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Study of selected molecular and cellular aspects of autoimmunity

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PATRICK BÄTTIG
Dipl. Natw. ETH
Born 15th May 1974
Citizen of Hergiswil b. Willisau, LU

Accepted on the recommendation of
Prof. Dr. S. Werner, examiner
Prof. Dr. H.M. Eppenberger, co-examiner
Dr. M.F. Bachmann, co-examiner

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SUMMARY

The immune system is a powerful tool to fight intruding pathogens. In order to efficiently recognize non-self antigens, the immune system generates a tremendous diversity of specificities. However, the antigen receptors are generated randomly, resulting also in auto-reactive specificity. The immune system therefore developed mechanisms to avoid or suppress this auto-reactivity at various checkpoints. One important checkpoint is the interaction of T cells with dendritic cells, since activation of T cells by immature DCs results in tolerance while mature DCs may trigger T cell immunity. Given the importance of DC maturation for immunity an alphaviral Delphi technology screen was performed [1], seeking for novel maturation factors. Using a one gene/well format assay applied to a DC-T-cell-co-culture, the chemokine CCL19 was identified. Further investigations revealed that CCL19 is indeed a potent DC maturation factor, leading to the upregulation of co-stimulatory molecules and the secretion of pro-inflammatory cytokines. Altogether these findings reveal a novel role for CCL19 in orchestrating immune responses and demonstrate the importance of DCs in the balance of self-tolerance and autoimmunity.

The T cell receptor is one of the key molecules in the adaptive immune system allowing differentiation between self and non-self. To prevent autoimmune diseases due to self-reactivity, the T cells undergo negative selection during maturation in the thymus. This leads to a restricted TCR repertoire, where self-reactive T cells are eliminated, a process described as central tolerance. Using altered peptide ligands derived from the p33 antigen from the lymphocytic choriomeningitis virus fused to virus-like particles as model antigens, the TCR repertoire of wild-type mice was analyzed. Whereas in TCR transgenic mice, only p33- and A4Y-APLs were efficiently recognized, wild-type mice showed no such prevalence. An *in vivo* killer assay revealed almost no cross-reactivity of p33 versus APLs. However, the cross-reactivity was sufficient to lead to dose- and virus-dependent viral protection. Analyzing the APLs in a transgenic mouse model for diabetes in which p33 is expressed by the β -islet cells of the pancreas, p33-VLP immunization induced autoimmunity whereas the APL-VLPs failed. Strikingly after depletion of regulatory T cells, APLs were able to induce diabetes. Thus, cross-reactive T cells fail to mediate autoimmunity unless the regulatory T cells were removed from the system.

Using the same model system of p33 and APLs, the concept called “original antigenic sin” was studied. Original antigenic sin describes the phenomenon that during a viral infection, arising escape mutants evade the immune system by inducing a strong response against the original virus, but no or only a weak response against themselves. In the experiment, immunization with APL-VLPs simulated initial infection with the original virus and later p33-VLP immunization mimicked escape mutant infection. To characterize the effector function of induced T cell responses, vaccinated mice were challenged with a recombinant vaccinia-p33 virus. Interestingly, initial APL-VLP immunization prevented a protective response generated by subsequent p33 vaccination. Hence the results are in accordance with the concept of the original antigenic sin.

Auto-reactive T cells are key players in the development of autoimmunity. Therefore the regulation or even elimination of these T cells would be a possibility to interfere with immune responses, especially self-destructing immune responses. The lectin galectin-1 is known to induce apoptosis in thymocytes and proliferating T cells, making it a candidate for the treatment of autoimmune diseases. But since galectin-1 acts as homodimeric molecule, galectin-1 is monomeric at concentrations below 1 μ M, high concentrations of the protein are required for activity. To circumvent the need for dimerization, two galectin-1 monomers were genetically linked to form a covalent dimer and the apoptotic activity of this new molecule was evaluated. The structurally optimized form of galectin-1 exhibited a ten-fold increased activity, making it to an attractive candidate for therapeutic intervention.

ZUSAMMENFASSUNG

Das Immunsystem ist ein mächtiges Werkzeug im Kampf gegen eindringende Pathogene. Es erzeugt eine riesige Diversität von Spezifitäten, um fremde Antigene effizient zu erkennen. Da die Spezifitäten aber zufällig generiert werden, ergeben sich auch solche mit Selbst-Reaktivität. Um das zu verhindern oder um die Selbst-Reaktivität zu unterdrücken, entwickelte das Immunsystem verschiedene Mechanismen. Ein solcher Mechanismus wirkt bei der Interaktion zwischen T-Zellen und dendritischen Zellen, denn abhängig vom Reifungsgrad der DC führt die Interaktion zu Toleranz (unreife DCs) oder T-Zell Immunität (reife DCs). In Anbetracht der Wichtigkeit der DC-Reifung für die Immunität wurde ein alphaviraler Delphi-Technologie-Screen [1] durchgeführt, um neue Reifungsfaktoren für DCs zu identifizieren. Ein „Ein-Gen/Well-Format-Assay“, angewendet auf DC-T-Zell-Kulturen, führte zur Identifizierung des Chemokins CCL19 als potenter T-Zell-Proliferation-Induzierungsfaktor. In weiteren Untersuchungen konnte gezeigt werden, dass CCL19 wirklich ein Reifungsfaktor für DCs ist, der zur Hochregulierung von aktivierenden Molekülen und zur Absonderung von entzündungsfördernden Zytokinen führt. Diese Befunde zeigen eine neue Rolle von CCL19 bei der Kontrolle von Immunantworten auf und betonen die Wichtigkeit von DCs im Gleichgewicht zwischen Toleranz und Autoimmunität.

Der T-Zell Rezeptor ist eines der Schlüsselmoleküle im Adaptiven Immunsystem. Er ermöglicht das Unterscheiden zwischen Selbst und Fremd. Um Selbst-Reaktivität zu verhindern, die zu Autoimmunität führen kann, unterziehen sich die T-Zellen der negativen Selektion während der Reifung im Thymus. Negative Selektion führt zu einem begrenzten TCR Repertoire, aus dem die selbst-reaktiven T-Zellen eliminiert wurden. Dieser Zustand wird als zentrale Toleranz bezeichnet. Mit veränderten Peptid Liganden (APLs) als Modell-Antigene, die vom p33 Antigen des Lymphocytic Choriomeningitis Virus abstammen, gebunden an virus-ähnliche Partikel, wurde das TCR Repertoire von Wildtyp-Mäusen analysiert. Während in TCR-transgenen Mäusen nur p33- und A4Y-APLs effizient erkannt wurden, zeigen Wildtyp-Mäuse keine solche Prävalenz. In einem *in-vivo*-Killer-Assay wurde nur wenig Kreuzreaktivität zwischen p33 und APLs gefunden. Trotzdem war die Kreuzreaktivität ausreichend, um zu Dosis- und Virus-abhängigem Viren-Schutz zu führen. In einem

transgenen Diabetes-Mausmodell (β -Insel Zellen des Pankreas exprimieren p33), führte p33-VLP Immunisierung zu Autoimmunität im Gegensatz zur APL-VLP Immunisierung. Induktion von Diabetes mit APLs war erst nach der Entfernung der regulatorischen T-Zellen möglich. Deshalb kann man folgern, dass kreuzreaktive T-Zellen nicht zur Induktion von Autoimmunität reichen, es braucht zusätzlich die Eliminierung der regulatorischen T Zellen aus dem System.

Mit dem gleichen p33/APL-Modellsystem wurde das Konzept der „original antigenic sin“ untersucht. „Original antigenic sin“ beschreibt das Phänomen, dass die Viren-Mutanten die während einer viralen Infektion auftreten, dem Immunsystem dadurch entfliehen, dass sie eine starke Antwort gegen den originalen Virus, aber keine oder nur eine schwache Antwort gegen sich selbst auslösen. Die APL-Immunisierung simulierte die Infektion mit dem ursprünglichen Virus und die p33-Immunisierung imitierte eine Infektion mit einer Viren-Mutante. Durch die Infektion mit einem rekombinanten Vaccinia-Stamm wurde die Schutzwirkung der induzierten T-Zell Antwort in den geimpften Mäusen charakterisiert. Interessanterweise, verhinderte eine vorangegangene APL-VLP Immunisierung die Errichtung einer schützenden p33 Immunantwort, was in Übereinstimmung mit dem Konzept der „original antigenic sin“ steht.

Selbst-reaktive T-Zellen sind Schlüsselspieler in der Entwicklung von Autoimmunität. Die Regulation oder gar die Eliminierung dieser T Zellen wäre eine Möglichkeit, um selbstzerstörerische Immunantworten zu verhindern. Für das Lektin Galectin-1 wurde gezeigt, dass es Apoptose in Thymozyten und proliferierenden T-Zellen induziert. Eine Fähigkeit, die es zu einem potentiellen Kandidaten für die Behandlung von Autoimmunerkrankungen macht. Aber weil Galectin-1 nur als Dimer aktiv ist und es bei Konzentration unter $1\mu\text{M}$ hauptsächlich als Monomer vorliegt, sind hohe Konzentrationen des Proteins für die Aktivität nötig. Um das Problem der Dimerisierung zu umgehen, wurden zwei Galectin-1 Monomere genetisch verbunden, um ein stabiles Dimer zu bilden und die Apoptose-Aktivität von diesem neuen Molekül wurde untersucht. Diese strukturell verbesserte Version von Galectin-1 weist eine zehnmal höhere Aktivität auf und ist deshalb ein attraktives Molekül für therapeutische Eingriffe bei Autoimmunerkrankungen.

INTRODUCTION

1. T CELLS

T cells are one subset of cells constituting the adaptive immunity. Together with the B cells they allow the immune system to specifically respond to various pathogens. Additionally, they are able to form an immunological memory, which allows the organism to mount a stronger and faster response to a second infection with the same pathogen. The T cell repertoire can be subdivided in different subtypes (Figure 1).

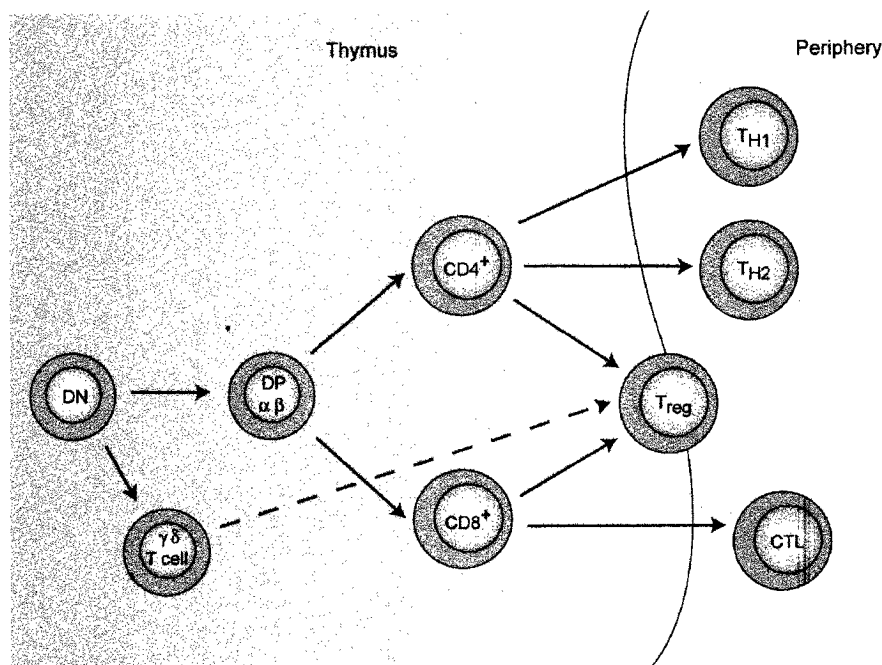


Figure 1. T cell subtypes: In the thymus T cells develop either into $\alpha\beta$ or $\gamma\delta$ T cells. The $\alpha\beta$ T cells are further subdivided into $CD4^+$ and $CD8^+$ T cells. After leaving the thymus the $CD4^+$ T cells develop in the periphery into T helper cells type 1 (T_{H1}) or type 2 (T_{H2}). The $CD8^+$ T cells develop in the periphery into cytotoxic T cells (CTLs). T_{regs} can be of either of the different subtypes of T cells. Some of the T_{regs} develop in the thymus and others arise in the periphery.

A first differentiation can be made by the expression of the T cell receptor (TCR), which is the definitive T-cell lineage marker. There are two defined types of

TCR: one is a heterodimer of a α and β subunit and the other consists of a γ and δ subunit [2,3]. Both receptors are associated with a set of five polypeptides, the CD3 complex, and form the TCR complex [4]. $\gamma\delta$ T cells are a minor portion (5-10%) of the blood T cells and their physiological function is still controversial. $\alpha\beta$ TCR expressing T cells comprise the rest (90-95%). The $\alpha\beta$ T cells are further distinguished by their expression of CD4 or CD8. The CD4 expressing T cells are mainly helping inducing immune responses and therefore called T helper cells (T_H). The second, CD8 positive, subset is predominantly cytotoxic and therefore named cytotoxic T lymphocytes (CTL). The CD4+ T cells recognize antigen presented by MHC class II molecules and the CD8+ T cells recognize antigens presented by major histocompatibility complex (MHC) class I molecules [5]. A small subset of the $\alpha\beta$ and the largest part of the $\gamma\delta$ are "double negative". All these populations can be further subdivided into functional subsets by the expression of surface markers or on the basis of cytokine production. For example, $\alpha\beta$ TCR expressing T cells are subdivided according to the expression of either CD28 or cytotoxic T-lymphocyte associated protein 4 (CTLA-4). Whereas engagement of CD28 on CD4+ T cells delivers a co-stimulatory signal, engagement of CTLA-4, which binds the same ligands on antigen presenting cells (APCs), provides a negative regulatory signal [6].

The T_H cells are subdivided into T_{H1} or T_{H2} cells according to their pattern of cytokine expression. T_{H1} cells are characterized by interleukin-2 (IL-2) and interferon- γ (IFN- γ) secretion, whilst T_{H2} cells secrete IL-4, IL-5, IL-6 and IL-10. T_{H1} cells are responsible for cell-mediated immunity, whereas T_{H2} are responsible for extracellular immunity [7].

An additional subset of T cells were initially named suppressor T cells and, after much debate, reintroduced as regulatory T cells (T_{reg}). These cells negatively regulate immune responses and may be important to prevent autoimmunity. T_{regs} can be of either subset, but the predominant T_{regs} are CD4+ T cells expressing the IL-2 co-receptor (CD25). They mediate their regulatory function via the secretion of cytokines like transforming growth factor- β (TGF- β) or by direct cell-cell interaction [8,9].

1.1. T cell receptor

The recognition of the antigen presented on MHC class molecules is the most important feature of T cells to guarantee a specific immune response. In contrast to antibodies the TCR does not recognize free antigen and furthermore the antigen has to be processed to shorter peptides prior presentation to the TCR.

Despite this difference in Antigen recognition, antibodies and TCRs share many similarities. They both belong to the Ig superfamily. They are both clonally distributed; each lymphocyte has a receptor with its own specificity. And the receptors are generated by somatic recombination from a limited number of germline genes [10].

Two different types of TCRs exist, the $\alpha\beta$ [2] and the $\delta\gamma$ TCR [3]. The $\alpha\beta$ TCR comprises a disulphide-linked heterodimer of a α (40-50 kDa) and β (35-47 kDa) subunit (Figure 2) [11]. The structure of the TCR was solved by X-ray crystallography [12]. Each polypeptide chain comprises two extracellular immunoglobulin-like domains of about 110 amino acids, anchored into the plasma membrane by a transmembrane peptide with a short cytoplasmic tail [13]. The amino acid sequence variability of the TCR resides in the N-terminal domains of the α and β polypeptides. The region of the greatest variability corresponds to immunoglobulin hypervariable regions, also known as complementary-determining regions (CDRs) [14]. These are clustered together to form an MHC-antigen binding site which is analogous to the antigen binding site on antibodies [15].

An unusual feature is that both subunits comprise positively charged residues in the transmembrane region. Interestingly, these residues are important for the assembly and the intracellular transport of the TCR complex [16]. Both the $\alpha\beta$ and the $\gamma\delta$ TCR are associated physically with a series of polypeptides, collectively called CD3 [4]. The CD3 components show no amino acid variability on different T cells and do not generate additional diversity. Functionally CD3 is important for signal transduction following antigen recognition by the TCR. CD3 comprises of four invariant polypeptides, called γ , δ , ϵ and ζ [17-20].

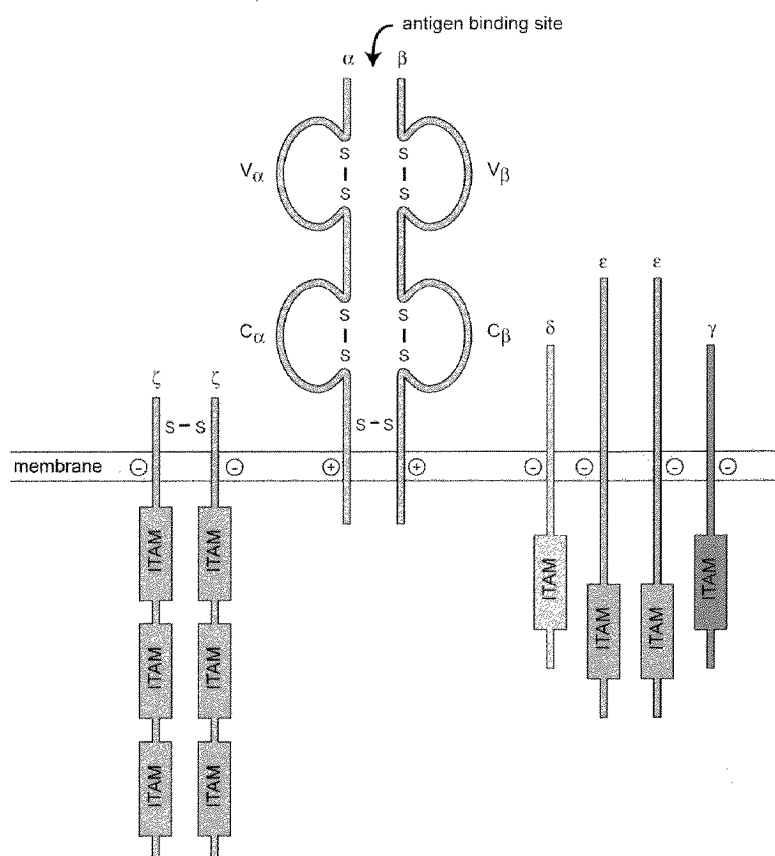


Figure 2. The T cell receptor: The T cell receptor complex consists of the α and the β (or γ and δ) chain of the T cell receptor and the proteins from the CD3 complex. The α and the β chain contains each a variable and a constant region. The variable regions together build up the binding site for the antigen. Furthermore they contain a positively charged transmembrane segment, which is important for the interaction with the proteins of the CD3 complex. CD3 consists of one γ , one δ , two ϵ and two ζ chains. The CD3 γ , δ and ϵ chains are closely related in their primary sequence. An important feature is the negatively charged transmembrane segment, which interacts with the TCR, and an ITAM sequence in the intracellular domain. The CD3 ζ chains contain only a small extracellular part, also a negatively charged transmembrane domain and a long intracellular part containing three ITAM motifs each. The ITAM motifs contain tyrosine residues which can be phosphorylated by specific kinases leading to a signal transduction to the nucleus.

The CD3-TCR complex is composed of one $\alpha\beta$ heterodimer, one γ and one δ chain and two ϵ and two ζ polypeptide chains. The CD3 chains are organized as heterodimers of $\gamma\epsilon$ and $\delta\epsilon$ and a homodimer $\zeta\zeta$ [21,22]. The CD3 γ , δ and ϵ chains are products of three closely linked genes which are related in their primary sequences [23]. They are members of the Ig superfamily, each containing an external domain followed by a transmembrane domain and a highly conserved cytoplasmic

tail. The transmembrane domains contain negatively charged amino acids which are important for the interaction with the positively charged amino acids from the TCR. *In vitro* mutation of these negatively charged amino acids prevents the assembly of the complex [16]. The cytoplasmic tail of each of the chains carries one immuno-receptor tyrosine activation motif (ITAMs) [24,25].

The CD3 ζ gene is encoded on a different chromosome than the other CD3 subunits and it is structurally unrelated to them [26]. The ζ chains consist of only a small extracellular part, a transmembrane segment, with negatively charged amino acids and a large cytoplasmic tail containing three ITAMs [24]. Altogether there may be 10 ITAMS present in the CD3 complex. The tyrosine residues in these motifs are a target for phosphorylation by specific protein kinases [27]. Mutation experiments have shown that the ITAMs and especially the tyrosines are essential for T-cell activation [28,29]. After the TCR is bound to the antigen-MHC, these motifs become phosphorylated within minutes.

The overall structure of the $\gamma\delta$ TCR is similar to that of its $\alpha\beta$ counterpart [30]. The major difference is their distinct anatomical locations. 95% of the peripheral T cells and the majority of the TCR-expressing thymocytes are bearing a $\alpha\beta$ TCR. The $\gamma\delta$ TCR T cells are abundant in various epithelia, such as epidermis, intestinal epithelium, uterus and tongue [31]. Furthermore differences in function and antigen recognition are proposed. Research to clear whether $\gamma\delta$ T cells recognize different classes of antigen than $\alpha\beta$ T cells showed that animals which are genetically deficient in $\gamma\delta$ cells generally have impaired resistance to some infections [32,33]. Some studies suggest that $\gamma\delta$ cells do not require antigens to be presented by classical MHC molecules at all [34,35].

1.1.1. Genes encoding the T cell receptor

The arrangement of the genes for the α , β , γ and δ chains of the TCR is very similar to the immunoglobulin heavy chain (Figure 3) [36]. The α and γ chain have sets of V genes and J genes while the β and δ loci have sets of V, D and J genes. The diversification of the TCR gene occurs by recombination between V, D and J segments [37]. In the mouse the δ chain locus is embedded between the V and J loci of the α chain locus. The α chain locus carries about 100 different V and also J segments leading to great diversity. The β chain locus and δ chain locus contain

additionally D segments, which allow a V-D-J arrangement but also V-J or V-D-D-J joins. The fact that the D segments are used in all three different reading frames adds some more diversity. The γ chain locus is arranged similar to the antibody light chain locus, with four C genes, each associated with one J gene, and one to four V genes [38].

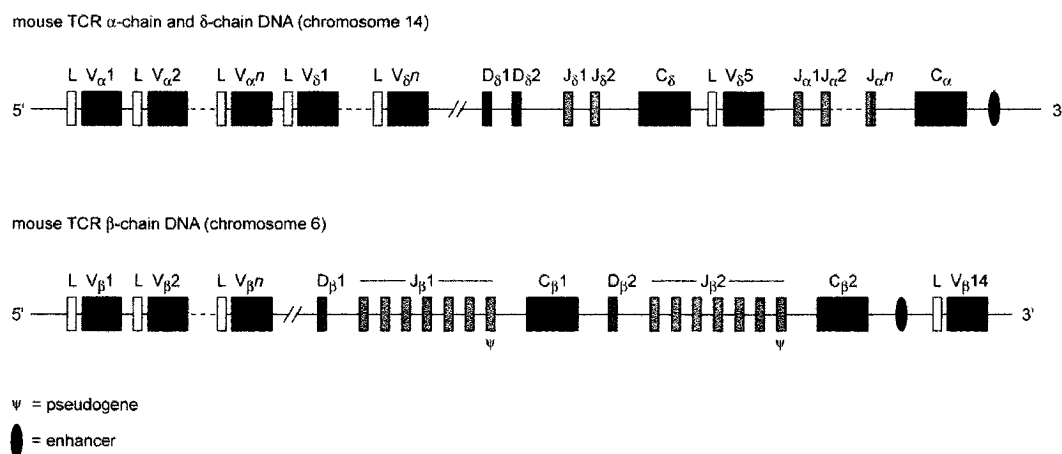


Figure 3. Genes encoding the T cell receptor: (adapted from Immunology, Janis Kuby) Shown are the TCR α , β and δ chain DNA. The α chain (also the γ) has sets of V and J genes, which are rearranged by recombination to build the variable region of the α chain. T cells express the product of a functional V-J-C recombination on the surface. The β chain (or δ) has additionally sets of D genes, which allows further variation of the variable region. The product on the cell can be a V-D-J-C, a V-J-C or a V-D-D-J-C join. The locus for the δ chain is embedded in the α chain locus.

