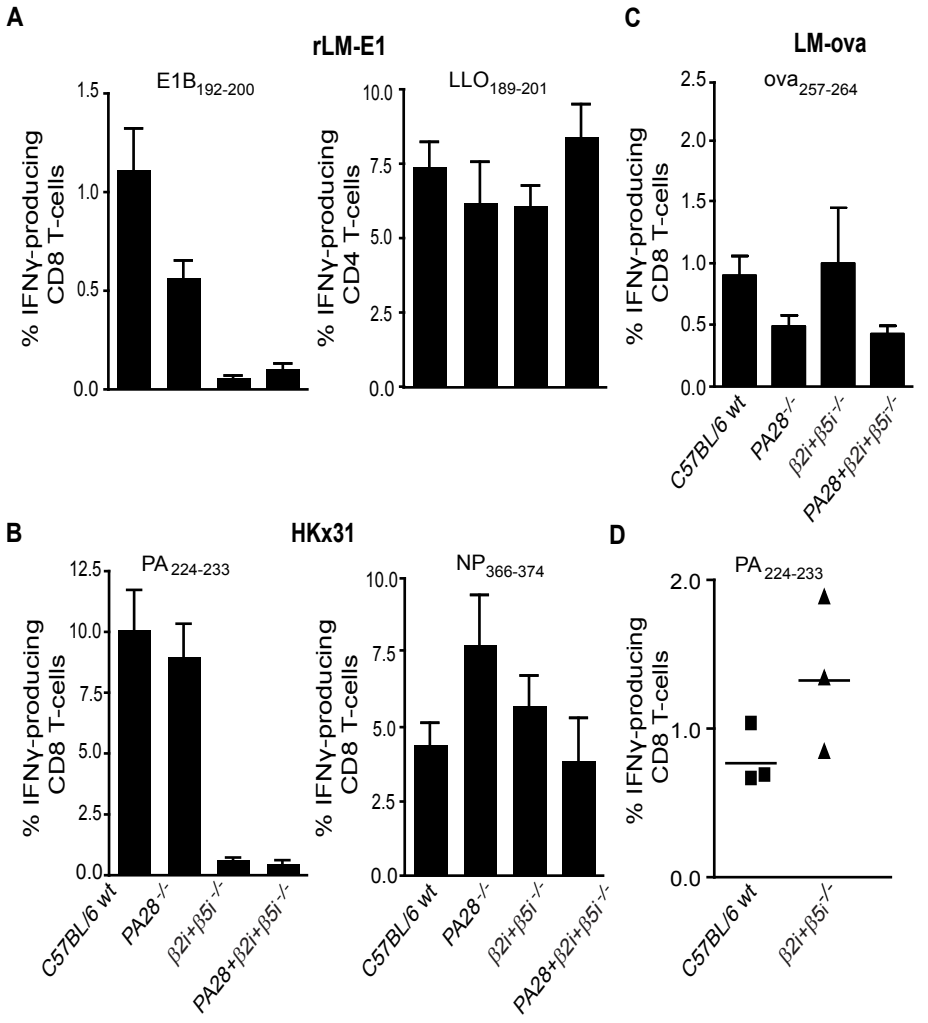


Figure 5. Effects of PA28 and immunosubunits on T-cell responses to rLM- and Influenza-derived antigens. B6 wt, PA28$^{-/-}$, β5i/LMP7+β2i/MECL-1$^{-/-}$ and PA28+β5i/LMP7+β2i/MECL-1$^{-/-}$ mice were infected with (A) 5x10^3 rLm-E1, (B) 5x10^4 U Influenza/HKx31 or (C) 1x10^5 rLM-ova or (D) immunized *i.v.* with $PA_{224-233}$-pulsed BM DCs. (A-D) Antigen-specific, IFNγ-producing CD4$^+$ and CD8$^+$ T cells were quantified. FACS gating strategy is shown in Supporting Information Figure 2. (A, C) Eight days after rLM-infection, the frequencies of $E1B_{192-200}$- or $ova_{257-264}$ -specific CD8$^+$ T cells or $LLO_{189-201}$-specific CD4$^+$ T cells were determined in the spleen by staining for CD8 or CD4 and intracellular IFNγ. (B) Percentages of $NP_{366-374}$ and $PA_{224-233}$ specific CD8$^+$ T cells in the BAL, 7 days after Influenza infection. (D) Percentages of $PA_{224-233}$-specific CD8$^+$ T cells in the spleen, 7 days after injection of peptide-pulsed DCs. Background responses measured in the absence of stimulating peptides were subtracted. Data show mean + SEM and are representative of two to four experiments with n = 2 to 8 mice per group (A-C) or values for individual mice, representative of one experiment (D).

After rLM-ova-infection, all mice responded vigorously to the immunoproteasome-independent $ova_{257-264}$ epitope (Figure 5C), with insignificant difference in the frequencies of ova-specific $CD8^+$ T cells between PA28-deficient and PA28-positive mouse groups. Thus, PA28 is not required for the activation of ova-specific $CD8^+$ T cells. In these experiments, the frequencies of $CD4^+$ T cells responding to the listeriolysin O–derived epitope $LLO_{189-201}$ were similar in all mouse groups, indicating that all mice had been infected and responses to this $CD4^+$ T-cell epitope were not affected by the absence or presence of immunosubunits and/or PA28 (Figure 5A and data not shown).

Responses to the Influenza HKx31-derived $NP_{366-374}$ and $PA_{224-233}$ epitopes were quantified 7 days after infection, in the bronchoalveolar lavage (BAL) (Figure 5B). Although all mice responded to $NP_{366-374}$, we failed to detect $PA_{224-233}$–specific $CD8^+$ T-cell responses in mice deficient for β5i/LMP7+β2i/MECL-1, as expected (40). The absence of a $PA_{224-233}$–specific response could not be explained by defects in the T-cell repertoire, as β5i/LMP7+β2i/MECL-1-deficient mice showed $PA_{224-233}$–specific $CD8^+$ T-cell responses comparable to wt mice that were immunized with GM-CSF expanded BM-derived DCs loaded with synthetic $PA_{224-233}$ peptide (Figure 5D). $PA28^{-/-}$ mice showed a robust response to $PA_{224-233}$ comparable to the response in wt B6 mice.

Taken together, we find that in contrast to the immunosubunits, PA28 has no dramatic qualitative effects on the size of $CD8^+$ T-cell responses mounted against several dominant Influenza- and rLM-derived epitopes.

Discussion

Stimulation of cells with pro-inflammatory cytokines or IFNγ upregulates the expression of the proteasome immunosubunits and of PA28. Although the immunosubunits are known to play an important role in epitope processing and to shape the immunodominance hierarchy of pathogen-specific $CD8^+$ T-cell responses, the impact of PA28 on immune defense still remains enigmatic. We here demonstrate that both the proteasome immunosubunits and PA28 fulfill a central function in the production of MHC class I ligands *in vivo*. Importantly, we find that spleen-derived lymphoid cells of mice that are gene-deficient for PA28 display lower MHC class I expression levels than wt mice, and observe a similar reduction in MHC class I levels on the cells of PA28+β5i/LMP7+β2i/MECL-1-deficient mice, in comparison to mice gene-deficient for the immunosubunits only (Figure 4, Table 1). These differences in MHC class I cell-surface levels become even more apparent after infection of the different mouse strains with rLM, a pathogen that targets the spleen (Figure 4) and induces the

secretion of Th1 cytokines, or after stimulation of B cells from uninfected wt or proteasome component-deficient mice with LPS, in cell culture (Figure 4). Thus, PA28 plays an important role in the production of MHC class I-binding peptides in lymphoid cells. Hence, in quantitative terms, PA28 is the second most important component of the proteasome system that contributes to the production of MHC class I ligands, surpassed only by β5i/LMP7, whose absence is responsible for the reduction in class I levels observed in mice that lack β5i/LMP7+β2i/MECL-1 (29, 31). Although β2i/MECL-1 (and β1i/LMP2) play an important role in the processing of a variety of epitopes (31, 36, 43), their effects are not substantial enough to influence overall MHC class I expression (31). Thus, both proteasome immunosubunits and PA28 should be considered as integral components of the MHC class I antigen processing machinery. They play an important role in the production of high-affinity peptides required to maintain the basal MHC class I cell-surface levels on lymphoid cells, as well as in providing additional ligands to stabilize newly synthesized MHC class I molecules following cytokine exposure.

The finding that PA28-deficiency leads to reduced MHC class I cell-surface levels both on immunoproteasome-positive and -negative cells, on which MHC expression is diminished already due to the absence of immunosubunits (Figure 4), indicates that the effects of the immunosubunits and PA28 are additive. Thus, these proteasome components enhance the production of different MHC class I ligands. This is consistent with the different working mechanisms of PA28 and the catalytic immunosubunits. PA28 binds to the 20S complex, leading to coordinated double cleavages and apparently thereby an enhanced generation of MHC class I ligands (23), whereas the immunosubunits change the 20S cleavage site preferences. In particular, the replacement of the constitutive subunit β5 by β5i/LMP7 leads to enhanced cleavage at the C-terminus of hydrophobic residues, which are critical anchors for binding to MHC class I molecules (34). In addition, enhanced proteolytic activity in the presence of immunoproteasomes may lead to enhanced antigenic peptide generation (21), although our previous studies failed to show any differences in kinetics of presentation of two immunoproteasome-independent epitopes between rLM-infected β5i/LMP7+β2i/MECL-1-deficient and -sufficient DCs *in vitro* (1).

Remarkably, deficiency of PA28+β5i/LMP7+β2i/MECl-1 reduces MHC class I expression only to approximately 53 % on unstimulated cells compared with wt and these levels can still be upregulated by exposure to LPS or cytokines. Thus, IFNγ-induced expression of other components of the antigen processing pathway such as of β1i/LMP2 (which, however, poorly assembles into 20S complexes in the absence of β5i/LMP7 (44)) or of aminopeptidases may lead to the production of additional peptides that stabilize the newly synthesized MHC class I molecules on stimulated PA28+β5i/LMP7+β2i/MECL-1-deficient cells. Alternatively, the increase in the numbers of cell-

surface MHC class I /peptide complexes may result from enhanced synthesis of MHC class I heavy chains and thus a renewed balance between gain and loss of MHC class I molecules, complexed with low- affinity peptides, from the cell surface. In support of this, addition of synthetic $ova_{257-264}$ to LPS blast cultures restores the H2-K^b levels on PA28+β5i/LMP7+β2i/MECL-1-deficient cells to nearly wt levels (data not shown), indicating that MHC class I molecules traffic to the cell surface also in cells that lack these proteasome components.

Importantly, in contrast to the profound effects of immunosubunits on the generation of specific epitopes and the fine specificity of pathogen-specific $CD8^+$ T-cell responses, the contributions of PA28 to peptide presentation had little impact on the sizes of the responses to Influenza- and rLM-derived epitopes. Taken together, all of four dominant $CD8^+$ T-cell epitopes tested triggered robust responses in PA28-deficient mice. The previous studies have shown that PA28 enhances 20S-mediated processing of the ova-derived $ova_{257-264}$ epitope (27, 45). However, our data do not show any significant differences in responses to this epitope in PA28-deficient compared with PA28-positive mice (Figure 5). Thus, reduced $ova_{257-264}$ presentation in the absence of PA28 has no or only minor effects on $CD8^+$ T-cell activation. This contrasts to the dramatic reduction in size of $CD8^+$ T-cell responses in immunoproteasome-deficient mice to immunoproteasome-dependent epitopes of which two ($E1B_{192-200}$ and $PA_{224-233}$) were included in this study.

It has been suggested that the hsp90 chaperone provides an alternative pathway for epitope supply to MHC class I molecules in cells that lack PA28 expression, and also in PA28-positive lymphoid cell lines unless stimulated with IFNγ (27). More recent studies, however, demonstrated that hsp90 binds proteasome-generated protein fragments to protect these from further degradation, and makes such fragments available for MHC class I loading (46, 47). Thus, hsp90 acts downstream of the proteasome, which was confirmed also for PA28-deficient cells (28). Also our finding that MHC class I expression levels are reduced on PA28-deficient compared with wt spleen cells implies that any compensatory effects of hsp90 on the peptide pool available for MHC class I binding in the absence of PA28 are partial at most, and is consistent with the notion that the hsp90 pathway does not produce new MHC class I ligands but enhances the presentation of peptides that are produced by the proteasome.

Taken together, we here show that both PA28 and immunosubunits play an important and additive role in proteasome-mediated processing of intracellular proteins into peptides that bind to MHC class I molecules. The role of PA28 in the production of class I ligands is much greater than expected, based on previous studies (25). We showed previously that the early kinetics of MHC class I antigen processing following infection are strongly influenced by the immunosubunits and determine the

immunogenicity of individual antigenic peptides (1). Similar to the immunosubunits, PA28 also enhances MHC class I presentation of specific epitopes (like $ova_{257-264}$) at early time points after infection. Nevertheless, consistent with previous findings (25), our analyses of rLM and Influenza-specific $CD8^+$ T-cell responses indicate that the effects of PA28 on antigen processing do not have any dramatic effects on the size and fine specificity of $CD8^+$ T-cell responses to these pathogens. Remarkably, a recent study showed that, like immunoproteasomes, PA28 is also involved in the degradation of oxidized proteins. Therefore, these components of the proteasome system may also play a role beyond antigen processing and immune recognition, in cellular adaptation to oxidative stress (21, 48).

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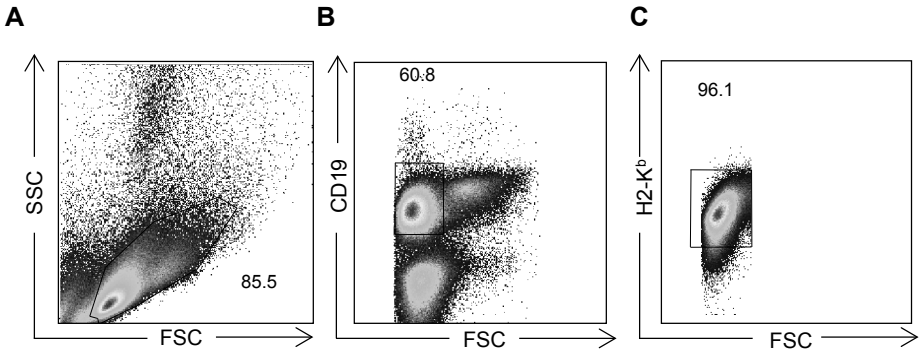
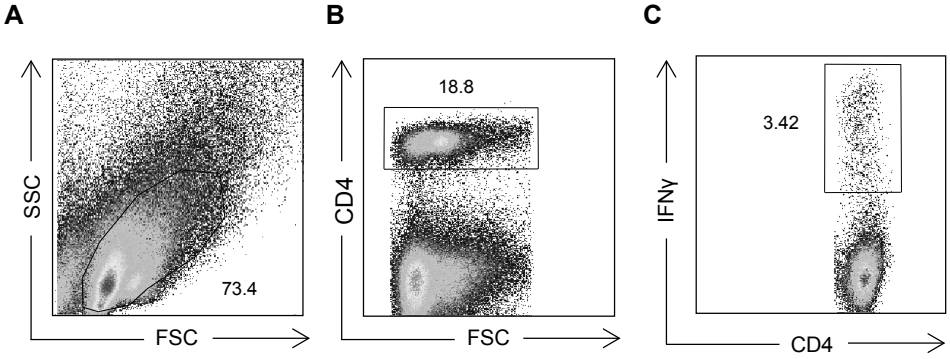
Supporting Information



Supporting Information Figure 1. Gating strategy for detection of MHC class I expression on B cells. Shown are B cells in the spleen of an uninfected wt mouse. Red blood cell-depleted spleen cell suspensions were stained with PE-conjugated anti-CD19 antibody and APC-conjugated anti-H2-Kb antibody. (A), Representative FACS plot with gating on live lymphocytes. (B), Gating of PE-positive B cells within the live gate. (C), K^b expression on CD19-positive B cells.



Supporting Information Figure 2. Gating strategy for detection of antigen-specific IFNγ-positive T cells. Shown are LLO196-201-reactive CD4 T cells in the spleen of an rLM-infected β5i/LMP7+β2i/MECL-1-/- mouse. After 6 hrs of stimulation with LLO196-201 peptide, cells were stained with APC-conjugated anti-CD4 antibody. Intracellular IFNγ was detected with PEconjugated anti-IFNγ antibody. (A), Representative FACS plot with gating on live lymphocytes. (B), Gating of APC-positive CD4 T cells within the live gate. (C), IFNγ positive CD4 T cells as percentage of total CD4 T cells in the live gate.

CHAPTER 3

The proteasome immunosubunit MECL-1 is a T-cell-intrinsic factor influencing homeostatic expansion

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Abstract

Homeostatic regulatory mechanisms maintain the constant ratios between different lymphocyte subsets in the secondary lymphoid organs. How this dynamic equilibrium is achieved, in particular following the clonal expansion and subsequent contraction of different cells after infection, remains poorly understood. Expression of the proteasome immunosubunits has been shown to influence not only major histocompatibility complex (MHC-I) antigen processing and thereby T-cell responses, but also the CD4/CD8 T-cell ratios in lymphoid organs. We examined the relationships between these different immunosubunit-mediated effects in mice of varying proteasome subunit composition during infection with *Listeria monocytogenes*. Mice that lacked the immunosubunit multicatalytic endopeptidase complex-like 1 (MECL-1) maintained enhanced CD4/CD8 T-cell ratios during infection, while MHC-I surface levels resembled those in wildtype (wt) mice. LMP7 gene-deficient mice, on the other hand, showed a reduced MHC-I expression, while their splenic CD4/CD8 ratios were similar to these in wt mice. Remarkably, analysis of bone marrow-chimeric immunosubunit gene-deficient mice, reconstituted with a mixture of wt and LMP7+MECL-1-deficient bone marrow, revealed that the LMP7+MECL-1-deficient T-cell population maintained a higher CD4/CD8 T-cell ratio than the wt T-cell population before, during and after infection and T-cell memory formation. Since in these mice the immunosubunit-positive and immunosubunit-negative T-cell populations were selected in the same thymus and expanded in the same lymphoid environments, our findings indicate that MECL-1 influences the homeostatic equilibrium between T-cell subsets, not through indirect extra-cellular signals, such as MHC-I expression or the cytokine milieu, but through direct effects on T-cell-intrinsic processes.

Introduction

In secondary lymphoid organs, the ratios between different types of leukocytes are remarkably stable. Even during most infections, when lymphoid organs expand and then retract, this homeostatic balance remains well preserved. The mechanisms leading to this balance remain poorly understood.

Analyses of gene-deficient mice lacking the expression of different proteins involved in the MHC-I antigen processing pathway, such as the proteasome immunosubunits, have indicated that this pathway influences the ratio between CD4 and CD8 T cells. The proteasome is an abundant cellular protease that degrades both short- and long-lived intracellular proteins and thereby regulates many cellular processes (reviewed in reference 15). One function of proteasomes involves the generation of peptides that bind to MHC-I molecules, which is effected through the degradation of self- and foreign proteins. In order to optimize the processing of antigenic peptides and thereby the induction of CD8 T-cell responses, 20S proteasomes in infected tissues are equipped with three cytokine-inducible proteasome subunits (i.e., the immunosubunits LMP2/iβ1, MECL-1/iβ2 and LMP7/iβ5), which replace their constitutive homologues (β1, β2 and β5) in the cellular proteasome population (8, 17). The same three inducible subunits are constitutively expressed in both immature and mature dendritic cells (10, 11).

Over the recent years, different functions have been ascribed to the proteasome immunosubunits. Their incorporation into the proteasome complex modifies the 20S cleavage specificity and thereby the repertoire of peptides generated (9, 16, 18-20), MHC-I cell surface expression levels (6), and the specificity of CD8 T-cell responses (3, 5). In addition, the proteasome immunosubunits have been reported to influence T-cell repertoire selection (1, 3) and CD4/CD8 T-cell ratios (2, 6).

In particular, the question of how the expression of the three proteasome immunosubunits influences the relative frequencies of CD4 and CD8 T cells remains enigmatic. Since the expression of the immunosubunits influences the liberation of MHC-I ligands and thereby the repertoire of MHC-I-presented peptides on the cell surface, one could argue that the expression of the immunosubunits in the thymus during T-cell maturation could influence the CD4/CD8 T-cell ratios in the secondary lymphoid organs mainly through effects on peptide processing. Such a notion is supported by a recent publication describing a thymus-specific proteasome subunit (tβ5) that prevents the incorporation of LMP7/iβ5 into proteasomes in the cortical tissue of the thymus (12). Mice gene-deficient for this thymus-specific proteasome subunit showed dramatic alterations in the positive selection of CD8 T cells, which strongly influenced the CD4/CD8 T-cell ratio in the secondary lymphoid organs as well. How-

ever, since this proteasome subunit replaces an immunosubunit, this finding also suggests that the immunosubunits are less likely to influence the CD4/CD8 T-cell ratios on the level of the positive selection of CD8 T cells in the thymus.
Thus, in order to unravel whether the different effects of the proteasome immunosubunits on T-cell regulation can be explained solely by the altered liberation of MHC-I ligands, in particular in the thymus, or whether proteasome functions unrelated to antigen processing are involved in this process, we analyzed the T-cell compartments of uninfected and *Listeria monocytogenes*-infected mixed bone marrow (BM)-chimeric and gene-deficient mice that differed with respect to proteasome subunit composition. Our studies show that the observed effects of immunosubunit expression on CD4/CD8 T-cell ratios can neither be explained by altered MHC-I antigen processing and peptide presentation in the thymus nor by alterations in T-cell-proliferative capacity. Instead, they are a direct result of MECL-1 expression in the T-cell subsets.

Materials and Methods

Mice and infections

B6 and B6.SJL (CD45.1) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). LMP7+MECL-1 double-gene-deficient B6 mice (2) were maintained by in-house breeding under standard conditions. F_2 offspring was generated by crossing of LMP7+MECL-1 double-gene-deficient B6 mice with wildtype (wt) B6 mice. The *L. monocytogenes* strain rLM-E1 was grown in brain heart infusion medium (BD Biosciences, Franklin Lakes, NJ) supplemented with 250 µg/mL spectinomycin and harvested while in log phase. For primary infection, 6- to 12-week-old female mice were inoculated *i.v.* in the tail vein with $0.1xLD_{50}$ (5×10^3 rLM-E1 bacteria) in 100 µL PBS. All experiments involving animals were approved by the Institutional Committee on Animal Resources of the University of Rochester Medical Center (Rochester, NY) or the Committee on Animal Experiments of the University of Utrecht Veterinary School.

Construction of BM-chimeric mice

BM cells, flushed from the femurs of donor mice, were depleted of mature T lymphocytes by incubation with anti-CD4 mAb (clone GK1.5) and anti-CD8 mAb (clone 3-55) and subsequently with Guinea pig complement (Invitrogen), added at a concentration of 4.5 µg/mL for 30 min. Recipient mice were irradiated with 7 Gy as a single dose from an X-ray irradiator, reconstituted via the tail vein with 10^7, 1-to-1-mixed BM cells of CD45.1^{pos} B6.SJL and CD45.2^{pos} LMP7+MECL-1-deficient donor mice

(mixed BM-chimeric mice) or with 10^7 BM cells of CD45.1^{pos} B6.SJL or CD45.2^{pos} LMP7+MECL-1-deficient donors (single-chimeric mice), and then allowed to reconstitute for 28 days until additional experiments were performed.

Western blot analysis

Splenocytes (20×10^6 to 40×10^6) were washed twice with PBS and lysed in 100 µL of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA pH 8.0, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 6 µg/mL aprotinin, 7 µM pepstatin A, 10 µM leupeptin) for 20 min on ice, followed by three cycles of freezing and thawing. The lysates were cleared by centrifugation (15 min at 14,000 rpm) at 4°C and quantified by determining the optical density at 280 nm. Aliquots of 100 µg were electrophoresed on 12% SDS polyacrylamide gels and blotted onto nitrocellulose membranes, and proteins were visualized by Ponceau red staining, using standard procedures. The blots were blocked for 1 h in blocking buffer (PBS with 10% horse serum, 5% (w/v) dry milk and 0.4% Tween 20) at room temperature, incubated overnight at 4°C in a 500-fold dilution of anti-mouse MECL-1 or a 1:1000 dilution of anti-mouse LMP7 rabbit antiserum in PBS with 2% dry milk and 0.1% Tween-20, and developed with horseradish peroxidase-conjugated goat-anti-rabbit IgG and enhanced chemiluminescence according to the manufacturer's instructions (Roche, Indianapolis, IN).

Antibodies and flow cytometry

The monoclonal antibodies used in this study included FITC-, PE-Cy5- and APC-conjugated anti-mouse CD8α (clone 53-6.7), PE- and APC-conjugated anti-mouse CD4 (clone GK1.5), FITC-conjugated anti-mouse TCRβ (clone H57-597), PE-conjugated anti-mouse CD19 (clone MB19-2), PE-conjugated anti-mouse IFNγ (clone XMG1.2), PE-Cy5-conjugated anti-mouse CD45.1 (clone A20), FITC- and PE-conjugated anti-mouse CD45.2 (clone 104), biotin-conjugated anti-B220 (clone RA3-6B2), biotin-conjugated anti-H-2K^b (clone AF6-88.5), and PE- and APC-conjugated streptavidin. All these reagents were purchased from eBioscience, San Diego, CA. To analyze spleen cell subsets, mice were sacrificed at the time points indicated in the figure legends, and their spleens were collected and pressed through a cell strainer to prepare single-cell suspensions. Samples of 1×10^6 to 5×10^6 spleen cells were resuspended in ice-cold PBS with 1% bovine serum albumin and 0.02% NaN_3 (PBA buffer), incubated with anti-mouse CD16/CD32 (clone 2.4G2) to block Fc-receptors for 10 min, then with the appropriate fluorochrome-conjugated antibodies or with biotin-conjugated anti-class I antibody for 30 min, and then with streptavidin-PE or -APC, for 30 to 60 min on ice. The cells were analyzed on a FACScalibur (BD Biosciences, Franklin Lakes, NJ), using Cellquest software.

Synthetic peptides

Synthetic peptides corresponding to the adenovirus type 5-derived epitopes $E1A_{234-243}$ and $E1B_{192-200}$, and the *Listeria monocytogenes*-derived epitopes $LLO_{296-304}$ and $LLO_{189-201}$ were purchased from Invitrogen, Carlsbad, CA.

Analysis of T-cell responses

At the time points specified in the figure legends, mice were sacrificed and their spleens were collected. To quantify the percentages of splenic CD4 and CD8 T cells specific for the different rLM-E1-derived epitopes, approximately 10 x 10^6 erythrocyte-depleted splenocytes were incubated for 6 h with or without 500 nM synthetic peptide, in 1 mL RPMI medium (RPMI with 10% FCS [HyClone Laboratories, Logan, UT], 2 mM L-glutamine, 30 µM 2-mercaptoethanol and penicillin/streptinomycin), containing 50 µg/mL gentamicin and 9 µM monensin (eBioscience, San Diego, CA). Thereafter, cells were stained with FITC-conjugated anti-mouse CD8 or APC-conjugated anti-mouse CD4 antibody in the presence of anti-CD16/32 (clone 2.4G2), fixed with 2% paraformaldehyde, and then stained with XMG1.2-PE in the presence of 0.5% saponin, to detect intracellular IFNγ. The cells were analyzed on a FACS-Calibur.

Results

Differential roles of the individual proteasome immunosubunits in MHC ligand generation and T-cell expansion are maintained during infection

To explore the respective effects of LMP7 and MECL-1 on antigen processing and CD4/CD8 T-cell frequencies during an ongoing immune response, when the immunosubunits are highly expressed and immunoproteasome function is expected to be needed, we crossed LMP7+MECL-1- gene-deficient with control (wt) B6 mice. The F_2 offspring (5 $LMP7^{pos}$+$MECL\text{-}1^{neg}$, 6 $LMP7^{neg}$ +$MECL\text{-}1^{pos}$ and 25 $LMP7^{pos}$+$MECL\text{-}1^{pos}$ mice, as determined by Western blotting [data not shown]) were infected with a sublethal dose of the *Listeria monocytogenes* strain rLM-E1 (5).

Analysis of spleen cell populations of uninfected and infected mice at the peak of the CD8 T-cell response to Listeria infection showed that the cell surface levels of MHC-I molecules were reduced on splenic lymphocytes of both infected LMP7 gene-deficient and uninfected and infected LMP7+MECL-1 double-gene-deficient mice (Figure 1A and data not shown). In contrast, no reduction of MHC-I expression was observed on cells of infected MECL-1-deficient mice (Figure 1A). Thus, from our data, we infer that LMP7 plays a predominant role in the generation of high-affinity class I ligands during infection, whereas MECL-1 has a relatively moderate effect on

the repertoire of class I-presented peptides .

Analysis of the ratios of CD4 to CD8 T cells showed that these were significantly enhanced in MECL-1-deficient mice compared to LMP7-deficient and control (LMP7^{pos}+MECL-1^{pos}) mice (P = 0.0043; two-tailed Mann-Whitney test), both when infected (day 7) and in the absence of infection (Figure 1B). Thus, consistent with previous observations (1, 2), our data indicate that the absence of LMP7 leads to reduced MHC-I expression, whereas the expression of MECL-1 determines the CD4/CD8 T-cell ratios found in the secondary lymphoid organs. This difference in T-cell ratios between MECL-1$^{-/-}$ and wt mice is maintained even during an ongoing T-cell response. The use of F_2 littermates in these experiments excluded the possibility that observed differences between gene-deficient and control B6 mice were due to genetic differences.

The predominant role of LMP7 in MHC-I ligand production is most likely explained by the fact that LMP7-containing proteasomes preferentially cleave after hydrophobic residues (20), leading to enhanced generation of MHC-I binding peptides. The effects of MECL-1 on CD4/CD8 T-cell ratios are more difficult to explain but apparently do not result from effects of MECL-1 on MHC-I cell surface levels.

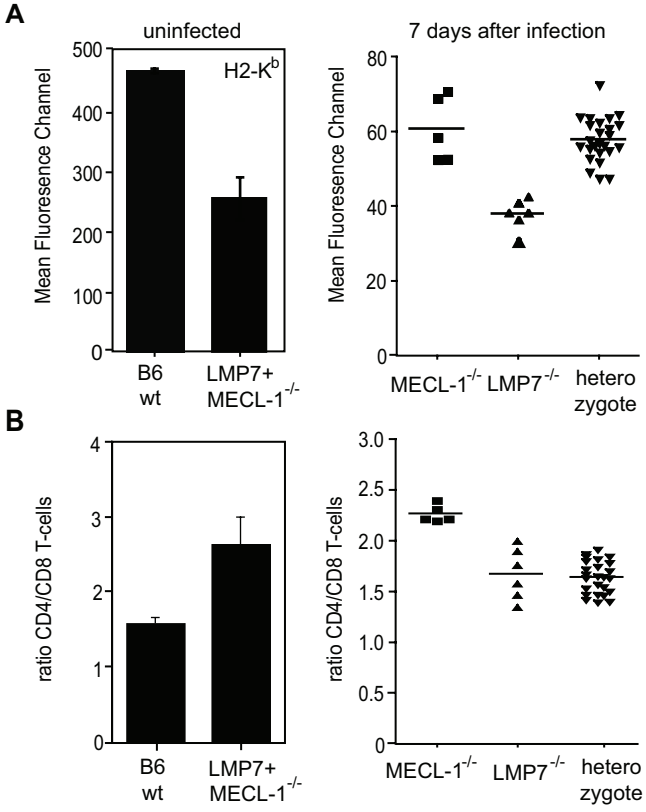

Figure 1. Effects of LMP7 and MECL-1 on MHC-I cell surface expression (A) and CD4/CD8 ratios (B) in uninfected and *L. monocytogenes*-infected mice. Splenocytes of age-matched B6 wt, MECL-1$^{-/-}$, LMP7$^{-/-}$ and LMP7+MECL-1$^{-/-}$ mice were stained with anti-CD19-APC, biotin-conjugated anti-H-2K^b, and SAV-PE (uninfected mice; n = 4); anti-CD19-PE, biotin-conjugated anti-H-2K^b, and SAV-APC (infected mice) (A); or anti-CD4-FITC and anti-CD8α-APC antibodies (B) and analyzed by flow cytometry. Mean fluorescence channels of the CD19$^+$ populations (A) and CD4/CD8 T-cell ratios (means ± SD, n = 5 or 6) (B) are depicted.

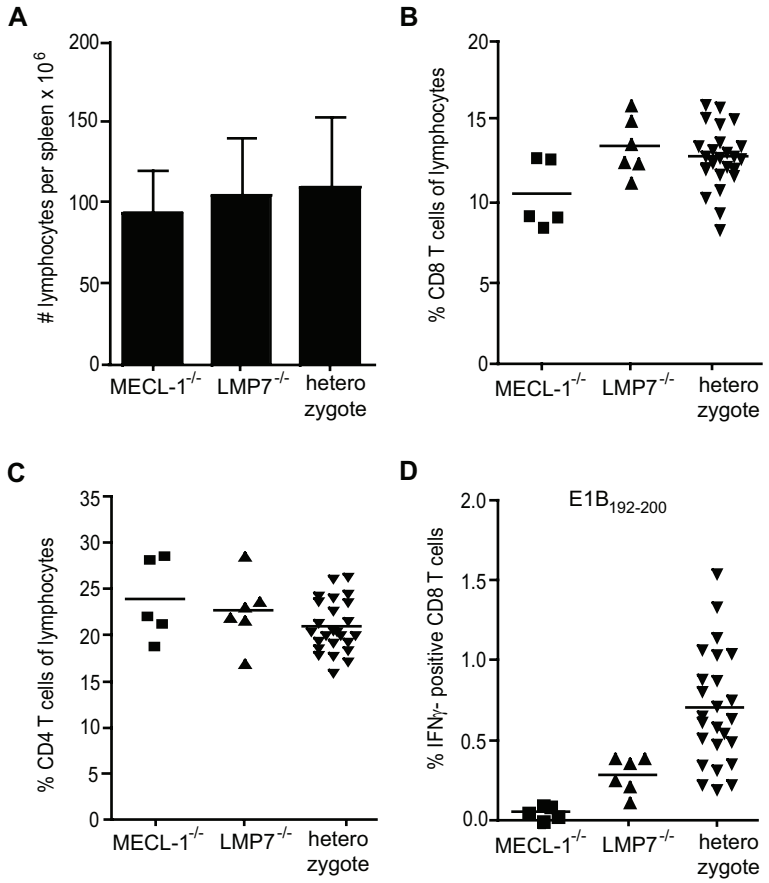

Figure 2. Effects of LMP7 and MECL-1 in Listeria-infected mice. F_2 offspring of B6 wt x LMP7+MECL-1$^{-/-}$ mice was infected *i.v.* with 5 x 10^3 rLM-E1 bacteria. The spleens were collected at day 7 after infection and analyzed for the presence of LMP7 and/or MECL-1 by immunoblot analysis, using specific rabbit antisera (x-axis). (A) Total numbers of splenocytes per spleen (means ± SD). (B and C) Cells were stained with anti-CD8α-APC and anti-CD4-FITC to determine the relative frequencies of CD8 and CD4 T cells, respectively. (D) Cells were incubated with 500 nM synthetic $E1B_{192-200}$ for 6 h in the presence of monensin, and the frequencies of $E1B_{192-200}$-specific CD8 T cells were determined by staining for CD8 (anti-CD8α-FITC) and intracellular IFNγ (IFNγ-PE). Background, detected in samples incubated without peptide, was subtracted.

Expression of MECL-1 is necessary for the priming of $E1B_{192-200}$-specific immune responses

Remarkably, in contrast to observations in uninfected immunosubunit-deficient mice (6), the spleen sizes of the infected F_2 littermates failed to correlate with the absence or presence of either of the immunosubunits (Figure 2A). Also, although the CD4/CD8 ratios were consistently enhanced in MECL-1$^{-/-}$ mice (Figure 1D), the absolute

percentages of splenic CD4 and CD8 T cells (Figure 2B and C) fluctuated in all mouse groups. Thus, although there was a slight tendency toward lower CD8 T-cell frequencies in MECL1$^{-/-}$ mice, we failed to find any statistically significant differences between the sizes of the CD8 T-cell populations of the different mouse groups (Figure 2B). Therefore, the increase in the CD4/CD8 ratio in the absence of MECL-1 is not explained simply by a reduced positive selection of CD8 T cells in these mice.

We previously generated a recombinant Listeria strain, rLM-E1, that secretes a modified form of Listeria p60, encompassing two adenovirus-derived CD8 T-cell epitopes (5). One of these two epitopes, E1B$_{192\text{-}200}$, is generated in an immunoproteasome-dependent fashion (5). To examine whether the absence of LMP7 and/or MECL-1 affects the CD8 T-cell response to rLM-E1-derived E1B$_{192\text{-}200}$, the infected F_2 littermates were analyzed for the presence of E1B$_{192\text{-}200}$ T cells by IFNγ-intracellular staining and flow cytometry. These analyses revealed CD8 T-cell responses to E1B$_{192\text{-}200}$ in both control and LMP7-deficient, but not MECL-1 deficient, mice (Figure 2D). Thus, MECL-1 appears to be essential for the processing of this epitope. Caudill and coworkers (2) previously showed a partial deficit in the maturation of MECL-1-containing proteasomes in LMP7 gene-deficient mice. Consistent with this finding, as well as with our own observation that the spleens of LMP7^{neg}+MECL-1^{pos} F_2 mice contained relatively little MECL-1 (data not shown), the levels of E1B-specific responses in LMP7-deficient mice were significantly lower than those in control mice ($P = 0.0081$) (Figure 2D).

Taking the data together, while the expression of MECL-1 does not influence the overall quantity of cellular peptides presented by MHC-I molecules or the absolute frequencies of CD8 T cells in infected mice, its expression can have a significant influence on the presentation of specific pathogen-derived epitopes and therefore on the CD8 T-cell response to infection.

Differences in homeostatic CD4 and CD8 T-cell expansion between immunosubunit-deficient and -expressing T cells in mixed BM-chimeric mice

To further examine whether expression of MECL-1 in T cells themselves or in their cellular interaction partners (such as thymic or peripheral professional antigen presenting cells [pAPC]) influences homeostatic T-cell expansion, we reconstituted irradiated LMP7+MECL-1-deficient mice with a mix of BM derived from wt B6/SJL (CD45.1$^+$) and from LMP7+MECL-1-deficient (CD45.2$^+$) mice. In these mixed BM-chimeric mice, T cells that derive from either source of BM will mature and differentiate in the same thymic and lymphoid environments. Analysis of the different CD45.1$^+$ and CD45.2$^+$ lymphocyte subsets in the spleens of mixed BM-chimeric mice by flow cytometry 28 days after reconstitution showed the expected reduction of MHC-I expression on CD45.2$^+$ (LMP7+MECL-1$^{-/-}$) cells compared to CD45.1$^+$ (wt) cells (Figure

3A). Remarkably, these experiments further revealed enhanced ratios between CD4 and CD8 T cells of CD45.2^{+} (immunosubunit-deficient) origin in comparison to ratios between CD4 and CD8 T cells of CD45.1^{+} (wt) origin (Figure 3B). Thus, the differences in ratios between T-cell subsets as detected in gene-deficient versus wt mice (Figure 1) are maintained when those T cells mature, differentiate and expand in the same thymic and peripheral lymphoid environments. Most interestingly, at both day 7 and day 46 after infection of these BM-chimeric mice with *L. monocytogenes*, the CD4/CD8 T-cell ratios remained significantly higher in the CD45.2^{pos} than in the CD45.1^{pos} population (Figure 3B). An enhanced ratio between CD4/CD8 T cells was also observed in wt recipients transplanted with immunosubunit-deficient BM (Figure 3C), further indicating that the recipient background does not influence the T-cell distribution. Taken together, these findings strongly suggest that MECL-1 influences the CD4/CD8 T-cell ratios directly through T-cell-intrinsic regulatory mechanisms and not via other cells that interact with these T cells, either in the thymus or in the secondary lymphoid organs.

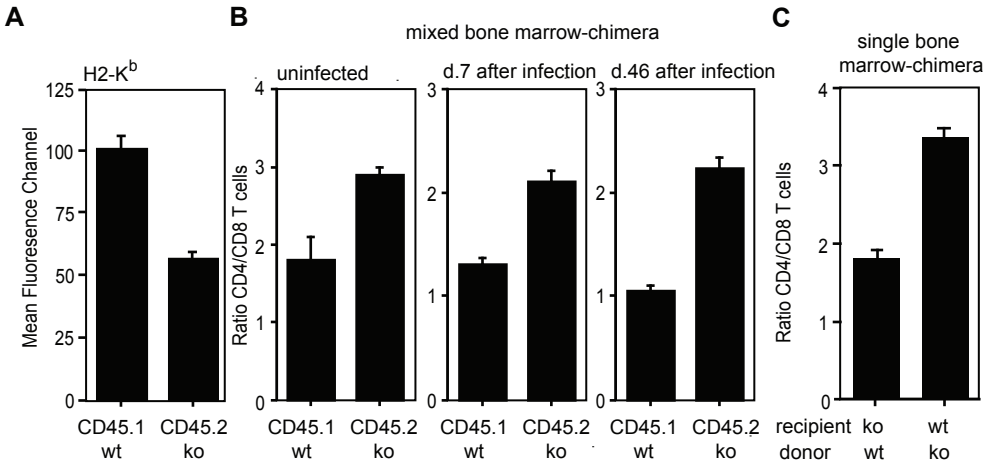

Figure 3. MHC-I cell surface levels and CD4/CD8 T-cell ratios of LMP7+MECL-1^{pos} and LMP7+MECL-1^{neg} T-cell subsets in mixed BM-chimeric mice. Lethally irradiated LMP7+MECL-1$^{-/-}$ mice were reconstituted with a mixture of BM from B6.SJL (wt; CD45.1^{pos}) and LMP7+MECL-1$^{-/-}$ (ko, CD45.2^{pos}) mice and were infected with rLM-E1 28 days later. (A) Splenocytes of mixed BM-chimeric mice were stained at day 28 after BM injection with fluorochrome-conjugated anti-CD45.1, anti-CD45.2, anti-CD19, and biotin-conjugated anti-H-2K^{b} and SAV-APC to determine the H-2K^{b} expression levels on the CD45.1 and CD45.2 B-cell subsets. Mean fluorescence channels are depicted (means ± SD, n = 2). (B) Relative frequencies of CD45.1^{pos} and CD45.2^{pos} CD4 and CD8 T cells in the spleens of mixed BM-chimeric mice were determined at day 28 after BM injection and at days 7 and 46 following Listeria infection by staining with fluorochrome-conjugated anti-CD45.1, anti-CD45.2, anti-TCRβ, anti-CD4, and anti-CD8α antibodies. CD4/CD8 T-cell ratios are depicted (means ± SD, n = 2 to 5). The data are representative of two independent experiments. (C) Relative frequencies of CD4 and CD8 T cells in the spleens of CD45.1^{pos}, wt recipients reconstituted with CD45.2^{pos}, LMP7+MECL-1$^{-/-}$ BM and LMP7+MECL-1$^{-/-}$ recipients reconstituted with wt BM (means ± SD, n = 4).

Immunosubunit-deficient and -expressing T cells exert similar antigen-specific proliferation in mixed BM-chimeric mice

Although LMP7+MECL-1-deficient lymphocytes were found to hyperproliferate following stimulation with polyclonal mitogens *in vitro* (2), hyperproliferation was not detected in the sole absence of MECL-1 and therefore is unlikely to explain the enhanced CD4/CD8 T-cell ratios detected in mice lacking MECL-1 (1, 2). Nevertheless, enhanced T-cell expansion may bias the CD4/CD8 T-cell ratios during an ongoing immune response. To explore whether the absence of LMP7 and MECL-1 influences T-cell proliferative capacity under physiological conditions as well, we infected mixed BM-chimeric, LMP7+MECL-1-gene-deficient mice with rLM-E1. Quantifications of T cells specific for the rLM-E1-derived CD8 T-cell epitope $E1B_{192\text{-}200}$ and CD4 T-cell epitope $LLO_{189\text{-}201}$, at day 7 after infection, showed that the $CD45.1^{pos}$ and $CD45.2^{pos}$ T-cell subsets responded in largely similar fashions (Figure 4). Thus, after antigen-specific activation, LMP7+MECL-1-deficient ($CD45.2^{pos}$) and control ($CD45.1^{pos}$) T cells expanded at comparable rates. Of note, due to inefficient processing in the absence of MECL-1, rLM-E1-infected LMP7+MECL-1-deficient mice were not able to respond to the p60E1-derived $E1B_{192\text{-}200}$ epitope (Figure 2D) (5). Therefore, in the chimeras, all $E1B_{192\text{-}200}$-specific CD8 T cells of wt and of knock-out origin must have been primed by antigen-presenting $CD45.1^{pos}$ (wt) pAPC. Consequently, due to the presence of both LMP7+MECL-1-deficient ($CD45.2^{pos}$) and control ($CD45.1^{pos}$) pAPC, $E1B_{192\text{-}200}$ responses in mixed BM-chimeric mice were smaller (Figure 4) than those observed in wt mice (Figure 2D).

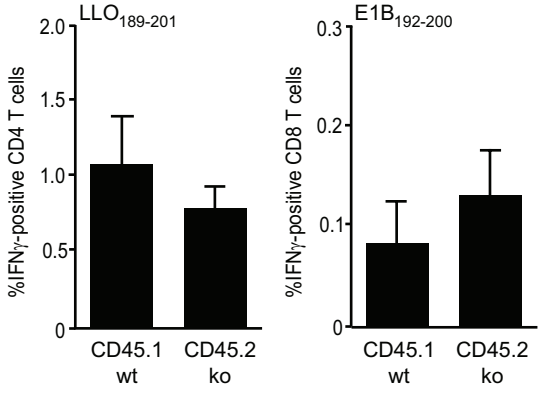

Figure 4. T-cell responses in rLM-E1-infected mixed BM-chimeric mice. Mixed BM-chimeras were infected with rLM-E1, and the relative frequencies of $CD45.1^{pos}$ and $CD45.2^{pos}$ T cells in the spleens specific for the rLM-E1-derived CD8 T-cell epitope $E1B_{192\text{-}200}$ and CD4 T-cell epitope $LLO_{189\text{-}201,}$ were determined by intracellular IFNγ staining (means ± SD, n = 5). Background, detected in samples incubated without peptide, was subtracted.