V(D)J recombination is tightly regulated in the context of lymphocyte development, exhibiting lineage, developmental stage, and allele specificity. Although the recombination at TCR and Ig loci is mediated by the same recombinase complex, the rearrangement of TCRs is limited to T cells, whereas the complete rearrangements of Ig genes are limited to B cells [39]. Within the cell lineage, recombination is regulated temporally and in a stage-dependent manner [40]. For example, TCR β rearrangement occurs in DN thymocytes before TCR α rearrangement, which occurs in DP thymocytes. Moreover, in a given lymphocyte, only one of two alleles usually undergoes functional rearrangement, a process known as allelic exclusion (Figure 4) [39].

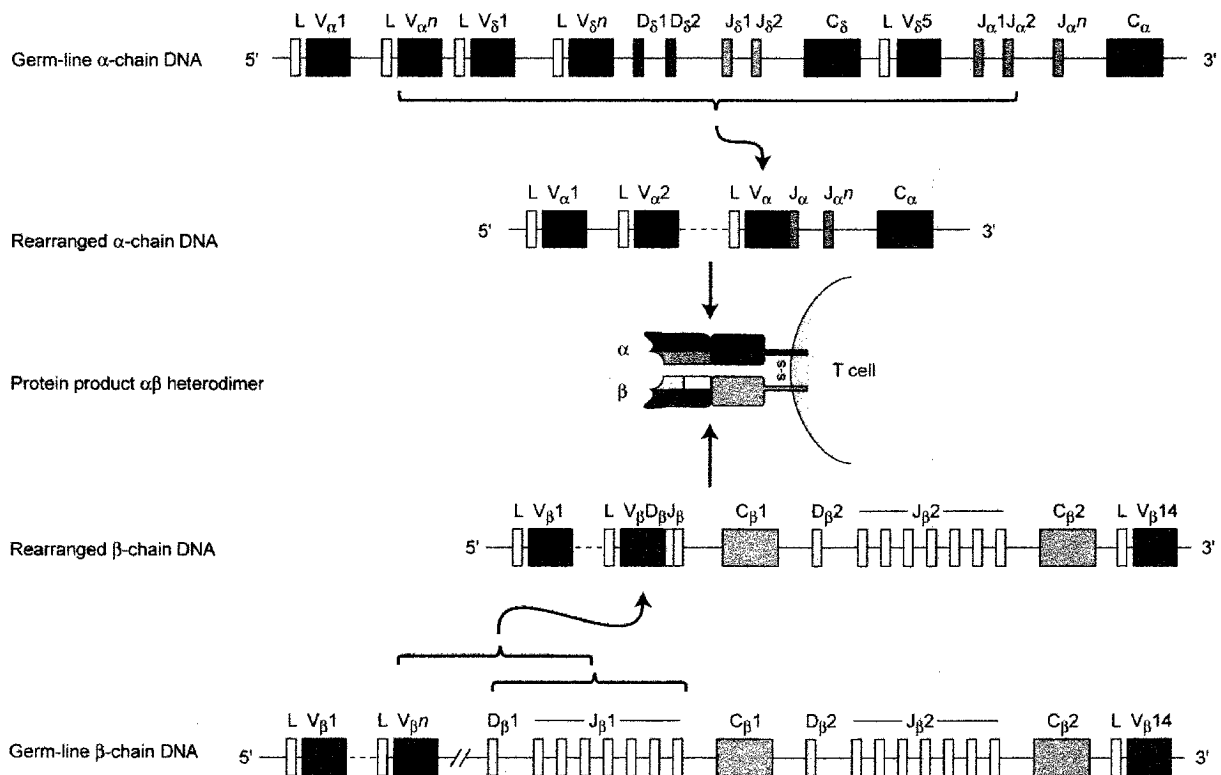


Figure 4. Gene rearrangement yielding a functional $\alpha\beta$ T cell receptor:(adapted from Immunology, Janis Kuby) The α chain undergoes a variable V-J joining, leading to the variant region of the α chain. Added to the V-J is the C segment building a complete α chain. The β chain undergoes two variable-region joinings: First D to J and the V to DJ. Or as mentioned alternatively also VJ or VDDJ recombinations are possible. The rearrangement is dependent on the RAG gene products and is tightly regulated in the context of T cell development, lineage and allele specificity.

In contrast to B cells where somatic mutation provides additional immunoglobulin diversity, TCR diversity is achieved only by rearrangement of TCR genes. This may be linked with the need to maintain tolerance to self and recognition of MHC by T cells. However, the generated diversity is tremendous. It was estimated that about 2.9×10^{22} different $\alpha\beta$ TCRs are possible. And assuming that 99 % of the sequences are rejected owing to coding for auto-antigens or other defects, this would still give 2.9×10^{20} possible murine TCRs, and this with less than 10^9 T cells leaving the thymus in a life-time of a mouse [41].

1.2. T cell development

Lymphoid progenitors arise from hematopoietic stem cells in the bone marrow (BM) and migrate via the blood to the thymus. In the thymus they lose the potential for B cell [42-44] and natural-killer-cell development [45]. The result is a double negative (DN; no CD4 and CD8), committed T cell precursor. DN thymocytes can be further subdivided according to their surface expression of CD44 and CD25: DN1, CD44+CD25-; DN2, CD44+CD25+; DN3, CD44-CD25+; and DN4, CD44-CD25- (Figure 5) [46].

T cells can either express the $\gamma\delta$ or $\alpha\beta$ TCR. The $\alpha\beta$ is the more dominant and starts from DN3 cells expressing a pre-TCR- α chain [47-49]. The Pre-TCR- α chain, encoded by a non-rearranging locus, pairs with the TCR β chain, which is the product of a set of somatic DNA-rearrangements, which are Recombination Activating genes (RAG1 and RAG2) dependent [50,51]. At the T cell surface the pre-TCR- $\alpha\beta$ is associated with a collection of proteins (the CD3/ ζ complex) that is involved in the proximal signal transduction [52]. Active signaling is required for further thymocyte development [53].

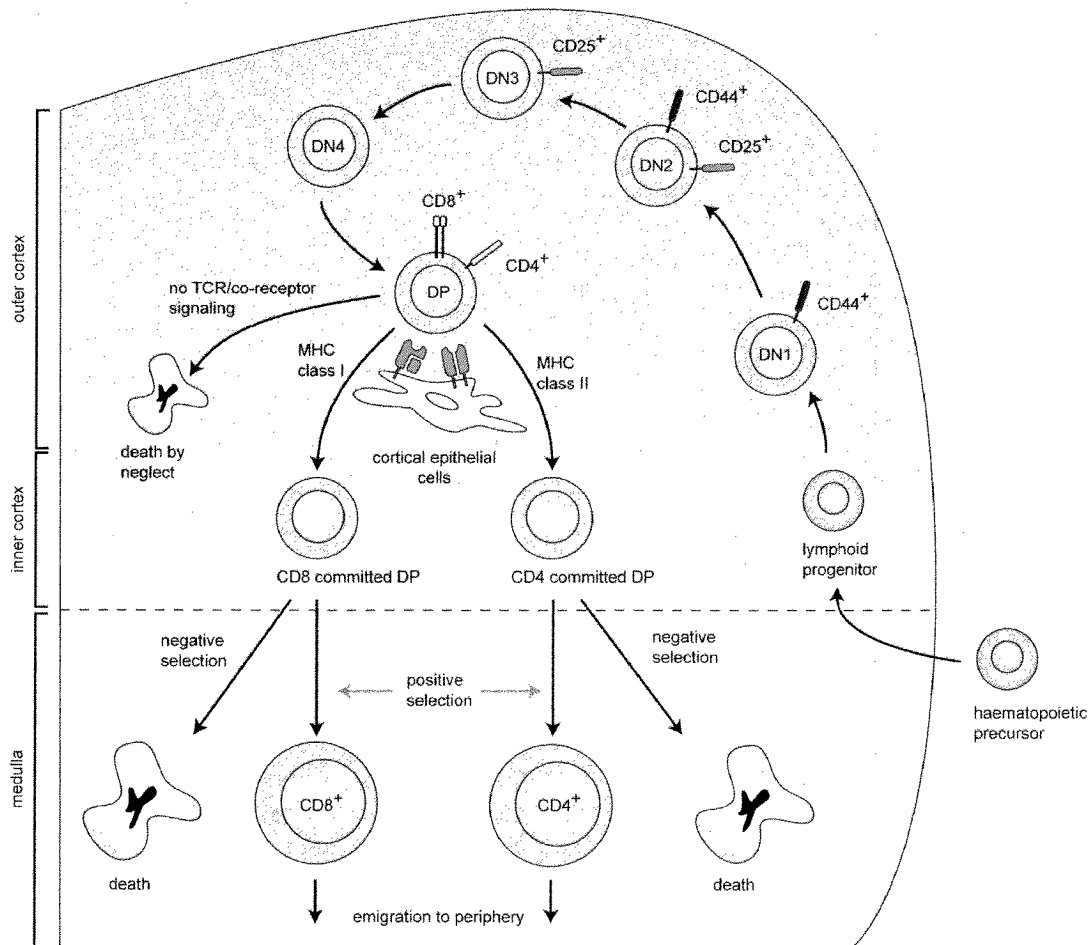


Figure 5. T cell development in the thymus: (adapted from Gemain et al [54]) Committed lymphoid progenitors from the bone marrow arrive in the thymus. Early committed T cells lack expression of the TCR, CD4 and CD8, and are termed double negative (DN; no CD4 and CD8) thymocytes. DN thymocytes can be further subdivided into four stages of differentiation (DN1, CD44⁺CD25⁻; DN2, CD44⁺CD25⁺; DN3, CD44⁻CD25⁺; and DN4, CD44⁻CD25⁻). During the progression from DN2 to DN4, the cells express the pre-TCR, which is composed of the non-rearranging pre-T α chain and a rearranged TCR β -chain. After successful pre-TCR expression, the cells progress to the double positive state, with replacement of the pre-TCR α -chain with a newly rearranged TCR α chain, which yields a complete $\alpha\beta$ TCR. The double positives then interact with cortical epithelial cells that express a high density of MHC class I and II molecules associated with self-peptides. The fate of the DP thymocytes depends on the signaling induced by the interaction of the TCR with the MHC-self-peptides ligands. Insufficient signaling results in death by neglect. Too much signaling promotes negative selection by acute apoptosis. Intermediate level of signaling initiates effective maturation (positive selection). Thymocytes that express a TCR binding antigen in MHC class I context become CD8⁺ T cells, whereas those that express TCRs that bind antigen in a MHC class II context develop to CD4⁺ T cells. These cells then are exported to the peripheral lymphoid sites.

T cells that emerge from β selection undergo some cell divisions and after recombination at the TCR- α locus they express the mature $\alpha\beta$ TCR. They also start

to express the co-receptor molecules CD4 and CD8 developing into a population of CD4+CD8+ (DP; double-positive) cells. In the thymus this immature T cell population constitutes 90% of the T cells [55]. In the cortical region of the thymus, these double-positive T lymphocytes are exposed to epithelial cells that express MHC class I and II molecules. T cells that do not recognize self-MHC are deleted via programmed cell death (death by neglect, ~90 % of the cells), whereas T cells that bind to self-MHC molecules survive (positive selection), differentiate into either single-positive CD4 or CD8 cells (depending on whether they bind MHC class II or class I, respectively), and migrate into the medulla of the thymus [56].

In the medulla, single-positive cells are exposed to auto-antigens in the presence of self-MHC molecules. Those T cells that bind self-antigens with high affinity are deleted (negative selection) via TCR-mediated programmed cell death [57]. Auto-reactive T lymphocytes that are not deleted in the thymus (due to low affinity binding or responsiveness to cryptic antigens, i.e., self-antigens not present in the thymus during normal development) are usually anergized or suppressed in the peripheral lymphoid tissues. The mature T cells leave the thymus via the bloodstream and enter the secondary lymphatic organs and wait for their appointment with the DCs.

1.3. T cell activation

T cell signaling uses the signal transduction pathways that are common to many other cell types, including B cells. A key principle is the clustering of receptors upon ligand binding. This leads to activation of the associated tyrosine kinases which phosphorylate tyrosine residues in the cytoplasmic tails of the clustered receptors, followed by the recruitment of additional kinases and signaling molecules, resulting in a cascade of activated kinases. These signaling cascades result in activation of transcription factors which lead to the induction of specific gene expression (reviewed in [58], [59] and [60]).

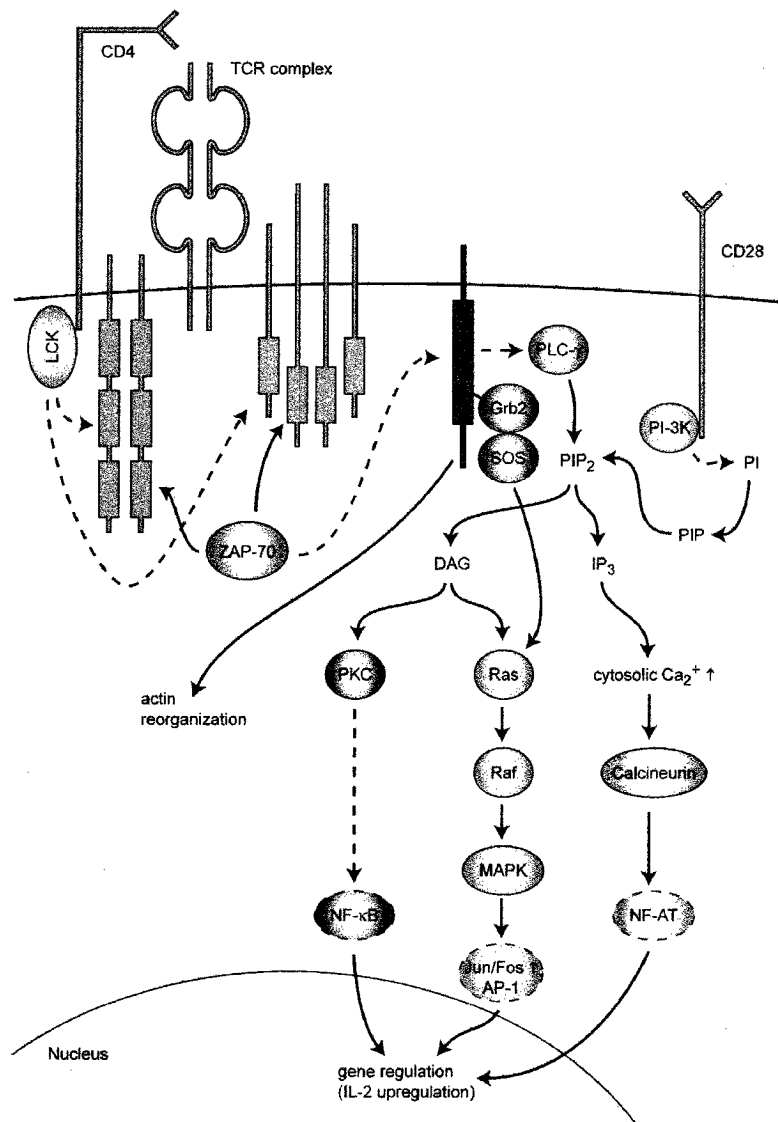


Figure 6. TCR-mediated signaling: After ligation of the TCR with antigen-MHC complex, the tyrosine kinase LCK gets activated and starts to phosphorylate the ITAM domains in the CD3 complex. The phosphorylation of the ITAM allows other kinases like ZAP-70 the binding to them. By trans- or auto-phosphorylation events these kinases are activated and start to phosphorylate adapter molecules like the linker for activated T cells (LAT). Phosphorylated LAT allows the binding of SH2 motif containing proteins, which leads to the activation of phospholipase C (PLC- γ). PLC- γ cleaves phosphatidylinositol bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol trisphosphate (IP₃). Additionally LAT activation leads to actin reorganization events. IP₃ releases Ca²⁺ from intracellular stores leading to the activation of Ca²⁺- dependent enzymes like calcineurin, which phosphorylates the transcription factor NF-AT. The phosphorylation allows NF-AT to enter the nucleus and to turn on gene transcription. Simultaneously DAG initiates the Ras/Raf/MAPK pathway leading to Jun/Fos upregulation, leading to AP-1 regulation inducing IL-2 production. Additionally DAG activates protein kinase C (PKC), which activates the transcription factor NF- κ B. NF- κ B also turns on gene transcription. The co-stimulatory molecule CD28 is associated with the phosphoinositide-3 kinase which converts after activation phosphatidylinositol (PI) into phosphatidylinositol 3-phosphate (PIP), a

preliminary stage of PIP_2 . All the signaling events end up in the nucleus inducing gene transcription, with IL-2 as one of the most important gene products leading to auto-activation.

The TCR is associated with the CD3 complex and CD4 or CD8. Recognition of an antigen-MHC complex by the TCR and ligation of co-stimulatory molecules initiates the signaling events. The earliest events comprise tyrosine phosphorylation, involving tyrosine kinases of the Src family, particularly Lck associated with CD4 and CD8, and fyn which phosphorylate the ITAMs in the γ , δ , ϵ and ζ chains of the CD3 complex [61]. Phosphorylation of the ITAMs initiates a series of steps schematically shown in Figure 6. Phosphorylation of the ITAMs allows the association with other kinases including ZAP-70, which then gets activated by trans- or auto-phosphorylation [62,63]. ZAP-70 phosphorylates adapter proteins like the linker for activated T cells (LAT) (reviewed in) [64]. The phosphorylation of LAT allows the binding of Src homology region 2 (SH2) containing proteins, which leads to the activation of phospholipase C (PLC- γ) and also leads to actin reorganization events [65].

PLC- γ initiates two distinct pathways both leading to further downstream signaling by the cleavage of phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol trisphosphate (IP_3). IP_3 releases Ca^{2+} from intracellular (ER) stores to activate calcium-dependent enzymes such as calcineurin. Calcineurin phosphorylates the transcription factor nuclear factor of activated T cells (NF-AT) which causes its translocation to the nucleus. Meanwhile DAG activates protein kinase C, which then activates the transcription factor NF- κ B [66]. Additionally DAG also activates the Ras/Raf/MAPK pathway leading to the transcription of fos and jun, immediate early genes for cell division, and activation of the promoter AP-1 which acts with NF-AT on the IL-2 gene [67]. Alternatively the Ras/Raf/MAPK pathway can be initiated by growth factor receptor-bound protein 2 (Grb2)-SOS complex, which also get activated by association with the LAT molecule [68]. The co-stimulatory molecule CD28, when cross-linked with B7 molecules on the surface of the APC, also adds additional activation signals, specifically by the activation of a Phosphoinositide-3 kinase (PI-3K), which phosphorylates phosphatidylinositol (PI) converting it to phosphatidylinositol 3-phosphate (PIP), a preliminary stage of PIP_2 [69,70].

All the signaling events induce the transcription of genes in the nucleus. The most important gene activated is IL-2. This allows the T cells to remain activated by autocrine stimulation since IL-2 receptor expression is also induced upon T cell activation [71,72].

The exact requirements in order to get activation upon antigen recognition are still under debate. Although it is known that a single TCR-MHC/antigen interaction is not sufficient, the exact amount of TCRs necessary to be triggered in order to activate the T cell is unknown. The story is further complicated by the fact that other stimulatory signals may influence the requirements. By the study of T cells, antigens were classifiable in agonists and antagonists, depending on their ability to activate specific T cell clones. Especially the p33 peptide from lymphocytic choriomeningitis virus (LCMV), and variants thereof were analyzed for agonistic or antagonistic properties on p33-specific T cells [73,74].

1.4. Cytotoxic T lymphocytes (CTL)

Cell-mediated cytotoxicity is an essential defense against intracellular pathogens, including viruses and some bacteria. Besides the central role played in the defense against pathogens, CTLs are also very important for the clearance of tumor cells. In modern medicine they are responsible for the destruction of allogenic tissue grafts, for example after organ transplantation. Natural killer (NK) cells and also some myeloid cells can also mediate cytotoxicity. CTLs recognize specific antigens presented by MHC molecules. Most CTLs are CD8⁺ and recognize the antigen presented on a MHC class I molecule, but about 10% of MHC-restricted cytotoxic T cells are CD4⁺ and recognize antigen presented on class II molecules. The most important role of CTLs is the elimination of virus infected cells. Virtually all nucleated cells express MHC class I molecules on the surface and start to present viral peptides after infection. Antigen specific CD8⁺ T cells are then able to recognize these cells and eliminate them by cell lysis. Several viruses have evolved mechanisms to avoid recognition by CTLs. For example, they reduce the expression of MHC class I molecules in infected cells, as shown for the human cytomegalovirus [75].

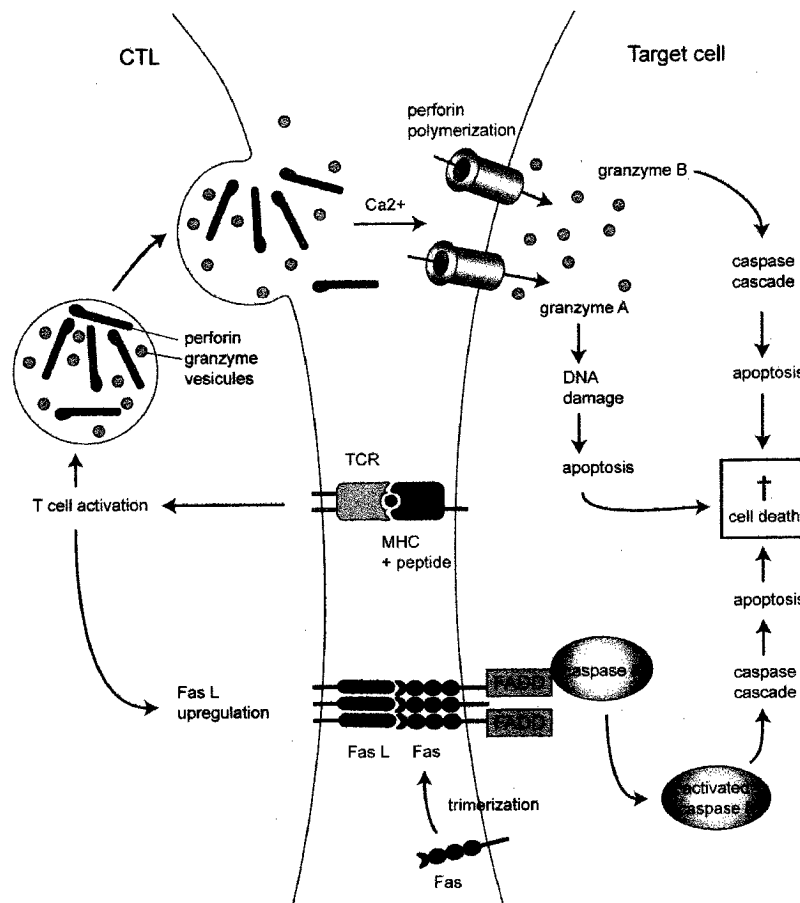


Figure 7. Cytotoxic T cell mediated killing: After a cytotoxic T cell (CTL) has recognized a non-self antigen on the surface of a target cell, it can initiate killing by two different pathways. **1.** The perforin/granzyme pathway: CTL carry vesicles containing granzymes and perforin. After activation, the content of the vesicles are released into the cleft between the two cells. Perforin polymerizes in a Ca^{2+} -dependent manner leading to pores in the target cell membrane. Through these pores the granzymes enter the cell and start the apoptosis program in the cell. Granzyme A leads to DNA damage and impairs the repair mechanisms which lead to apoptosis and the granzyme B, similar to caspase 8 induces the caspase cascade leading to apoptosis. **2.** The Fas –FasL-pathway: FasL is upregulated on the CTL and binds to Fas on the surface of the target cell. This initiates trimerization of Fas, allowing the association of transducing molecules like FADD. FADD recruits caspase 8, which gets activated by cleavage and initiates the caspase cascade leading to apoptosis of the target cell.

The CTLs have evolved several different pathways to induce cell death in the target cells (reviewed in) [76]. Cells can die in two ways – by necrosis or apoptosis.

Apoptosis is a highly ordered, physiological process, in which the cells are systematically disassembled. Mitochondria lose membrane integrity and cytochrome C leaks into the cytoplasm [77]. Then the nuclear DNA is cleaved into fragments by endonucleases. The cells detach from their neighbors and the cytoplasm and the

nucleus condense. Finally, the cell membrane starts to form blebs and the cell fragments into condensed apoptotic bodies. Apoptotic cells attract mononuclear phagocytes and are rapidly taken up by phagocytosis and broken down in phagolysomes. Most important for mediation of apoptosis in cells are the caspases. Caspases are a group of proteases, which cleave their substrates on the C-terminal side of an aspartate residue. They are produced in a proenzyme form and become activated by cleavage into two or three subunits. Several caspases together are forming a caspase cascade leading to the destruction of the cell in an apoptotic way [78]. Therefore apoptosis can be induced extremely fast in the target cell, then the caspases are present in an inactive proenzyme form, and they just need to be activated by cleavage to act synergistically on the destruction of the cell.