Discussion

The initial finding that proteasomes in cells with antigen-processing function exchange their catalytic machinery to incorporate three cytokine-inducible subunits strongly suggested an important function for the immunoproteasome formed in this way in antigenic peptide generation and CD8 T-cell activation. However, although immunoproteasomes play an essential role in the processing of multiple pathogen-derived, immunodominant antigenic peptides and determine the fine specificity of T-cell responses (3, 5), recent studies have indicated that the immunosubunits may regulate CD8 T-cell immunity at multiple levels, possibly through diverse mechanisms (2, 3). Analyses of immunosubunit-deficient mouse strains have revealed that the absence of either subunit can modify the T-cell repertoire, presumably by effects on positive and negative selection of T cells in the thymus (12, 14), which was also seen as an explanation for the enhanced CD4/CD8 T-cell ratios detected in mice that lack MECL-1 expression (1-3, 14). In contrast to this assumption, we showed here that MECL-1 affects the T-cell compartment (i.e., CD4/CD8 T-cell ratios) through a mechanism that operates independently of the epitope processing function of proteasomes or of thymic selection processes.

Importantly, we found that the CD4/CD8 T-cell ratios in the MECL-1-deficient T-cell population were enhanced, not only in uninfected, but also in infected mice during an ongoing T-cell response. This effect was observed in both MECL-1-deficient and mixed BM-chimeric mice after infection with different Listeria strains (Figure 1B and 3B and data not shown) and selectively affected the MECL-1-deficient T-cell population (Figure 3B). Unlike previously reported observations in uninfected mice (1, 2), we found that the absolute numbers of CD8 T cells or CD8 percentages were neither consistently nor significantly reduced in spleens of infected MECL-1-deficient mice compared to MECL-1^{pos} mice (Figure 2B and data not shown), implying that the enhanced CD4/CD8 T-cell ratios in the former mice cannot be explained by differences in relative expansion of the CD4 and CD8 T-cell subset.

We previously found that LMP7+MECL-deficient mice are incapable of mounting CD8 T-cell responses to the rLM-E1-derived E1B$_{192\text{-}200}$ epitope due to delayed presentation of this antigenic peptide on immunoproteasome-deficient pAPC (5). Our present studies (Figure 2D) ascribe this failure to the absence of MECL-1. Despite earlier observations that MECL-1 and LMP2 incorporate in preproteasome complexes in a mutually codependent fashion (7, 13), LMP2 incorporation is at best slightly reduced in both MECL-1 and LMP7+MECL-1- deficient mice (2). Thus, LMP2 cannot be responsible for defects in epitope processing or CD4/CD8 ratios, as detected in MECL-1 gene-deficient mice. In contrast, the absence of LMP7 diminishes the ef-

ficiency of MECL-1 incorporation dramatically (4). Still, LMP7-deficient mice raise significant responses to the E1B epitope, and moreover, ratios between CD4 and CD8 T cells are normal in these mice. Thus, these data indicate that even a relatively inefficient incorporation of single immunosubunits, such as MECL-1, into the cellular proteasome population already has pronounced effects.

Our analyses of mixed BM-chimeric mice showed that differences in ratios between MECL-1-deficient CD4 and CD8 T cells and MECL-1-expressing CD4 and CD8 T cells were maintained when these T-cell populations developed within the same mixed BM-chimeric mouse. This finding excludes the possibility that an altered repertoire of self-peptides presented in the thymus/secondary lymphoid organs or other external differences between MECL-1-deficient and MECL-1^{pos} mice may explain the altered regulation of T-cell expansion in the former mice. Taken together, this leaves the alternative explanation that MECL-1, which is constitutively expressed in T cells (2), directly influences homeostatic expansion, perhaps by effects on the degradation of CD4 or CD8 T-cell-specific transcription or (anti-)apoptotic factors.

In summary, we show here that the immunosubunit MECL-1 regulates T- cell immunity in different ways. On one hand, MECL-1 is essential for the processing and presentation of selected MHC-I-presented peptides, which has effects both on induction of CD8 T-cell responses (Figure 2D) and on selection of the CD8 T-cell repertoire (1). On the other hand, we have provided evidence that the effects of MECL-1 on CD4 or CD8 T-cell expansion are entirely unrelated to its role in antigen processing. Our findings suggest that MECL-1 influences the homeostatic regulatory processes that maintain the relative proportions of both T- cell subsets, through a T-cell-intrinsic mechanism independent of thymic or lymphoid interaction partners.

Acknowledgements

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CHAPTER 4

Immunoproteasome-deficiency has no effects on NK cell education, but confers lymphocytes into targets for NK cells in infected wildtype mice

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Abstract

NK cells are part of the innate immune system and contribute to the eradication of virus infected cells and tumors. NK cells express inhibitory and activation receptors that interact with their respective ligands on target cells, and their decision to kill is based on the balance of signals received through these receptors. Presence of MHC class I molecules during education, recognized by inhibitory receptors, influences the responsiveness of peripheral NK cells. We here demonstrate that mice with reduced MHC class I levels on lymphoid cells, due to deficiency of immunoproteasomes, have responsive NK cells in the periphery. Furthermore, we show that transferred immunoproteasome-deficient splenocytes are tolerized in wt recipients, however, are rejected when the recipients are virally infected, dependent on the presence of NK cells. Together, these results indicate that expression of reduced MHC class I levels is sufficient to protect these cells from rejection by wt NK cells, but this tolerance is broken by infection, which induces an NK cell-dependent rejection of immunoproteasome-deficient cells.

Introduction

Natural Killer (NK) cells are part of the innate immune system and play a role in the eradication of viruses and tumors. NK cells express a variety of germ-line encoded inhibitory and activating receptors that interact with their respective ligands on target cells (1). Upon interaction with a potential target cell, the NK cell's decision whether to attack this cell is determined by the sum of signals received through these receptors (2). MHC class I molecules are recognized by inhibitory receptors, and therefore, NK cells attack cells that lack cell surface MHC class I (3). This 'missing self' hypothesis would imply that NK cells from mice that lack MHC class I expression, like β_2m- or TAP-deficient mice, reject their own cells. This is, however, not the case because developing NK cells are educated in a MHC class I-dependent process called 'licensing' or 'disarming', resulting in responsive NK cells in the periphery (4, 5). NK cells from mice that lack MHC class I expression are therefore hyporesponsive (6, 7). The recognition of MHC class I by murine NK cells is largely dependent on inhibitory receptors of the Ly49 family (8). The responsiveness of peripheral NK cells is thus determined by presence of MHC class I on the target cell, and by expression of Ly49 on the NK cell. This responsiveness is tunable like a rheostat, thus, changes in strength of inhibitory signals during education quantitatively tune NK cell activity (9-11). Consequently, mice that express different types and combinations of MHC class I alleles have functionally different NK cells as more inhibitory signals during education result in increasingly responsive NK cells (9, 12). Joncker et al. showed that NK cell responsiveness correlated with the number of inhibitory self-MHC class I receptors on NK cells (11).

The 'missing self' hypothesis dictates that NK cells kill cells that lack self-MHC class I on the cell surface. This principle is important for NK cell mediated eradication of viruses that down-regulate MHC class I of the host cell to avoid CD8 T-cell recognition (13). Brodin et al. studied to what extent MHC class I down-regulation was required on the target cell to induce NK cell mediated killing (14). Varying MHC class I levels on cells derived from $TAP^{-/-}$ mice by incubation with different concentrations of peptide, they found that rejection occurred when MHC class I levels dropped below 20%. In addition, cells from MHC class I hemizygous mice, which displayed strongly reduced MHC class I levels, were not rejected by wt NK cells. These results indicate that MHC class I levels are normally present in excess, and that a large reduction of surface MHC class I is required to induce NK cell mediated killing. The expression level of MHC class I molecules on the cell surface is influenced, amongst others, by peptide supply. Upon peptide binding to MHC class I in the endoplasmic reticulum, a stable complex is formed that is transported to the cell surface. The proteasome

is the major proteolytic enzyme complex that is responsible for the degradation of proteins into peptides that bind to MHC class I molecules. Most cells express the constitutive proteasome that contains three subunits with catalytic capacity, namely β1, β2 and β5. However, in lymphoid cells and cells exposed to cytokines during inflammation (e.g. IFNγ) (15) these proteolytic subunits are replaced by their homologues immunosubunits, iβ1(LMP2), iβ2(MECL-1) and iβ5(LMP7) to form the so called immunoproteasome. Immunoproteasomes have altered cleavage site preference that enhances the generation of a significant number of antigenic peptides (16-18). Mice that are deficient for immunoproteasomes therefore express reduced levels of MHC class I on the cell surface, which is mainly due to the absence of iβ5/LMP7 that causes a defect in peptide supply (19).

Since the responsiveness of peripheral NK cells is influenced by the presence of MHC class I during education, we here set out to determine whether immunoproteasome-deficient mice, that express lower levels of surface MHC class I, have responsive NK cells in the periphery. And furthermore, since cells with reduced surface MHC class I are prone to NK cell mediated rejection, we also assessed whether immunoproteasome-deficient cells are NK cell-dependently rejected after transfer into wt recipients.

Previous studies have shown that transferred immunoproteasome-deficient CD8 T cells could barely be recovered from virally-infected recipients (20, 21). A detailed study from Moebius et al. showed that immunoproteasomes are essential for survival of T cells in infected mice, and this explains the disappearance of transferred immunoproteasome-deficient T cells in infected recipients (21). They excluded a role for NK cell-mediated rejection by showing that transferred immunoproteasome-deficient CD8 T cells can be recovered from uninfected recipients. We here used a different approach to study whether NK cells reject transferred immunoproteasome-deficient cells in infected recipients.

In the present study we found that immunoproteasome-deficient mice have responsive NK cells in the periphery. We furthermore found that transferred immunoproteasome-deficient splenocytes were tolerized in wt recipients. However, upon infection of recipients, immunoproteasome-deficient cells were rejected and we showed that this was NK cell-mediated. Thus, immunoproteasome-deficient splenocytes are NK cell-dependent rejected only in infected, but not in naïve recipients.

Materials and methods

Mice, infection and poly(I:C) stimulation

B6.SJL (CD45.1), CD45.1.2 (F_1 of B6 x B6.SJL), RAG1$^{-/-}$ and β5i/LMP7+β2i/MECL-

$1^{-/-}$ (30) were all maintained by in-house breeding under standard conditions. RAG1+β5i/LMP7+β2i/MECL-1 gene-deficient mice were generated by crossing of $RAG1^{-/-}$ with β5i/LMP7+β2i/MECL-$1^{-/-}$ mice. Infection with Influenza virus (A/HK/x31; H3N2) was performed *i.n.* under light isofluorane anesthesia with 10^5 50% egg infective dose in 30 µL PBS as described (31). For stimulation *in vivo*, mice were injected *i.v.* with 100 µg poly(I:C) in PBS 24 h before sacrifice. Mice were used between 7 to 14 weeks of age and all animal experiments were approved by the Committee of Animal Experiments of the University of Utrecht.

Cell isolation, staining and flow cytometry

Mice were sacrificed by cervical dislocation, spleens were excised, leukocytes were obtained by pressing through a 70-µM cell strainer (BD Biosciences), and red blood cells were removed by ammonium chloride lysis. Staining of surface markers with the indicated antibodies was performed in the presence of Fc block (2.4G2) for at least 20 minutes on ice. For intracellular staining, cells were fixed with 2% paraformaldehyde for 20 minutes at room temperature, and intracellular staining was performed in the presence of 0.5% saponine for 1 hour at 4°C Antibodies were purchased from eBioscience [anti-CD49b (DX5), -CD27 (LG.7F9), -CD11b (M1/70), -CD11c (N418), -H2-K^b (AF6-88.5.5.3), -CD19 (MB19-1), -NKp46 (29A1.4), -CD45.2 (104), -IFNγ (XMG1.2), -CD69 (MB19-1)] or from Biolegend [anti-LAMP-1 (1D4B), -TCRβ (H57-597), -CD45.1 (A20)]. Samples were measured on a FACSCantoII (BD Biosciences) and analyzed with FlowJo software (Tree Star).

***Ex vivo* stimulation of NK cells**

High protein binding EIA/RIA plates (Costar) were left uncoated or were O/N coated with anti-NKG2D (10 µg/ml) and anti-NKp46 (10 µg/ml) in PBS, and ~0.5 x 10^6 splenocytes from $RAG1^{-/-}$ or from RAG1+β5i/LMP7+β2i/MECL-$1^{-/-}$ mice were incubated on these plates in the presence or absence of 10 µg/mL IL-2. For CD69 measurements, cells were incubated for 6 hours, and for LAMP-1 and IFNγ measurements, cells were incubated for 2 hours in the absence of and an additional 6 hours in the presence of 10 µM monensin and 1,25 µg LAMP-1.

Adoptive transfer

Splenocytes of CD45.1 and β5i/LMP7+β2i/MECL-$1^{-/-}$ were mixed and ~15 x 10^6 cells were transferred *i.v.* into CD45.1.2. recipients that were untreated, NK cell depleted, or Influenza virus infected as indicated. NK cells were depleted by *i.p.* (day -1 and day 4) and *i.v.* (during adoptive transfer) administration of 25 µL anti-asialo GM1 antisera (Wako) in 200 µL in PBS. Recipients were sacrificed 8 days after adoptive transfer, and the recovery of CD45.1 and CD45.2 cells was measured by flow cy-

tometry as described.

Results

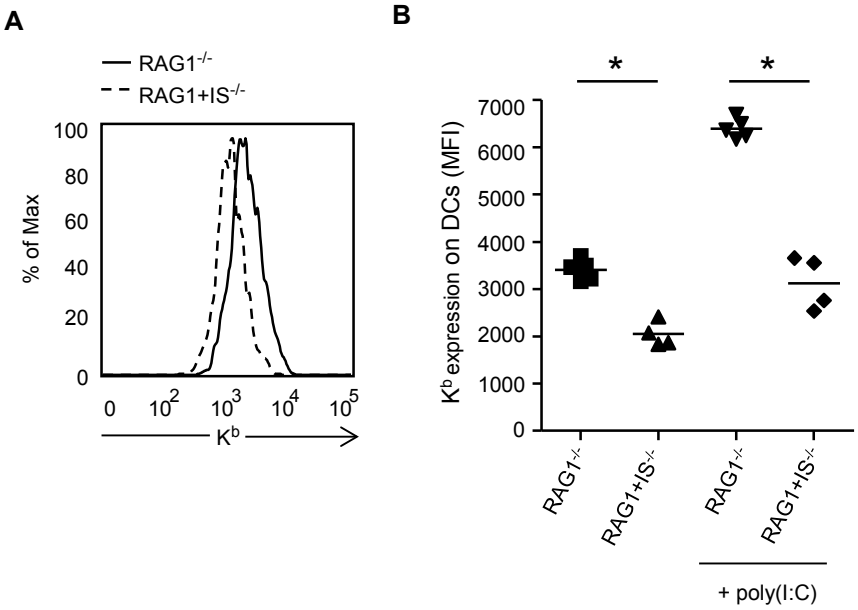

Figure 1. Effect of immunoproteasome-deficiency on constitutive expression and activation-induced upregulation of MHC class I molecules. Splenocytes of untreated or poly(I:C) treated RAG1$^{-/-}$ and RAG1+β5i/LMP7+β2i/MECL-1$^{-/-}$ (RAG1+IS$^{-/-}$) mice were analyzed for the expression of H2-K^{b} on DCs (CD11c^{hi}CD11b^{int}). (A) Representative histogram of untreated mice showing H2-K^{b} expression on DCs. (B) H2-K^{b} mean fluorescent intensity (MFI) on DCs of individual mice. Data are representative of two independent experiments, n= 4 to 6 mice per group. Statistical analysis was performed using a Mann-Whitney *U* test. *, P<0.05

Proteasome immunosubunit-deficiency leads to reduced MHC class I expression

During development, NK cell education depends on interactions between MHC class I molecules and NK cell-expressed inhibitory receptors. It has been shown previously that mice deficient for immunoproteasomes express reduced levels of cell-surface MHC class I molecules (19, 22, 23). To investigate whether immunoproteasomes, by reducing MHC class I expression, alter NK cell education, we used LMP7+MECL-1-deficient mice (immunosubunit knockout, IS$^{-/-}$) bred onto a RAG1-

deficient background (RAG1$^{-/-}$). Consistent with previous data in immunocompetent B6 mice, amounts of MHC class I H2-K^{b} molecules expressed on splenic DC of RAG1+IS$^{-/-}$ mice were considerably lower than those on DC of RAG-1-deficient controls (Figure 1A). Infection of mice with *Listeria monocytogenes* upregulates MHC class I expression in infected tissue (22, 23). In line with these findings, treatment of RAG1$^{-/-}$ and RAG1+IS$^{-/-}$ mice with the immunostimulator poly(I:C) led to upregulation of H2-K^{b} expression on DC of both RAG1$^{-/-}$ and RAG1+IS$^{-/-}$ mice (Figure 1B). However, upregulation of H2-K^{b} in RAG1+IS$^{-/-}$ was reduced compared to that of RAG1$^{-/-}$ mice. The reduced ability of immunosubunit-deficient DC to upregulate MHC class I expression is consistent with our previous observations of H2-K^{b} expression on B-cells of immunoproteasome-deficient mice, and reflect a defect in the supply of high affinity peptides available for binding to MHC class I molecules (23).

Responsiveness of peripheral NK cells in immunosubunit-deficient mice

The responsiveness of peripheral NK cells is quantitatively tuned by the number of MHC class I molecules that are present during NK cell development (9-11). We therefore assessed the responsiveness of peripheral NK cells in immunosunit-deficient mice, which have reduced MHC class I levels. We first measured some general aspects and consistently found that the numbers of NK cells in the spleens of RAG1+IS$^{-/-}$ mice were significantly lower than in RAG1$^{-/-}$ mice (Figure 2A). We furthermore analyzed CD27 and CD11b expression on NK cells from RAG1+IS$^{-/-}$ and RAG1$^{-/-}$ mice to define their maturation status. The most immature NK cells are CD11b^{-}CD27^{-} (R1) and during maturation they become CD11b^{-}CD27^{+} (R2), CD11b^{+}CD27^{+} (R3) and finally CD11b^{+}CD27^{-} (R4), respectively (24, 25). Comparing the two groups of mice, we found that the expression of these markers were highly comparable (Figure 2B, C). To assess the responsiveness of NK cells, we stimulated splenocytes from RAG1$^{-/-}$ and RAG1+IS$^{-/-}$ mice with plate-bound antibodies against NKp46 and NKG2D and measured CD69 upregulation, the production of IFNγ, and degranulation using LAMP-1. Although the levels of CD69, IFNγ, and LAMP-1 were increased on antibody-stimulated NK cells, there were no significant differences between the two groups of mice (Figure 2E-H). Furthermore, when IL-2 was added during incubation, NK cells from RAG1$^{-/-}$ and RAG1+IS$^{-/-}$ mice upregulate CD69, IFNγ, and LAMP-1 in a similar fashion (Figure 2E-H). Together, these results show that NK cells from RAG1+IS$^{-/-}$ mice are normally responsive in the periphery.

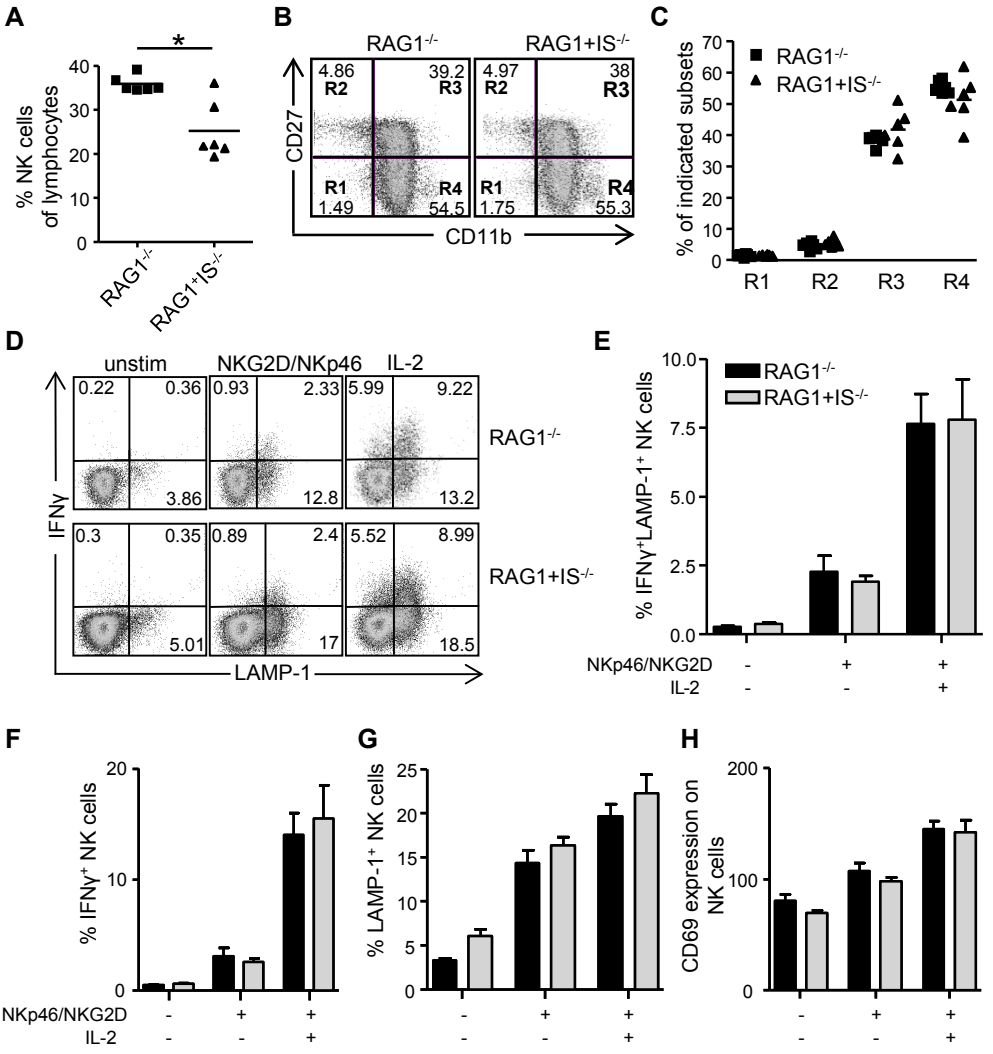

Figure 2. Immunoproteasome-deficient mice have responsive peripheral NK cells. (A-C) Splenocytes of RAG1$^{-/-}$ and RAG1+IS$^{-/-}$ mice were analyzed by flow cytometry (A) Frequencies of NK cells (DX5^{+}NKp46^{+}) as percentage of total lymphocytes. (B,C) Expression of CD11b and CD27 on DX5^{+}NKp46^{+} NK cells. (B) Representative FACS plots and (C) graph showing percentages of subsets for individual mice for the regions indicated in (B). (D-H) Splenocytes of RAG1$^{-/-}$ and RAG1+IS$^{-/-}$ mice were incubated on plates coated with anti-NKG2D and anti-NKp46, in the presence or absence of IL-2, or left unstimulated. Frequencies of IFNγ^{+}, LAMP-1^{+} NK cells and CD69 mean fluorescent intensity (MFI) on NK cells were determined by flow cytometry. (D) Representative FACS plots showing IFNγ^{+} and LAMP-1^{+} NK cells. (E-H) Bars showing the percentages of IFNγ^{+}LAMP-1^{+} NK cells (E), IFNγ^{+} NK cells (F), LAMP-1^{+} NK cells (G), and CD69 MFIs on NK cells (H). Results are shown as mean ± S.E.M. Data are representative for 2 independent experiments with 6 mice per group. Statistical analysis was performed using Mann-Whitney U test. *, P<0.05.

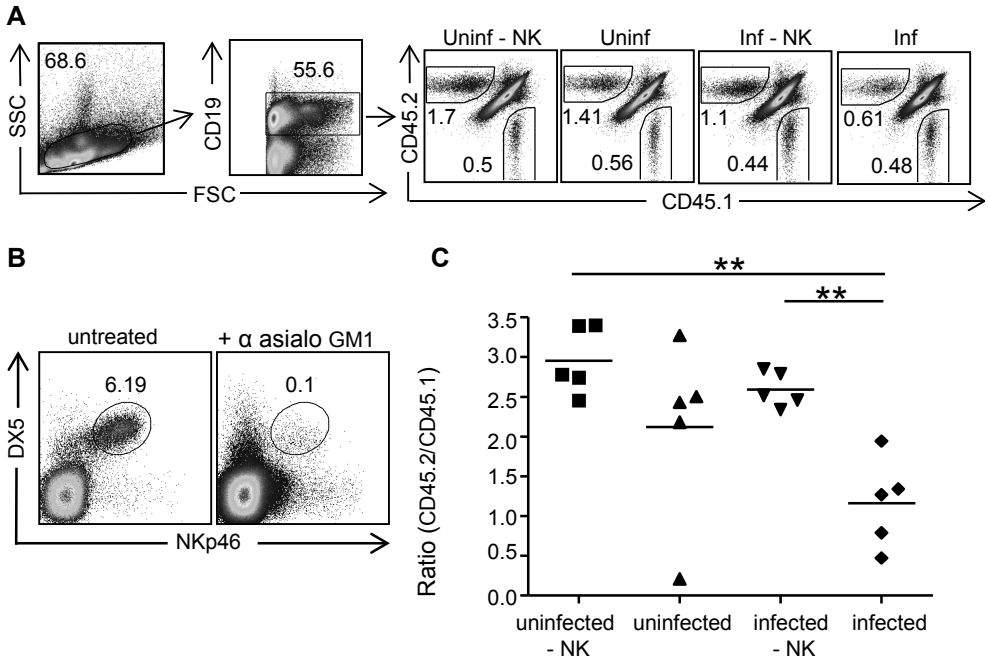

Figure 3. Rejection of immunosubunit-deficient cells in influenza infected mice. Splenocytes of congenic CD45.1 (wt) and β5i/LMP7+β2i/MECL-1$^{-/-}$ (IS$^{-/-}$) mice were 1:1 mixed based on total cell numbers, and transferred *i.v.* into untreated or anti-asialo GM1 treated CD45.1.2 recipients, that were subsequently infected *i.n.* with influenza or left uninfected. Recipient mice were sacrificed 8 days later and spleens were analyzed. (A) Representative FACS plots showing gating strategies and percentages of recovered CD45.1^{+} (wt) and CD45.2^{+} (IS$^{-/-}$) cells of CD19^{+} cells in the different groups: uninf – NK (uninfected, anti-asialo GM treated), uninf (uninfected, untreated), inf – NK (influenza infected, anti-asialo GM treated), inf (influenza infected, untreated). (B) Representative FACS plots gated on TCRβ^{-} cells showing staining for DX5 and NKp46 on splenocytes from untreated and anti-asialo GM treated mice. Cells in the gate are DX5^{+}NKp46^{+} NK cells. (C) Ratio of CD45.2 (IS$^{-/-}$)/ CD45.1 (wt) calculated by dividing absolute numbers of CD19^{+} CD45.2^{+} (IS$^{-/-}$) cells by absolute numbers of CD19^{+} CD45.1^{+} (wt) cells. Results are representative for 2 independent experiments with 5-6 mice per group. Statistical analysis was performed using a Mann-Whitney *U* test. **, P<0.01

NK cell-dependent rejection of immunosubunit-deficient cells in infected recipients

Several groups have previously reported that immunosubunit-deficient cells are rejected after transfer into infected wt recipients (20, 21). Here we investigated whether this rejection is caused by wt NK cells. For this, splenocytes from congenic wt CD45.1^{+} and CD45.2^{+} LMP7+MECL-1$^{-/-}$ (IS$^{-/-}$) mice were mixed and transferred *i.v.* into wt CD45.1.2 recipients that were either treated with anti-asialo GM1 to deplete

NK cells, which was shown to be efficient (Figure 3B), or left untreated. Subsequently, the recipients were either infected *i.n*. with Influenza virus or were left uninfected. At day 8 after infection, mice were sacrificed, and percentages of donor-derived B cells were determined by FACS analysis (Figure 3A). In uninfected recipient mice, numbers of recovered immunosubunit-deficient CD45.2 B cells were higher than numbers of CD45.1 cells (Figure 3C), which is due to the higher frequency of B cells in $IS^{-/-}$ mice and reflects the situation before injection after mixing splenocytes 1:1 based on numbers of total cells. The relative numbers of $IS^{-/-}$ B cells were similar in NK cell depleted and undepleted mice, indicating that NK cells from naïve recipients tolerated the $IS^{-/-}$ cells (Figure 3C). However, after infection with Influenza virus, relative percentages of recovered $IS^{-/-}$ cells were highly decreased. This decreased recovery of $IS^{-/-}$ cells was gone when recipients were NK cell depleted, which suggests that this rejection was NK cell-dependent (Figure 3C). Thus, although $IS^{-/-}$ cells are tolerized by NK cells from naïve recipients, infected recipients reject $IS^{-/-}$ cells NK cell-dependently.

Discussion

Previous studies have shown that defects in MHC class I antigen processing that reduce MHC class I cell surface expression, such as caused by for example a lack of β_2m or tapasin, are accompanied by hyporesponsiveness of peripheral NK cells (6, 7). In the current study, we show that immunoproteasome-deficient mice, despite having partially impaired MHC class I cell surface expression, have responsive peripheral NK cells. We furthermore show that adoptively transferred immunoproteasome-deficient splenocytes are tolerized by wt NK cells in naïve mice, however, are rejected by NK cells in Influenza virus infected mice. Thus, decreased MHC class I cell surface expression due to a lack of immunoproteasome expression, enhances the susceptibility of cells to lysis by activated wt NK cells.

We decided to assess the responsiveness of NK cells of immunoproteasome-deficient mice, since NK cells are educated in a MHC class I-dependent fashion, and the level of MHC class I influences the NK cell responsiveness (9-11). Although immunoproteasome-deficient mice have lower MHC class I levels (Figure 1), they have normally responsive NK cells in the periphery (Figure 2 D-H). These results seem in contrast with the rheostat model, which states that the responsiveness of peripheral NK cell is tuned by the amount of inhibitory signals received during education. Höglund's group studied the quantitative aspect of NK cell education by using mice that expressed different MHC class I alleles (9, 10) and found a relation between the inhibitory input during education and NK cell function in the periphery. Joncker

et al. showed that the number of receptors expressed for self-MHC class I on NK cells correlated with their responsiveness (11). However, in a recent paper, Jonsson et al. studied education of Ly49A^{+} NK cells quantitatively by using a group of MHC congenic mice (26). These mice expressed different MHC class I alleles on the same genetic background. By using Ly49A tetramers, they measured the affinity of these different MHC class I alleles for Ly49A, and compared this to educational capacity. Although their results were largely concordant to the rheostat model, these authors showed that optimal NK cell education already occurred with moderate-binding MHC class I ligands. Furthermore, H2-D^{d} and H2-D^{k} hemizygous mice, that showed a reduction in MHC class I levels of roughly 50%, had educated NK cells in the periphery, supporting a low saturation threshold for NK cell education. These findings are in agreement with our results in RAG1+IS$^{-/-}$ mice, where the decrease in MHC class I cell surface levels is just under 50%, in the absence of immune stimulation.

Since immunoproteasome-deficient mice express lower levels of MHC class I levels on the cell surface, we also tested whether these cells are targets for NK cell-mediated rejection upon adoptive transfer into wt recipients. We found that immunoproteasome-deficient B cells of iβ5/LMP7+iβ2/MECL-1-deficient B6 mice, which displayed a roughly 30% reduction in MHC class I levels, were tolerated by NK cells in wt B6 mice. This is in line with a report by Brodin et al., showing that cells of different types of MHC class I hemizygous mice, which have up to 60% reduced MHC class I levels, are tolerized by wt NK cells (14). In these studies, NK cell-mediated rejection occurred only when MHC class I levels dropped below 80% of their original level.

Recent studies already showed that adoptively transferred immunoproteasome deficient T cells failed to survive in infected recipients (20, 21). To test whether NK cells are responsible for this loss of T cells, we adoptively transferred immunoproteasome-deficient splenocytes into NK cell depleted and untreated recipients, which were subsequently infected with Influenza virus. Although immunoproteasome-deficient cells were tolerized in naïve recipients, our results showed that they were NK cell-dependently rejected in infected recipients (Figure 3C). This finding complements the study by Moebius et al., who tested different mechanisms for T-cell loss, and concluded that immunoproteasomes were essential for survival and expansion of T cells in infected mice. In these studies NK cell-mediated rejection was considered as unlikely, as transferred immunoproteasome-deficient CD8 T cells were tolerated by uninfected recipients, and because also LMP2- and MECL-1-deficient T cells were lost, although to a lesser extent than LMP7-deficient cells. From these and our studies, we conclude that the reason for loss of immunosubunit-deficient cells in infected wt mice is complex and involves multiple mechanisms, including NK cell mediated rejection and survival/expansion disadvantage (21).

Sun and Lanier have shown that $\beta_2m^{-/-}$:wt mixed BM-chimeric mice lose tolerance

upon MCMV infection, resulting in NK cell-mediated rejection of $\beta_2m^{-/-}$ cells. (27). This result resembles our finding, although we detected NK cell-dependent rejection of an otherwise tolerated cell-population, while $\beta_2m^{-/-}$ cells are rejected by NK cells in a naïve mouse. Although infection induces priming in the LN and thereby renders NK cells fully functional (28), we doubt whether this as such leads to NK cell-mediated rejection of an otherwise tolerated graft. NK cells attack cells according to the balance of activating and inhibitory ligands, thus, following either upregulation of activating or downregulation of inhibitory ligands (29). We suggest that in immunoproteasome-deficient cells, upregulation of activating ligands proceeds as in wt cells while upregulation of MHC class I molecules is relatively poor (Figure 1), and thereby renders these cells susceptible to NK cell-mediated rejection. We are currently in the process of testing this hypothesis.

In conclusion, we here have shown that immunoproteasome-deficient mice, despite having lower levels of MHC class I molecules, have normally responsive NK cells in the periphery. We furthermore showed that tolerance towards immunoproteasome-deficient splenocytes is broken upon infection. Elucidation of the underlying mechanism for infection-dependent rejection of immunoproteasome-deficient cells is subject to further studies.

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CHAPTER 5

The bone marrow functions as the central site of proliferation for long-lived NK cells

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submitted

Abstract

NK cells are innate lymphocytes that play an important role in immune defense against viruses. We investigated the dynamics of natural killer (NK) cell responses following intranasal respiratory virus infection. We show that NK cells rapidly immigrate into the airways following infection with Influenza virus or respiratory syncytial virus (RSV), but find no evidence of proliferation either at the site of infection or in the draining lymph nodes. Instead, we find that the bone marrow (BM) is the primary site of proliferation of both immature and mature NK cells during infection. Moreover, using an adoptive transfer model, we demonstrate that a proportion of peripheral, long-lived and phenotypically mature NK cells migrate back to the BM and there proliferate, both homeostatically and in response to virus infection. Thus, the BM is not only a site of NK cell development, but also an important site for proliferation of long-lived mature NK cells.

Introduction

Natural killer (NK) cells are innate lymphocytes that provide early protection against viral infection and tumor growth. The activation status of NK cells is determined by a balance of signals delivered through their activating and inhibitory receptors (1). Upon activation, NK cells can kill target cells and produce cytokines that tune the immune response. Although being part of the innate immune system, recent studies indicated that at least a proportion of NK cells is not short-lived (2), and acquires a memory-like phenotype (3-5). Although NK cells are known to lack gene rearrangement of their receptors, they express a variety of germ-line encoded receptors for recognition of target cells. For example, in some mouse strains, the stochastically expressed activating receptor Ly49H recognizes the murine CMV (MCMV) encoded protein m157 (6-9). Infection with MCMV induces preferential proliferation of Ly49H positive NK cells (10) that subsequently gain adaptive traits and are able to respond during recall infection (4). Thus, NK cells can become long-lived, however at the moment, it is unknown where such long-lived NK cells reside. This may be at the site of the former infection or in a more central location, with the possibility to rapidly re-locate to the site of (re-)infection.

Many studies indicate that NK cells play a pivotal role in control of respiratory virus infection. Intranasal infections of mice with respiratory syncytial virus (RSV) or Influenza virus leads to influx of NK cells in the lungs (11-13). Gene-deficient mice that lack the NK cell activating receptor NKp46 show an enhanced susceptibility to Influenza virus infection (12) and, during RSV infection, lung NK cells produce cytokines and are cytotoxic (11). Moreover, NK cell depletion prior to RSV infection skews the CD4 T-cell response towards a T helper 2 phenotype, which is associated with immunopathology (14).

In the current study, we have determined the kinetics of NK cell expansion, contraction and survival in response to respiratory virus infection. Surprisingly, we find that not the lung or draining lymphoid tissues but the bone marrow (BM) is the primary site of NK cell proliferation during infection. Using an adoptive transfer model, we further demonstrate that the BM contains not only immature NK cells but also mature, long-lived NK cells that migrate back from the periphery to undergo both homeostatic and infection-induced proliferation in the BM.

Material and Methods

Mice and infection

B6 and BALB/c mice were purchased from Charles River. B6.SJL (CD45.1) and

CD45.1.2 mice were bred in-house under standard conditions. Mice were used between 7 to 17 wks of age. *I.n.* infections with RSV or Influenza virus were performed under light isofluorane anesthesia. RSV A2 was grown on BSC-1 cells, and mice were infected with 5 x 10^6 pfu. Influenza virus infection (A/HK/x31; H3N2) was performed with 105 50% egg infective dose in 30 µL PBS as described (15). All animal experiments were approved by the Committee on Animal Experiments of the University of Utrecht.

BrdU incorporation

To measure in vivo proliferation, 0.8 mg/mL BrdU (Sigma Aldrich) was added to the drinking water of naïve and infected mice during the first 6 days of infection. The drinking water was protected from light exposure and changed daily. Mice were sacrificed after 14 days, and organs were harvested. A BrdU pulse was given to uninfected, infected or recipient mice by administration of BrdU i.p (0.8 mg in 200 µL PBS) and i.n. (0.8 mg in 50 µL PBS), after isofluorane anesthesia. The mice were sacrificed 1 hour later and organs were harvested.

Sample collection and tissue preparation

Mice were sacrificed by i.p. injection of sodium pentobarbital and lymphocytes were obtained from the spleens, mediastinal lymph nodes (MLN), livers, lungs, blood, BM and lungs or BAL. BM cells were obtained by flushing the femurs and tibiae. Lungs and liver were perfused with PBS before excision. Lungs were minced and incubated in PBS containing collagenase (2.4 mg/mL; Roche Applied Science) and DNase (1 mg/mL; Roche Applied Science) for 30 minutes at 37°C. Single-cell suspensions were prepared by passage through cell strainers and lymphocytes were isolated using lympholyte-M (Cederlane) according to manufacturer's instructions. Liver lymphocytes were prepared as described (16), with the exception that lympholyte-M (Cederlane) was used for density separation. Single cell suspensions of spleen and MLN were prepared using cell strainers. Red blood cells were removed from the spleen, BM and blood by ammonium chloride lysis.

Antibodies and flow cytometry

Before staining of cells, Fc receptors were blocked with anti-CD32/CD64 (2.4G2) for 10 minutes at 4°C. Staining was performed for at least 20 minutes at 4°C. For intracellular staining with anti-Ki-67, anti-IFNy or anti-Granzyme B, cells were fixed with 2% paraformaldehyde for 20 minutes at room temperature, and intracellular staining was performed in the presence of 0.5% saponine in PBS for 1 hour at 4°C. For intracellular staining of BrdU, cells were fixed, permeabilized overnight in 0.5% saponine, and stained for 1 hour at 4°C. For figure S1, BAL cells were first culti-

vated 1:1 with YACs for 6 hours in the presence of monensin, and then stained as described. Fluorochrome-conjugated antibodies were purchased from eBioscience [CD69 (H1.2F3), CD49b (DX5), TCRβ (H57-597), NK1.1 (PK136), CD27 (LG.7F9), CD3ε (145-2C), NKp46 (29A1.4), KLRG1 (2F1), CD62L (MEL-14), CD45.2 (104), CD45.1 (A20), IFNy (XMG1.2)], anti-Brdu (PRB1) was obtained from Molecular Probes, anti-Granzyme B (GB12) from Caltag and anti-Ki-67 (B56) from BD Biosciences. Samples were measured on a FACSCalibur or FACSCantoII (BD Biosciences) and analyzed with FlowJo software (Treestar).

NK cell isolation, cell labeling and adoptive transfer

NK cells were enriched from cell suspensions of peripheral organs (lung, liver, spleen) of naïve mice or mice that were infected with RSV or Influenza virus 2 to 4 wks earlier, using an NK-cell isolation kit (Miltenyi Biotec). For adoptive transfer, 0.3-0.7 x 10^6 purified cells were injected *i.v.* into recipient mice. In some experiments, prior to transfer, cells were labeled by incubation with 5 μM CFSE (Invitrogen) in PBS at room temperature for 10 minutes. CFSE was quenched with FCS and cells were washed twice with PBS before injection.

Results

Respiratory virus infection induces NK cell influx into the airways

To determine the kinetics and phenotype of NK cell responses to respiratory virus infection, we infected mice intranasally with two different respiratory viruses: mouse-adapted Influenza virus strain A/HK/x31 (H3N2) and human RSV. Infection with either virus induced a rapid influx of NK cells into the airways (Figure 1A and B). Relative proportions of NK cells peaked at day 2, and then slowly declined. NK cells recovered from the bronchoalveolar lavage (BAL) expressed CD69 (Figure 1C), thus had an activated phenotype, and produced IFNγ upon restimulation (Figure S1). Thus, intranasal infection with two different respiratory viruses leads to influx of activated NK cells into the airways with very comparable kinetics and phenotype.

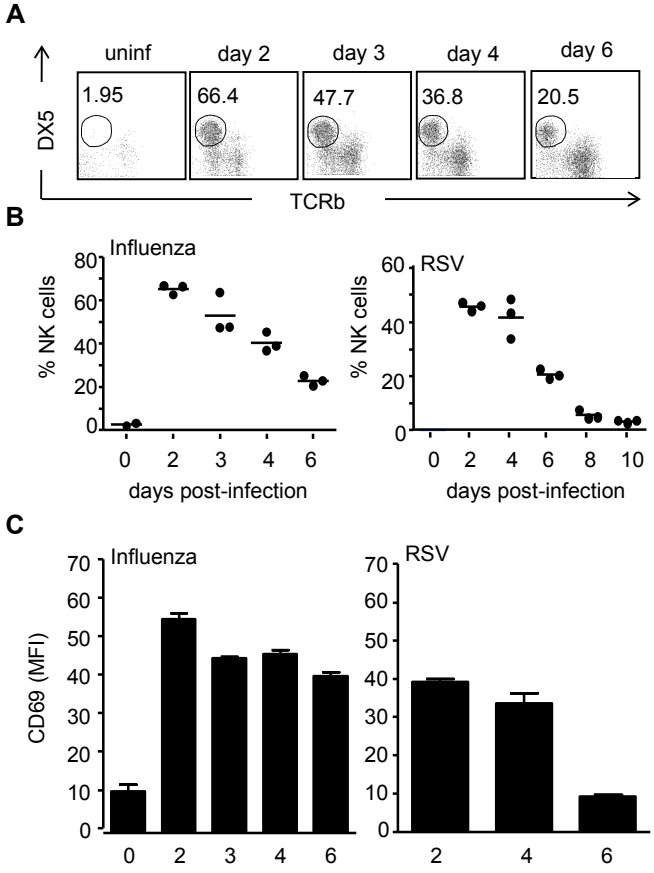

Figure 1. Respiratory virus infection induces influx of activated NK cells into the airways. BALB/c mice were infected i.n. with Influenza virus or RSV, and the presence and activation of NK cells (TCRβ-DX5+) in the BAL was determined by flow cytometry at the indicated days post-infection. (A) Representative FACS plots showing NK cells as percentages of total live lymphocytes in the BAL after influenza virus infection. (B) Percentages of NK cells in the BAL, at the indicated days after Influenza virus or RSV infection. (C) Mean fluorescence intensities (MFI) of CD69 expression on NK cells in the BAL. Results are shown as mean + SEM.

NK cell proliferation during respiratory virus infection takes place mainly in the BM

To determine whether the influx of NK cells into the airways might be due to enhanced proliferation or to enhanced recruitment from other organs, we studied whether NK cells proliferate in response to respiratory virus infection. We first supplied BrdU continuously in the drinking water of mice over the course of the infection (day 1-6). Mice were sacrificed two wks after infection and BrdU incorporation by NK cells was determined by flow cytometry. We found an increased percentage of BrdU+ NK cells in spleen and BM of infected compared to the uninfected animals (Figure 2A). Thus, although infection induced NK cell proliferation, two wks after the infection, hardly any BrdU+ NK cells were found in the lungs.

To determine the site of proliferation more precisely, we administered BrdU i.p. and i.n. to uninfected mice and mice that had been infected 1.5 days earlier, thus, just before NK cells peaked in the BAL. Organs were harvested one hour later to determine BrdU incorporation. Strikingly, in both mouse groups hardly any BrdU incorporation was detected in NK cells recovered from the lungs and the BAL (Figure S2A). We also determined the expression levels of the proliferation marker Ki-67, and found that in particular the BM harbored high frequencies of Ki-67+ NK cells, which were even further increased upon infection (Figure 2B and C). When we pulsed mice with BrdU 4.5 days after infection, increased numbers of BrdU+ (Figure 2D) and Ki-67+ (Figure S2B) NK cells were detected in the BM of infected compared to uninfected mice. In contrast, both at day 1.5 and 4.5 after infection, hardly any BrdU+ NK cells were detected in the BAL, lungs and draining lymph node (Figure 2A, C and D), which strongly suggested that proliferation of NK cells was minimal in these organs. The lack of BrdU incorporation was unlikely to be due to a lack of recovery of proliferating NK cells or inaccessibility of the cells for BrdU, since T cells in these organs had incorporated BrdU in their DNA at day 4.5 post-infection (p.i.) (Figure 2F). Remarkably, in contrast to NK cells, infection did not lead to increased proportions of BrdU positive T cells in the BM. Taken together, these data indicate that respiratory virus infection induces increased proliferation of NK cells in the BM, but not at the site of infection.