In comparison necrosis is a less ordered event, in which dying cells fall apart releasing their contents, thereby leading to macrophage activation and inflammation [79].

One pathway of cytotoxicity used by CTLs is the perforin/granzyme pathway (Figure 7, upper panel). Perforin is a monomeric pore-forming protein that is structurally and functionally related to the complement component C9 [80-82]. Granzymes are a collection of serine esterases, which act on intracellular pathways that trigger apoptosis and DNA degradation [83-85]. Granzyme B has the same specificity as the caspases. In order to activate the apoptotic pathway in target cells granzymes need to enter the cell. This is the point where the perforin and the granzymes act synergistically. Upon target cell recognition by a CTL, perforin and granzymes are released into the cleft between the two cells. Perforin monomers are incorporated into the target cell membrane and polymerize to form transmembrane channels [86], through which granzymes enter the target cell and induce apoptosis. Granzyme B triggers apoptosis by caspase cleavage [87], whereas Granzyme A initiates a caspase-independent cell death pathway, causing nicks in the DNA [88,89]. Interestingly, despite the close contact of CTL and target cell, perforin does not affect the CTL. This may be explained by proteoglycan which inactivates perforin through binding or by cathepsin B which was found to inactivate perforin by cleavage [90,91].

Additionally CTLs may induce apoptosis through members of the tumor necrosis factor (TNF) receptor family (Figure 7, lower panel). The ligand of Fas (FasL), one

member of this group, is expressed on mature CD4⁺ and CD8⁺ T cells after activation [92]. Ligation of Fas induces trimerization of the Fas molecules on the cell surface, followed by association with adaptor proteins, like FADD which recruits and activates caspases 8 and 10 [93-96]. Most CD8⁺ T cells have vesicles containing TNF and lymphotoxin, which can be released on target cells and act similar as FasL on Fas but on TNFR-1 (CD120a). They cause receptor trimerization leading to adaptor protein (TRADD, FADD, RIP and RAIDD) recruitment, which initiates caspases activation. These adaptor proteins carry so called death domains (DD) which are important for the recruitment of caspases (reviewed in) [97].

1.5. T_{H1} versus T_{H2}

Naïve T_H cells can differentiate into at least two functional classes of cells during an immune response [5]. T_{H1} cells, first characterized by IFN- γ secretion, and T_{H2} cells, which secrete IL-4 (reviewed in [98,99]). T_{H1} cells are responsible for cell-mediated immunity, whereas T_{H2} cells are responsible for extracellular immunity. In addition to IFN- γ , T_{H1} cells also secrete TNF- β and IL-2 [7]. The T_H cells are also important in the antibody class switching. T_{H1} promote the production of IgG2a, opsonizing and complement fixing antibodies. Furthermore, they promote macrophage activation, antibody-dependent cell-mediated cytotoxicity and delayed-type hypersensitivity [100].

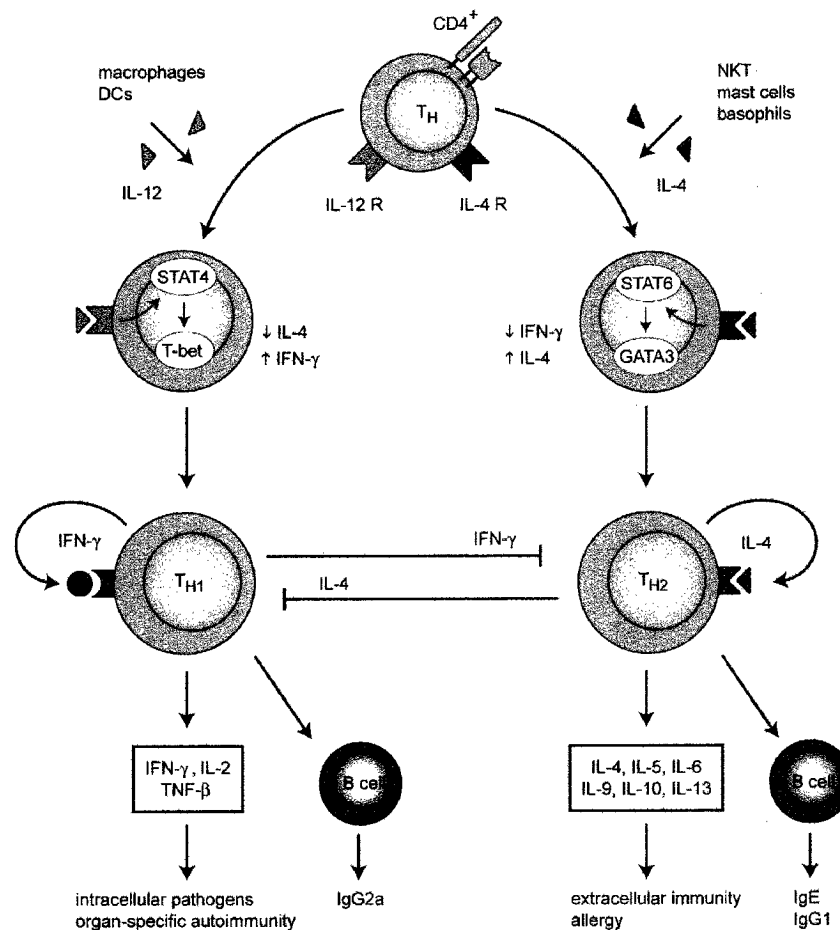


Figure 8. Schematic representation of induction and regulation of T_{H1} and T_{H2} cells: (adapted from Liew et al [101]) A precursor T_H can differentiate into T_{H1} or T_{H2} cells depending primarily on the cytokine environment. Interleukin-12 (IL-12) drives T_{H1} cells, whereas IL-4 promotes T_{H2} cells. By ligation of the IL-12 receptors the T_{H1} program, leading over signal transducer and activator of transcription 4 (STAT4) and the molecule T-box expressed in T cells (T-bet), is initiated. IL-4 induces T_{H2} program over STAT6 and GATA3. T-bet induces the down-regulation of IL-4 production and initiates IFN-γ production, an autocrine T_{H1} promotion factor. On the other hand GATA3 induces the opposite, with IL-4 as autocrine T_{H2} promotion factor. IL-4 and IFN-γ block T_{H1} respectively T_{H2} responses. Main mediators of T_{H1} responses are IFN-γ, IL-2 and TNF-β. The typical T_{H2} cytokines are IL-4, IL-5, IL-6, IL-9, IL-10 or IL-13. T_{H1} cells induce IgG2a isotype switch and are important for the defence against intracellular pathogens. Associated with T_{H1} response is organ-specific autoimmunity. T_{H2} cells induce IgE and IgG1 isotype switch and are important in the extracellular immunity. Associated with T_{H2} response is allergy.

T_{H2} cells are characterized by the production of IL-4 and IL-5 with IL-6, IL-9, IL-10 and IL-13 also commonly produced [102]. These cells provide optimal help for humoral immune response, including IgG1 and IgE isotype switching and mucosal immunity, stimulation of mast cell and eosinophil growth and differentiation and IgA synthesis [100].

Both subsets of T_H cells have been implicated in pathological responses. T_{H1} cells can mediate organ-specific autoimmunity and T_{H2} cells have been implicated in the pathogenesis of asthma and allergy [103]. Considering that T_{H1} cytokines inhibit T_{H2} cells and vice versa, the immune response tends to settle either into a T_{H1} or T_{H2} type of response.

T_{H1} and T_{H2} can further be distinguished by surface marker expression. Chemokine receptors CXCR3 and CCR5 are preferentially expressed on T_{H1} cells [104,105] and whilst chemokine receptors CXCR4, CCR3, CCR4 CCR7 and CCR8 are associated with T_{H2} cells [105,106].

Several factors influence the decision whether a CD4+ T cell develops into T_{H1} or T_{H2} . Dependent on the site of antigen presentation, the co-stimulatory molecules provided [107,108], the properties of the immunogen, the peptide-density and binding affinity, a T_{H1} or T_{H2} response is induced. Furthermore it may depend on the host genetic background and finally on the cytokine profile and balance of cytokines evoked by the antigen. For example, high peptide density on APCs was shown to favor T_{H1} and low densities favor T_{H2} responses [109].

With defined *in vitro* culture systems and T cells from TCR transgenic mice, the development of either T_{H1} or T_{H2} cells was extensively studied. Whereas IL-4 was found to be essential for the induction of T_{H2} cells from naïve precursors (Figure 8, right panel) [110,111], IL-12 was shown to play the same role in the development of T_{H1} cells (Figure 8, left panel) [112,113]. IL-12 and IL-4 originate both from the innate immune response. NKT cells, mast cells or basophils secrete IL-4 [114-116] and macrophages and DCs produce IL-12 [117-119]. In T_{H1} differentiation, signaling by IL-12 receptor leads to activation of STAT4 (signal transducer and activator of transcription 4) [120] and induction of a unique T_{H1} transcription factor called T-bet (T-box expressed in T cells) [121], which upregulates IFN- γ and downregulates IL-4 and IL-5 expression. In contrast IL-4 induces T_{H2} -cell development through STAT6

[122,123], which activates GATA3 [124], leading to the upregulation of IL-4 and IL-5 and down regulation of IFN- γ expression.

However, although a strong polarization is found *in vitro*, *in vivo* responses tend to be a mixture of both, T_{H1} and T_{H2}.

1.6. Regulatory T cells

As mentioned earlier, T cells undergo an intrathymic selection process where T cells with high avidity for self-antigens are eliminated. This process called central tolerance is crucial to avoid the development of autoimmunity. However, this process is not complete. For example, self-antigens exist which are not present in the thymus, hence self-reactive T cell clones specific for these self-antigens cannot be eliminated. It was postulated that the incompleteness of central tolerance is important to guarantee a complete immune repertoire [125]. The incompleteness includes a high price, namely, the potential to develop autoimmunity. To avoid this, the immune system developed additional mechanisms to prevent auto-reactive immune responses. One very important mechanism is mediated by T_{regs}.

What are regulatory T cells?

Although they are not an uniform population they can be grouped into adaptive and naturally occurring regulatory T cells (Figure 9). The natural T_{reg} cells are generated during the T cell development in the thymus [126]. They show diverse TCR usage and are potentially capable of recognizing self-antigens [127]. Once generated the thymic T_{reg} cells are exported to the periphery, where they have been postulated to prevent activation of self-reactive T cells [128]. The thymus induced T_{regs} are CD4+ and typically CD25+, additionally they express CTLA-4 and GITR (glucocorticoid-induced TNF-receptor family related protein) [128,129]. Unlike conventional T cells, which upregulate CD25 upon activation, the T_{regs} maintain expression of CD25 [130]. CD4+CD25+ T cells, which constitute ~10% of peripheral murine CD4+ T cells, possess potent regulatory activity *in vitro* [9,131] and *in vivo* [8,132]. They show a partially anergic phenotype, they proliferate poorly upon TCR stimulation and their growth is dependent on exogenous IL-2 [133]. CD4+CD25+ T cells are shown to inhibit autoimmune diabetes in mice [128] and rats [134]. The stimuli that are required

for the generation of the natural T_{regs} in thymus are incompletely defined. Nevertheless, CD28 ligation [135] and strong antigenic signals [136] were shown to be necessary.

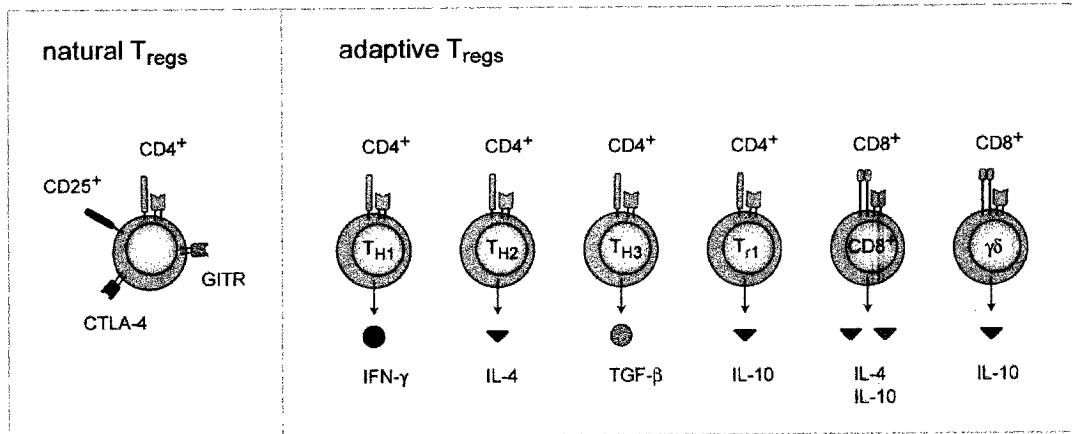


Figure 9. Regulatory T cell subsets: regulatory T cells can be subdivided into natural T_{regs} and adaptive T_{regs} . The natural T_{regs} arise during T cell development in the thymus, and are $CD4^+CD25^+$. Negative regulation by natural T_{regs} is transduced by cell-contact over molecules like CTLA-4 and GITR. Adaptive T_{regs} can be of different origin. Described in the literature are T_{H1} or T_{H2} cell, which regulate immune responses. Furthermore T_{H3} cells which mediate regulation by TGF- β , or T_{r1} cells which regulate immune reactions by IL-10 secretion are additional subtypes. But also $CD8^+$ T cells and $\gamma\delta$ T cells are found to mediate immune regulation.

Adaptive regulatory T cells originate from the thymus and can be either derived from classical T cell populations or natural T_{regs} . The level of CD25 expression is variable, depending on disease setting and the site of regulatory activity [137]. Whereas the natural T_{regs} mediate their function via cell-cell contact, the adaptive T_{regs} exert their function mainly in a cytokine dependent manner [138-140]. Furthermore, in contrast to the natural T_{regs} , they need further antigen exposure-induced differentiation in the periphery to be active [141]. Examples of adaptive regulatory T cells are T_{H1} cells, which prevent experimental autoimmune encephalomyelitis (EAE) by secretion of IFN- γ and avoiding a T_{H2} response [142]. Or T_{H2} cells, which suppress T_{H1} mediated induction of diabetes by the secretion of IL-4 [143]. The $CD4^+ T_{H3}$ cells suppress induction of autoimmunity by the secretion of TGF- β [144]. Other adaptive T_{regs} are $CD4^+T_{r1}$ (IL-10 mediated) [145,146], $CD8^+ T$

cells (by IL-10 or IL-4 secretion) [147,148] or CD8+ $\gamma\delta$ T cells (IL-10 mediated) [149]. From this list it gets quite obvious that there is no uniform population of T_{reg} s. Their most important feature is to inhibit ongoing immune responses by the secretion of regulatory cytokines or by direct cell-cell interaction.

2. DENDRITIC CELLS

DCs are the most potent professional APCs found in the immune system [150]. They were named based on their dendrite-like cellular branches, which are typical for mature DCs. Like macrophages and other phagocytes, DCs are part of the innate immune system and their main function is to capture antigens, process and present them to the cells of the adaptive immune system thereby inducing a strong immune response. Whilst the innate immune system is evolutionarily old and already developed in invertebrate organisms [151,152], the adaptive immune system is evolutionarily relative new and developed during vertebrate evolution. DCs evolved as link between the two branches of the immune system in vertebrates. The innate immune system, as found in invertebrate organisms like worms and insects has at its disposition already several defense mechanism, as there are [153 649]: self-recognition, phagocytic cells, encapsulation mechanisms and inducible antimicrobial factors. The main innovations in vertebrates during the development of the adaptive immune system were the invention of antigen receptors allowing specific immunoreactions and as consequence thereof the immunological memory. For the first time in evolution the vertebrate immune system was able to respond specifically to pathogens, and the immunological memory allowed protection against repetitive infections with the same or a similar pathogen. The defense mechanisms of the innate immune system are crucial for the defense against pathogens early after infection. Together with the adaptive immune system it gives the vertebrates the possibility to clear its body from pathogens, with the DCs as an important key mediator between the two parts of the immune system. To do so, several different specialized subtypes of DCs developed and the mechanisms and pathways of capturing of pathogen, presenting to the cells of the adaptive immune system and activation of the adaptive immune system are very diverse.

2.1. Dendritic cell development

DCs occur in different subsets, with different functions and also different origins. DCs share their origin with the myeloid and lymphoid cells, they all develop from a pluripotent stem cell in the BM (Figure 10). Most DCs are believed to be of myeloid origin, but evidences were found that some DCs shared early steps of development with B cells [154,155] and the CD8⁺ DCs of the thymus shared early precursors with T cells [156]. After this findings all CD8⁺ mouse DCs were thought to derive from lymphoid-restricted precursors and all CD8⁻ DCs to derive from myeloid-restricted precursors, leading to the terms “lymphoid” and “myeloid” DCs. But this concept was recently challenged. Mutant mice, which cannot form T cells, still produced thymic CD8⁺ DCs [157,158]. After the isolation of myeloid-restricted and lymphoid-restricted precursors from the BM it was shown that both could produce DCs, and each precursor could produce all the splenic and thymic DC subtypes [159-161]. Therefore the final surface phenotype of DCs is not determined by its myeloid or lymphoid origin. The fact that CD8⁻ and CD8⁺ DCs require different cytokines and express different transcription factors may support this concept [162-165]. Phenotypic and functional criteria have allowed classification of murine DCs into six main subpopulations. DCs in the lymphoid tissue can be subdivided into a CD8⁻ and CD8⁺ cell population [166]. CD8⁻ DCs can be further divided in CD4⁻CD8⁻ and CD4⁺CD8⁻ subsets [167,168]. In the lymph nodes there exists an additional DC population that expresses intermediate levels of CD8 [169]. This DC population may be derived from one of two other main DC populations, the epidermal langerhans cells or from dermal DCs, which are exclusively found in the skin [170]. Finally expression of B220, an isoform of CD45, characterizes the plasmacytoid DCs, which are found in all lymphoid organs and have the potential to produce large amounts of type I interferon in response to viral infection [171]. In addition these cells have been proposed to be involved in the maintenance of T-cell tolerance by inducing the differentiation of T_{regs} [172]. Besides these main populations of DCs, additional subsets have been described in specific organs of the mouse; however, their physiological significance remains to be determined.

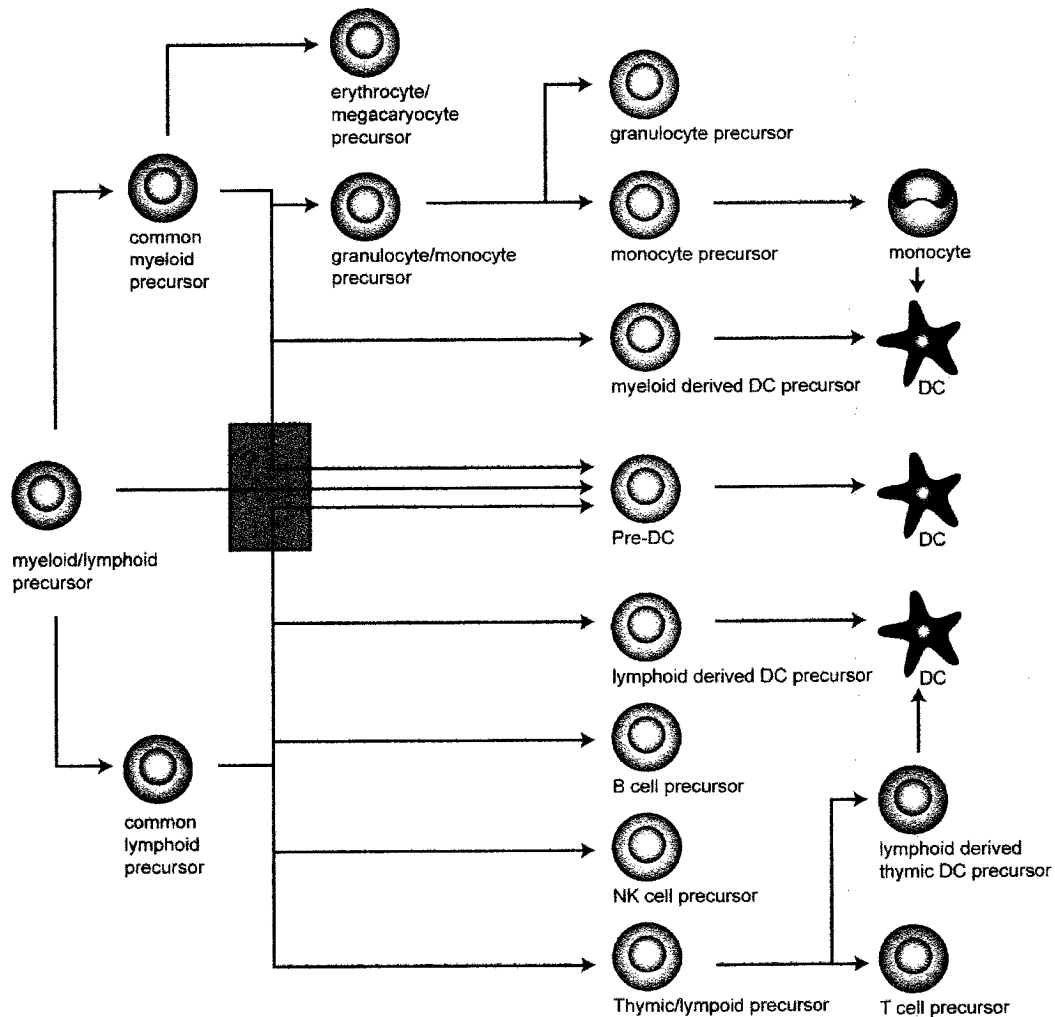


Figure 10. Theoretical model of the development of dendritic cells. (Adapted from Ardavin et al [173]) The differentiation of DCs has been proposed to proceed directly through myeloid and lymphoid derived precursors, and through circulating common DC precursors (pre-DCs). Additionally, there are other pre-stages leading to DCs. On the myeloid side there are a myeloid derived DC precursor and also a monocyte precursor leading to DCs. The lymphoid side offers a lymphoid derived precursor and the thymic lymphoid precursor, which can develop into DCs.

2.2. DC function

DCs are involved in many different processes. They exist in two major differentiation states, immature or mature (Figure 11). In the immature state their main function is to capture antigens, process them into small peptides and to load

them on MHC I or II molecules. After antigen capturing immature DCs (imDC) undergo maturation if induced by appropriate factors. Whilst maturation is ongoing DCs start to migrate to the lymphoid tissues. During the migration, co-stimulatory molecules are up-regulated and the secretion of cytokines is induced. Once the DC has reached the lymphoid organs, it has fully matured and has become an efficient antigen presenter for T and B cells [174-176].

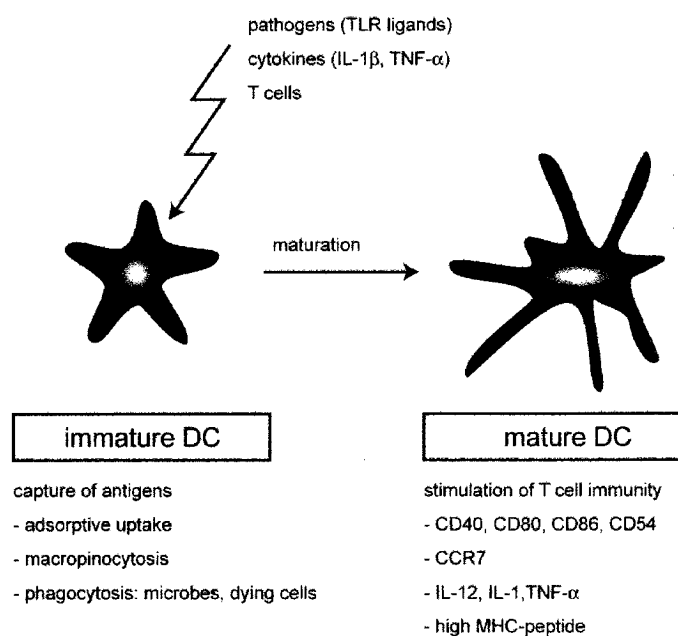


Figure 11. Immature and Mature DC: DCs are found in two different functional states: immature and mature. The main function of immature DCs is the capturing of antigen. This is done by adsorptive uptake, macropinocytosis or phagocytosis. After activation by danger signals like pathogens, cytokines or T cells, the immature DC mature. Mature DCs stimulate T cell immunity by the presentation of antigen and the upregulation of co-stimulatory molecules like CD40, CD80 and CD86 or the secretion of pro-inflammatory cytokines. Furthermore CCR7, an important receptor for migration is upregulated.

ImDCs have been long regarded as sentinels of the immune system, which patrol the body for intruders. Recent studies suggest that imDCs also actively maintain tolerance to self-antigens [177]. Three features of imDCs led to this conclusion. First, imDCs can efficiently capture apoptotic cells and present self-antigens to T cells [177]. Second, as imDCs are continuously produced and widely distributed in the body, they can constantly sample different self-antigens and present them to T cells. Third, imDCs do not undergo maturation when they capture self-antigen under physiological conditions. Indeed recent findings suggest that imDCs have the capacity to induce peripheral self-tolerance by promoting naïve T cells into IL-10 producing T regulatory/suppressor cells [178,179].

After infection or uptake of antigen, imDCs undergo rapid maturation. Pattern recognition receptors, such as the Toll like receptors (TLR), expressed by imDCs allow them to recognize microbial antigens, which induces the maturation program [180,181]. Maturing DCs rapidly lose endocytotic activity and increase surface expression of MHC class I and II-peptide complexes [182]. Additionally they up regulate the surface expression of adhesion and co-stimulatory molecules (CD40, CD54, CD80 and CD86) [183-185], and secrete pro-inflammatory cytokines like IL-1, IL-12 or TNF- α [118]. Maturing DCs start to express the chemokine receptor CCR7 [186], which drives their migration into the lymphatics and to the lymph nodes through the CCR7 ligands CCL21 and CCL19 [187]. In the lymphoid tissue the mature DCs screen for antigen-specific naïve T cells and induce primary T cell mediated immune responses [188].

2.2.1. Capturing of the antigen

ImDCs reside somewhere in the body as sentinels waiting to capture foreign antigen coming from bacteria, virus or other invaders. Or, alternatively, as mentioned above, they consume apoptotic self cells to establish self-tolerance. The first step to fulfill their function is antigen-uptake. ImDCs are therefore equipped with different possible pathways to take up pathogens or apoptotic cells. They capture antigen by phagocytosis [189] similar to macrophages, by macropinocytosis [190], which allows to take up small vesicle droplets coming from apoptotic cells or by receptor-mediated endocytosis [191]. Especially endocytosis, allows imDCs, to specifically take up defined antigen. For example antibody or complement opsonized bacteria can be

actively taken up by endocytosis via the Fc or complement receptors on the surface of imDC [192]. Other proteins important for receptor-mediated endocytosis are the C-type lectins, which allow sugar specific binding of bacteria and subsequent uptake [193,194]. Furthermore microbial lipopeptides can be taken up by TLRs expressed on imDCs (TLR1, TLR2) [195,196].

2.2.2. Processing of the antigen and loading on to the MHC class molecules

After uptake, the antigen has to get processed to be presented to the cells of the adaptive immune system. Whereas peptides are presented on MHC class I or II molecules, glycolipids are presented on CD1 [197,198], MHC class I molecules are present on virtually all nucleated cells in the body. Peptides presented by MHC class I molecules are normally derived from endogenously produced proteins. In the pathological situation peptides derived from viruses, intracellular bacteria or tumor antigens are also presented. The presentation of foreign antigen, usually leads to killing of the infected cells by specific CTLs. The class I route starts with the proteasome, which is an ancient protein machinery able to process proteins targeted for degradation by ubiquitinylation (see Figure 12, left side) [199]. The proteasome consist of four stacks of seven units each, resulting in a hollow cylinder through which the proteins from the cytosol are passed and cut in smaller pieces by the proteolytic activity inside the proteasome [200]. Interestingly some of the subunits of the proteasome are found in close vicinity to MHC genes, indicating a close developmental history [201,202]. The subunits encoded in the MHC region are incorporated into the proteasome after cellular stimulation with IFN- γ [203,204]. Thus, two types of proteasomes exist, the constitutive proteasomes, which are expressed in all somatic cells, and the immunoproteasomes, which are expressed under the influence of cytokines such as IFN- γ . Recently was shown that the additional subunits in the immunoproteasome are important for the specificity of the immune response [205,206].

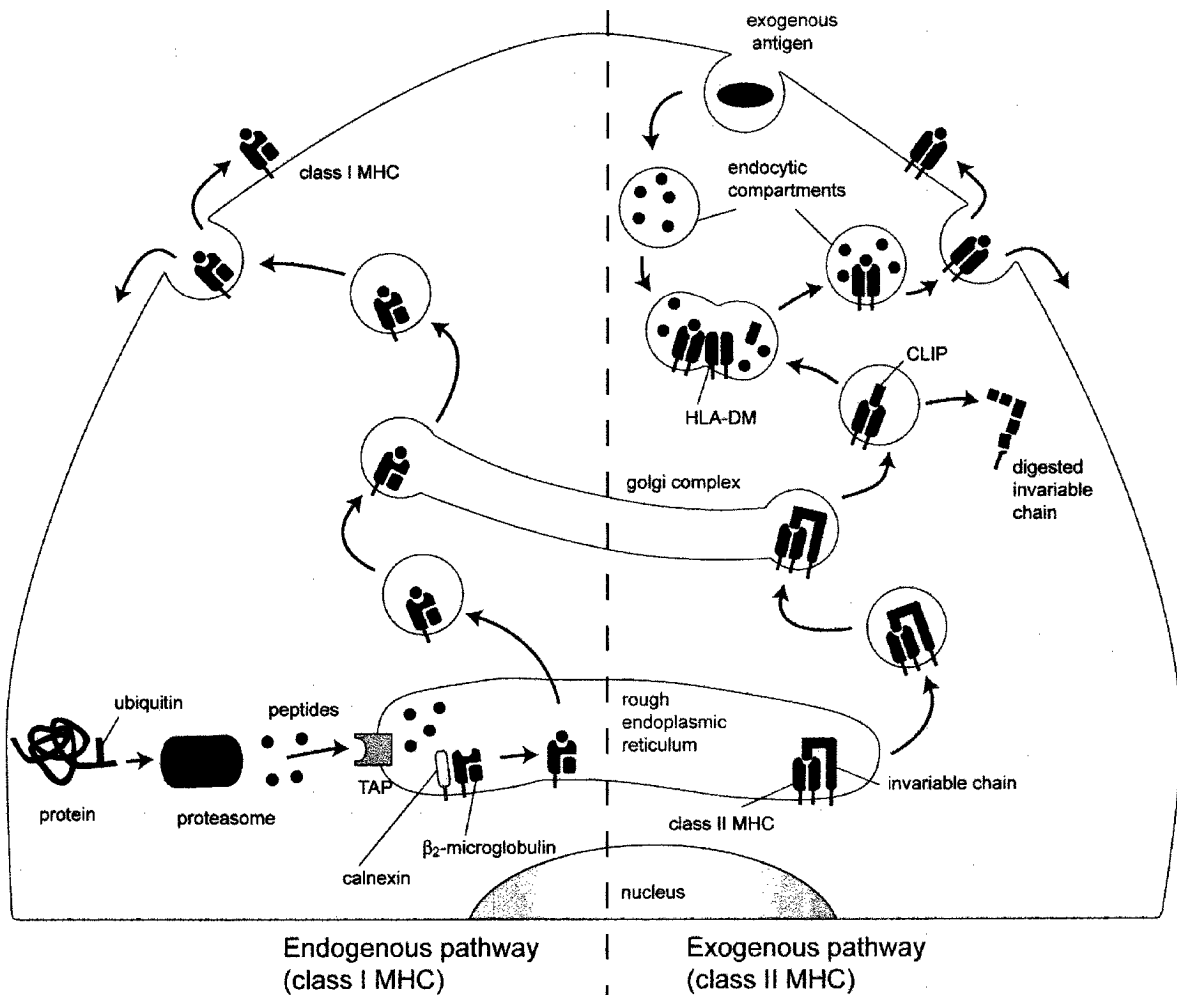


Figure 12. Model of separate antigen presenting pathways for endogenous and exogenous antigens: (adapted from Immunology, Janis Kuby) Endogenous antigen is processed by the proteasome, the peptide transported to the ER by the TAP transporter, and loaded on the MHC class I molecules. By vesicle transport the peptide-loaded MHC class I molecules get to the surface, where they present its load to CD8+ T cells. In the exogenous pathway, the antigen is taken up by phagocytosis. And the antigen gets cleaved in the endocytic compartments. Meanwhile the MHC class II molecule gets build in the ER and associated with the invariable chain. Then the MHC class II gets transported to the endocytic compartments, where the invariant chain gets cleaved to CLIP, staying associated until exchange with peptide, a reaction catalyzed by HLA-DM. The loaded MHC class II molecule reaches the cell surface and presents the antigen to CD4+ T cells.

The majority of antigenic peptides originate from newly synthesized proteins [207-209]. The proteasome is the enzyme complex that generates the correct carboxyl terminus of the MHC class I ligands. However many antigenic peptides are generated as amino-terminal-extended precursors. Since optimal peptides that bind to MHC class I molecules are 8-10 residues long and generally possess hydrophobic or positively charged C termini [210], they require amino-terminal trimming by aminopeptidases located either in the cytosol or in the ER [211-214]. For example one report describes a cytosolic leucine aminopeptidase, which is able to cleave a single residue from the amino terminus of a precursor peptide [211]. Additionally, also ER luminal peptidases were described [215-217].

TAP1 and TAP2 proteins are responsible for the transport of the peptides from the cytosol into the lumen of the ER [218,219]. Both proteins belong to the superfamily of ABC transporters, comprising a large number of membrane proteins, transporting a diverse set of molecules across membranes in an ATP-dependent manner (for review see) [220]. The peptide transport by the TAP heterodimer is a multistep process [221]. In a fast association step, the peptide binds to TAP [222], followed by a slow isomerization of the TAP complex [223]. It has been suggested that this structural reorganization triggers ATP hydrolysis and peptide translocation across the membrane.

In the ER the peptides are loaded on the MHC class I molecules. The MHC class I molecule is first complexed with chaperones, which allows a proper binding to the beta2-microglobulin [224]. After the MHC class I molecule is completed and stabilized by chaperones, tapasin guides it to the TAP transporter, where the peptides processed by the proteasome are directly bound to the MHC class I molecule [225-227]. Then the MHC class I molecule leaves the ER in micro vesicles through the golgi apparatus and reach the surface of the cell [228], where they can interact with the TCR of CD8+ T cells, which may be activated to kill the target cell.

The most important antigen presenting pathway in DCs is the class II route. Normally, peptides derived from exogenous proteins are presented by MHC class II molecules [229,230]. In contrast to MHC class I molecules the MHC class II molecules are exclusively present on APCs. APCs actively take up antigens, process and load them on MHC class II (Figure 12, right side). The antigens get encapsulated in the endosome or phagosome. Then they are processed and degraded in the late

endosome or liposome [231]. This is performed by pH dependent proteolytic enzymes like endopeptidases and exopeptidases, like the cathepsins [232]. Later the MHC class II molecules arrive in the vesicles containing the processed peptides from the pathogens [233].

The MHC class II molecules are synthesized in the ER out of three chains. Two transmembrane chains (alpha and beta) and an invariant chain [234] which covers the binding cleft of the MHC class II molecule and prevents peptides processed by the proteasome and actively transported to the ER from being loaded on the MHC class II molecule. This avoids a mixture of the class I and II pathway. However, such cross-presentation has been reported and postulated to be important for the establishment of a protective CTL response [235,236]. The invariant chain gets processed later in the transport from the ER to the late endosome where the peptides have been generated, which are then loaded on the MHC class II molecule. The invariant chain gets first processed by proteolytic enzymes into CLIP, a peptide that remains bound to the MHC. CLIP is then removed and exchanged against a pathogen-derived peptide in the late endosome [237,238]. This step is catalyzed and supported by another molecule called HLA-DM, which is homologous to MHC class II and acts as a chaperone [239-242]. The peptide loaded MHC class II molecule then reaches the cell surface and presents the peptide to CD4+ T cells.

As mentioned above there is a third pathway of presentation. Namely the presentation of hydrophobic peptides and glycolipids on the class I-like MHC called CD1. CD1 MHC molecules are built by an alpha chain homologous to MHC class I, which pairs with beta2 microglobulin to form a complete antigen presenting class I molecule (reviewed in [243]).

2.2.3. Migration to the lymphatic organs

In order to exert their function, DCs have to migrate to the lymphoid tissue to present their antigen to T and B cells. DCs find their way from the BM to the specific tissue by the guidance of chemokines. ImDCs express a variety of chemokine receptors, including CCR1 [244], CCR5 [245], CCR6 [246,247] and CXCR1 [248], which may participate in the recruitment of DCs to the inflamed tissue [249]. Differential expression may also contribute to the selective recruitment of imDCs to the different organs (Figure 13).

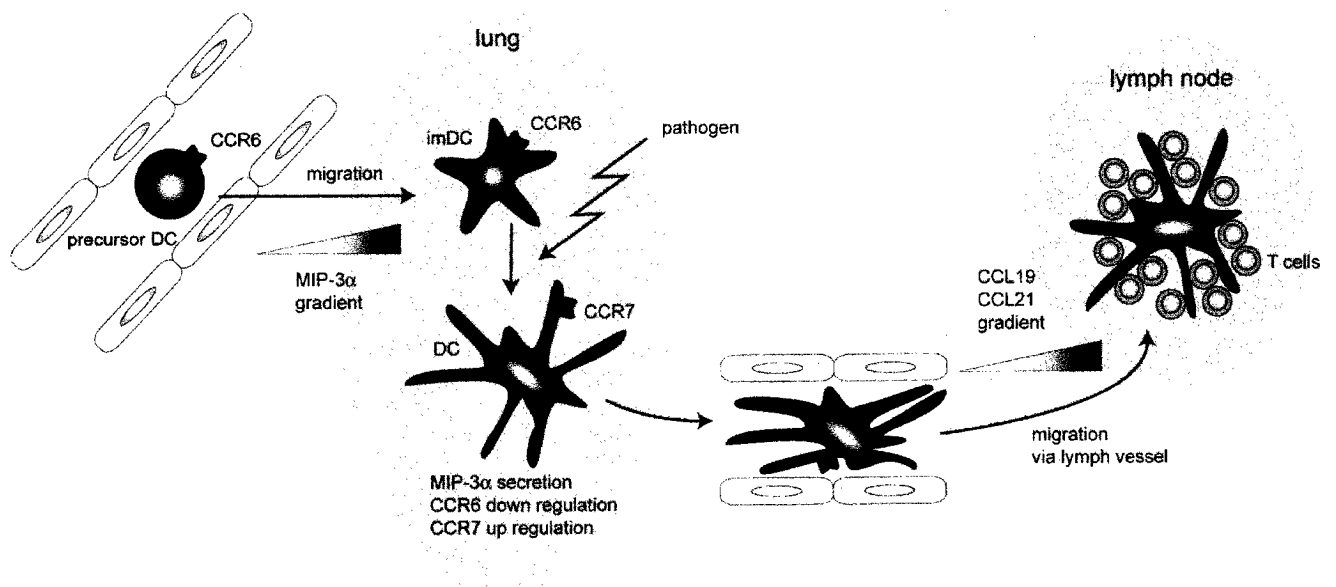


Figure 13. Trafficking of DCs: (adapted from Bleijs et al [250]) DC precursors are attracted from blood to tissue (here lung) by cytokines and chemokines (here MIP-3 α). Following transendothelial migration, the DCs capture antigen, get activated and start to mature. During maturation the receptor for MIP-3 α , CCR6 gets down-regulated, and the receptor for CCL19 and CCL21, CCR7 gets upregulated. To attract further DCs, the mature DC starts to secrete MIP-3 α . The secretion of the chemokine renders the mature DC unresponsive for the lung homing chemokine and allows the DC to follow the CCL19, CCL21 gradient guiding them to the lymph node. Arrived in the Lymph node the DC presents antigen to T cells inducing proliferation of antigen specific T cells.

For example, CCR6, the receptor of MIP-3 α , is expressed at high levels on imDCs in the lung [251,252]. In line with this is constitutive expression of MIP-3 α in the lung and in the liver [253]. Several organs express different chemokines for the steady-state recruitment of imDCs. During inflammation expression of additional chemokines is turned on – yet the mature DCs emigrate from the tissue into draining lymphoid organs. Therefore, there have to be pathways to make maturing DCs unresponsive to these recruitment chemokines, in order to enable them to migrate against the stream. Indeed, the DCs have evolved two different procedures for this task: First they down-regulate the chemokine receptors for the tissue-driving chemokines making them unresponsive [254]. Second they start to secrete these chemokines themselves [252], which acts as back-draft potentiating the recruitment of imDCs to the inflamed tissue [255].

The up-regulation of the CCR7 receptor on mature DCs is crucial for their migration to the lymphoid tissue [256,257], which renders them responsive to the chemokines CCL19 [258] and CCL21 [259,260]. CCL21 is expressed at high levels by high endothelial venules (HEV) in lymph node (LN) and by stromal cells in T cell areas of LNs, spleen and Peyer's patches [261-263]. CCL19 is secreted by a subset of DCs in T cell areas of lymphoid tissue [264]. Both were found to be important in T cell migration [260,264] and they are found to guide DCs to the lymphatic organs [251,252,265,266]. For CCL21 the function as migration driving force for DCs was found by experiments with plt mice, which carry a defect in the CCL21 gene. This mutation affects the expression of CCL21 and also negatively regulates CCL19 expression [267,268].

After the DCs left the inflamed tissue and enter the lymph vessel system, they follow the CCL21 and CCL19 gradient guiding them to the lymphatic organs. Once arrived there, they enter the organs (Figure 14). P-, E- or L-selectins allow the DCs to tether to the endothelium [269], which is essential for the later firm adhesion by leukocyte function-associated antigen-1 (LFA-1) and very late activation antigen 4 (VLA-4) [182,270], which bind to the vascular cell adhesion molecules intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [271] and final extravasation [272]. In the lymphatic organs the presentation and activation of specific T cells starts [273].

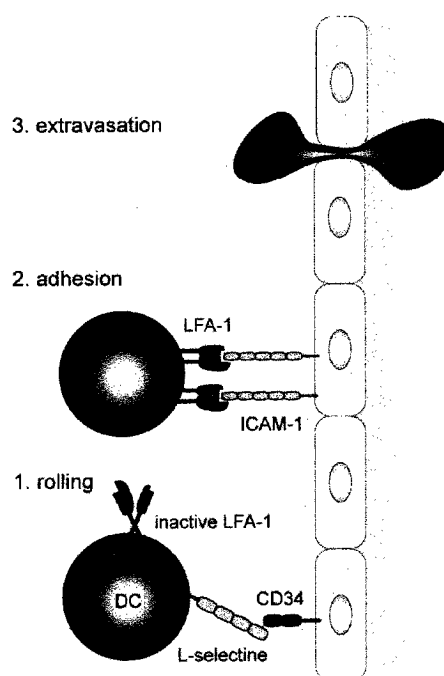


Figure 14. Extravasation of DCs into tissue: (adapted from Bleijs et al [250]) First the DCs start to roll along the endothelia of the tissue. The rolling is initiated by weak binding interactions between CD34 and L-selectin. Later a firm adhesion takes place by LFA-1-ICAM-1 interactions. And finally the DC extravasates through the endothelia into the tissue.

2.2.4. Interaction with T cells

The DCs display the captured and processed antigen to T cells in draining lymphoid organs. The TCR α and β chain dimer complex of T cells is responsible for specific recognition of peptide presented by MHC class I or II molecules on DCs. Additional molecules are enhancing this specific interaction (Figure 15).

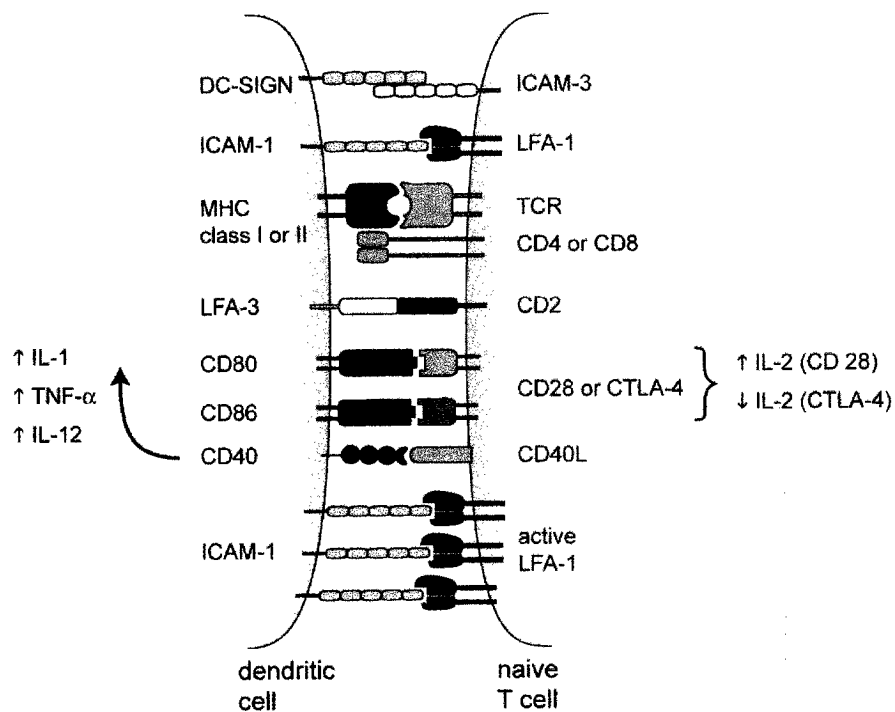


Figure 15. DC-T cell interaction: (adapted from Bleijs et al [250]) In the lymph nodes the DCs interact with T cells. First adhesion is mediated DC-SIGN-ICAM-3 interactions, thereby allowing subsequent ICAM-1-LFA-1 interactions. After full ligation of the TCR with the MHC molecules, LFA-1 on T cells is activated and allows a high affinity ICAM-1-LFA-1 interaction. The interaction of the two cells is further stabilized by LFA-3-CD2 interactions. The CD80/CD86-CD28/CTLA4 interaction mediates a second signal either stimulatory or inhibitory on T cells. Similarly the CD40-CD40L interaction induces activation of the DC, leading to cytokine secretion.

The initial contact between T cell and DCs is mediated by adhesion molecules like the β_2 -integrin LFA-1 on T cells and ICAM-1 on DCs [274]. Recently, a novel DC-specific adhesion receptor (DC-SIGN: DC-specific ICAM-3 grabbing non-integrin) has been identified, which binds with high affinity to ICAM-2 [275] and ICAM-3 [276] on T cells. Especially ICAM-3 is important in the initiation of an immune response [277]. The transient binding allows the T cells to encounter a large number of MHC/peptide complexes on different APCs. In the absence of a specific interaction the contact is short-lived and the T cells migrate to the next DC. This wandering of the T cells from one DC to the next was nicely shown in a recent study, where the movements of T cells and DCs were measured in a lymph node *in situ* [278]. But if the peptide on the MHC molecule is recognized by the TCR of the T cell, a specific contact is formed leading to the formation of an immunological synapse [279]. The TCR-MHC complex,

together with CD2-LFA-3 interactions is situated in the center of the synapse and LFA-1-ICAM-1 interactions are forming the outer ring. Following ligation of the TCR, LFA-1 on T cells is activated enhancing its ability to interact with ICAM-1. This stabilizes the immunological junction through the high avidity LFA-1-ICAM-1 and CD2-LFA-3 interactions [280,281].

However, these interactions are not sufficient to activate T cells. Additionally a second signal transmitted by co-stimulatory molecules on DCs is needed to efficiently activate the T cell. The most potent co-stimulatory molecules are the members of the B7-family, CD80 and CD86 [282]. CD80 and CD86 are members of the Ig superfamily and bind to CD28 and CTLA-4 on T lymphocytes [283], thereby delivering an activation or inhibitory signal [284,285]. CD28 is the main co-stimulatory ligand expressed on T cells [286]. CD28 stimulation has been shown to lead to an increased and prolonged expression of IL-2 and other cytokines [287,288]. This interaction is believed to be important for induction of an effective immune response and preventing tolerance [289]. But studies with CD28 knock-out (KO) mice clearly showed that CD28 triggering is not obligatory, as they are still responding to antigen [290]. Other co-stimulatory signals may substitute the co-stimulatory signal delivered by CD28/B7 like ICOS/ICOSL or OX40/OX40L [291,292]. CTLA-4, the alternative ligand for the B7 molecules, is an inhibitory receptor, leading to reduced IL-2 production [293].