Our data so far could not rule out that respiratory viral infection mainly induced enhanced development of NK cells in the BM. We therefore examined the maturation status of dividing NK cells by measuring CD27 and CD11b expression (17, 18). Both in uninfected and infected mice, BrdU+ NK cells in the BM were represented in all four subsets ($CD11b^{low}CD27^{low}$, $CD11b^{low}CD27^{high}$, $CD11b^{high}CD27^{high}$ and $CD11b^{high}CD27^{low}$), including the mature $CD11b^{high}CD27^{low}$ subset (Figure 2E), suggesting that both immature and mature NK cells proliferate in the BM.

In conclusion, our results indicate that an intranasal infection with a respiratory virus

induces enhanced NK cell proliferation and that most of these NK cells proliferate in the BM.

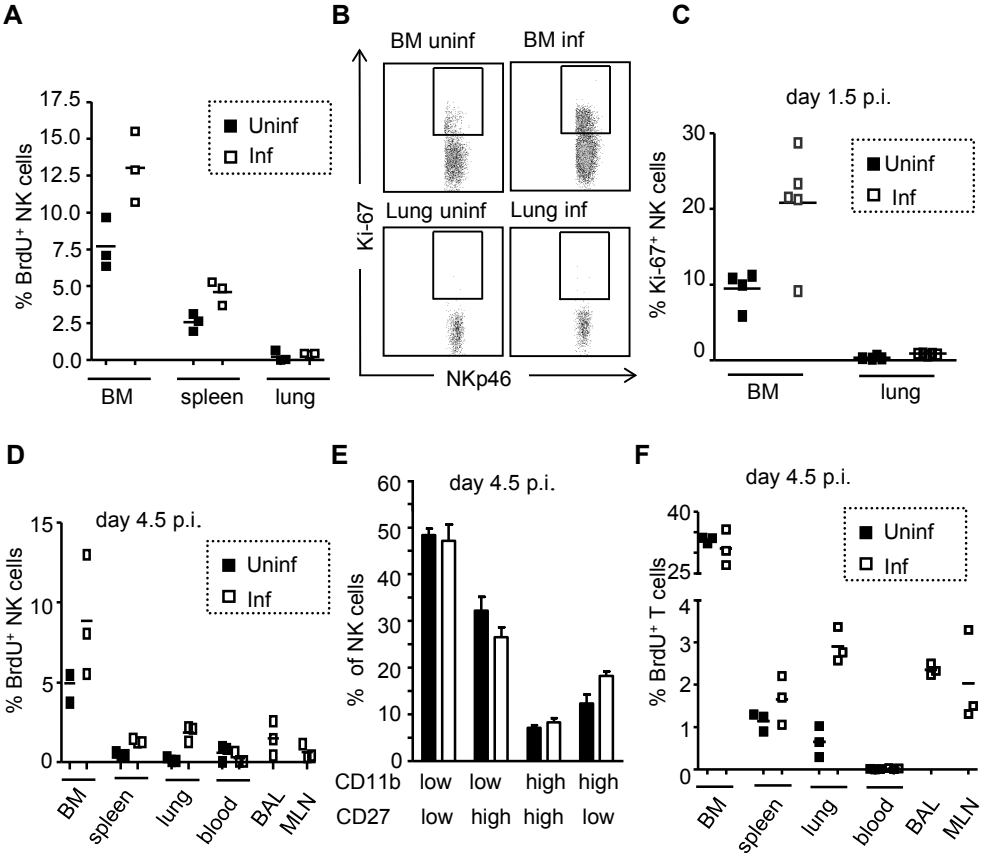

Figure 2. Respiratory virus infection induces increased NK cell proliferation in the BM. (A) B6 mice were infected (inf) with RSV or left uninfected (uninf). BrdU was added to the drinking water from day 1 to 6, and administered i.n. and *i.p* at day 1.5 post-infection (p.i.). Fourteen days later, NK cells from the BM, spleen and lung were analyzed for intracellular BrdU content. (B-F) BALB/c mice were infected with RSV or left uninfected, and BrdU was administered *i.n.* and *i.p*. at day 1.5 or 4.5 post-infection. Mice were sacrificed 1 hr later. Percentages of Ki-67+ NK cells or BrdU+ NK cells in the indicated organs were determined by flow cytometry. (B) Representative FACS plots and (C) percentages of Ki-67+ NK cells in the BM and lung at day 1.5 p.i. (D) Percentages of BrdU+ NK cells in the indicated organs at day 4.5 p.i. (E) Maturation status of dividing (BrdU+) NK cells at day 4.5 p.i. Results are shown as mean + SEM. (F) Percentages of BrdU+ T cells (TCRβ+) in the indicated organs at day 4.5 p.i.

Transferred, long-lived NK cells have a mature phenotype and undergo both homeostatic and infection-induced proliferation in the BM

The BM is a known place for NK cell development, however, our data so far suggest that also NK cells with a mature phenotype proliferate in the BM. To further determine where mature NK cells proliferate, we infected mice with Influenza virus, and

2 wks later transferred NK cells purified from the periphery (lung, liver, spleen) into naïve congenic mice. We could still recover these NK cells from recipient mice 4 to 5 wks after transfer, indicating that they are long-lived. While transferred NK cells preferentially homed back to their site of origin, part of these peripheral NK cells migrated to the BM (Figure 3A). The recovered transferred NK cells had a lower expression of CD27 (Figure 3B) and a higher expression of killer cell lectin-like receptor G1 (KLRG1) (Figure 3C) than endogenous NK cells in all organs analyzed, indicating that transferred cells had a mature phenotype (18, 19). Interestingly, in the spleen and BM a proportion of the transferred NK cells had downregulated CD62L (Figure 3D) and transferred NK cells had higher granzyme B (GzmB) expression compared to endogenous peripheral NK cells (Figure 3E). These data indicate that long-lived NK cells are phenotypically different from endogenous NK cells.

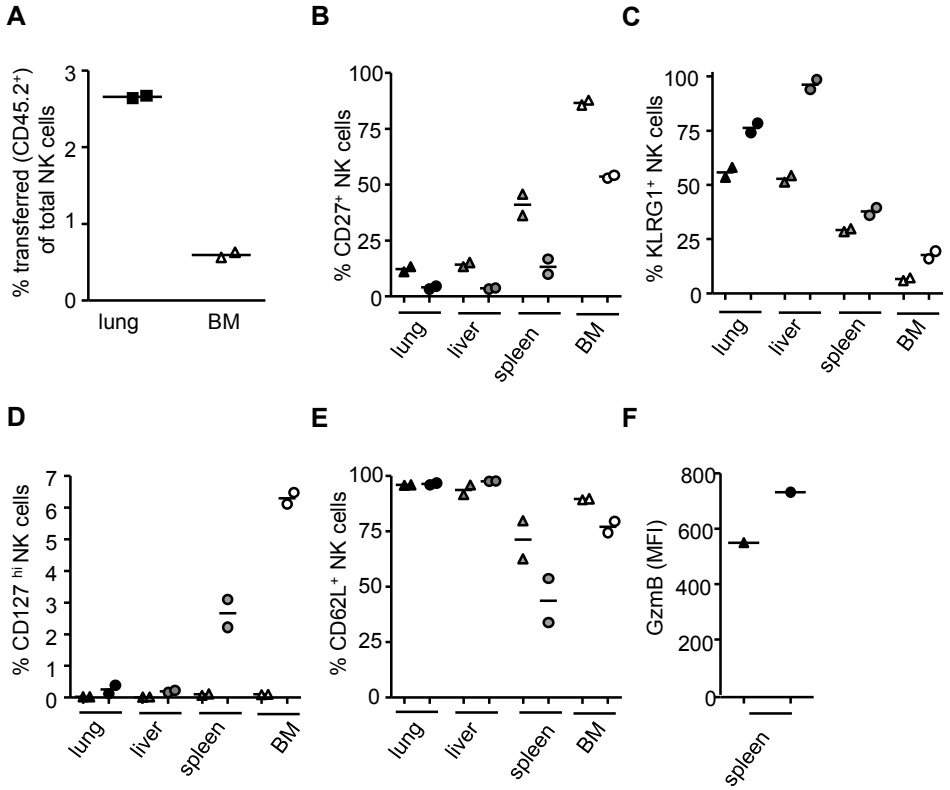

Figure 3. Mature long-lived NK cells have a distinct phenotype. (A-E) CD45.2 mice were infected with Influenza virus and 14 days later, NK cells purified from the lung, liver and spleen were transferred into naïve CD45.1.2 mice. (A) CD45.2 NK cells as percentage of total NK cells, recovered 3 wks after transfer from the lungs and BM of CD45.1.2 recipient mice. (B-E) Phenotype of transferred, CD45.2 (circles) and endogenous, CD45.1.2 (triangles) NK cells, recovered from the indicated organs. Presented data were obtained using pooled cells of 2 to 3 (A-D) or 5 (E) mice.

To determine where mature long-lived NK cells proliferate, we transferred carboxyfluorescein diacetate succinimidyl ester (CFSE) labeled peripheral NK cells from the liver, spleen and lungs of Influenza virus-infected mice into congenic recipient mice. Analysis of the CFSE content 4 to 5 wks after transfer showed that only a small percentage of NK cells recovered from the peripheral organs of the acceptor mice, i.e. lung, liver and spleen, had undergone division (Figure 4A and B), and of those that had divided, most had undergone not more than one division. In contrast, most of the transferred NK cells recovered from the BM had undergone multiple divisions. Similar results were seen when cells of RSV infected or naïve mice were transferred (Figure S3).

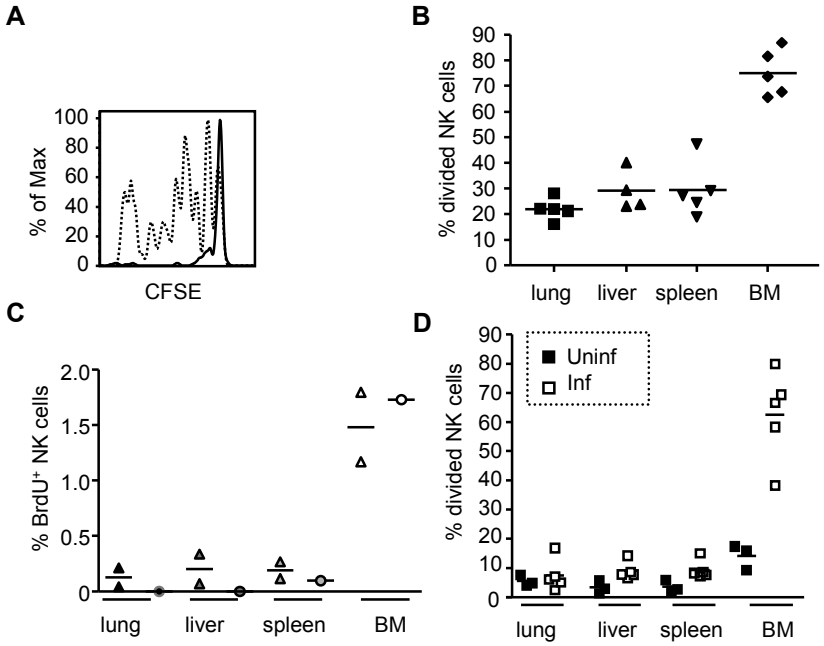

Figure 4. Mature, long-lived NK cells proliferate in the BM. (A-B) NK cells enriched from peripheral organs (lung, liver, spleen) of Influenza virus-infected CD45.2 mice, 2 wks after infection, were labeled with CFSE and transferred into CD45.1.2 mice. CFSE profiles of transferred NK cells were determined 4 to 5 wks later. (A) Representative CFSE profile of transferred NK cells in the BM (dotted line) and lung (solid line). (B) Percentages of divided NK cells in the organs indicated. (C) Percentages of BrdU+ endogenous, CD45.1.2 and transferred, CD45.2 NK cells in indicated organs of CD45.1.2 recipient mice, 3 wks after adoptive transfer of peripheral NK cells of Influenza virus infected CD45.2 mice. Mice received BrdU *i.p.* and *i.n.*, 1 hr before being sacrificed. Presented data were obtained using pooled cells of 2 to 3 mice and background, measured in cells of BrdUneg mice, was subtracted. (D) NK cells enriched from peripheral organs (lung, liver, spleen) of Influenza virus-infected CD45.2 mice, 3 to 4 wks after infection, were labeled with CFSE and transferred into CD45.1.2 mice. Recipient mice were left uninfected or infected 13 days later with Influenza virus, and sacrificed at day 7 post-infection. CFSE profiles of transferred NK cells in different organs were determined. Depicted are percentages of divided, transferred NK cells in the indicated organs of individual recipient mice.

To address the question whether long-lived NK cells indeed proliferate in the BM and not only preferentially home back there after division, we transferred peripheral NK cells from Influenza virus infected mice and 3 wks later we gave a BrdU pulse for one hour. When comparing BrdU incorporation of transferred NK cells in different organs, we exclusively detected BrdU+ NK cells in the BM (Figure 4C). Remarkably, in a similar experiment in which peripheral, CFSE labeled NK cells were transferred, we found that the proportion of divided, transferred NK cells in the BM dramatically increased following respiratory virus infection (Figure 4D). From these data we infer that a proportion of mature NK cells migrates to the BM where they are maintained and able to react to virus infection with a proliferative response.

Discussion

Although it is well-established that NK cells play an important role in immune protection to viral infection, relatively little is known about the kinetics of NK cell responses to most viral pathogens. In the current study we investigated the response of NK cells to respiratory viral infections. We found that upon infection, NK cells rapidly immigrated into the airways, however, did not detectably proliferate there. Instead, proliferation occurred preferentially in the BM. Surprisingly, NK cell proliferation was increased upon infection and appeared not to be restricted to naïve NK cells, as also mature, $CD11b^{high}CD27^{low}$ NK cells proliferated. In specific, using an adoptive transfer model, we found that a proportion of a population of long-lived NK cells migrated to the BM and there underwent both homeostatic and infection-induced proliferation. Thus, although the BM harbors high amounts of immature, developing NK cells, our data indicate that it is also the central site of proliferation for long-lived NK cells.

The transfer model used to determine NK cell fate was based on recent publications that showed that NK cells can become long-lived after hapten, cytokine or viral exposure, suggesting an adaptive memory-like feature of these cells (3-5). Following transfer from respiratory virus infected mice into naïve recipients, we were able to recover NK cells at least up until 4 to 5 wks later, indicating that a proportion of the transferred cells were long-lived. Remarkably, a phenotypically similar long-lived NK cell population could be recovered from mice that had received NK cells of uninfected mice (Figure S3), perhaps because the mouse colonies used here were maintained under standard conditions, thus not strictly pathogen-free. Transferred, long-lived NK cells that were recovered from recipient mice were distinguishable from endogenous NK cells by higher KLRG1 and lower CD27 expression, further indicating their mature phenotype (17, 18).

As demonstrated in Figure 2A-D, respiratory virus infection induces increased NK

cell proliferation in the BM but barely in the periphery. In particular at day 4.5 after RSV infection (Figure 2F), T cells derived from the draining lymph node, lung and BAL, had incorporated BrdU indicating that they were proliferating, whereas hardly any BrdU-positive NK cells were detectable in these organs. Thus, while T-cell proliferation occurs in the lymphoid tissues draining the site of infection, most NK cells proliferate in the BM upon respiratory virus infection. Intriguingly, this finding resembles previous findings during bacterial infection, where monocytes move from the BM into infected tissue, and are subsequently replenished in the BM from progenitors (20, 21).

Previous studies on interactions between NK cells and Influenza virus infected cells, by the group of Mandelboim, have shown that NK cells recognize Influenza virus hemagglutinin (HA) through the activating receptor NKp46, which leads to target cell killing (22). Mice that lack NKp46 die more readily of Influenza virus infection than wt mice, despite similar increases in NK cell numbers in the lungs (12). These data suggest that NKp46 ligation may lead to activation but not proliferation. Our finding that NK cells do not proliferate in the lungs upon intranasal Influenza virus infection further confirms this proposition. In contrast, stimulation of the activating receptor Ly49H through MCMV m157 leads to selective proliferation of Ly49H positive NK cells after infection (10). These different outcomes of receptor ligation might result from differences in the signal pathways used by Ly49H and NKp46, which signal through the adaptor protein DAP12 (23) and the FcεRIγ and CD3ζ (24), respectively.

In conclusion, based on previous publications and our current, own data we propose the following model for NK cell development and responses to respiratory virus infection. Immature NK cells develop in the BM and, following virus entry in the periphery and inflammation, are recruited to the site of infection. Emigrated NK cells are activated in the periphery and there participate in viral clearance and regulation of both innate and adaptive responses. In the BM, NK cell emigration is likely to lead to temporal NK cell lymphopenia, which induces NK cell proliferation and replenishment. After pathogen clearance, a proportion of activated NK cells becomes long-lived and can migrate to the BM where they 1), undergo homeostatic proliferation; and 2), rapidly proliferate following re-infection. Both proliferative processes lead to progenitor cells that migrate back out into the periphery. The advantage of long-lived NK cells in relation to immune protection is presently not fully understood. Remarkably, previous studies have shown that also memory T cells migrate to and undergo homeostatic proliferation in the BM (25-27) and that non-proliferating memory T cells are nonspecifically recruited to the lung airways following infection with heterologous respiratory viruses, probably to provide cytokines for early viral control (28, 29). Analogous to T cells, also long-lived NK cells may be recruited nonspecifically to sites of infection and provide anti-viral and/or immune stimulatory cytokines that

lead to enhanced control of virus infection. This proposition will be the subject of future studies.

Acknowledgements

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Supporting Information

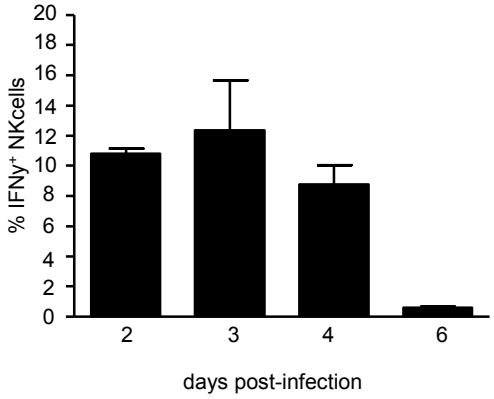

Figure S1 Respiratory virus infection induces influx of activated NK cells into the airways. BALB/c mice were infected *i.n.* with Influenza virus and the BAL was harvested at the indicated days post-infection. The cells were restimulated with YAC cells in the presence of monensin for 6 hrs, and intracellular IFNγ was measured by flow cytometry. Percentages of IFNγ+ NK cells are shown as mean + SEM.

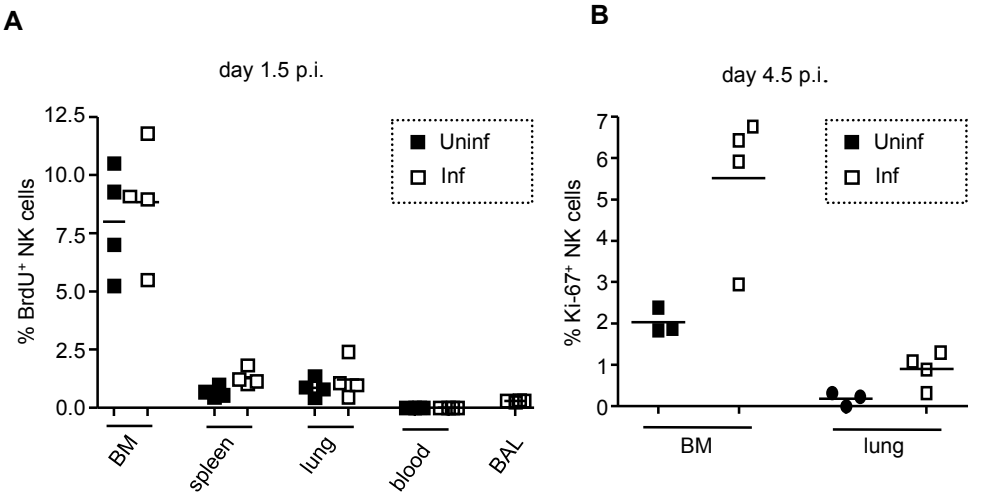

Figure S2 Respiratory virus infection induces increased NK cell proliferation in the BM. BALB/c mice were infected with RSV or left uninfected, and BrdU was administered *i.n.* and *i.p.* at day 1.5 or 4.5 post-infection. Mice were sacrificed 1 hr later. Percentages of Ki-67+ NK cells or BrdU+ NK cells in the indicated organs were determined by flow cytometry. (A) Percentages of BrdU+ NK cells in the indicated organs of individual mice at day 1.5 p.i., (B) Percentages of Ki-67+ NK cells in the BM and lungs at day 4.5 p.i.

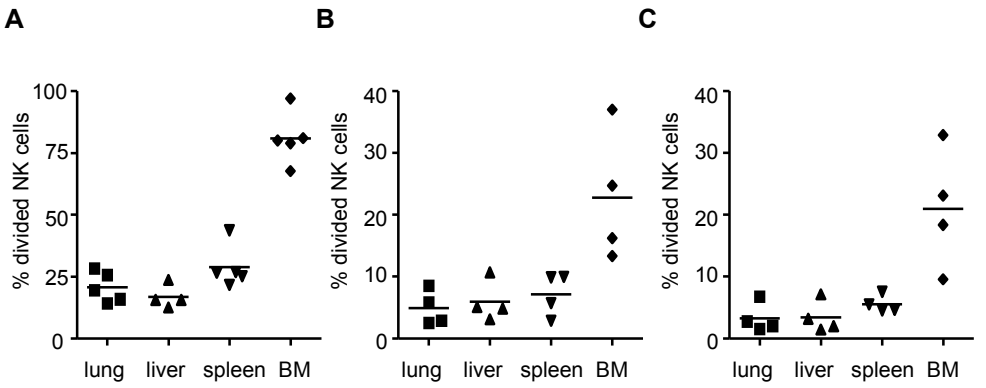

Figure S3 Mature long-lived NK cells divide mainly in the BM. (A), NK cells enriched from peripheral organs (lung, liver, spleen) of uninfected CD45.1 mice were labeled with CFSE and transferred into CD45.1.2 mice. CFSE profiles of transferred NK cells were determined 4-5 wks later. (B, C), NK cells enriched from lung, liver, spleen and BM of Influenza virus-infected CD45.1 (B) or RSV-infected CD45.2 (C) mice, 3-4 weeks after infection, were labeled with CFSE and transferred into CD45.1.2 mice. CFSE profiles of transferred NK cells were determined 10 days later. Depicted are percentages of divided, transferred NK cells in the indicated organs of individual recipient mice.

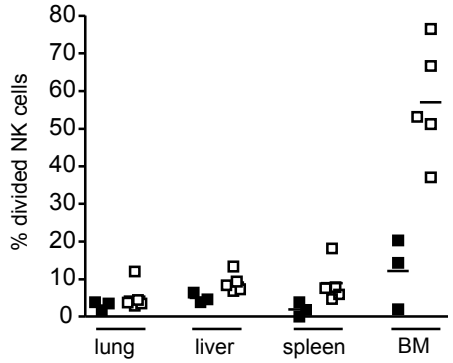

Figure S4 Respiratory virus infection increases proliferation of mature long-lived NK cells in the BM. (A), NK cells enriched from peripheral organs (lung, liver, spleen) of uninfected CD45.1 mice were labeled with CFSE and transferred into CD45.1.2 mice. Recipient mice were infected 13 days later with Influenza virus or left uninfected, and sacrificed 7 days p.i. CFSE profiles of transferred NK cells were determined. Depicted are percentages of divided, transferred NK cells in the indicated organs of individual recipient mice.

CHAPTER 6

Pathogen-specific requirements for the processing of antigens that prime CD8 T-cell responses

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in preparation

Abstract

CD8 T cells play an essential role in containing intracellular infections and tumor growth. CD8 T cells recognize complexes of MHC class I molecules with antigenic peptides, which are mainly processed by the catalytic protease complex the proteasome. CD8 T-cell responses are assumed to be primed by non-infected professional antigen presenting cells (pAPCs) like DCs. However, where processing of the antigenic epitopes that are presented on the pAPCs occurs remains unknown. DCs could take up antigens to process and present them, or infected cells could pre-process the antigens and deliver them in a processed form to DCs for presentation to CD8 T cells. To reveal the exact mechanism of antigen delivery to DCs, we have established an infection model in which we measure the size of CD8 T-cell responses to immunoproteasome-dependent epitopes, derived from recombinant *Listeria monocytogenes* or Influenza, in mice that express or lack the IFNγ-inducible proteasome subunits (immunoproteasome subunits) in specific cell types only. We found that epitopes derived from Listeria and Influenza both require processing in bone marrow-derived cell types in order to induce CD8 T-cell responses. However, Influenza-derived epitopes have to be processed in $CD11c^+$ DCs only, whereas Listeria-derived epitopes can also be processed by cell types other than $CD11c^+$ DCs. Thus, our data show that the antigen processing pathways leading to the generation of epitopes that prime CD8 T-cell responses vary for different pathogens, and indicate that also other cell types than DCs can process those antigens that prime CD8 T-cell responses.

Introduction

CD8 T cells play an important role in immune protection against intracellular pathogens and tumor growth. CD8 T cells reacting to cellular infection recognize peptides derived from pathogen proteins that are presented on the cell surface of the infected cell by MHC class I molecules. The presented peptides are processed mainly by the proteasome, an intracellular multicatalytic protease complex. In most cells, the enzymatic activity of proteasomes is exerted by three constitutively expressed subunits β1, β2, and β5 that are part of the 20S proteasome. In lymphoid cells and cells exposed to (pro)inflammatory cytokines, these catalytic subunits are replaced by their inducible homologues β1i (LMP2), β2i (MECL-1) and β5i (LMP7) resulting in the formation of so-called immunoproteasomes. Incorporation of immunoproteasome subunits was shown to change the cleavage preferences of 20S proteasomes (1) and to enhance the generation of a significant number of antigenic peptides (2, 3).
While pathogen-specific effector CD8 T cells recognize their peptide antigen on any infected cells, priming of naïve CD8 T cells requires the presentation of antigenic peptides by professional antigen presenting cells (pAPCs) that provide costimulation. Many studies have shown that mainly DCs are responsible for CD8 T-cell priming (4-6). There are two main pathways for CD8 T-cell activation, direct presentation and cross-presentation. Direct presentation is a task of infected pAPCs, where peptides presented in the MHC class I molecules are derived from antigens, synthesized by or secreted into the cytosol of the pAPC itself (7). Alternatively, if the APC is not infected, it needs to capture exogenously synthesized antigens for display on their MHC class I molecules. This mechanism is termed cross-presentation, which is described to occur predominantly by DCs that are well equipped to capture antigen that then probably is translocated into the cytosol (8). It has been suggested that CD8 T cells specific for peptides derived from antigens that are rapidly degraded by host cell proteasomes are primed primarily by infected pAPCs, whereas CD8 T cells specific for peptides of stable antigens may be primed also by cross-presentation (9, 10). The extent to which each of these pathways contributes to priming of the overall, pathogen-specific CD8 T-cell response during infection remains unclear, unless the infecting pathogens possesses mechanisms for evasion of either direct presentation (11, 12) or cross-presentation (13), in which case the alternative pathway is followed.
Listeria monocytogenes is an intracellular bacterium that invades the cytosol of infected cells. CD8 T cells play an important role in the clearance of Listeria from infected hosts and provide long-term protective immunity (14). Although both secreted and non-secreted bacterial antigens can prime CD8 T-cell responses (14,

15), protective immunity is provided by CD8 T cells recognizing the bacterial virulence factors that are secreted into the cytosol of the infected cell, probably because only these proteins are processed into MHC class I presented T-cell epitopes (16). Previous studies into the pathways leading to priming of Listeria-specific CD8 T-cell responses have shown that dying neutrophils function as antigen donors for cross-priming pAPC that activate naïve CD8 T cells specific for non-secreted bacterial proteins (15). Both cross-priming (17, 18) and DC (4, 5) have been implicated also in priming of naïve CD8 T cells recognizing the secreted Listeria proteins. On the other hand, the immunodominant target of the CD8 T-cell response in BALB/c mice and the only MHC class Ia-presented target so far identified in the C57BL/6 mice are both derived from listeriolysin O, a protein that is rapidly degraded by the infected host cells (19, 20) and therefore presumably less likely to prime CD8 T cells through the cross- presentation pathway.

In former studies, using the recombinant *Listeria monocytogenes*-E1 (LM-E1) strain, we defined a relationship between antigen processing kinetics and the dominance hierarchy of CD8 T-cell responses (21). In specific, we found that one of the epitopes processed from the rLM-E1-secreted p60-E1 hybrid protein, $E1B_{192\text{-}200}$, elicited a dominant CD8 T-cell response in infected wt mice, but failed to elicit any response in immunoproteasome-deficient mice, due to the delayed processing kinetics of the peptide.

To gain more insight into the site of antigen processing and the exact mechanism by which antigens are delivered to pAPCs, we have established an infection model in which CD8 T-cell responses to immunoproteasome-dependent epitopes derived from recombinant *Listeria monocytogenes* are measured in mice that either lack or express the immunosubunits in specific cell types only. By making use of several different combinations of bone marrow-chimeric mice, our data show that both Listeria- and Influenza-derived epitopes require processing in BM-derived cell types. However, processing of Listeria-derived epitopes can be performed by cell types other than $CD11c^+$ DCs, whereas Influenza-derived epitopes are processed in $CD11c^+$ cells only. Thus, this study suggests that the antigen processing pathways leading to the generation of epitopes that prime CD8 T-cell responses vary for different pathogens, and do not necessarily involve DCs.

Materials and methods

Mice and infections

Recombinant *L. monocytogenes* rLM-E1 was grown in brain-heart infusion medium (BD Biosciences) supplemented with 250 µg/mL spectinomycin and harvested while

in log phase. Mice were inoculated *i.v.* into the tail vein with a sublethal dose of 5 x 10^3 rLM-E1 in 100 µL PBS. For infection with Influenza Virus strain HKx31, mice were anesthetized with isofluorane and then infected *i.n.* with 5 x 10^4 hemagglutination units in 30 µL PBS.
C57/BL6 (B6) and BALB/c mice were purchased from Harlan and Taconic respectively. B6 iβ5/LMP7+iβ2/MECL-1 gene-deficient mice (22) were maintained by in-house breeding under standard conditions. CD11c-DTR/GFP mice (4) were obtained from Dirk Busch in Munich. For infection, 6- to 12-week-old female mice were used. All experiments involving animals were approved by the Committee on Animal Experiments of the University of Utrecht Veterinary School.

Construction of BM-chimeric mice
6- to 12-week-old female recipient mice were irradiated with 10 Gy as a single dose from an X-ray irradiator. Irradiated recipients were reconstituted *i.v.* into the tail vein with 10^7 wt B6 or iβ5/LMP7+iβ2/MECL-1 gene-deficient BM cells or 10^7 1:1 mixed BM cells derived from iβ5/LMP7+iβ2/MECL-1 gene-deficient and CD11c-DTR/GFP mice or iβ5/LMP7+iβ2/MECL-1 gene-deficient and BALB/c mice. BM cells were isolated from femurs and tibias of donor mice and depleted of T cells by incubation with a mixture of the monoclonal antibodies anti-CD4 (clone GK1.5) and anti-CD8 (clone 3-55) followed by Guinea pig complement (Invitrogen) at a concentration of 4.5 µg/mL for 30 minutes. Infection of chimeric mice was performed the earliest 6 weeks after reconstitution.

DC depletion
Depletion of DCs in BM-chimeric mice that had been reconstituted with CD11c-DTR/GFP derived BM cells was performed by *i.p.* injection of 10 ng/gr bodyweight diphtheria toxin (Sigma) in PBS one day prior to infection and once again with infection. The efficacy of DC depletion was evaluated by FACS analysis one day after infection. Splenocytes of DT-treated and untreated BM-chimeric mice were stained with anti-mouse CD11c and $CD11c^+$ GFP expressing populations were analyzed (data not shown).

IFNγ ELISPOT
ELISPOT assays were performed in 96-well MAIP ELISPOT plates (Millipore) coated with 2 µg/mL AN18 (anti-mouse IFNγ) in 50 µL of PBS O/N at 4˚C.Wells were then washed and blocked with RPMI medium supplemented with 10% FCS, glutamate, antibiotics and 30 µM β-mercaptoethanol for 30 min at 37˚C. Splenocytes or BAL cells were incubated with or without 2 µM synthetic peptides ($E1A_{234-243}$ and $E1B_{192-200}$ for LM-E1 infected mice and $NP_{366-374}$ and $PA_{224-233}$ for Influenza Virus HKx31 in-

fected mice) in 100 µL RPMI medium per well for 6 h at 37˚C. Thereafter, plates were washed with PBS plus 0.01% Tween 20 (PBST), and IFNγ was detected by incubation with 2 µg/mL biotinylated anti-IFNγ, clone XMG1.2, (BD Pharmingen), followed by 1 µg/mL alkaline phosphatase-conjugated streptavidin (Jackson) in PBST supplemented with 2% BSA. The assay was developed with the Vector AP substrate kit (Vector Laboratories). Plates were then washed, dried and analyzed using the Elvis plate reader and software.

Intracellular cytokine staining

Percentages of $E1A_{234-243}$, $E1B_{192-200}$, $NP_{366-374}$ and $PA_{224-233}$-specific CD8 T cells were determined by intracellular cytokine staining and FACS analysis as described (21). In brief, approximately 10 x 10^6 erythrocyte-depleted lymphocytes were incubated for 6 h with or without 500 nM synthetic peptide in 1 mL RPMI medium containing 50 µg/mL gentamicin and 9 µM monensin. Thereafter, cells were stained with an FITC-conjugated anti-mouse CD8 antibody (clone 53-6.7) in the presence of the anti-CD16/32 (clone 2.4G2), fixed with 2% paraformaldehyde and then stained with XMG1.2-PE (anti-IFNγ) in the presence of 0.5% saponine. Cells were analyzed on a FACSCalibur.

Results

Expression of immunoproteasome subunits in bone marrow-derived cells is essential for processing of immunoproteasome-dependent epitopes

In previous studies we have shown that mice lacking the immunoproteasome subunits iβ5/LMP7 and iβ2/MECL-1 raise similar robust responses to the immunoproteasome-independent epitope $E1A_{234-243}$ as wt mice, but fail to elicit a CD8 T-cell response to the immunoproteasome-dependent epitope $E1B_{192-200}$ after infection with recombinant *Listeria monocytogenes* strain rLm-E1 (21, 23). Similarly, infection of mice with Influenza strain HKx31 resulted in clear CD8 T-cell responses in the BAL to the $NP_{366-374}$ epitope in wildtype (wt) mice and iβ5/LMP7+iβ2/MECL-1-deficient mice. However, no CD8 T-cell responses to the immunoproteasome- dependent epitope $PA_{224-233}$ were detected in mice deficient for the immunoproteasome subunits (23). The absence of $E1B_{192-200}$ and $PA_{224-233}$ responses could not be explained by defects in the T-cell repertoire of iβ5/LMP7+iβ2/MECL-1-deficient mice (21, 23). Therefore, the recombinant *Listeria monocytogenes* strain and the Influenza HKx31 strain containing both an immunoproteasome-dependent and -independent epitope, provide a useful tool in unravelling the role of different cell types in antigen processing.

In order to study which cells are essential for the processing of the different epit-

opes, we generated bone marrow (BM)-chimeric mice in which we reconstituted lethally irradiated wt and iβ5/LMP7+iβ2/MECL-1-deficient mice with iβ5/LMP7+iβ2/MECL-1-deficient and wt BM, respectively. As a control, BM- chimeric mice were generated of wt mice that were reconstituted with wt BM and iβ5/LMP7+iβ2/MECL-1-deficient mice reconstituted with iβ5/LMP7+iβ2/MECL-1-deficient BM. Six weeks later, mice were infected *i.v.* with a sublethal dose of rLm-E1. Quantification of CD8 T-cell responses to the rLM-E1$B_{192\text{-}200}$ epitope in the spleen of infected mice showed that only mice that were reconstituted with BM derived from wt donor mice were able to respond to the E1$B_{192\text{-}200}$ epitope (Figure 1A), while wt mice (and iβ5/LMP7+iβ2/MECL-1 deficient mice) that received BM from immunoproteasome-deficient mice failed to respond to the immunoproteasome- dependent E1$B_{192\text{-}200}$ epitope (Figure 1A). Comparable results were obtained after infection of BM-chimeric mice with Influenza. As shown in figure 1B, only BM- chimeric mice that received wt BM cells could elicit CD8 T-cell responses to the immunoproteasome-dependent $PA_{224\text{-}233}$ in the BAL, whereas wt mice reconstituted with iβ5/LMP7+iβ2/MECL-1 deficient BM did not respond. These results indicate that the presence of proteasome immunosubunits in BM derived cells is essential for the processing of immunoproteasome-dependent epitopes in order to mount CD8 T- cell responses.

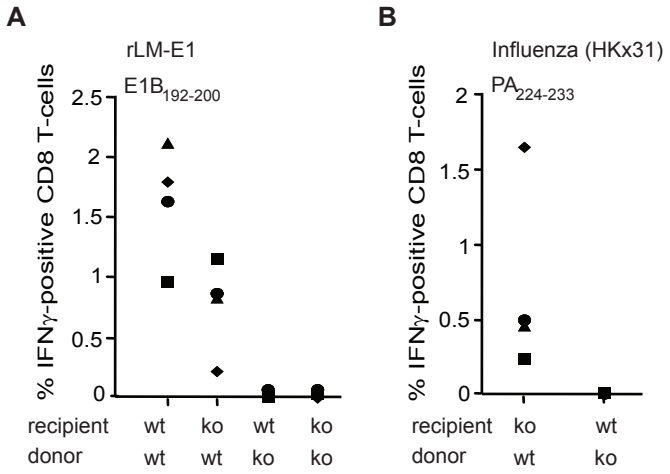

Figure 1. Expression of immunoproteasomes in BM-derived cells required for induction of CD8 T-cell responses to immunoproteasome-dependent epitopes. Lethally irradiated wt and iβ5/LMP7+iβ2/MECL-1-deficient mice were both reconstituted with either wt BM or iβ5/LMP7+iβ2/MECL-1-deficient BM. Six weeks after BM injection, BM-chimeric mice were infected *i.v.* with 5 x 10^3 rLm-E1 (A) or *i.n* with 5 x 10^4 U Influenza-HKx31 (B). On day 8 after rLM-E1 infection frequencies of E1B-specific CD8 T cells were determined in the spleen by staining for CD8 and intracellular IFNγ (A). (B) Percentages of PA-specific CD8 T cells in the BAL, 7 days after Influenza infection. Background responses measured in the absence of stimulating peptides were subtracted. Values for individual mice are shown.

Processing of Listeria-derived epitopes can be performed in cells other than DCs, whereas Influenza-derived epitopes require DCs only

To study the role of DCs in epitope processing, we used the CD11c-DTR transgenic mouse system developed by Jung et al.(4). In these mice, CD11c expressing DCs can be depleted *in vivo* by injection of diphtheria toxin (DT).

Iβ5/LMP7+iβ2/MECL-1-deficient mice were lethally irradiated and reconstituted with a 1:1 mixture of BM derived from iβ5/LMP7+iβ2/MECL-1-deficient mice and CD11c-DTR/GFP mice. After depletion of DCs by DT treatment, these mice could express immunoproteasome subunits in all hematopoietic cell types but DCs. To determine whether DCs are essential in processing of Influenza derived epitopes, DT-treated and untreated mixed BM-chimeric mice were infected with Influenza and CD8 T-cell responses were quantified. Both treated and untreated mice were able to mount responses to the immunoproteasome-independent epitope $NP_{366-374}$ (Figure 2A). However, no response to the immunoproteasome-dependent epitope $PA_{234-243}$ could be detected in the DT-treated mice, whereas the untreated mice did respond to this epitope Figure 2B). These data indicate that Influenza-derived epitopes have to be processed by $CD11c^+$ DCs in order to induce efficient CD8 T-cell responses.

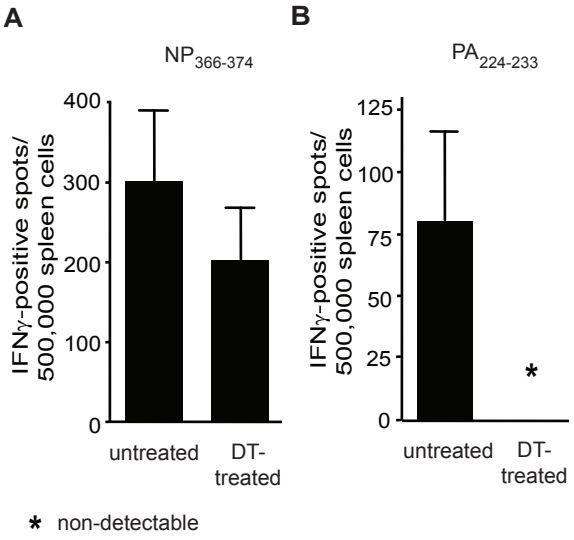

Figure 2. Effect of DC depletion on generation of Influenza-derived epitopes. Lethally irradiated iβ5/LMP7+iβ2/MECL-1-deficient mice were reconstituted with a 1:1 mixture of BM derived from iβ5/LMP7+iβ2/MECL-1-deficient and CD11c-DTR/GFP mice. Six weeks after BM injection, BM-chimeric mice were treated with DT (10 ng/gr bodyweight) or left untreated, and infected *i.n.* with 5 x 10^4 U Influenza-HKx31. At day 8 after infection CD8 T-cell responses to $NP_{366-374}$(A) and $PA_{224-233}$(B) were determined in spleens by IFNγ ELISPOT. Background responses measured in the absence of stimulating peptides were subtracted. Shown are means +/- SEM.

To our surprise, however, the situation was different when BM-chimeric mice that had either been treated with DT or had been left untreated were infected with rLM-E1. As expected, both treated and untreated mice showed good responses to the immunoproteasome-independent $E1A_{234-243}$ epitope (Figure 3A). Furthermore, however, responses to the immunoproteasome-dependent $E1B_{192-200}$ epitope were also detected in DT-treated and untreated mice. Since in the DT-treated animals only DCs remain that cannot express the immunosubunits, these data indicate that essential antigen processing steps leading to the generation of Lm-E1-derived epitopes can also be performed by cell types other than DCs (Figure 3B).

Taken together, the antigen processing pathways leading to the generation of epitopes that prime CD8 T-cell responses appear to vary for different pathogens, and do not necessarily involve DCs.

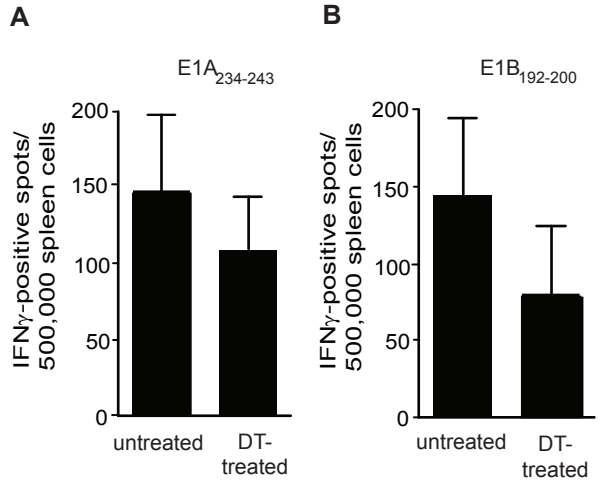

Figure 3. Effect of DC depletion on generation of Lm-derived epitopes. Lethally irradiated iβ5/LMP7+iβ2/MECL-1-deficient mice were reconstituted with a 1:1 mixture of BM derived from iβ5/LMP7+iβ2/MECL-1-deficient and CD11c-DTR/GFP mice. Six weeks after BM injection, BM-chimeric mice were treated with DT (10 ng/gr body weight) or left untreated, and infected *i.v.* with 5 x 10^3 rLm-E1. At day 8 after infection CD8 T-cell responses to E1A (A) and E1B (B) were determined in spleens by IFNγ ELISPOT. Background responses measured in the absence of stimulating peptides were subtracted. Shown are means +/- SEM.

Immunoproteasome-expressing MHC class I mismatched pAPC are unable to provide epitopes to immunoproteasome-deficient pAPC

Several studies suggested that cross-priming and direct priming by infected DCs are the main mechanisms that activate naïve CD8 T cells (5, 24). To determine whether preprocessed fragments or epitopes are handed over to the priming pAPC, mixed

BM-chimeric mice with a 1:1 mixture of BM derived from iβ5/LMP7+iβ2/MECL-1-deficient ($H2^b$) and BALB/c ($H2^d$) mice were generated. In this way, these BM-chimeric mice are able to process proteasome dependent epitopes due to the presence of immunoproteasome subunits in cells derived from BALB/c mice. However, BALB/c derived APCs are unable to present the epitopes due to the MHC class I mismatch. In this experimental set-up, neither cross-priming nor direct priming of infected pAPCs is possible. However, a hand-over of preprocessed fragments from immunosubunits-expressing BALB/c DCs to immunosubunit-deficient, H2-K^b-expressing DCs remains an option. BM-chimeric mice (iβ5/LMP7+iβ2/MECL-1-deficient mice reconstituted with either wt BM, iβ5/LMP7+iβ2/MECL-1-deficient BM or with a mixture of iβ5/LMP7+iβ2/MECL-1-deficient and BALB/c BM) were infected with rLM-E1 or Influenza. Consistent with our previous data, CD8 T-cell responses to $E1B_{192\text{-}200}$ and $PA_{234\text{-}243}$ were clearly detected in the control BM-chimeric mice that received wt BM and responses were absent in BM-chimeric mice reconstituted with iβ5/LMP7+iβ2/MECL-1-deficient BM (Figure 4 A, B). Figure 4 further shows that in the mixed BM-chimeric mice, no CD8 T-cell responses were elicited to the $E1B_{192\text{-}200}$ and $PA_{234\text{-}243}$ epitope. These data suggest that the MHC class I mismatched BALB/c-derived immunoproteasome-expressing pAPCs, had not provided sufficient amounts of preprocessed epitope precursors to the immunoproteasome-deficient H2-K^b-expressing pAPCs to prime efficient CD8 T-cell responses in the MHC-mismatched BM-chimeric mice. This indicates that this potential way of antigen-delivery does not play an important physiological role during the immune responses to these two pathogens.