Other surface molecules important in the recognition of the MHC-peptide molecules by the TCR are CD4 and CD8. The two major subsets of T cells, T_H or CTLs express CD4 or CD8, respectively. They act as co-receptors in the recognition of the MHC complex. CD8 interacts with MHC class I and CD4 with MHC class II (reviewed in [294]). Molecules that activate DCs upon engagement are also important. CD40, which is the best described example, is a member of the TNF receptor superfamily. Its ligand, CD40L, a TNF- α like molecule, is expressed on activated T lymphocytes [295]. The CD40-CD40L interaction initiates a retrograde activation signal on DCs which leads to the up-regulation of CD80 and CD86 [296].

Furthermore the secretion of cytokines like IL-1, TNF- α or IL-12 by the DCs is induced, supporting the activation of T cells and guiding their development [297-300]. IL-1 is a pluripotent immunomodulatory cytokine that has an initiating role in cellular and humoral immunity in the periphery [301]. IL-1 consists of two molecular species,

IL-1 α and IL-1 β , both of which exert similar, but not completely overlapping, biological functions through the IL-1–type I receptor [302]. IL-12 consists of two chains, a p40 protein and a p35 protein, which are both required for activity [303]. Mouse DCs are known to secrete functional IL-12. IL-12 is believed to bias the immune response towards a T_{H1} type [119,298]. The cytokine TNF- α and its receptors play key roles in the development of the immune system and in immune regulation, inflammation and autoimmunity [304]. Due to the ability of TNF- α to induce the production of other inflammatory cytokines and the ability to activate macrophages it is important during the process of inflammation [305]. This altogether starts the adaptive immune system, leading to an efficient T_H or CTL response.

3. AUTOIMMUNITY

The immune system evolved in order to protect higher organisms from infection with a tremendous variety of microorganisms like bacteria, viruses, protists, worms and fungi. Furthermore mechanisms evolved which prevent the development of cancer. Additionally, the innate immune system also disposes apoptotic cells or other waste from the normal cellular household. Although the immune system is designed to protect the organism, under certain circumstances it gets beyond control leading to massive damage to the organism by self-destruction. In these cases the immune system, instead of fighting against pathogens, develops to the worst foe itself by the induction of autoimmunity. The "horror autotoxicus" of the organism, as it was called by Paul Ehrlich at the beginning of the last century, is real.

Autoimmune disorders can be classified in organ-specific and systemic diseases. Organ-specific diseases are characterized by cell-mediated immune reactions leading to massive organ destruction. Important mediators of disease are T_{H1} cytokines such as IL-2 and IFN- γ and the effector responses tend to occur via cell-mediated immune responses such as killing by CTLs or through IgG and IgM antibodies directed Fc receptor-mediated killing. In contrast systemic autoimmune disorders are characterized by elevated levels of T_{H2} cytokines such as IL-4, IL-5, and IL-10 and important features are widespread circulation of auto-antibodies and immune complex deposition. Tissue damage is initiated by opsonization with antibody followed by complement-mediated cell lysis [306]. Some autoimmune syndromes demonstrate both organ-specific and systemic components. A classical example for an organ-specific autoimmune disease is Hashimoto's thyroiditis. These patients have developed auto-antibodies against thyroglobulin which react with the thyroid leading to highly localized lesions [307]. In contrast in the systemic lupus erythematosus (SLE), a systemic disease, the sera from patients react with many tissues in the body, since one of the dominant antibodies is directed against cell nucleus components [308,309].

But why is there self-reactivity in the repertoire of our immune system?

Because the immune system requires a wide diversity for its receptors to recognize the different pathogens, the adaptive immune system generates B cell receptors (BCRs) and TCRs randomly by gene rearrangement or even somatic mutation. This tremendous diversity raises the danger of auto-reactive antigen receptors, leading to self-reactivity. And actually a small number of auto-reactive B and T cells constitute a normal part of the immune cell pool, since the production of auto-antibodies is also frequently observed in normal healthy individuals. To avoid this self-reactivity, the immune system evolved several mechanisms summarized as self-tolerance. Autoimmune disorders are a direct result from the breakdown of immunologic tolerance. Therefore induction of self-tolerance is an important mechanism for the prevention of autoimmune disease and nature developed several different pathways to establish and maintain self-tolerance. Tolerance can be broadly classified in two groups: central tolerance and peripheral tolerance.

3.1. Central Tolerance

Central tolerance refers to the clonal deletion of self-reactive B and T cells during their maturation in the BM (B cells) [310,311] or the thymus (T cells) (Figure 15, upper panel) [55,312-315].

Auto-reactive B cells are eliminated at the stage of immature B cells, when they already express an antigen specific BCR [316-318]. Functional BCRs bind to extracellular molecules and initiate antigen specific cytoplasmic signaling [319-322]. If the BCR does not bind antigen, the signaling stays on basal level and the B cell is released in the peripheral circulation. If the immature B cell encounters antigen capable of crosslinking its BCR, this indicates that it responded to an auto-antigen and the B cell will be blocked from further development [323,324]. However, some autoreactive B cell clones escape deletion and enter the circulation in an anergic state [325]. Such anergic cells can potentially be activated with pathogen-derived ligands that mimic auto-antigens [326]. It has to be admitted that the negative selection for B cells is much less stringent than for T cells.

Clonal deletion of self-reacting T cells has been extensively investigated. Several lines of evidence suggest that T lymphocytes that bear receptors for self-antigens are forced to undergo apoptosis during the maturation in the thymus, a process called

negative selection. Detailed recapitulation of the development of the T cells in the thymus can be found in the T cell section.

However the mechanisms leading to central tolerance are leaky. This is due to a compromise the immune system takes to assure a complete repertoire of B and T cells. To further reduce the risk of self destruction the immune system evolved the mechanism of peripheral tolerance.

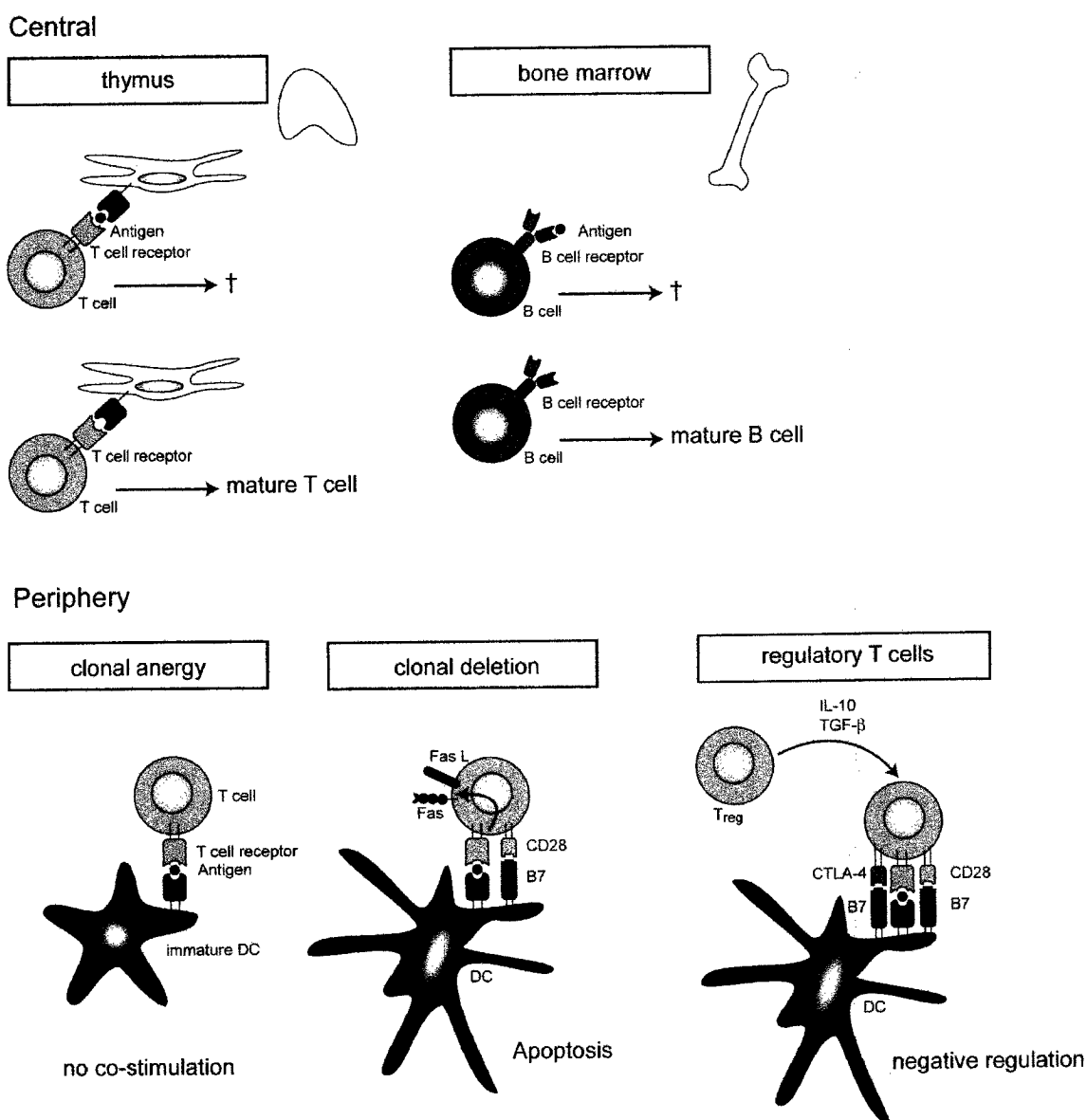


Figure 16. Central and peripheral Tolerance. Mechanisms leading to tolerance: Central tolerance: T and B cells undergo positive and negative selection in the thymus respectively in the bone marrow. In the thymus T cells were tested for their ability to recognize antigen presented on MHC molecules. T cells that recognize the MHC molecule are rescued (positive selection); T cells that recognize self-

antigen presented by MHC molecules are deleted (negative selection). In the bone marrow the B cells are tested for their self specificity, B cells binding to antigen are deleted.

Peripheral tolerance: In the periphery further mechanisms maintain tolerance: Clonal anergy: If self-antigen is presented by immature DCs, the specific T cells become anergic. Clonal deletion: Self-reactive T cells, constantly activated, upregulate the Fas molecule on the surface, which binds to FasL, leading to induction of apoptosis. Regulatory T cells: regulatory T cells can influence the activation of self-reactive T cells by the secretion of cytokines like IL-10 or TGF- β or by direct cell-cell contact (for example with CTLA-4).

3.2. Peripheral Tolerance

As outlined below, peripheral tolerance is induced by several different pathways (Figure 15, lower panel).

1. Clonal anergy: T cell anergy is a mechanism in which the lymphocyte is intrinsically functionally inactivated following an antigen encounter, but remains alive for an extended period of time in a hyporesponsive state. Anergy can be induced by submitogenic exposure to peptide antigen in the absence of a co-stimulatory signal provided by soluble cytokines or by interactions between co-stimulatory receptors on T cells and counter-receptors on antigen-presenting cells [289,327]. APCs do not upregulate these molecules prior to activation. Especially immature DCs which continuously sample and present auto-antigens to T cells [328,329], display low surface expression of MHC class I and II molecules and co-stimulatory B7 proteins and therefore induce tolerance to self-reactive T cells [179,182,330,331].

Other publications mention CTLA-4 and PD-1 as important mediators of anergy induction. Ligation of CTLA-4 was shown to be required for anergy *in vivo* [332] and PD-1 is highly expressed on anergic T cells [333]. Moreover although all cells present antigens on their surface the expression of co-stimulatory molecules is restricted to professional APCs. Hence, non-professional APCs are important mediators of tolerance to self antigens. Clonal anergy is also observed for B cells. If a B cell encounters an antigen in the absence of specific T help, the antigen-receptor is down-regulated and the cell can no longer be activated [334].

2. Clonal deletion by activation-induced cell death (AICD): Pathway of T cell suicide often involving upregulation of Fas ligand that binds to the death receptor Fas. It is postulated that repetitive TCR engagement in T cells specific for self-antigen may serve as trigger for AICD [335-342]. All Lymphocytes express Fas

(CD95), a member of the TNF-receptor-family. Fas ligand (FasL), structurally homologous to TNF, is expressed on activated T lymphocytes. This co-expression of the receptor-ligand pair, leads to Fas mediated apoptosis in activated T cells. The importance of this mechanism for the elimination of self-reactive T cells is highlighted by two mouse strains where Fas-FasL pathway is naturally deleted. Whereas Lpr mice have a mutation in the Fas gene [343], gld mice carry mutations in the FasL gene [344] and both mouse strains spontaneously develop a severe autoimmune disease resembling SLE. Interestingly also IL-2 was shown to play a crucial role in AICD, as T cells from mice that lack IL-2 or IL-2 receptor fail to undergo Fas-mediated AICD [338,345]. The ability of IL-2 to promote apoptosis was linked to STAT5-mediated induction of the Fas ligand [346]. Consistent with a role of IL-2 in peripheral tolerance, mice deficient in IL-2 production or signaling show autoimmunity [347,348].

3. Peripheral suppression by T cells: A subset of T cells called suppressor or T_{regs} have the ability to inhibit T cell responses [127,132]. This inhibition has been postulated to be transmitted mainly by cytokines, however surface receptors appear to be implicated as well. For example some T_{regs} constitutively express CTLA-4 [349], which counteracts the activation signal transmitted by CD28 [6]. Others secrete the inhibitory cytokines TGF- β and IL-10 [350,351]. Further evidence for the role of inhibitory cytokines in peripheral tolerance arise from observations made in TGF- β KO mice which display inflammatory lesions similar to those seen in some autoimmune diseases [352].

Also T_{H} cells have been shown to be implicated in the regulation of peripheral tolerance. For example, $T_{\text{H}2}$ cells have been implicated in mediating self-tolerance by regulating the pathogenic function of self-specific $T_{\text{H}1}$ cells. Certainly, cytokines produced by $T_{\text{H}2}$ cells can down-regulate $T_{\text{H}1}$ responses and vice versa [353-356].

Considering how vital the prevention of autoimmunity is for survival, several mechanisms evolved to prevent it. Like T helper cells are important regulators of humoral and cellular immunity, tolerance of self-reactive T cells is crucial for the prevention of autoimmune diseases. However, under certain conditions, tolerance can be broken and an autoimmune pathology may result. It has been suggested that T lymphocytes are the primary players in the initiation and perpetuation of both spontaneous and chemical-induced autoimmune diseases [357,358]. This has been shown in animal models in which autoimmunity could be transferred by adoptive

transfer. Moreover the transferred autoimmunity could be reversed by the selective depletion of T_H cells with specific antibodies or complement [359-361].

3.3. Mechanism leading to autoimmunity

Several different mechanisms can lead to autoimmunity. The development of autoimmune disease is not only dependent on a permissive genetic background but other factors such as viral, bacterial, or chemical insult may lead to self-reactivity. Often development of autoimmune disease results from a combination of several different events. Although most pathways leading to autoimmunity bypass the mechanisms leading to peripheral tolerance some circumvent the central tolerance.

3.3.1. Release of isolated auto-antigens

Auto-reactive T cells with specificity for self-antigens absent in the thymus during the early stages of T-cell development may escape thymic deletion. These antigens generally have relatively low circulating levels or are anatomically sequestered in specific tissues (e.g., myelin basic protein or thyroglobulin) where vascular and/or cellular basement membranes constitute an effective barrier that prevents access of the antigen to auto-reactive T cells. Destruction of these basement membranes and following availability of antigen may lead to the induction of autoimmune diseases. Induction of organ-specific autoimmune diseases following tissue trauma have been frequently reported and likely occur via tissue damage that results in the availability of previously isolated antigens, as in the case of orchiditis following vasectomy [362,363]. Infection with tissue-tropic pathogens such as viruses may induce similar autoimmune phenomenon, and these infections also provide the additional stimulus of the production of soluble mediators and co-stimulatory molecules important in the perpetuation of the immune response. This is best exemplified in rodents and humans who develop diabetes after infection with Coxsackie B viruses [364].

3.3.2. Breakdown of T cell anergy

Self-reactive T cells which escape clonal deletion in the thymus are rendered anergic if they meet their specific antigen without additional activation by co-stimulatory molecules or cytokines. But T cell tolerance may be broken by the

delivery of a co-stimulatory signal. This may occur after infections resulting in tissue necrosis and local inflammation. For example in multiple sclerosis (MS) up-regulation of B7.1 molecules could be detected in the central nervous system of patients [365]. Similarly induction of B7.1 has been observed in the synovium of patients with rheumatoid arthritis (RA) [366]. Finally, similar observations were made in a transgenic mouse model of insulin-dependent (type1) diabetes mellitus. In this model a viral peptide from the LCMV is expressed under the control of the insulin promoter as a transgene on islet β cells [367]. Normally, these mice do not develop autoimmunity, but if B7.1 is co-expressed on the β -islet cells, peripheral tolerance is broken and autoimmunity develops [368].

3.3.3. Chemical Alteration of Self-Peptides

T lymphocytes that bind with low affinity to self-antigens in the thymus may not be deleted. However, these T cells are normally functionally anergized in the periphery. But responses to and presentation of these self-peptides can be enhanced under certain conditions. Some metals induce autoimmune disease via the creation of new high-affinity binding sites for MHC molecules on chemical-bound self-peptides, allowing activation of previously anergized T cells [369]. Expression of altered nucleolar proteins appears to be an important step in the development of mercuric chloride (HgCl_2)-induced autoimmunity in a rodent model of SLE [370]. In addition, many drugs induce autoimmunity via formation of hapten-induced auto-antibodies. For example halothane induce reactions in which hapten-specific T cells provide help to antibody-producing B cells that recognize the modified hapten but not the native form of the self-protein [371-373].

3.3.4. Defective Activation-Induced cell death (AICD)

In AICD auto-reactive T cells undergo apoptosis via the Fas-FasL pathway induced by persistent activation. Therefore, defects in this pathway may rescue auto-reactive T cells which then induce autoimmunity as described for the spontaneous KO mice for these two molecules, which have a similar phenotype to the SLE. In SLE patients rarely mutations in the Fas or FasL gene are observed, however other defects in AICD may contribute to human autoimmune diseases. For example one report shows that T cells from Lupus patients become resistant to apoptosis through

the upregulation of cyclooxygenase-2 (COX-2), which is an inhibitor of the Fas-FasL pathway. Notably, the gene encoding COX-2 is located in a lupus-susceptibility region on chromosome 1. Further the authors could show that COX-2 inhibitors were able to suppress the production of pathogenic auto-antibodies to DNA by causing autoimmune T-cell apoptosis [374].

3.3.5. Defective T cell-mediated suppression

In animal models the loss of the T_{regs} can lead to the induction of autoimmunity. The idea that regulatory T cells can limit the function of auto-reactive T and B cells is quite attractive. It offers the possibility to treat autoimmune diseases by generation and application of such cells to patients. At least in animal models this approach was successful. It was shown that these cells were able to prevent autoimmune colitis in a mouse model [375]. The authors show that chronic activation of both human and murine CD4⁺ T cells in the presence of IL-10 gives rise to T_{regs} with low proliferative capacity, producing high levels of IL-10, low levels of IL-2 and no IL-4, which suppress the proliferation of CD4⁺ T cells in response to antigen and prevent colitis induced in SCID mice by pathogenic CD4⁺CD45RB(high) T cells. In a second study the authors generated T_{regs} from peripheral naïve CD4⁺ T cells and showed that transfer of these cells in a murine asthma model prevented house dust mite-induced allergic pathogenesis in lungs [376]. But T_{regs} are not representing a uniform population of cells, which can be easily defined by unique surface markers. Therefore the successful use of these cells as a tool to treat autoimmune diseases still needs further improvement.

3.3.6. Molecular Mimicry

Many peptide fragments of infectious agents are homologous with host proteins and perhaps induce organ-specific autoimmune responses. It is well known that rheumatic heart disease sometimes follows a streptococcal infection. And indeed it was found that a membrane protein on the β -hemolytic streptococcus bacterium has a high degree of homology with cardiac myosin, and antibodies that target the bacterium also cross-react with cardiac muscle and induce rheumatic fever [377,378]. *Yersinia enterocolitica*, a bacterium normally associated with food poisoning outbreaks, has also been associated with various autoimmune diseases. Increased

levels of antibodies to Yersinia have been demonstrated in patients with Graves disease or autoimmune thyroiditis [379,380]. These antibodies cross-react with a variety of thyroid antigens [381,382]. The most compelling data supporting this concept are coming from studies with myelin basic protein-reactive T cell clones, derived from patients with MS. There, it was found that T cell clones seem to react even more strongly with viral peptides than peptides derived from the myelin basic protein [383].

3.3.7. Polyclonal Activators

Superantigens activate T cells through the variable domain of the TCR β chain. This distinctive mode of T cell activation, together with the ability of superantigens to bind to a wide variety of MHC class II molecules, leads to activation of large numbers of T cells irrespective of their MHC/peptide specificity [384]. The *Mycoplasma arthritidis* superantigen stimulates cytokine production and up regulates MHC class II expression in human T cell lines [385] and stimulates T lymphocytes with arthritis-associated TCR- β chains [386]. In a rodent model, *Mycoplasma arthritidis* superantigen stimulated T_H cells, resulting in polyclonal B-cell activation and/or differentiation of antigen-specific B cells [387]. Thus, autoimmunity may result from unspecific activation of T and/or B cells via superantigens. Such a mechanism may contribute to the pathogenesis of type I diabetes in some cases [388].

3.3.8. Exposure of Cryptic Self and epitope spreading

During the past years it got obvious that molecular sequestration of antigens is much more common than the anatomic sequestration. For each protein just few antigenic determinants are effectively processed and presented to T cells. During development the T cells specific for self-antigens are either deleted or anergized. But the T cells for cryptic self-antigens did not get deleted. Therefore if the cryptic antigens are presented in an optimal way, an immune response against self can be induced. But the mechanisms for rendering a cryptic antigen optimal for presentation are not well understood [389].

Another mechanism important for the exaggeration of autoimmune diseases is epitope spreading. During the development of an autoimmune disease a shift can be

observed from the antigenic epitope originally inducing autoimmunity to others which also start to trigger autoimmunity. As shown for EAE in mice, where the autoimmune response shifted from the original antigen to epitopes released as a result of acute tissue damage due to the immunopathogenesis of relapsing clinical episodes [390].

3.3.9. Genetic Factors

Familial studies suggest a clear association between genetics and autoimmune diseases, particularly those with an organ-specific pathology [391]. Further, although concordance rates between identical twins can be relatively low depending on the disease, this may be explained by non-identity in immune repertoires because of TCR and Ig gene recombination, variations in receptor assembly, and somatic mutation of B-cell receptors. The most clearly established genetic association is with specific alleles within the MHC gene complex. Certain haplotypes (HLA-B8, DR2-DR5) tend to be associated with certain autoimmune diseases (reviewed in [392,393]). However, a specific MHC haplotype is not sufficient for development of autoimmune disease, and autoimmunity-associated haplotypes are found in individuals with no clinical signs of disease.

There are also a number of non-MHC genes that may contribute to autoimmunity. Many studies have examined the possibility that predisposition for certain autoimmune diseases in the human population may lie in the germline genes for the TCR (reviewed in [394,395]). The strongest evidence for TCR involvement arises from studies of sibling pairs with recurrent/relapsing MS. They shared specific TCR- β haplotypes at a frequency much higher than would be expected based on random segregation [396]. TCR-gene polymorphisms have also been associated with disease susceptibility [397].

RESULTS

4. EXPRESSION CLONING IDENTIFIES THE CC-CHEMOKINE CCL19 AS POTENT ACTIVATOR OF DENDRITIC CELLS

Patrick Bättig¹, Monika Bauer¹, Urs Eriksson², Christiane Ruedl¹, Ute Lässig¹, Roger Beerli¹, Klaus Dietmeier¹, Lidia Ivanova¹, Thomas Pfister¹, Lorenz Vogt¹, Manfred Kopf³, Philippe Saudan¹ and Martin F. Bachmann¹

¹Cytos Biotechnology AG, Wagistr 25, 8952 Zurich-Schlieren, Switzerland

²Department of Research and Medicine, University Hospital, Petersgraben 4, CH-4031 Basel, Switzerland

³Molecular Biomedicine, Department of Environmental Sciences, Swiss Federal Institute of Technology, CH-8092 Zurich, Switzerland

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4.1. Abstract

Dendritic cells (DCs) are key regulators for the induction of immune responses. They capture antigen, process it and, upon activation, migrate to lymphoid organs where T cell priming occurs. Using an alpha-viral expression cloning technology for the identification of proteins that enhance T cell proliferation in a DC-T cell co-culture system, we isolated the chemokine CCL19 as potent inducer of proliferation. Subsequent studies showed that CCL19 enhanced T cell proliferation by inducing maturation of DCs, resulting in up-regulation of co-stimulatory molecules and the production of the pro-inflammatory cytokines IL-1 β , IL-12 and TNF- α . CCL19-stimulated DCs were highly immunogenic *in vivo* and were able to cause autoimmune myocarditis if pulsed with myosin-derived peptides. Our findings suggest CCL19 as a potent natural adjuvant for the activation of DCs, and suggest that chemokines not only orchestrate DC migration but also regulate their immunogenic potential.