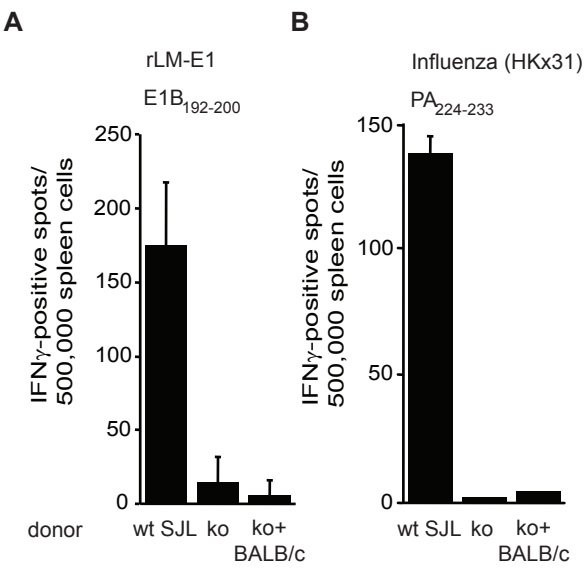

Figure 4. The role of cross-dressing in providing epitopes to pAPC for presentation to CD8 T cells. BM-chimeric mice were generated by reconstituting lethally irradiated iβ5/LMP7+iβ2/MECL-1-deficient mice with BM derived from wt, iβ5/LMP7+iβ2/MECL-1-deficient or a mixture of iβ5/LMP7+iβ2/MECL-1-deficient and BALB/c mice. Six weeks after reconstitution mice were infected *i.v.* with 5 x 10^3 rLM-E1 (A) or *i.n.* with 5 x 10^4 U Influenza HKx31. Spleens were analyzed on day 8 after infection for CD8 T-cell responses to $E1B_{192\text{-}200}$ (A) and $PA_{224\text{-}233}$ (B) by IFNγ ELISPOT. Background responses measured in the absence of stimulating peptides were subtracted. Shown are means +/- SEM.

Discussion

It has generally been assumed that CD8 T-cell responses to intracellular pathogens are primed by DC that either have been infected or cross-present antigens acquired from infected, “antigen donor” cells. Using a novel model in which the antigenic peptides used as read out can only be processed by a subset of BM-derived cells only, we show that during Listeria infection BM-derived cells other than DC can prime CD8 T-cell responses to a secreted antigen.

Our model is based on our previous observations that mice lacking the proteasome immunosubunits β5i/LMP7 and β21/MECL-1 fail to mount CD8 T-cell responses to the rLM-E1-derived $E1B_{192\text{-}200}$ eptope, following infection, due to poor generation of this epitope in the absence of immunoproteasomes (21, 25, 26). Thus, by constructing different BM-chimeric mice in which either the periphery or (specific cells in the) lymphoid compartment expressed the proteasome immunosubunits, we were able to determine the role of different cell types in antigen processing and CD8 T-cell priming during rLM-infection. Former studies into the role of different cell types in priming of Listeria-specific cell responses have established that CD8 T-cell activation requires the interaction with antigen-presenting BM-derived APC (27, 28). Jung et al. (4), using DTR tg mice in which DC are ablated upon injection of diphtheria toxin, narrowed this further down to a strict requirement for DC. However, later studies demonstrated that CD8α DC, the DC subset presenting Listeria antigens following infection (5), are required also to transport the bacteria into the spleen (29), the site of CD8 T-cell priming during Listeria infection (30, 31). In the present studies we avoided conditions where DC were entirely absent, by including antigen processing-defective, immunosubunit-deficient BM in all chimeric mice.

Following systemic infection, Listeria mainly infects the spleen and liver, where the bacteria enter phagocytic cells including DC, macrophages, neutrophils and hepatocytes. Because our model tests which cell types *process* the pAPC-presented peptides that prime Listeria-specific CD8 T cells, in contrast to previous models that tested which cells *presented* the antigens, we first determined the respective roles of BM-derived and non BM-derived cells in activation of rLM-derived $E1B_{192\text{-}200}$-specific CD8 T cells following infection. Thus, assuming that BM-derived pAPC prime CD8 T-cell responses, the antigens presented on the priming pAPC could derive from infected cells of the peripheral tissue, most likely infected hepatocytes in case of rLM infection. However, in agreement with previous studies (27, 28), our data demonstrated that induction of E1B-specific CD8 T-cell responses required immunosubunit expression in BM-derived cells but not in the peripheral tissue (Figure 1). Thus, the periphery, despite being a significant harbour of pathogen, does not serve as antigen

donor for CD8 T cell-priming pAPC. To re-evaluate the role of DC in T-cell activation, we then constructed mixed BM-chimeric mice using LMP7+MECL-1-deficient recipients that were transplanted with a mixture of LMP7+MECL-1-deficient and CD11c DTR tg BM. Thus, these BM-chimeric mice express the immunosubunits in all BM-derived cells with the exception of DC. Strikingly, we found that these mixed BM-chimeric mice still mounted E1B-specific CD8 T-cell responses following infection (Figure 3), indicating that DC are not essential at least for the processing of CD8 T-cell priming E1B epitopes during rLM infection. In the same experiment, in the absence of immunoproteasome-positive DC, priming of CD8 T-cell responses to an immunoproteasome-dependent Influenza-derived CD8 T-cell epitope ($PA_{224-233}$) following Influenza infection was fully abrogated. The latter observation is in agreement with previous studies showing that DC are essential for priming of Influenza-specific CD8 T-cell responses (32) and further confirm the complete ablation of immunoproteasome-positive DC in the mixed BM-chimeras, as shown also by FACS analysis (data not shown).

The finding that immunosubunits and thus epitope processing in DC is not required for induction of rLM-specific CD8 T cells leaves the possibility that DC that acquire preprocessed antigen may function as CD8 T-cell responses priming pAPC. At the same time, such a scenario seems rather unlikely, since preprocessed fragments are highly likely to be rapidly degraded, while cross-presentation is assumed to require stable antigens that are processed by the receiving pAPC (9). On the other hand, it has also been suggested that unstable bacterial products could be a prominent source of antigen, which then as well would require further processing by proteasomes of the receiving cross-presenting DC (18). Indeed, immunosubunit-deficient recipient mice that are reconstituted with a mix of immunosubunit-deficient BM and immunosubunit-positive but MHC mismatched BM fail to respond to rLM-E1-derived $E1B_{192-200}$, arguing against the possibility that immunosubunit-deficient DC acquire preprocessed antigen in this model.

Dolan et al. (33) reported that CD8 T cells can be primed by a third mechanism termed cross-dressing. Cross-dressing involves the transfer of peptide-MHC class I complexes derived from dead donor cells to DCs, which subsequently present the complexes to CD8 T cells. Recently, it was however shown that cross-dressed DCs can only activate memory CD8 T cells but not naïve T cells, at least after viral infection (34). In our model we did not investigate memory responses, but cannot formally exclude a role for cross-dressing in priming of naïve T cells in our model systems.

Taken together, our findings indicate that pAPC, other than DC, can process those antigens that prime CD8 T cells during Listeria infection. At the same time, our data should not be interpreted as excluding a role for DC in priming of CD8 T-cell responses during Listeria infection, but as evidence of an alternative pathway that operates

at the same time.

To our knowledge, this is the first clear demonstration of a non-DC mediated route of CD8 T-cell priming during infection. Nevertheless, more studies have shown a direct role, mainly for macrophages, in CD8 T-cell priming. For example, Pozzi et al. (35) have demonstrated that both peptide-loaded DC and peptide-loaded macrophages directly activate antigen-specific CD8 T cells upon transfer into mice. In addition, Neuenhahn et al. showed that injection of Listeria-infected macrophages can trigger anti-Listeria CD8 T-cell responses in mice, although in these studies the authors did not examine whether the injected macrophages functioned as priming pAPC or as antigen donors for other pAPC (29). Although our studies do not show which non-dendritic cell type primes Listeria-specific CD8 T cells in our model, a role for macrophages is highly likely. First, these cells are highly infected during rLM infection, and efficiently present the secreted bacterial antigens, which are the main target of Listeria-specific CD8 T-cell responses. Alternatively, proteasome-dependent cross-presentation was in fact first shown for macrophages, thus, also uninfected macrophages that cross-present LM antigens may prime the responding CD8 T cells.

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CHAPTER 7

Summarizing discussion

Summarizing discussion

CD8 T cells are key players in the elimination of intracellular pathogens. Activation of naïve CD8 T cells requires the degradation of pathogen-derived antigenic peptides and their presentation by MHC class I molecules on the cell surface of professional antigen presenting cells (pAPCs). Although pathogens contain many potential antigenic peptides that could elicit a CD8 T-cell response, protective CD8 T-cell responses are only directed towards a very small amount of (sub)dominant peptides. The ubiquitin proteasome system is involved in the generation of most of these peptides. Proteolysis of antigens by the IFNγ-inducible immunoproteasome with or without proteasome regulator PA28 has been shown to accelerate the generation of many peptides, resulting in effective responses to these determinants. Different mechanisms probably contribute to the selection of immunoproteasome generated peptides as dominant epitopes for a CD8 T-cell response, such as changed cleavage site preferences that enhance generation and presentation of epitopes, enhanced degradation of oxidized nascent proteins that contribute to rapid presentation and elimination of other potentially dominant determinants through immunosubunit-mediated destuction. In this thesis, both the role and the effects of immunoproteasomes and PA28 in different immune effector functions were studied.
The relevance of these studies and the impact of immunoproteasomes on different cellular processes will be discussed here.

Effects of IFNγ-inducible components of the proteasome system on peptide generation

IFNγ is an important modulator of the antigen processing pathway as it not only induces formation of immunoproteasomes but also stimulates the induction of proteasome activator PA28. These components of the proteasome system have more characteristics in common. They both were shown to highly accelerate the generation of certain antigenic peptides (in infected cells) (1) and more or less similar to the immunosubunits, PA28 dramatically enhances the usage of the specific cleavage sites. Furthermore, turnover of synthetic polypeptide substrates containing MHC class I binding peptides was accelerated in the presence of PA28, similar to immunoproteasomes (1, 2). In contrast, immunoproteasomes have been shown to enhance the generation of a significant number of antigenic peptides, whereas PA28 seems to affect the generation of only a small number of epitopes (3). The effect of PA28 was also shown to be allele-specific, supporting preferably the generation of epitopes that can be presented by K^b or L^d molecules (4). The inducible components of the proteasome system, PA28 and the immunosubunits, are both expressed in

pAPCs and in cells exposed to inflammatory cytokines. However, the effects of these components on antigen processing for presentation in MHC class I molecules and CD8 T-cell responses were only studied separately. In chapter 2, we used different gene-deficient mice (PA28$^{-/-}$, β5i/LMP7+β2i/MECL-1$^{-/-}$ and PA28+β5i/LMP7+β2i/MECL-1$^{-/-}$) to investigate the effects of the inducible components of the proteasome system on immune responses. Previous studies already reported a diminished expression of MHC class I molecules in mice lacking immunosubunits, which is mainly due to the absence of β5i/LMP7 (5, 10). We demonstrated that mice deficient for PA28 displayed lower MHC class I expression than wt mice and found a similar reduction in MHC class I expression in PA28+β5i/LMP7+ β2i/MECL-1-deficient mice compared to mice lacking only β5i/LMP7+β2i/MECL-1 (chapter 2, Figure 4 and Table 1). This indicates that both PA28 and the immunosubunits play an important role in the production of MHC class I ligands, which means that the role of PA28 in generation of peptides is much greater than so far appreciated. Our finding that deficiency of PA28 results in decreased levels of MHC class I molecules both in the presence or absence of immunoproteasomes, indicates that the effects of both components of the proteasome system are additive and that PA28 and the immunoproteasome enhance the generation of MHC class I ligands through different mechanisms.

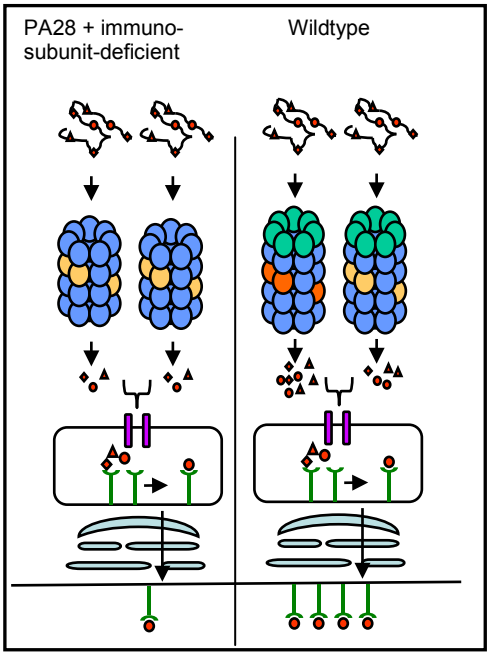

Figure 1. Summarizing scheme of chapter 2. PA28 and the immunosubunits use different mechanisms to enhance the supply of peptides for MHC cass I-binding. Mice that lack both proteasome components therefore have strongly reduced MHC class I levels.

Immune responses in gene-deficient mice

Immunoproteasomes can be essential in the processing of specific epitopes, leading to strongly decreased or impaired CD8 T-cell responses to these epitopes in the absence of immunoproteasome subunits (6, 7) (chapter 2, 3; E1B). The CD8 T-cell responses to the immunoproteasome-dependent recombinant *Listeria monocytogenes*- and Influenza-derived epitopes $E1B_{192-200}$ and $PA_{224-233}$ were dramatically reduced in β5i/LMP7+β2i/MECL-1-deficient mice (chapter 2). In contrast, in PA28-deficient mice we could still detect efficient CD8 T-cell responses to the four rLM- and Influenza-derived CD8 epitopes that were tested in chapter 2, which is consistent with previous findings (2). This indicates that the contribution of PA28 to peptide processing and presentation of these epitopes does not considerably affect CD8 T-cell responses. Furthermore, we did not find significant differences in responses to the ovalbumin-derived epitope $ova_{257-264}$ in PA28-deficient mice compared to wt mice, although previous studies have shown that PA28 enhances proteasome-mediated processing of the $ova_{257-264}$ epitope (8, 9). So absence of PA28 may lead to reduced presentation of the ova epitope, the effects on the CD8 T-cell response to this and other epitopes are rather mild.

Gene-deficient mice are more susceptible to pathogens

Although CD8 T-cell responses that were measured in the gene-deficient mice were quite comparable, unless the epitope was immunoproteasome-dependent, we noticed enhanced susceptibility to both Listeria and Influenza in some experiments. After infection with Lm-OVA both PA28 and PA28+immunosubunit-deficient mice showed more signs of illness than wt or immunosubunit-deficient mice. Bacterial counts in the spleens of Lm-OVA infected β5i/LMP7+β2i/MECL-$1^{-/-}$ mice were 10-fold higher than in PA28+β5i/LMP7+β2i/MECL-1-deficient mice at day 3 after infection. When the experiment was repeated with Lm-wt including wt mice we measured bacterial counts in both the livers and spleens. In both organs the bacterial counts were higher in PA28+β5i/LMP7+β2i/MECL-1-deficient mice than in immunoproteasome-deficient mice. The latter group, however, seemed to be more resistant than wt mice. Thus, a lack of PA28 enhanced, whereas a lack of immunosubunits decreased the susceptibility of mice to Listeria infection, indicating that these two components of the proteasome system play different roles in the immunopathogenesis of Listeria infection.

Furthermore, we measured higher viral loads in the lungs of Influenza infected immunosubunit- and PA28+β5i/LMP7+ β2i/MECL-1-deficient mice than in wt or PA28-deficient mice. Consistently, we detected higher viral loads in mice lacking PA28 and the immunosubunits compared to wt mice at day 4 and 7 after Influenza infection. Depletion of either or both NK cells and CD8 T cells did not increase the viral

load in the lungs 4 days after Influenza infection. However, at day 7 after infection PA28+β5i/LMP7+β2i/MECL-1-deficient mice depleted of CD8 T cells showed a higher viral load compared to non-depleted PA28+β5i/LMP7+β2i/MECL-1-deficient mice or CD8 T-cell depleted wt mice, although the results were not significant.
Together, it seems that mice deficient for PA28 or both PA28 and the immunosubunits have more problems in controlling infections, and thus are more susceptible to certain pathogens. To find out why mice deficient for PA28 and the immunosubunits have higher bacterial counts in the liver and spleen and higher viral loads in the lungs after Influenza infection respectively, more extended studies should be performed.

The role of MECL-1 in T-cell repertoire selection and homeostatic expansion
Mice deficient for the immunoproteasome subunit MECL-1 show an increased CD4/CD8 T-cell ratio (10, 11) (chapter 3). Because the immunoproteasomes are expressed in the thymus (12, 13) these findings were explained by effects on positive and negative selection in the thymus. In chapter 3, we generated mixed bone marrow (BM)-chimeric mice to follow the relative expansion of CD4 and CD8 T cells derived from wildtype (wt) and LMP7+MECL-1-deficient mice in one recipient. These studies showed that the CD4/CD8 T-cell ratios in immunoproteasome-deficient T cells remained enhanced in BM-chimeric LMP7+MECL-1-deficient mice, reconstituted with both wt and LMP7+MECL-1$^{-/-}$ BM, excluding environmental factors or thymic selection as a cause for the altered CD4/CD8 T-cell balance in MECL-1$^{-/-}$ mice. Therefore, we concluded that MECL-1 influences the regulatory processes that maintain the balance between CD4 and CD8 T-cell subsets through a T-cell intrinsic mechanism. Thus, while MECL-1 was shown to be essential for the processing and presentation of specific MHC class I-presented peptides effecting both the induction of CD8 T-cell responses (chapter 3, Figure 2D) and CD8 T-cell repertoire selection (11), the effects of MECL-1 on CD4 or CD8 T-cell expansion are caused by a so far unknown T-cell intrinsic mechanism.

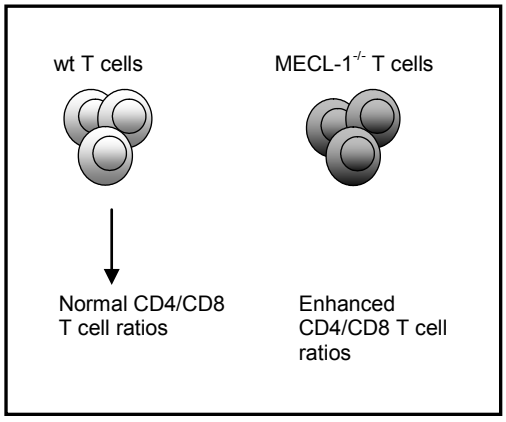

Figure 2. Summarizing scheme of chapter 3. MECL-1-deficiency leads to enhanced CD4/CD8 T-cell ratios through an unknown, T-cell intrinsic mechanism.

Does reduced MHC class I expression influence NK cell function?

Activation of NK cells is regulated by expression of different inhibitory and activating receptors at the NK cell surface (14, 15). MHC class I molecules are ligands for the inhibitory receptors of NK cells, so the presence of MHC class I during education is important for NK cell function. Mice deficient for immunoproteasome subunits express reduced levels of MHC class I (chapter 2) (9), which could influence the education of NK cells. Therefore, in chapter 4, we examined whether immunoproteasome-deficient mice have educated NK cells in the periphery. The maturation states of NK cells in RAG1$^{-/-}$ and RAG1+LMP7+MECL-1$^{-/-}$ mice were highly comparable. Furthermore, the effector function of NK cells of RAG1$^{-/-}$ that also lacked the immunosubunits, measured by production of LAMP-1 and IFNγ after antibody stimulation, was similar to NK cells from RAG1$^{-/-}$ mice in untreated and poly(I:C) stimulated mice. So immunoproteasome-deficient mice have functional NK cells in the periphery.

Immunoproteasome-deficient CD8 T cells that were transferred into wt mice could hardly be recovered when recipients were virally infected (2, 16, 17). Moebius et al. suggested that immunoproteasomes are important for the survival of T cells in infected mice and claimed that the rejection of T cells was NK cell-independent, because immunoproteasome-deficient T cells were recovered from uninfected recipients. Also our data showed that splenocytes from immunoproteasome-deficient mice that were transferred into wt recipients were tolerized in uninfected recipients, however, they were rejected when these wt mice were infected with Influenza. Using depleting antibodies, rejection was shown to be NK-cell dependent. So, in contrast to Moebius et al., we found NK cell-dependent rejection of β5i/LMP7+β2i/MECL-1-deficient cells in Influenza infected recipients. Thus, probably several different mechanisms could be involved in rejection of transferred immunoproteasome-deficient cells.

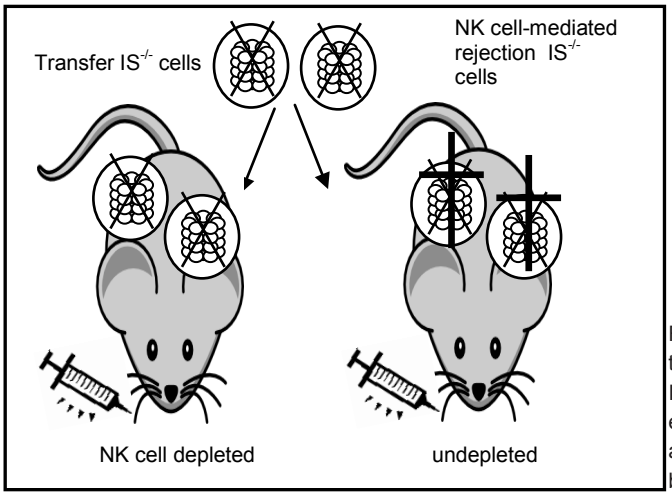

Figure 3. Summarizing representation of chapter 4. Transferred IS$^{-/-}$ cells are tolerized in wt recipients, but upon infection IS$^{-/-}$ cells are rejected, which is mediated by NK cells.

Antigen processing pathways *in vivo*

Naïve T cells require presentation of antigenic peptides by pAPCs that provide co-stimulation, in order to be primed while pathogen-specific effector CD8 T cells are activated by any infected cell. The main pathways used for activation of CD8 T cells are direct presentation by infected pAPCs and cross-presentation. It is still unclear which of these pathways contributes the most to priming of the specific CD8 T-cell response during an infection. In previous studies it has been established that priming of Listeria-specific CD8 T-cell responses requires interaction of CD8 T cells with BM-derived APCs that present the antigens (18, 19). Jung et al. demonstrated that mice from which $CD11c^+$ DC were depleted failed to mount CD8 T-cell responses following Listeria infection (20). However, later studies showed that DCs are essential for entry of Listeria into the spleen (21, 22). Thus a failure to infect the spleen may have contributed to the observed lack of CD8 T-cell priming in the spleen of DC-depleted infected mice. Several studies have suggested a role for macrophages in priming of CD8 T-cell responses (21, 23). In chapter 6, using recombinant Listeria monocytogenes containing an immunoproteasome-dependent epitope as an infection model, we studied which cell types process and deliver the peptides presented on pAPCs that prime CD8 T-cell responses. Our previous data showed that mice deficient for the proteasome immunosubunits β5i/LMP7 and β2i/MECL-1 poorly generate the rLM $E1B_{192\text{-}200}$ epitope after infection and therefore fail to elicit $E1B_{192\text{-}200}$-specific CD8 T-cell responses (24-26). By using BM-chimeric mice we found that induction of E1B-specific CD8 T-cell responses requires expression of immunoproteasomes in BM-derived cells (chapter 6, Figure 1), in concordance with other studies (18, 19), excluding a role for peripheral cells as antigen donors. Furthermore, we found that DCs are not essential for processing of the E1B epitope to prime CD8 T cells after rLM infection. For this we generated mixed BM-chimeric mice using β5i/LMP7+β2i/MECL-1-deficient recipient mice that were reconstituted with a mixture of β5i/LMP7+β2i/MECL-1-deficient and CD11c DTR tg BM. After injection of diphtheria toxin, only BM-derived cells other than DC expressed immunoproteasomes required to process $E1B_{192\text{-}200}$. Upon infection with rLM-E1 we could detect $E1B_{192\text{-}200}$-specific CD8 T-cell responses in the mixed BM-chimeric mice, indicating that other cells than DCs could process the epitope. As it was shown that DC are essential for priming of Influenza-specific CD8 T-cell responses (27), we used Influenza infection as a control in these experiments. Mixed BM-chimeric mice did not mount CD8 T-cell responses to the immunoproteasome-dependent $PA_{224\text{-}233}$ epitope, indicating that the DC population was indeed entirely depleted after DT-injection.