4.2. Introduction

Dendritic cells (DCs) are key initiators of innate and adaptive immunity [398,399]. They are potent antigen processing and presenting cells with the unique ability to stimulate primary immune responses and boosting secondary immune reactions [400]. Immature DC precursors exit the BM and circulate via the bloodstream to reach their target tissues, taking up residence at sites of potential pathogen entry in a physiological stage that is specialized for antigen capture [273]. After uptake of antigen by phagocytosis, macropinocytosis or via receptor-mediated endocytosis [182,401], antigen is processed and peptides thereof are presented on the cell surface associated with the MHC class I or II molecules [402]. After receiving maturation inducing signals either directly from pathogens or via inflammatory stimuli, DCs change the expression pattern of chemokine receptors, allowing them to leave the peripheral tissue and to migrate to draining lymphoid organs [403]. Upregulation of CCR7 during maturation renders DCs sensitive to two chemoattractants, MIP-3b/CCL19 and 6kine/CCL21, which direct the migration [187,404]. Arrived in the T cell regions of lymphoid organs, DCs can induce both activation and proliferation of specific CTLs and T_H cells via presentation of immunogenic peptides in association with self-MHC class I and II molecules, respectively [270,399]. Besides protection against pathogens, DCs appear to be central to the regulation, maturation, and maintenance of a cellular immune response to cancer [405,406]. In contrast, in the absence of an activation stimulus, DCs induce T cell tolerance rather than activation [177,178,407,408]. Thus, regulation of DC-activation is central for the establishment of protective T cell responses as well as for the maintenance of T cell tolerance [409].

Pathogen-mediated APC activation is usually transduced via the Toll like receptor (TLR) family [410-412]. TLR recognize invariable patterns associated with pathogens like LPS (TLR4) [413,414], double-stranded RNA (TLR3) [415], flagellin (TLR5) [416] or bacterial DNA (TLR9) [417,418]. But APC activation is also induced by pro-inflammatory cytokines like TNF- α [419], IL-1 β [420,421] or IL-7 [422] secreted from the surrounding inflamed tissue. Members of the TNF superfamily, in particular CD40L and Trance/RANKL are additional and potent stimuli for the maturation of DCs both *in vitro* and *in vivo* [423-425]. Recently, chemokine receptors

can have been shown to be directly involved in induction of DC-maturation. Specifically, ligation of CCR5 by toxoplasmosis-derived cyclophilin led to production of IL-12 in DCs [426]. The importance of DC-maturation for the generation of protective T cell responses is highlighted by the fact that several viruses interfere with activation of DCs. Poxviruses inhibit DCs maturation by lowering the expression of CD86 level, leading to reduced T cell co-stimulation [427]. Along a similar line, measles virus engages the CD46 molecule on DCs, inhibiting the expression of IL-12 [428].

To identify novel proteins capable of regulating T cell responses by activation of DCs, we performed an alphavirus based expression cloning campaign. We used sindbis virus as carrier of cDNA from activated splenocytes normalized against naïve splenocytes and generated by infection of BHK cells a protein library [1]. Sindbis is a single-stranded RNA virus with positive polarity belonging to alphavirus genus. Using a bipartite system, where one RNA molecule encodes the structural proteins and the other encodes the protein of interest plus the viral replicase, it is possible to generate replication competent encoding genes or even libraries of interest.

We report here the identification of mouse CCL19 as a potent maturation factor for DCs and demonstrate that CCL19 can be used to activate DCs *ex vivo* to induce *in vivo* an autoimmune disease in a myocarditis model. These findings indicate that induction of DC maturation is an important property of CCL19 and suggest that chemokines may not only organize the migration of DCs but also directly regulate their ability to prime T cell responses.

4.3. Material and Methods

4.3.1. Mice

BALB/c and C57BL/6 mice were purchased from Harlan (Horst, The Netherlands) and used at the age of 8–14 wk. DO11.10 TCR-transgenic mice were obtained from the breeding colony at the Institut für Labortierkunde (University of Zurich, Zurich, Switzerland). Animals were kept under SPF conditions.

4.3.2. Construction of normalized cDNA libraries

RNA was isolated from LCMV-activated mouse splenocytes of C57BL/6 mice. Tester cDNA was produced from polyA⁺ RNA using the template switch protocol [429]. Photobiotinylated polyA⁺ RNA from naïve splenocytes was used as driver for the normalization. Therefore 10 mg RNA was mixed with 30 mg photobiotin acetate and irradiated for 20 minutes using a 300W Phillips Reflector Floodlamp. Free photobiotin was removed. For hybridization, 2 mg tester cDNA was mixed with 2 mg biotinylated driver polyA⁺ RNA and 0.2 mg oligo-dT (20mer), ethanol precipitated, and resuspended in formaldehyde hybridization buffer (80% formaldehyde, 25 mM HEPES pH 7.5, 250 mM NaCl, 5 mM EDTA). Hybridization was carried out in an Eppendorf Mastercycler personal. After an initial denaturation step, nucleic acid hybridization was allowed to proceed for 16 hours at 42 °C. Unhybridized single-stranded cDNA was purified by streptavidin-mediated removal of RNA/DNA hybrids and excess driver RNA. Double-stranded cDNA was produced by PCR, digested with the restriction endonuclease Sfi1 (Roche) and cloned into the alphaviral expression vector pDelSfi. The library plasmid linearized with NotI or PaeI and the helper plasmid pDHEB [430] linearized with EcoRI were subjected to SP6 RNA polymerase-mediated *in vitro* transcription using the mMessage mMachine™ kit (Ambion). Library RNA was co-electroporated with an equimolar amount of helper RNA into 10⁷ Baby hamster kidney (BHK) cells. 18 hours post transfection, cell supernatants were harvested and the viral titers determined.

4.3.3. Generation of protein library

BHK cells were infected with the normalized viral library at a multiplicity of infection of 0.1. Two hours later, cells were washed and further incubated in the presence of a neutralizing anti-Sindbis antibody to avoid superinfection. 8 hours post-infection cells were detached and stained for the expression of sindbis virus proteins and single cell sorted into 96-well plate containing BHK cells. Plates were then incubated for 3 days at 37 °C, until full cytopathic effect had developed. A master plate with 50 µl supernatant containing replication competent viruses was made and stored at -80 °C. The remaining supernatants were UV irradiated in order to inactivate viruses and then used for the DC-T cell proliferation assay. The procedure is schematically shown in Figure 1A.

4.3.4. Preparation of dendritic cells and T-cells

Immature mouse dendritic cells (DC) were isolated from spleens and mesenteric lymph nodes of naïve BALB/c mice as described [431]. Purified DCs were pulsed with 4nM of OVA-peptide (Ovalbumin (OVA) 323-339; ISQAVHAAHAEINEAGR). OVA-peptide-specific CD4⁺-T cells were isolated from spleens of naïve DO11.10 mice using MACS beads according to the instructions of the supplier (Miltenyi Biotec). Bone marrow derived dendritic cells (BM-DCs) were prepared from bone marrow of naïve BALB/c mice as described elsewhere [432].

For DC maturation, cultures were pulsed for 4h or 18h with TNF-α (50 ng/ml; R&D systems) or LPS (0.1 µg/ml; Sigma-Aldrich; E. coli, 026:B6) with or without anti-CD40 (clone3/23, 5 µg/ml; BD PharMingen) or FLAG-CCL19 (0.33 µg/ml or 0.99 µg/ml). Alternatively these samples were treated with 10 µg/ml proteinase K (Sigma-Aldrich) for 45 min at 37°C in DPBS (containing Ca²⁺ and Mg²⁺) and afterwards boiled at 95°C for 10 min.

4.3.5. Functional expression screening of DC maturation factors

Maturation of dendritic cells was determined via their capacity to induce T cell proliferation in the presence of specific antigen. Briefly, 30000 naïve OVA-peptide pulsed dendritic cells/well were seeded in 96-well round bottom plates. 50 µl of UV-irradiated viral supernatant containing one protein of the normalized activated mouse spleen library was added and the plates were incubated at 37°C. As controls

supernatants containing virally expressed TNF- α or supernatants from SR5-infected BHK cells were used. After 18-20 hours 50000 naïve OVA-specific CD4⁺ cells were added to each well and the plates were further incubated for 2.5 days. ³H-thymidine (1mCi/well) was added for the final 14 h of culture. Cells were harvested using a multiple-well harvester and ³H-thymidine incorporation was determined in a scintillation counter. Viral supernatants leading to maturation of dendritic cells resulting in high ³H-thymidine incorporation into CD4⁺ cells were reanalysed in a second proliferation assay for confirmation.

4.3.6. RT-PCR rescue of positive supernatants

RT-PCR was performed on supernatants from wells which showed increased proliferation rates. Therefore viral RNA was isolated from supernatant using the QIAmp Viral RNA Kit (Qiagen). RNA was transcribed into cDNA using SUPERSRIPTTM II RNase H- reverse transcriptase (Invitrogen Life Technologies). Complementary RNA was removed by RNase H treatment. The genes were amplified by PCR using Expand High Fidelity PCR System (Roche) and then sequenced.

Further subcloning of CCL19 into a pCEP-N-FLAG-C vector with gene specific primers bearing a BamHI-site at N-terminus (5'-atcgcgGATCCCGGTGCTAATGATGCGGAAGA-3') and a NheI-site at the C-terminus (5'-ctatactagctagctcaAGACACAGGGCTCCTTCTGG-3') was performed. This construct was then used for protein production.

4.3.7. Expression and purification of the recombinant proteins

293-EBNA cells were seeded at a density of 4×10^6 cells/ 90mm plate in DMEM (GIBCO BRL) containing 10% FCS (GIBCO BRL) 1 day prior to transfection. The cells were then transfected with lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation. One day after transfection cells were passed under puromycin selection. The resistant population was then further expanded. Serum-free supernatants were collected and an affinity purification with ANTI-FLAG[®] M2-Agarose-sepharose Affinity Gel (SIGMA) was performed according to the manufacturers manual. For elution the Flag-tagged protein FLAG peptide (Asp-Tyr-

Lys-Asp-Asp-Asp-Asp-Lys) 100 µg/ml in PBS was used. After extensive dialysis against PBS, the proteins were stored in PBS at 4°C. The protein concentrations were determined by Bradford (Biorad).

4.3.8. CFSE in vitro proliferation assay

CFSE was purchased from Molecular Probes (Eugene, OR). BM-DCs were activated as described above for 4h. Then DO11.10 transgenic CD4⁺ T cells were added, obtained by positive MACS MicroBeads isolation (Miltenyi Biotec) with a purity of at least 90%. The T cells were labelled 0.5 µM CFSE by incubation at 37°C for 10 min. After labelling, the cells were subsequently washed with PBS at 4°C. After four days incubation cells were harvested, stained with PE labelled anti-CD4 mab (0.1 µg/ml; BD PharMingen) and the CD4⁺ T cells were analysed for CFSE staining by FACS analysis.

4.3.9. Staining for FCM and ELISA

The following antibodies were used for staining: FITC-conjugated anti-CD86, FITC-conjugated anti-CD80, PE-conjugated anti-CD11c, allophycocyanin-conjugated anti-TNF-α, biotin-conjugated anti-IL-1β, biotin-conjugated anti-IL-12 Abs (all BD Pharmingen), ALEXA633-conjugated Streptactin (IBA). Dead cells were stained with PI and gated out. Intracellular cytokine stainings were performed according to standard protocols.

For detection of IL-1β and IL-12 produced from BM-DCs, cells were cultured for 18h with indicated stimuli. Then the supernatants were analyzed by sandwich ELISA for IL-1β (R&D Systems) and IL-12 (BD PharMingen). As standards recombinant IL-1β and IL-12 (both R&D systems) were used.

4.3.10. Induction of experimental autoimmune myocarditis

BM-DCs were generated as described [432]. In order to minimize spontaneous maturation of DCs, cultures were supplemented with IL-10 (10 ng/ml). For the induction of experimental autoimmune myocarditis, DCs were loaded with a murine heart muscle specific peptide derived from α-myosin H chain [433]. DCs were treated subsequently with different stimuli including 0.1 µg/ml LPS plus 5 µg/ml anti-CD40 ab

or 0.33 µg/ml Flag-CCL19 for 4 h and injected i.p. (2×10^5 cells/mouse) on days 0 and 2 and 4. Non-stimulated DCs were injected as negative control. On day 10 mice were sacrificed and the hearts were removed and the grade of myocarditis was determined by histology.

A vague increase in interstitial cellularity was not considered sufficient for diagnosis. Myocarditis was scored on a semi-quantitative scale using grades from 0 to 4 (0, no inflammatory infiltrates; 1, small foci of inflammatory cells between myocytes or inflammatory cells surrounding individual myocytes; 2, larger foci of >100 inflammatory cells or involving >30 myocytes; 3, >10% of a myocardial cross-section involved; and 4, >30% of a myocardial cross-section involved).

4.4. Results

4.4.1. Functional cloning of CCL19 as inducer of T cell proliferation in DC-T cell mixed culture assays

As DCs are crucial for the induction of immune responses, we set out to identify novel DC maturation factors using an alphaviral screening technology.

We generated a normalized activated spleen cDNA library and transferred it into an alphaviral expression vector. Originally the RNA was extracted from spleens of LCMV-infected BALB/c mice, transcribed in cDNA and normalized against splenocyte RNA as described in Materials and Methods. The cDNA library was transformed into a viral library by *in vitro* transcription and electroporation together with a viral helper construct.

Following the protocol shown in Figure 1A BHK cells were infected with an MOI of 0.1 and single infected cells were sorted on subconfluent BHKs in 96 well plates. This led to the generation of a protein library within 3 days, where each well corresponds to the expression of one different protein. The protein containing supernatants were harvested and remaining virus inactivated by UV-light. Spleen derived DCs were treated with the supernatants over night and pulsed with OVA peptide. The next day OVA-specific CD4⁺ T cells were added. Proliferation was assessed 2.5 days later by ³H-Thymidine incorporation.

The average counts per plate were calculated and every well which showed a count/min value two standard deviations above the average was further evaluated, the virus recovered and the cDNA sequence determined (Figure 1B). Out of the library 10000 genes in total were tested for induction of T cell proliferation in the assay. CCL19 was identified two times. One plate where CCL19 was identified is shown in Figure 1B.

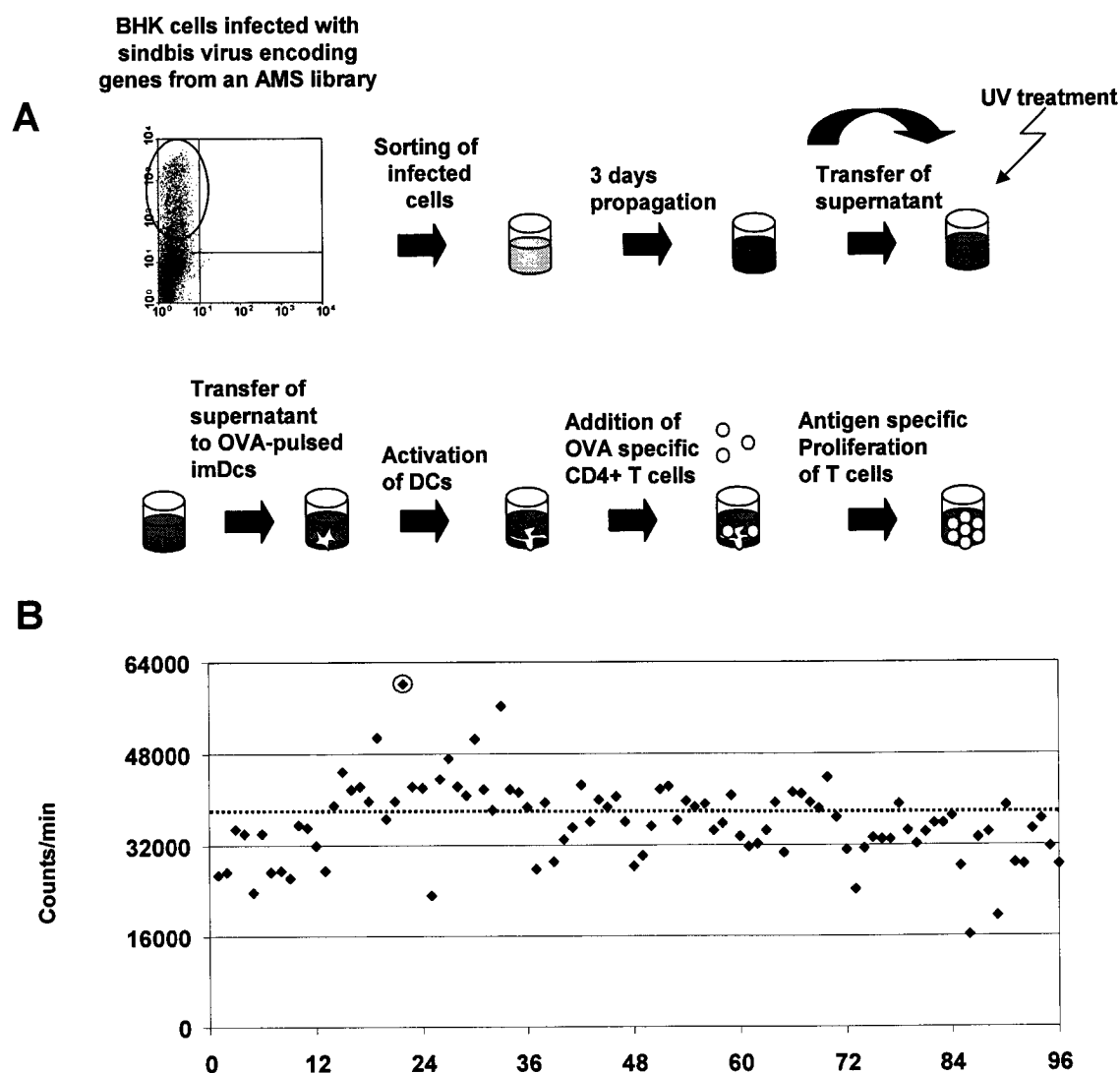


Figure 1(17). Outline of the screening strategy and CCL19 as one of the results of the screening campaign: **A.** BHK cells were infected with a MOI of 0.1 with sindbis virus encoding genes from an activated mouse spleen library. Infected cells (stained with anti-sindbis specific antibody) were sorted one cell/well on BHK cells seeded in 96 well plates. After 3 days propagation the supernatant of the infected wells were harvested, UV treated and transferred on OVA-peptide pulsed spleen-derived DCs. After over night treatment OVA-specific MACS-beads purified CD4⁺ DO11.10 T cells were added to the wells. Read-out for activation of DCs was incorporation of H³-thymidine into proliferating T cells. **B.** A 96 well plate is shown were one well showed substantially increased proliferation of OVA-specific CD4⁺ T cells (encircled point). RNA from the positive well was extracted, transcribed by a RT-PCR step into DNA and sequenced. The virus encoded gene turned out to be CCL19. Y-axis counts/min, X-axis location on the 96 well plate. Pointed line indicates counts/min average of the plate.

4.4.2. CCL19 induces maturation of dendritic cells

To reconfirm that CCL19 is inducing proliferation in the DC-T cell co-culture system, the RNA from the virus was recovered, transcribed in DNA and the sequence cloned into the alphaviral vector. Virus was generated, BHK cells were infected and the supernatant was retested (Figure 2A). As we were interested in DC activation factors, we wanted to exclude a possible direct effect of CCL19 on T cells. Therefore the supernatant was tested directly on T cells showing no effect on the proliferation (Data not shown). From this we concluded that CCL19 influences DCs directly. To test, whether CCL19 may enhance T cell proliferation by activating DCs, we treated spleen derived DCs with the supernatants over night and analyzed them for surface expression of CD40 and CD86 by FACS staining with specific antibodies. CD40 and CD86 are both important molecules for T cell activation and are up-regulated upon DC maturation. We found a significantly higher part of mature DCs in cultures treated with supernatants from SR-CCL19 infected BHK cells compared to DC cultures treated with supernatants from empty virus infected BHK cells (Figure 2B and 2C). Therefore we conclude that CCL19 indeed induces maturation in DCs.

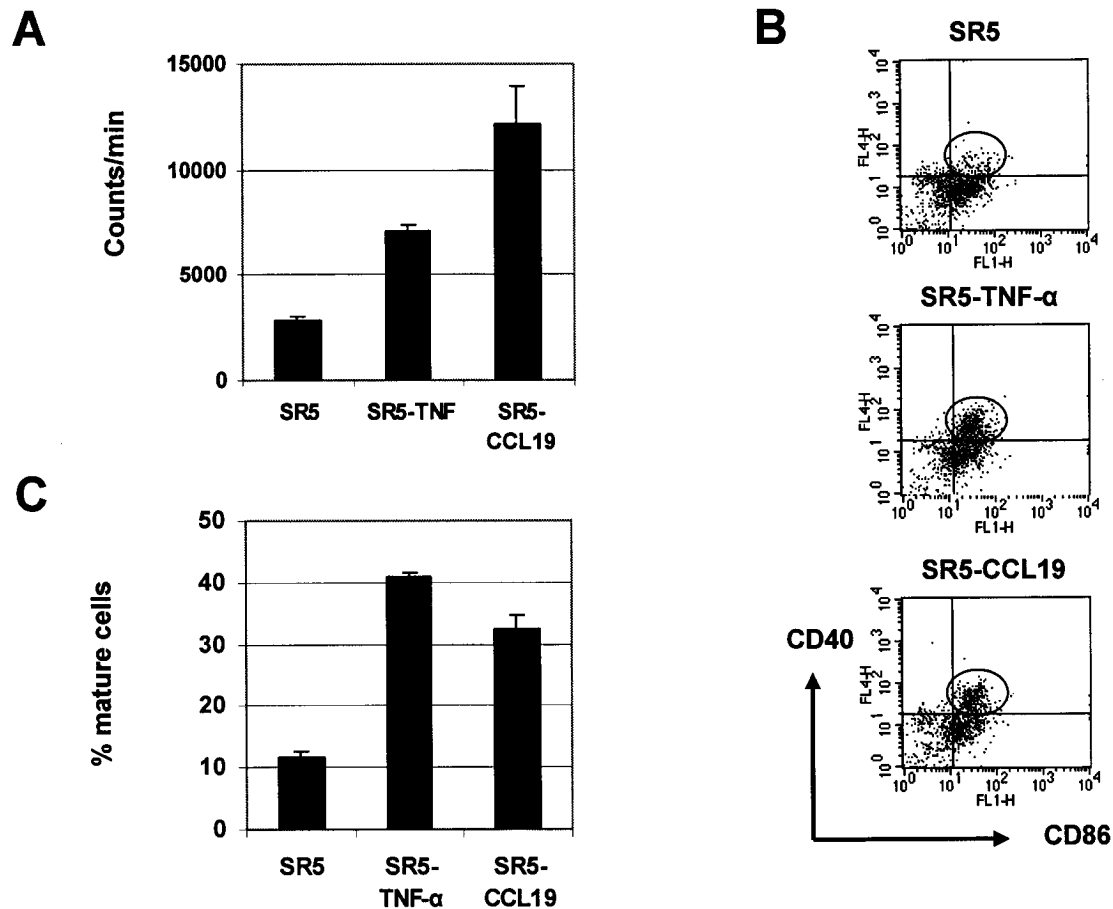


Figure 2(18). Reanalysis approved effect of SR-CCL19 on T cell proliferation and showed effect on DC maturation state: **A.** new supernatant of the recovered SR-CCL19 was produced and retested in the DC-T cell coculture screening system. Reproducibly SR-CCL19 was found to induce increased proliferation. SR-CCL19 was compared to SR5 and SR-TNF- α . Counts/min are shown on the Y-axis. The stimuli were shown on the X-axis. The error bars represent the standard deviation of threeplicates. **B and C.** Spleen derived DCs were treated over night with UV-treated SR5, SR-TNF- α or SR-CCL19 supernatants. Then, the DCs were analysed by FACS staining for CD40 and CD86 surface expression. The amount of mature DCs was determined as shown by the oval gate. % mature cells were shown on the Y-axis. The stimuli were shown on the X-axis. The error bars represent the standard deviation of duplicates.

4.4.3. Purified CCL19 enhances proliferation of T cells in the DC-T cell co-culture system

To test whether the effects found were specific for CCL19 and not an artefact from the alpha-viral system, CCL19 was sub-cloned into an eukaryotic expression

vector. A N-terminal FLAG-tagged CCL19 protein was produced and purified over an anti-FLAG column (Figure 3A). The purified protein was used for all further experiments. First the proliferation promoting quality in the DC-T cell co-culture system was retested. As presenter cells bone marrow derived DCs were taken and the proliferation for three different OVA peptide concentrations was tested. DCs were pulsed with the peptide for 1 h and then treated with the different stimuli for 4 h. Then the OVA-specific T cells were added and the proliferation was measured after 3 days by (3H)-thymidine incorporation (Figure 3B). In a similar experiment T cell proliferation was assessed by CFSE-staining. The DCs were treated as above and incubated for four days with CFSE labelled T cells. In both experiments CCL19 induced the same extent of proliferation as LPS/aCD40 and TNF- α and clearly more than PBS treated DCs (Figure 3C).

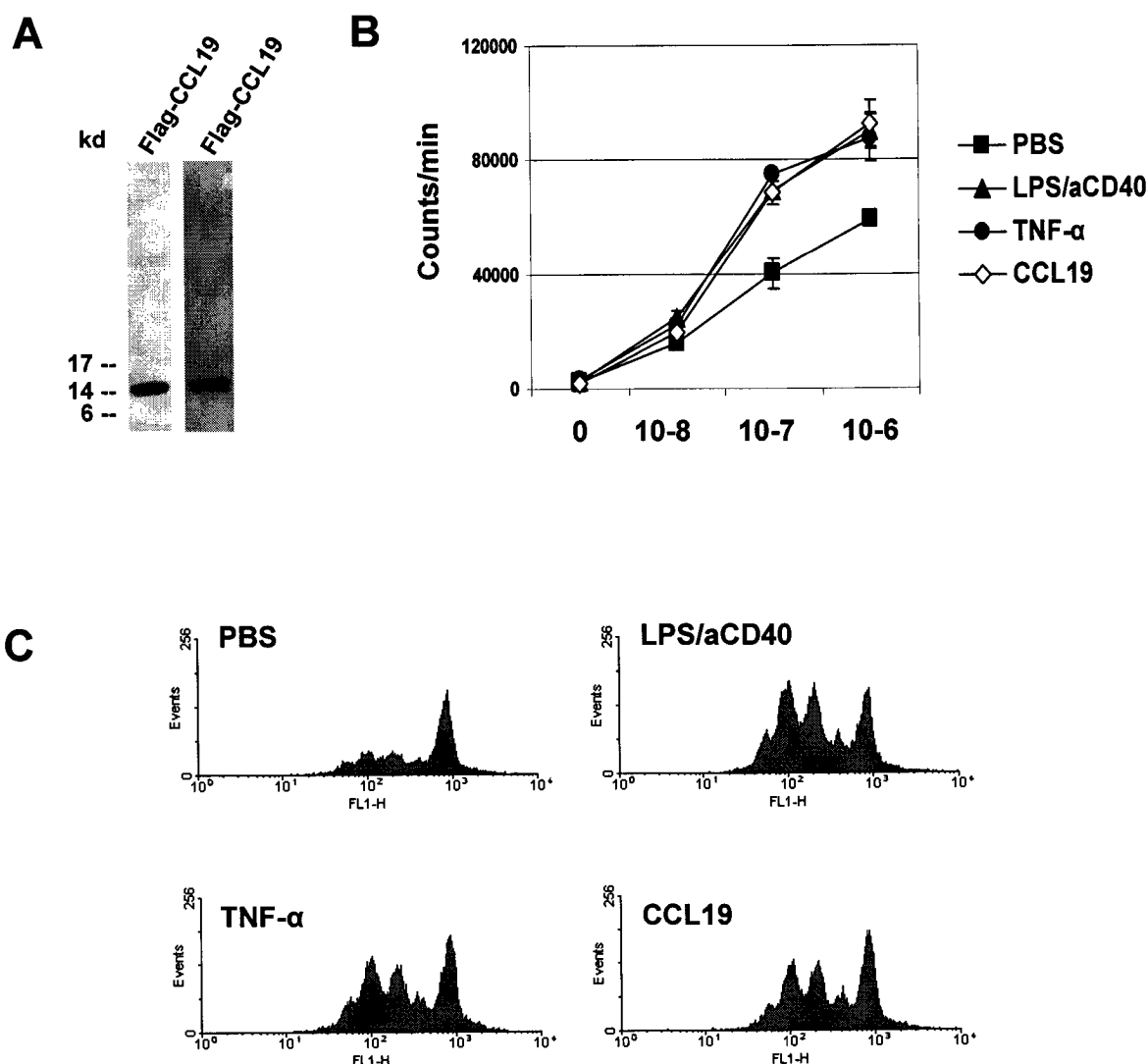


Figure 3(19). Purified eukaryotic CCL19 induces peptide-specific proliferation in BM-DC-T cell cocultivation assay: **A.** CCL19 was subcloned in an eukaryotic expression vector to produce a C-terminal FLAG-tagged CCL19 protein. EBNA cells were transfected and supernatant was harvested and the FLAG-CCL19 was purified over an anti-FLAG column. The protein was analyzed on SDS-PAGE (left) and on a western blot stained with a anti-FLAG ab (right). **B.** OVA-pulsed BM-DCs were treated with PBS, LPS/aCD40 ab (0.1 µg/ml/ 5 µg/ml), TNF-α (50 ng/ml) or FLAG-CCL19 (0.33 µg/ml) for 4h. OVA-specific transgenic CD4⁺ T cells were added and the proliferation was assessed by H3-Thymidine incorporation. Y axis shows incorporated H³-thymidine and the x axis shows the used OVA peptide concentration. **C.** OVA-pulsed (10⁻⁸M) BM-DCs were activated as mentioned under A, then OVA-specific CFSE-labelled CD4⁺ T cells were added and after 4 days incubation analysed for CFSE staining.

4.4.4. CCL19 induces up-regulation of co-stimulatory molecules and the production of cytokines in DCs

The differentiation into mature DCs is associated with the loss of receptors that are involved in antigen uptake, but instead co-stimulatory molecules are up-regulated which are important for interaction with and signal transduction on T cells. By the concomitant production of cytokines the DCs further promote the induction of an immunological response to a pathogen attack. In order to analyze the maturation program triggered in DCs by CCL19, we determined up-regulation of CD40 and CD86 and the levels of the pro-inflammatory cytokines interleukin-1 beta (IL-1β), interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF-α), which are important molecules for protective immunity as well as in immune diseases.

We therefore tested for the surface expression of some co-stimulatory molecules after CCL19 treatment. DCs were treated over night and the surface expression of CD40 and CD86 was assessed by FACS. To exclude that the found effect was due to LPS contamination of the used protein samples the samples were also treated with proteinase K and subsequent heat denaturation. CCL19 was found to induce upregulation of CD86 and CD40 (Figure 4A) in a LPS independent fashion, shown by the loss of function after proteinase K treatment. Similar upregulation of CD86 and CD40 was found for CCL19 or TNF-α and LPS treatment (Figure 4A). After proteinase K treatment activation by the protein samples (CCL19 and TNF-α) was

abrogated, whereas the effect of LPS was not altered. Analyzing the supernatants of the stimulated DCs in an IL-1 β and IL-12 specific sandwich ELISA, it was found that CCL19 induced strong production of the two cytokines (Figure 4B). The induction of IL-12 production was similar to LPS treated DCs, but the IL-1 β production was much stronger than found for LPS treatment. In comparison to TNF- α treatment CCL19 induced clearly higher levels of IL-12 and IL-1 β . Interestingly a recombinant form of CCL19 expressed in bacteria failed to induce DC-maturation, indicating that post-translational modifications of CCL19 may be important for its DC stimulation properties.

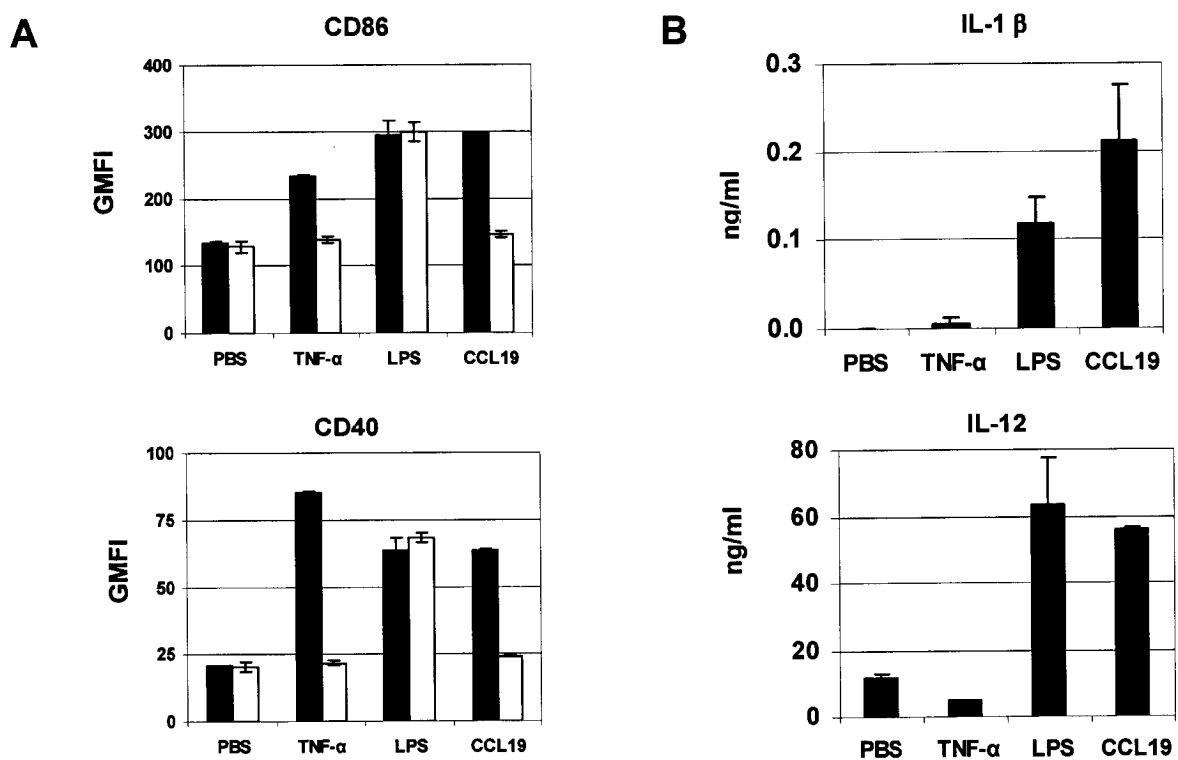


Figure 4(20). Purified eukaryotic CCL19 induces maturation of BM-DCs and the secretion of IL-1 β and IL-12: **A.** shows the induced CD86 and CD40 surface expression on BM-DCs treated with various stimuli. The geometric mean of CD86 or CD40 surface expression is shown on the Y-axis. The treatment of the BM-DCs is shown on the X-axis. The error bars represent the standard deviation of duplicates. One typical experiment is shown. **B.** Induction of IL-1 β and IL-12 secretion in BM-DCs stimulated with PBS, TNF- α (50 ng/ml), LPS (1 μ g/ml) or FLAG-CCL19 (0.33 μ g/ml). 18h after addition of stimuli the supernatant of these cells were harvested and analyzed with a sandwich-ELISA for the presence of IL-1 β and IL-12. The amount of secreted cytokine was quantified by the comparison to a

dilution of recombinant IL-1 β respectively IL-12. The stimulus used is shown on the X-axis and the amount of secreted cytokine on the Y-axis. The error bars represent the standard deviation of three replicates.

4.4.5. Induction of autoimmunity by CCL19-stimulated DCs

In order to examine the *in vivo* relevance of the maturation signal which CCL19 imparts on DCs, a myocarditis autoimmune disease model was investigated. In the model used, it was shown that DCs pulsed with a heart specific peptide and activated by LPS plus anti-CD40 ab were able to induce myocarditis in BALB/c mice. For the induction of the autoimmune disease DCs have to be activated simultaneously via two separate pathways, the first signal via TLR4 and the second signal via CD40. One signal alone fails to prime DCs for autoimmunity [434].

In order to test CCL19 in this system, we pulsed DCs with a heart specific peptide and activated them for 4 h either with LPS/anti-CD40 ab, PBS, or CCL19. Activation of DCs was confirmed by analysis of co-stimulatory molecule expression and cytokine production (Figure 5 and not shown). 200'000 of the DCs were injected i.p. in BALB/c mice on day zero, two and four with five mice per group. On day 10, mice were killed, the heart taken and evaluated for macroscopic signs of myocarditis. Further analysis was performed on the basis of sections from fixed hearts, which were analyzed and scored by a specialist in a double-blind fashion (Table 1).

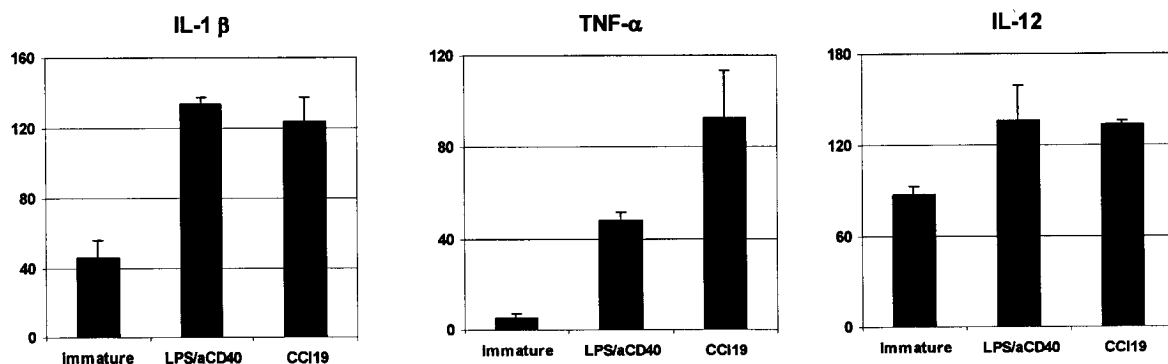


Figure 5(21). Intracellular cytokine expression: BM-DCs treated with PBS, LPS/ α CD40 ab (0.1 μ g/ml/5 μ g/ml), FLAG-CCL19 (0.33 μ g/ml) for 8 hours were analyzed for intracellular cytokine expression. Following the protocol described in the methods section, an IL-1 β , IL-12 and TNF- α specific staining was performed and analyzed. On the y-axes the geometric mean of intracellular cytokine expression is shown and the x-axes the used stimulus is shown.

As expected [434] LPS/anti-CD40 ab treated DCs induced strong myocarditis in the mice. While PBS-treated DCs failed to cause disease, CCL19 induced strong signs of myocarditis. Thus, CCL19 alone was able to stimulate DCs sufficiently strongly for disease induction, an observation usually only made if two signals are combined.

Table 1. Prevalence and Severity of myocarditis in BM-DC injected mice.

activation	DC injection	Prevalence (day 10)	median severity
none	Days 0, 2, 4	1/5 ^{a,b}	0 (0, 0, 0, 0, 2)
LPS/ α -CD40	Days 0, 2, 4	5/5 ^a	2.5 (2, 2.5, 3, 2, 3)
CCL19	Days 0, 2, 4	5/5 ^b	2 (2, 2.5, 2, 2, 2)

^aP<0.0001. ^bP<0.005 (t-test)

4.5. DISCUSSION

Regulation of dendritic cell maturation is essential for the balance between maximal protective T cell responses and minimal immunopathology. Here we show that CCL19, a chemokine attracting DCs from the periphery to T cell regions of lymphoid organs, is a strong maturation signal for DCs, surpassing in its potency CD40 both *in vitro* and *in vivo*.

A key function of chemokines and their receptors is the orchestration of leukocyte migration. For example, chemokines regulate segregation of T areas from B areas in lymphoid organs and the traffic of lymphocytes between the two areas. Chemokines are also instrumental for induction of local inflammation. Leukocytes traverse the endothelium of blood vessels and migrate into inflamed tissues in a three step process: recognition of endothelium by selectins, activation by chemokines followed by firm attachment by integrins. Naïve, resting T cells express the chemokine receptor CCR7, which binds CCL19 and CCL21. These two chemokines are constitutively produced in lymphoid organs and attract T cells to the T regions of these organs. Intriguingly, DCs, upon activation, also express CCR7, and become responsive to CCL19/CCL21, which attracts them to the T regions for optimal T cell priming. It is thought that this mechanism is responsible for the migration of Langerhans cells from the skin to lymphoid organs upon activation. However, chemokine receptors may not only be responsible for cellular migration but also for activation of dendritic cells. Specifically, CCR5, the receptor for MIP-1 α [435], MIP-1 β , RANTES and HIV gp 120 [436], also recognizes cyclophilin derived from toxoplasmosis [426]. This triggers a potent activation program in DCs leading to massive production of cytokines, in particular IL-12. This CCR5-mediated activation of DCs results in induction of a protective T_{H1} response, ridding the pathogen. However, if overshooting, this response may also result in immunopathology [437]. The present study shows that maturation of DCs and the production of pro-inflammatory cytokines may be an important component of chemokine biology. Specifically, CCL19 was identified in a global screen for proteins that activate DCs, as a strong inducer of dendritic cell maturation. In fact, CCL19 was more potent than TNF inducing the release of pro-inflammatory cytokines such as IL-1. This indicates

that CCL19 is a key maturation signal for DCs and not just one protein more able to mediate activation of DC. This notion is corroborated by the finding that CCL19-matured but not anti-CD40 treated DCs were able to induce autoimmune myocarditis [434]. Hence, CCL19 is not only more potent at inducing the production of pro-inflammatory cytokines in DCs but is also powerful in terms of generating specific T cell responses *in vivo*.

The data presented here suggest the following model: Langerhans cells and other DCs in peripheral tissues scavenge antigens and, upon encountering pathogens or an inflammatory milieu, undergo an initial activation step. This leads to upregulation of CCR7, recruiting the DCs to lymphoid organs. During this migration, CCL19 induces full maturation of DCs rendering them capable of effectively triggering potent T cell responses once they reach lymphoid organs. Chemokines, such as CCL19, therefore not only orchestrate immunity through guiding the leukocytes along the complex pathways of the lymph and blood circulation but also through regulation of DC maturation. Thus, CCL19 ensures both that antigens reach lymphoid organs and that these antigens are presented in a maximally immunogenic context.

5. INDUCTION OF AUTOIMMUNITY AS A FUNCTION OF EPI TOPE STRENGTH AND IMMUNE REGULATION

Patrick Bättig*, Philippe Saudan*, Tazio Storni*, Awen Gallimore† and Martin F. Bachmann*

*Cytos Biotechnology AG, Wagistrasse 25, 8952 Zürich-Schlieren, Switzerland

†Medical Biochemistry and Immunology, University of Wales College of Medicine, Cardiff, CF14 4XN , UK

Address correspondence to: martin.bachmann@cytos.com

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5.1. Abstract

T cell responses are regulated by the affinity/avidity of the T cell receptor for the MHC/peptide complex, available costimulation and duration of antigenic stimulation. Altered peptide ligands are usually recognized with a reduced affinity/avidity by the T cell receptor and are often able to only partially activate T cells *in vitro* or may even function as antagonists. Here we assessed the ability of altered peptide ligands derived from peptide p33 of lymphocytic choriomeningitis virus to mediate lysis of target cells *in vivo*, confer anti-viral protection and cause autoimmune disease. In a normal host, *in vivo* cross-reactivity between altered peptide ligands was rather limited and even strongly cross-reactive cytotoxic T lymphocytes were only able to mediate moderate anti-viral protection. In a transgenic mouse model expressing lymphocytic choriomeningitis virus glycoprotein in the islets of the pancreas, altered peptide ligands induced a transient insulinitis but failed to induce autoimmune diabetes. Strikingly, however, if immune homeostasis was disturbed by depletion of regulatory CD25⁺ T cells, diabetes and increased blood glucose levels was triggered not only by the full agonist p33 but also by an altered peptide ligand derived from it. Thus, autoimmunity is regulated by affinity/avidity and regulatory T cell thresholds.

5.2. Introduction

Regulation of T cell response is influenced by many parameters. Amongst the most important factors are duration of antigenic stimulation and available costimulation delivered by APCs, in particular dendritic cells (DCs) [409,438-440]. An additional key factor is how well the T cells recognize their antigen, which is mostly dictated by the affinity/avidity of the TCR for the MHC/peptide complex [441,442]. Of particular importance appears to be the off-rate of the TCR-MHC/peptide interaction, since proper TCR-triggering probably requires a minimal time-span [443,444]. Detailed *in vitro* studies have shown that weakly recognized peptides fail to induce efficient T cell responses and activate T cells only partially or even exhibit an antagonistic function [445,446]. The mechanism for this partial T cells activation has been intensively studied and it has been shown that partial T cell agonists do not induce full phosphorylation of the CD3 ζ -subunit and fail to recruit ZAP-70 [447,448], apparently because the TCR falls off the MHC/peptide complex too quickly for full TCR triggering [443] or perhaps dimerization [449].

An important *in vivo* role of partial agonists/antagonists during protective immune responses has also been postulated. Specifically, it has been suggested that virus or parasite variants that express antagonistic peptides have a selective advantage in the host [450-452]. Moreover, it has been argued that a pre-existing CTL response inhibits the development of a CTL response against a variant peptide, a phenomenon coined "original antigenic sin" [453]. There is also evidence for a role of cross-reactive T cells in the generation of autoimmunity, a hypothesis summarized under the term of "molecular mimicry" [454-457]. The idea is that pathogens that express antigenic epitopes highly homologous to host antigens may cause autoimmune disease. Despite an impressive body of evidence for molecular mimicry, the topic remains controversial, mostly due to the difficulties in distinguishing experimentally between cross-reactive T cells induced by mimicry and self-antigen specific T cells induced due to a local cell lysis and a pro-inflammatory milieu [458].

Here we wanted to study directly the efficiency of such cross-reactive T cells to mediate anti-viral protection and cause autoimmunity. As a model system, we used peptide p33 derived from the lymphocytic choriomeningitis virus (LCMV) and variant peptides derived thereof [74,459]. In order to induce strong immune responses with

the peptides, we fused them genetically to virus-like particles (VLPs) derived from the hepatitis B core antigen. We have previously shown that such VLPs, if combined with bacterial DNA rich in non-methylated CG motifs (CpGs) or anti-CD40 antibodies, induce potent CTL responses that surpass responses induced by recombinant vaccinia virus [460]. In order to cover a broad spectrum of cross-reactivities, we chose altered peptide ligands (APLs) previously found in a TCR-transgenic system to be a strong partial agonist (A4Y), a weak partial agonist (V4Y) or antagonist peptides (S4Y/G4Y) [73]. While most peptides induced strong CTL responses against themselves, *in vivo* cross-reactivity was limited and only conferred partial anti-viral protection. Moreover, cross-reactive CTL epitopes triggered transient insulinitis but failed to cause autoimmunity in a normal host. However, autoimmune diabetes and increased blood glucose levels were induced if the immunological balance was disturbed by elimination of CD25⁺ cells. Thus, both the strength of the TCR-MHC/peptide interaction and the CD25⁺ regulatory T cell environment dictate the power of molecular mimicry.

5.3. Materials and Methods

5.3.1. Mice viruses and cell lines

C57BL/6 mice were purchased from Harlan (Horst, The Netherlands) at the age of 8–14 wk. 327 TCR-transgenic mice expressing a TCR specific for peptide p33 [461] and transgenic mice expressing LCMV-GP under the control of the rat insulin promoter (RIP-GP) [367] were kept under specific pathogen-free conditions at Cytos Biotechnology AG.

Recombinant vaccinia virus expressing LCMV-GP was grown and titrated on BSC40 cells as described [462]. To examine antiviral immunity, vaccinated female C57BL/6 were infected i.p. with 1.5×10^6 PFU(high) or 3×10^4 PFU(low) Vacc-GP. Five days later ovaries were collected, and the vaccinia titers were determined. LCMV isolate WE was originally obtained from Dr. R. M. Zinkernagel (Institute of Experimental Immunology, University Hospital, Zurich, Switzerland) and propagated on L929 cells. To examine antiviral immunity, vaccinated female C57BL/6 were infected i.v. with 200 PFU LCMV. Five days later spleens were collected, and the LCMV titers were determined on MC57 fibroblasts as previously described [463].