DCs could still be the priming pAPC during Listeria infection, when they acquired pre-processed antigen from other (immunoproteasome expressing) cells. To address the role of antigen transfer, we generated mixed BM-chimeric mice using β5i/LMP7+β2i/

MECL-1-deficient recipients that were reconstituted with a mixture of immunosubunit-deficient and immunosubunit-expressing MHC mismatched BM. These latter cells could process but do not present the $E1B_{192-200}$ epitope. However, we failed to detect $E1B_{192-200}$-specific CD8 T-cell responses upon rLM infection, indicating that handover of pre-processed fragments to immunoproteasome-deficient DCs is rather unlikely. These results are in line with data obtained by Norbury et al., who found that cross-priming requires stable proteins that are processed by the receiving pAPC, and by Janda et al., where unstable bacterial products derived from infected antigen donor cells were found to be processed by cross-presenting DC (28, 29).
So in this chapter, we have shown that pAPCs other than DCs are likely to be able to prime CD8 T-cell responses after Listeria infection. This does not exclude a role for DCs in CD8 T-cell priming, but at least indicates that there is an alternative pathway that can prime as well. The cells that are likely candidates to serve as the other "non-DC pAPC" are macrophages, which are mainly infected during Listeria infection and they present the Listeria-derived antigens that are the targets of Listeria-specific CD8 T-cell responses. In our studies we only focussed on Listeria infection and used Influenza as a control system and found that in the latter infection DCs were indispensable for CD8 T-cell priming. Which cells are involved in priming of CD8 T cells after infection with other pathogens remains to be elucidated. Probably, the site of infection, the tropism of the infecting agens, and the nature of available antigens play an important role for the outcome of effective CD8 T-cell responses.

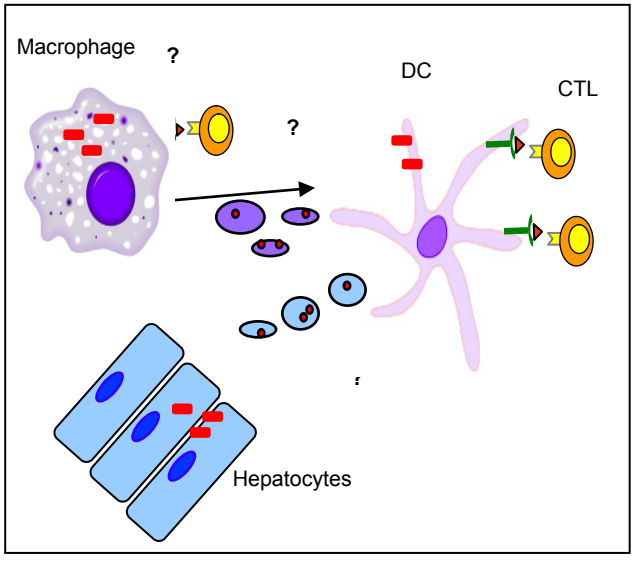

Figure 4. Schematic representation of chapter 6.
Antigen processing to prime CD8 T cells can be performed by DCs, but DCs are not always required. Macrophages might be involved, but hand-over of preprocessed fragments seems unlikely to be involved in CD8 T-cell priming.

Future perspectives for unraveling the function of immunoproteasomes

In addition to its role in antigen processing and generation of peptides for binding to MHC class I molecules, it has been suggested that the immunoproteasome subunits have important other functions, such as shaping the T-cell repertoire (2, 16, 30), T-cell expansion or survival (1, 2, 16, 17), maintenance of protein homeostasis under stress conditions (31) and influencing cytokine production (32, 33) (see chapter 1). Furthermore, a role for immunoproteasomes in pathogenesis of autoimmune diseases has been suggested. Specific inhibition of LMP7 with PR-957 blocked cytokine production and, thereby, reduced disease progression in mouse models of rheumatoid arthritis (32) as well as symptoms of dextran sulfate sodium (DSS)-induced colitis (34). In addition, DSS-induced colitis was strongly attenuated in mice deficient for either LMP2, LMP7 or MECL-1 (34, 35), perhaps due to altered function of innate immune cells in these mice. On the other hand, a very recent study by Zaiss et al. showed that proteasome immunosubunits expressed in inflamed peripheral tissues prevents development of autoimmune diseases mediated by CD8 T cells, suggesting that immunoproteasomes protect against autoimmune diseases (36).

Although extensive studies have increased our knowledge on antigen processing and proteasomes, still some questions remain about the role of subunits of (immuno)proteasomes in immune responses. What are the mechanisms underlying the possible functions of immunoproteasomes mentioned above? Which pathways are involved in e.g. cytokine regulation by immunoproteasomes? Do the individual proteasome subunits exert different functions? And why are the immunosubunits only constitutively expressed in lymphoid cells and inducible in peripheral tissues? Further studies with knockout mice and specific subunit inhibitors will enhance our knowledge of how cellular processes are regulated by immunoproteasomes. Immunosubunit-specific inhibition may further become useful tools in specifically targeting immunosubunits for therapeutic interventions.

In conlusion

The proteasome system plays an important role in regulation of immune responses through different mechanisms including MHC class I antigen processing. Incorporation of immunosubunits enhances the proteasome-mediated production of many antigenic peptides, which affects the specificity of CD8 T-cell responses. The findings presented in this thesis provide more insight in the role of immunosubunits and PA28 in antigen processing and immune recognition as well as in antigen processing pathways that are relevant for priming of CD8 T cells. This enhances our knowledge on potential targets for the development of CD8 T-cell activating vaccins.

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NEDERLANDSE SAMENVATTING

Het immuunsysteem

Dagelijks worden we blootgesteld aan ziekteverwekkers (zoals virussen, bacteriën en parasieten) die onze gezondheid in gevaar kunnen brengen. Bescherming tegen deze ongewenste indringers wordt geboden door het immuunsysteem, een ingewikkeld systeem dat bestaat uit verschillende soorten cellen die intensief samenwerken om ziekte te voorkomen. Voor elke immuunrespons (afweerreactie) is het belangrijk dat een pathogeen (de ziekteverwekker) of andere lichaamsvreemde stof herkend wordt en er daadwerkelijk een reactie optreedt om deze te verwijderen. Grofweg zouden immuunresponsen opgedeeld kunnen worden in twee categorieën: de aangeboren respons en de aangeleerde (aangepaste) specifieke respons. Hierbij zorgt de eerstgenoemde voor een snelle eerste reactie tegen ongewenste indringers die niet specifiek is. De specifieke respons daarentegen wordt gekenmerkt door specificiteit tegen bepaalde pathogenen en het vermogen om geheugen op te bouwen, waardoor een herhaalde infectie met hetzelfde pathogeen zal leiden tot een snelle, specifieke afweerreactie.

Herkenning van geïnfecteerde cellen

De witte bloedcellen (leukocyten) vormen een belangrijk onderdeel van het immuunsysteem en zij bevinden zich dan ook in het bloed en de lymfeklieren, locaties vanwaar zij zich gemakkelijk kunnen verplaatsen naar de weefsels en organen wanneer hun hulp daar hard nodig is. De B- en T cellen zijn leukocyten die belangrijk zijn voor de specifieke immuunrespons. Deze cellen herkennen bepaalde structuren (antigenen) door middel van een receptor en kunnen helpen bij het opruimen van pathogenen door antilichamen te maken (B cellen) of door geïnfecteerde cellen te vernietigen (T cellen). Om geïnfecteerde cellen daadwerkelijk te kunnen opruimen, moet het immuunsysteem de zieke cellen eerst herkennen. De cytotoxische T cel (CTL) is een type T cel dat hiervoor, net als de andere typen T cellen, een receptor heeft (de T cel receptor oftewel TCR) waarmee stukjes eiwit (peptiden) afkomstig van een pathogeen herkend kunnen worden. Deze peptiden worden aan de buitenkant van een cel gepresenteerd in zogenaamde major histocompatibility complex (MHC) moleculen. Een CTL heeft allemaal dezelfde receptoren die specifiek zijn om een bepaald peptide goed te herkennen. Wanneer die herkenning plaatsvindt, bindt de CTL aan de geïnfecteerde cel om hem te doden door middel van het uitscheiden van cytotoxische stoffen (stoffen die giftig zijn voor de cel).

Afbraak van eiwitten leidt tot peptiden

De productie van peptiden voor presentatie aan T cellen vindt plaats in de cel. In elke cel zijn vele (lichaamseigen) eiwitten aanwezig die belangrijk zijn voor de functie van een cel. Aminozuren zijn de bouwstenen van ieder eiwit en elk eiwit is dan ook

opgebouwd uit een unieke combinatie van de verschillende aminozuren die er zijn. Wanneer een eiwit overbodig is of beschadigd, zal het worden afgebroken tot kleine stukjes, de peptiden. Proteinases en peptidases zijn de enzymen die de eiwitten in stukjes “knippen”. Het proteasoom is het belangrijkste proteinase dat peptiden produceert. De peptiden worden meestal verder afgebroken tot losse aminozuren die weer worden gebruikt voor de opbouw van nieuwe eiwitten. Echter, een deel van de peptiden ontsnapt aan deze afbraak en komt terecht in het endoplasmatisch reticulum (ER), een onderdeel van de cel dat o.a. materialen binnen de cel transporteert. In het ER bevinden zich MHC moleculen die peptiden van een bepaalde lengte (8-11 aminozuren) goed kunnen binden en zodoende een complex kunnen vormen dat naar de oppervlakte van de cel kan worden getransporteerd voor presentatie aan de CTL. Een cel breekt niet alleen lichaamsvreemde eiwitten (bijv. van een virus of bacterie) af, maar ook lichaamseigen eiwitten. Hierdoor is er een kans dat een CTL bindt aan een peptide afkomstig van een lichaamseigen eiwit en zodoende een immuunrespons opwekt tegen een gezonde cel. Om deze vorm van auto-immuniteit te voorkomen, worden de T cellen tijdens hun ontwikkeling in de thymus getest. Wanneer een TCR goed bindt aan een complex van een lichaamseigen peptide in een MHC molecuul, zal deze T cel vernietigd worden (negatieve selectie). Zodoende blijven alleen de CTL over die geïnfecteerde cellen herkennen en zullen gezonde cellen niet aangevallen worden.

Het proteasoom is de grootste peptideproducent

Zoals hierboven al genoemd, is het proteasoom belangrijk voor de afbraak van eiwitten in de cel. Het proteasoom is een groot complex dat gevormd wordt door een kern (20S genaamd), bestaande uit vier op elkaar gestapelde ringen, die elk weer bestaan uit zeven verschillende onderdelen (de subunits). De buitenste ringen bestaan uit α-subunits en de binnenste ringen zijn opgebouwd uit β-subunits. De ruimte aan de binnenkant van de 20S kern is toegankelijk voor eiwitten die afgebroken moeten worden. Drie van de zeven β-subunits (β1, β2 en β5) zijn proteolytisch actief, wat betekent dat ze de binnengekomen eiwitten tot peptiden kunnen knippen. Het proteasoom is veelvuldig aanwezig in cellen en om goed functionerende eiwitten te beschermen tegen ongewenste afbraak, is de 20S kern van het proteasoom normaal gesproken afgesloten. Pas na het binden van een activator (19S, PA28) aan de 20S kern, wordt de tunnel geopend voor eiwitafbraak.

Inmiddels zijn er drie verschillende typen proteasoom bekend: het standaard of constitutieve proteasoom, het immunoproteasoom en het thymusproteasoom. Zij hebben verschillende proteolytisch actieve β-subunits. Zo horen β1, β2 en β5 bij het standaard proteasoom, maar zijn deze subunits in het immunoproteasoom vervangen door respectievelijk LMP2, MECL-1 en LMP7. Het thymusproteasoom lijkt

op het immunoproteasoom, maar heeft in plaats van LMP7 de subunit β5thymus (β5t) ingebouwd. Het standaard proteasoom is aanwezig in alle cellen, maar het immunoproteasoom komt vooral voor in cellen van het immuunsysteem (antigeen presenterende cellen (APC), natural killer (NK) cellen, B- en T cellen) of in cellen die blootgesteld zijn aan een signaalstof (cytokine) zoals interferon γ (IFNγ). Deze stof dient als communicatiemiddel tussen cellen en wordt door cellen geproduceerd wanneer er een infectie plaatsvindt, waardoor cellen aangespoord worden om immunoproteasomen aan te maken. De subunits van het proteasoom hebben allen een specifieke voorkeur voor het knippen van bepaalde aminozuurvolgorden in eiwitten. Hierdoor kunnen de verschillende proteasomen een divers repertoire aan peptiden produceren.

In dit proefschrift

Naast de subunits van het immunoproteasoom (LMP2, LMP7 en MECL-1) is ook proteasoom activator PA28 te induceren d.m.v. IFNγ. Samen spelen deze componenten van het proteasoomsysteem zowel een belangrijke rol bij de bewerking van eiwitten tot peptiden die binden aan de MHC klasse I moleculen als bij andere aspecten van immuunreacties.

In **hoofdstuk 2** werd gekeken naar de effecten van PA28 en de immunosubunits op de productie van MHC klasse I peptiden en de CTL responsen. Door afwezigheid van twee subunits van het immunoproteasoom (LMP7 en MECL-1), gaat de peptideproductie omlaag, wat resulteert in een dalende expressie van MHC klasse I moleculen aan het celoppervlak. Ook proteasoomactivator PA28 draagt bij aan de productie van MHC klasse I bindende peptiden. In deze studie werd gebruik gemaakt van muismodellen waarin PA28, de immunosubunits of beide afwezig waren. Uit de resultaten bleek dat de effecten van de immunosubunits en PA28 additief zijn, waardoor we kunnen concluderen dat beide componenten verschillende mechanismen gebruiken om de peptideproductie te verhogen. Verder werd gevonden dat het effect van PA28 op de productie van zogenaamde immunodominante epitopen (peptiden die herkend worden door een CTL) weinig invloed heeft op de CTL respons, terwijl de immunosubunits wel belangrijk zijn voor het genereren van enkele onderzochte epitopen om een CTL respons te induceren.

In **hoofdstuk 3** werd onderzocht wat de invloed van immunoproteasomen is op de regulatie van T cellen. Al in voorgaand onderzoek werd gevonden dat de ratio tussen CD4 en CD8 T cellen verhoogd is in muizen die de immunosubunit MECL-1 missen. Om te achterhalen waardoor deze verhoogde CD4/CD8 ratio veroorzaakt wordt, werden beenmerg chimere muizen gemaakt. De beenmergcellen van immunosubunitdeficiënte muizen werden vernietigd door bestraling, waarna een mix

van beenmerg afkomstig van wildtype en immunosubunitdeficiënte muizen werd teruggeplaatst (reconstitutie). Zodoende ontwikkelden T cellen afkomstig van beide soorten beenmerg zich in de beenmerg chimeren in dezelfde omgeving. Na analyse van de T cel populaties werden nog steeds verhoogde CD4/CD8 ratio's gevonden in de cellen afkomstig van de immunosubunitdeficiënte muizen. Dit geeft aan dat de verhoogde ratio blijft bestaan wanneer de ontwikkeling, maturatie en differentiatie van de T cellen plaatsvindt onder dezelfde omstandigheden en in dezelfde thymus. Deze resultaten suggereren dat MECL-1 direct via een T cel intrinsiek mechanisme invloed uitoefent op de CD4/CD8 ratio en dat dit niet het gevolg is van invloed van andere cellen of omgevingsfactoren.

De verlaagde expressie van MHC klasse I moleculen op immunosubunitdeficiënte cellen zou invloed kunnen hebben op de educatie van NK cellen, aangezien educatie van NK cellen plaatsvindt door interactie van MHC klasse I moleculen die binden aan de inhiberende (remmende) receptoren op NK cellen. Muizen die geen of zeer weinig MHC klasse I moleculen hebben zijn hyporesponsief. Daarom werd in **hoofdstuk 4** bestudeerd of de NK cellen van muizen met een verlaagde MHC klasse I expressie functioneel zijn. Zowel het fenotype (uiterlijk) als de functionaliteit van NK cellen afkomstig van wildtype en immunosubunitdeficiënte muizen werden met elkaar vergeleken. De expressie van markers die de maturatiestatus van NK cellen bepalen (CD27 en CD11b) waren gelijk in beide muizengroepen. Voor het meten van de functionaliteit van NK cellen werd de expressie van CD69, de productie van IFNγ en degranulatie d.m.v. LAMP-1 gemeten. In beide groepen werden geen grote verschillen gevonden, wat aantoont dat NK cellen van immunosubunitdeficiënte muizen functioneel zijn. Omdat wildtype NK cellen in staat zijn om MHC klasse I-deficiënte cellen af te stoten, werd in dit hoofdstuk onderzocht of immunosubunitdeficiënte cellen getolereerd worden in een wildtype omgeving. Hiervoor werden miltcellen van wildtype en immunosubunitdeficiënte muizen gemengd en een deel werd getransfereerd naar een muis met NK cellen en het andere deel werd getransfereerd naar een muis waarvan de NK cellen gedepleteerd waren. In beide gevallen werden de immunosubunitdeficiënte cellen teruggevonden, wat betekent dat deze cellen getolereerd werden. Echter, deze tolerantie werd verbroken wanneer de ontvangende muizen geïnfecteerd werden met influenza. Het percentage immunosubunitdeficiënte cellen dat werd teruggevonden na infectie was drastisch verlaagd. Doordat dit niet het geval was wanneer de ontvangende muizen geen NK cellen meer hadden door depletie, kan geconcludeerd worden dat de afstoting van de cellen afhankelijk is van NK cellen.
De bestudering van NK cellen kreeg een vervolg in **hoofdstuk 5**, waarbij de kinetiek van NK cellen na een respiratoire virusinfectie gevolgd werd. Hoewel NK cellen

zich kort na infectie met een respiratoir virus naar de longen begeven, werd er geen bewijs gevonden dat er NK cel proliferatie plaatsvindt op de plaats van infectie of in de dichtstbijzijnde lymfeklieren. Daarentegen werd gevonden dat proliferatie van NK cellen na infectie in het beenmerg plaatsvindt. In deze studie werd gebruik gemaakt van een transfer model, waarbij cellen van muizen geïnfecteerd met influenza werden getransfereerd naar een niet-geïnfecteerde ontvanger muis. Met dit model werd aangetoond dat een deel van de lang levende, mature NK cellen uit de periferie na transfer terug gaan naar het beenmerg om daar te prolifereren, zowel na infectie als in afwezigheid van een infectie. Het beenmerg blijkt naast de ontwikkelingsplek voor NK cellen ook een belangrijke plaats te zijn waar proliferatie van lang levende, mature NK cellen plaatsvindt.

Voor een goede CTL respons is presentatie van peptiden nodig door antigeen presenterende cellen (APCs), zoals dendritische cellen (DCs). Door gebruik te maken van eiwitten die epitopen bevatten die alleen geproduceerd worden in aanwezigheid van immunosubunits (een immunosubunit-afhankelijk epitoop), kon in **hoofdstuk 6** onderzocht worden welk celtype verantwoordelijk is voor eiwitbewerking en de aanlevering van bepaalde epitopen. Hiertoe werden beenmerg chimere muizen gegenereerd waarbij de immunosubunits tot expressie kwamen in specifieke celtypen. Door beenmerg chimere muizen te infecteren met Listeria (dat een immunosubunit-afhankelijk epitoop bevat) werd gevonden dat expressie van immunosubunits in cellen afkomstig van het beenmerg nodig is voor CTL responsen tegen het immunosubunit-afhankelijke epitoop. Hoewel DCs belangrijk zijn voor het opwekken van een goede CTL respons, werd er nog steeds een goede CTL respons gevonden tegen het immunosubunit-afhankelijke epitoop in Listeria in muizen waarvan de DCs gedepleteerd waren. In eenzelfde experiment waarbij beenmerg chimere muizen geïnfecteerd werden met het influenza virus, werd na DC depletie geen CTL respons gemeten tegen een immunosubunit-afhankelijk epitoop. Hieruit blijkt dat DCs niet altijd essentieel zijn voor het maken van een epitoop om CTL activatie teweeg te brengen. Ook kan geconcludeerd worden dat andere celtypen dan DCs betrokken zijn bij de productie van epitopen om CTL responsen te induceren.

De studies die in dit proefschrift zijn beschreven geven meer inzicht in de rol van de verschillende componenten van het proteasoomsysteem in antigeen bewerking en immuunresponsen. Ook dragen de resultaten bij aan een beter inzicht in de routes die gebruikt worden voor de antigeenbewerking die leiden tot activatie van een CTL. Deze bevindingen kunnen leiden tot ontwikkeling van doelgerichte, T cel activerende vaccins.

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Dankwoord

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Femke, hoe kun je leven zonder chocola??
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CURRICULUM VITAE
&
PUBLICATIONS

Curriculum Vitae

Natascha de Graaf werd op 21 december 1982 geboren in Delft. In 2001 behaalde zij het VWO diploma aan de christelijke scholengemeenschap Zandvliet te Den Haag. Aansluitend startte zij de studie Biomedische Wetenschappen aan de Universiteit van Leiden. Tijdens haar studie liep zij stage bij de afdeling Nierziekten van het Leids Universitair Medisch Centrum (LUMC), waar zij onder leiding van Dr. Andrea Woltman en Dr. Cees van Kooten onderzoek deed naar remming van DC maturatie door middel van CD40 bindende peptiden. Haar tweede stage werd gevolgd bij de afdeling Humane Genetica van het LUMC. Onder supervisie van Drs. Silvana van Koningsbruggen en Dr. Ir. Silvère van der Maarel werkte zij aan een project over de spierziekte FSHD getiteld: Beïnvloedt fosforylatie de subcellulaire localisatie van FRG1P? Bij de vakgroep Tumorimmunologie van de afdeling Immunohematologie en Bloedtransfusie van het LUMC werd de afstudeerstage gevolgd, waarbij onderzoek werd gedaan naar de identificatie van CTL epitopen die specifiek gegenereerd zijn door het constitutieve proteasoom, onder begeleiding van Drs. Nathalie Fu en Dr. Ferry Ossendorp. In 2008 werd het doctoraal examen behaald. Van november 2006 tot november 2010 was zij werkzaam als assistent in opleiding bij de afdeling Immunologie van de Faculteit Diergeneeskunde aan de Universiteit van Utrecht. Onder leiding van Dr. Alice Sijts leidde haar promotieonderzoek naar de IFNγ-induceerbare componenten van het proteasoom systeem tot dit proefschrift. Vanaf september 2011 is zij werkzaam op de afdeling Nierziekten van het LUMC.

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