5.3.2. Peptides, oligonucleotides, peptide-loaded tetramers, rat anti-mouse CD40, anti-CD25 Abs and VLPs

LCMV glycoprotein peptide p33–41 (KAVYNFATC) and the subvariants A4Y (KAVANFATC), V4Y (KAVVNFATC), G4Y (KAVGNFATC) and S4Y (KAVSNFATC) were synthesized by a solid phase method and were purchased from Eurogentec (Hestel, Belgium). Phosphorothioate-modified CpG-ODN was synthesized by Microsynth (Balgach, Switzerland). The following oligonucleotide sequence was used: 1668pt (5'-TCC ATG ACG TTC CTG AAT AAT-3') [460]. APL-loaded tetramers were purchased from Proimmune Limited (Oxford) and used according to the manufacturer's recommendations. Briefly, blood samples from 327 TCR-transgenic mice or peptide-VLP immunized C57BL/6 mice were stained with an anti-CD8 antibody and APL-loaded tetramers and analyzed by FACS. The hybridoma FGK45 producing anti-CD40 mAbs was originally provided by Dr T Rolink and the antibodies

were produced and purified in house. The hybridoma secreting anti-CD25 antibodies (PC61, rat IgG1) has been described previously [464]. Hybridomas were grown in culture and antibodies were purified by precipitation in saturated ammonium sulphate. Mice received 1mg of PC61 i.p. as indicated in the text.

The p33-VLP was generated as described in [460] using standard PCR methods, but instead of KAVYNFATM, KAVYNFATC was added. The p33 variants were fused the same way to the HBcAg (aa 1-183). The VLPs were produced and purified as described in [460]. The VLPs were analyzed with SDS-PAGE and on a 1% agarose gel.

5.3.3. *In vitro* and *in vivo* activation of p33-specific CD8+ T cells

For *in vitro* activation of p33-specific T cells, purified DCs obtained from spleens [465] were pulsed with different concentrations of p33, A4Y, V4Y, G4Y or S4Y peptide at 37°C for 1 h. After three washing steps, presenter cells (10^4 cells/well) were cocultured with Ag-specific transgenic CD8+ T cells (10^5 cells/well). After 2 days T cell proliferation was measured by ^3H -thymidine incorporation for 16 h (1 μCi /well).

For *in vivo* activation of p33-specific T cells, 327 TCR-transgenic mice were i.v. immunized with 30 μg p33-, A4Y-, V4Y-, G4Y-, S4Y-VLPs. Twenty-four hours later single-cell suspensions were prepared from spleens and incubated for 20 min at 37°C with anti-CD8 Cy5-conjugated Abs (BD PharMingen, San Diego, CA) to detect CD8+ p33-specific T cells. To analyze cell surface expression of the activation marker CD69, the cell suspensions were incubated with FITC-coupled anti-mouse CD69 mAbs (BD PharMingen). Live cells (1×10^4) were acquired in a FACSCalibur device and analyzed using WinMDI software version 2.8.

5.3.4. *In vivo* CTL assay

To assess *in vivo* cytotoxicity, target cells were loaded with the respective peptide (10^{-6}M), labelled with CFSE (Molecular Probes (Eugene, OR) [466] and injected into immunized mice. 4 hours later the percentage of specific lysis was calculated by comparison with lysis of unloaded CFSE labelled target cells. For the preparation of target cells, erythrocytes were removed from naive C57BL/6 spleen

and lymph node cell suspensions by osmotic lysis. The cells were then washed and split into two populations. One population was pulsed with 10^{-6} M p33 variants peptide, incubated at 37°C for 45 min, and labeled with a high concentration of CFSE (2.5 μ M) (CFSE_{high} cells). The second control target population was left without peptide and was labeled with a low concentration of CFSE (0.25 μ M) (CFSE_{low} cells). For i.v. injection, an equal number of cells from each population were mixed together, such that each mouse received a total of 2×10^7 cells in 150 μ l of PBS. Cells were injected into mice that had previously been immunized with VLPs carrying the p33 variants on the surface eight days before injection of cells. 4 h later cell suspensions were prepared from spleens and lymph nodes of the mice, analyzed by flow cytometry, and each population was detected by its differential CFSE fluorescence intensity. Up to 1×10^4 CFSE-positive cells were collected for analysis. To calculate specific lysis, the following formula was used: ratio = (percentage CFSE_{low}/percentage CFSE_{high}). Percentage specific lysis = [1 - (ratio unprimed/ratio primed) x 100] [467]

5.3.5. Assessment of autoimmunity

Glucose concentration in blood obtained from a tail vein was measured using Glucotrend active strips (Roche, Basel Switzerland). Mice were considered diabetic when glucose concentration exceeded 250 mg/dl for at least two consecutive days. After onset of diabetes or latest 15 days after immunization, the pancreas were removed, fixed in 4% buffered formaldehyde, and processed for H&E staining. Slides were coded and evaluated in a blinded fashion by a veterinary pathologist. For diagnosis of diabetes, an atrophy of islet cells and acute pancreatitis were considered.

5.4. Results

5.4.1. Generation of VLPs containing p33 variant epitopes

Peptide p33 (KAVYNFATC) and the variant peptides A4Y (KAVANFATC), V4Y (KAVVNFATC), S4Y (KAVSNFATC) and G4Y (KAVGNFATC) were fused to the C-terminus of the hepatitis B core antigen (HBcAg) by a three-leucine linker. SDS-Page analysis showed that the VLP subunits exhibited the expected size (Figure 1A) and agarose gel analysis [460] showed that the subunits assembled into particles (not shown). Most experiments performed previously with p33-derived variant peptides employed peptides with an M rather than a C at the C-terminus in order to prevent peptide dimer formation [468]. Since it has been reported that peptide p33 containing an M has slightly different properties for T cell activation than the wild-type p33 peptide containing the C [469], we confirmed in a first set of experiments that the C-versions of the peptides exhibit similar properties as those containing an M. As expected, peptide p33 was able to induce strongest proliferation of T cells derived from TCR-transgenic 327 mice followed by A4Y and V4Y. The two antagonistic peptides S4Y and G4Y showed no significant effects on T cell proliferation (Figure 1B). Moreover, VLP containing the variant peptides induced expression of CD69 in TCR transgenic mice, as a read-out for T cells activation, following the same hierarchy (Figure 1C). As observed previously, *in vivo* responses were weaker for APLs than those observed *in vitro* [470]. In addition, staining of TCR-transgenic T cells with tetramers loaded with either p33, A4Y, V4Y or S4Y revealed staining intensities paralleling the ability of the peptides to induce T cell activation (Figure 1D).

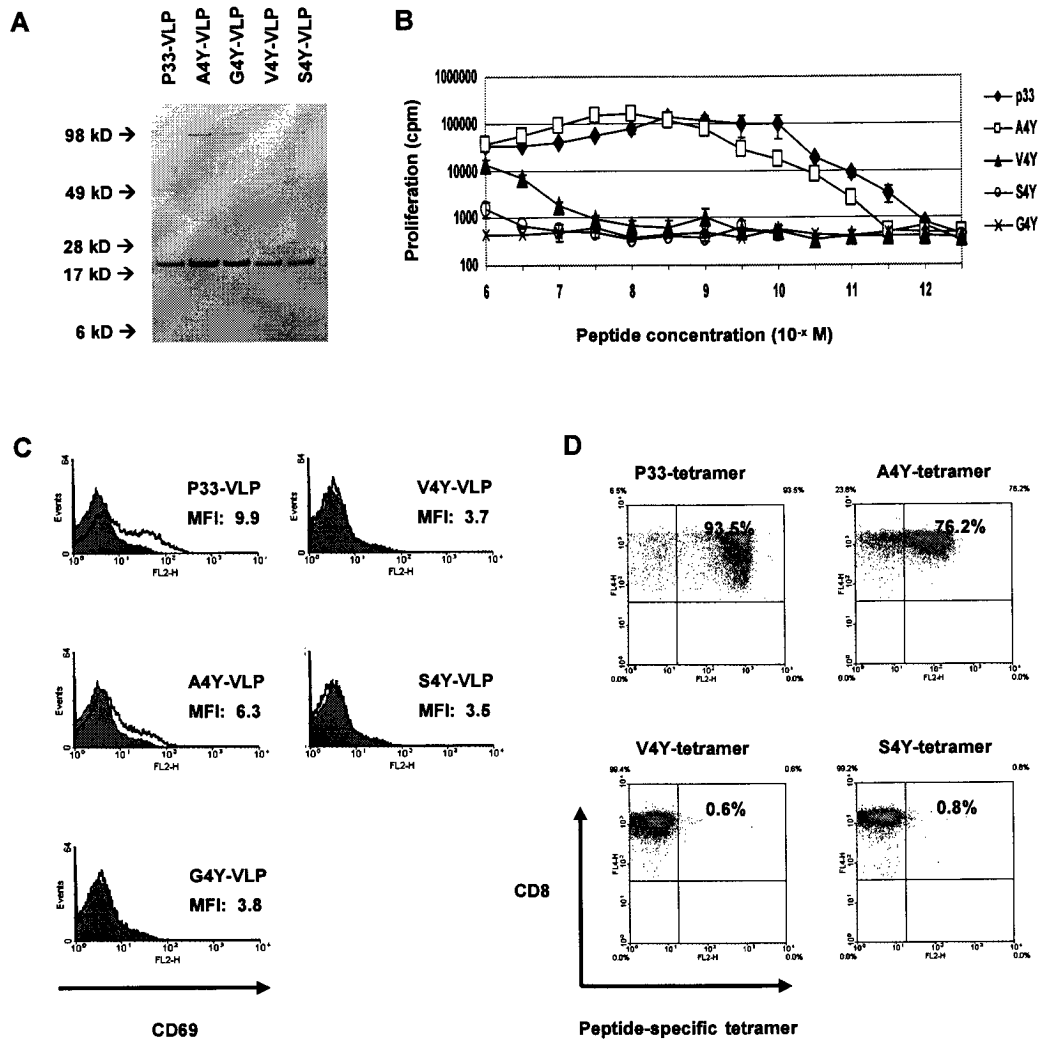


Figure 1(22). Characterization of recombinant VLPs bearing p33 variants: **A.** SDS-PAGE analysis of VLP monomers displaying LCMV p33 variants after gel filtration purification. **B.** DCs were pulsed with the indicated concentrations of p33, A4Y, V4Y, G4Y or S4Y peptide. Presenter cells were cocultured with CD8⁺ Ag-specific T cells for 2 days, and proliferation was assessed by [³H] thymidine incorporation. Error bars represent the SD of triplicate measurements. One representative experiment of three is shown. **C.** 327 TCR-transgenic mice were immunized i.v. either with 30 μ g of p33-, A4Y-, V4Y-, G4Y- or S4Y-VLPs. As a control mice were injected with PBS. One day after priming splenocytes were collected, stained with APC-labeled anti-CD8 mAbs and PE-labeled anti-CD69 mAbs and analyzed by flow cytometry. Overlays of T cells from the different VLP-immunised mice with T cells from PBS injected mice are shown. The mean fluorescence intensity (MFI) of the different cell populations in FL-2 is indicated in the respective histograms. **D.** Blood from 327 TCR-transgenic mice was stained with anti-CD8 and p33-, A4Y-, V4Y- or S4Y-loaded tetramers and analyzed by FACS. The APL used and the percentage of tetramer and CD8 double positive T-cells are indicated in the respective dot blots. One representative experiment is shown.

5.4.2. Weak *in vivo* cross-reactivity of the peptides

In order to test whether the variant peptides were able to induce CTL responses, C57BL/6 mice were immunized with the VLPs containing the variant peptides in the presence of CpG oligonucleotides [460]. Eight days later, at the peak of the response, frequencies of specific T cells were assessed by tetramer staining. Peptide p33, A4Y, V4Y or S4Y all induced high frequencies of specific T cells, ranging from 3.1% +/-1.0% for p33 to 11.0% +/- 1.8% for V4Y (Figure 2A). To further study the efficiency of the CTL responses induced, mice were vaccinated and perfused eight days later with CFSE labeled splenocytes loaded with the respective peptides. Unpulsed splenocytes with a lower CFSE intensity were cotransferred as an internal standard. The ratio of peptide pulsed versus control splenocytes was assessed 4 hours later by flow cytometry. Peptides p33, A4Y, V4Y and S4Y fused to VLPs were able to induce strong T cell responses as indicated by lysis of the respective peptide pulsed target cells, while peptide G4Y induced only an inefficient T cell response (Figure 2B). Similar results were obtained with MHC class I dimers loaded with the respective peptides (not shown). In contrast, lysis of splenocytes pulsed with cross-reactive peptides was very limited *in vivo* (Figure 2C). For example, in mice immunized with p33-VLPs, p33-pulsed splenocytes were efficiently lysed but not A4Y, V4Y, G4Y or S4Y pulsed splenocytes. Corresponding findings were obtained with the other peptides.

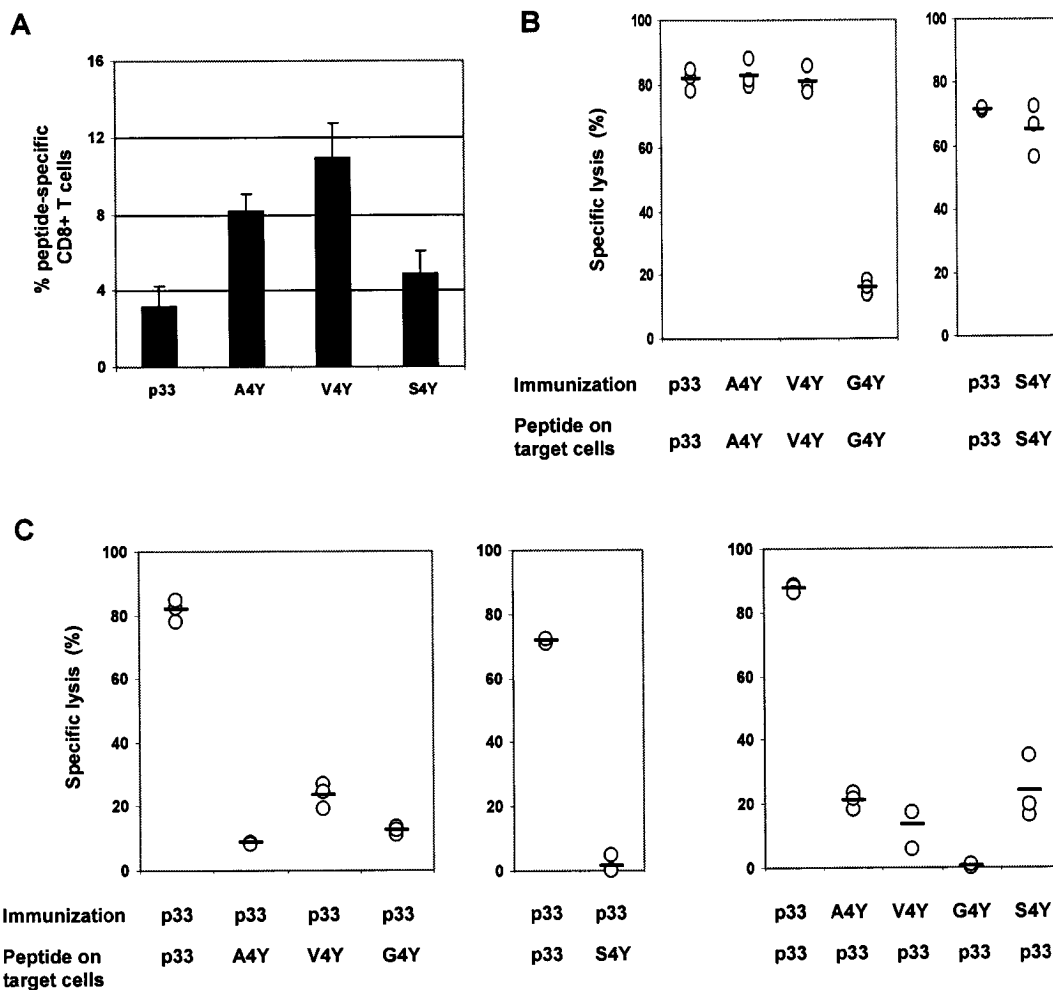


Figure 2(23). Induction of peptide-specific cytotoxic T cells by immunization with p33- and variants-VLP: C57BL/6 mice were immunized i.v. with either 30 μ g of p33-, A4Y-, V4Y-, G4Y- or S4Y-VLP with 20 nmol CpGs. As a control mice were injected with PBS and 20 nmol CpGs. Eight days later frequencies of specific T-cells and the efficiency of induced CTL responses were determined. **A.** Blood samples were stained with APL-tetramers and anti-CD8 antibody and analysed by FACS. The percentage peptide specific CD8+ T cells are shown on the Y-axis, and the peptide on the X-axis. Four mice were used per group, error bars represent the standard error of the mean. The different tetramers showed less than 0.2 % unspecific binding to naïve CD8+ T cells (data not shown). **B.** and **C.** A 1:1 mixture of CFSE labelled, peptide pulsed splenocytes (CFSE^{high}) and unpulsed splenocytes (CFSE^{low}) were injected i.v. into the immunized mice as target cells. After 4 h the mice were killed and spleen and LN analyzed for the presence CFSE^{high} (pulsed) and CFSE^{low} (unpulsed) cells. The response of each peptide against itself (**B**) and the cross reactivity of the different peptides were determined (**C**). *In vivo* cytotoxicity was determined as described in material and methods. Percentage specific lysis is shown on the Y-axis, the VLPs used for immunisation and the peptides loaded on target cells are shown on the X-axis. Circles represent individual mice and lines show average specific lysis in each group. Three mice were used per group. One of two comparable experiments is shown.

5.4.3. Anti-viral protection by cross-reactive T cells

The ability of cross-reactive T cells to mediate anti-viral protection was assessed next. Mice were immunized with the various VLPs together with CpG oligonucleotides and 9 days later, mice were challenged with recombinant vaccinia virus expressing LCMV-GP. Mice primed with p33-VLPs efficiently controlled viral replication (Figure 3). In contrast, none of the variant peptides was able to confer protection from high dose virus challenge (Figure 3A). Similar results were obtained if the VLPs were injected into mice in the presence of anti-CD40 antibodies (not shown). In order to test anti-viral protection under less stringent conditions, mice were immunized and challenged with a reduced viral dose, leading to 100-fold lower viral titers in ovaries of naïve mice (Figure 3B). Under these circumstances, complete protection was seen for the more strongly cross-reactive peptide A4Y and partial protection with the other agonistic peptide V4Y (Figure 3B). Protection from LCMV infection followed a similar pattern. Whereas p33, A4Y and V4Y induced good protection, G4Y and S4Y failed to do so (Figure 3C). Thus, variant peptides are able to mediate anti-viral protection albeit with limited efficiency.

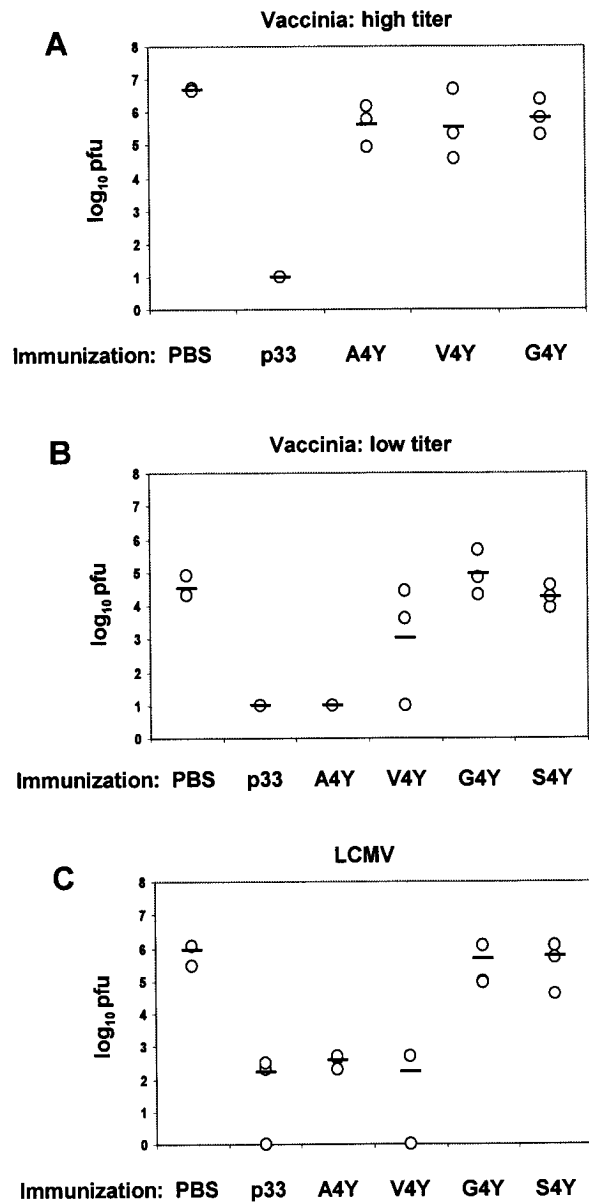


Figure 3(24). Pathogen and dose dependent protection after A4Y- and V4Y-VLP immunization: Groups of three C57BL/6 mice were immunized i.v. with either 30 μ g of p33-, A4Y-, V4Y-, G4Y- or S4Y-VLP and 20 nmol CpGs. As a control mice received PBS and 20 nmol CpGs. Nine days after priming mice were challenged i.p. either with a high dose 1.5×10^6 PFU (A) or a low dose 3×10^4 PFU (B) of Vacc-GP or i.v. with 200 PFU LCMV (C). Five days later ovaries (Vacc-GP) or spleens (LCMV) were isolated, and viral titers in the organs were determined. Circles represent individual mice and lines show average viral titers in each group.

5.4.4. Induction of autoimmunity

Transgenic mice expressing LCMV-GP under the control of the rat insulin promoter (RIP-GP) develop autoimmune diabetes upon infection with LCMV [367]. In a first set of experiments, we tested whether RIP-GP mice develop diabetes upon immunization with p33-VLPs. p33-VLPs induce poor T cell responses unless presented by activated DCs [460]. Thus, as expected, p33-VLPs failed to induce diabetes if given alone (not shown). However, if p33-VLPs were injected together with anti-CD40 antibodies, diabetes was rapidly induced with an onset between d-7 and d-13 (Figure 4). In addition, pancreatic islet cells were heavily infiltrated early after onset of diabetes and virtually absent a few days later (not shown). In contrast, variant peptides fused to VLPs failed to induce diabetes also if applied together with anti-CD40 antibodies (Figure 4). Thus, even for strongly cross-reactive peptides, such as A4Y or V4Y, no autoimmunity could be induced.

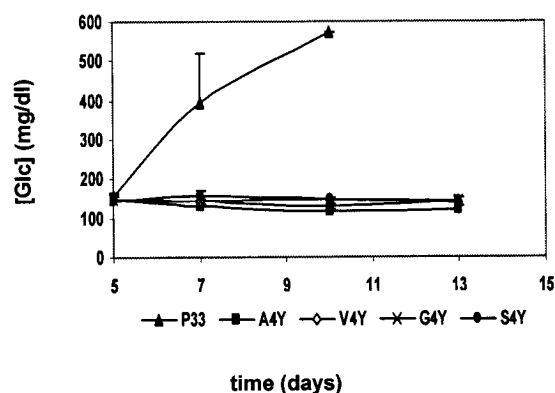


Figure 4(25). Induction of increased blood glucose level and autoimmunity by p33 but not by variant peptides: RIP-GP mice were immunized i.v. with either 100 μ g of p33-, A4Y-, V4Y-, G4Y- or S4Y-VLP and 100 μ g anti-CD40 antibodies. Blood glucose levels in these mice were monitored at different intervals starting 5 days after immunization. Three animals were used per group. P33-VLPs but not the variant peptides induced diabetes and significant elevation of blood glucose levels ($p < 0.001$).

It has been shown previously that regulatory T cells express the CD25 marker and depletion of these cells leads to increased resistance to tumor growth and T cell

responses [126,127,471-473]. In order to assess whether perturbation of the normal regulatory homeostasis of the immune system may lead to induction of autoimmunity by variant peptides, mice were depleted of regulatory T cells by injection of 500 μ g anti-CD25 antibodies on day four and five after immunization with the peptide A4Y fused to VLPs plus anti-CD40 antibodies or WT-VLPs plus anti-CD40 antibodies. Note that specific T cells, including p33-specific T cells, lose expression of CD25 during regular immune responses within 4 days [408,474]. Thus, the treatment should lead to selective loss of regulatory T cells while sparing effector T cells. Strikingly, in the absence of regulatory T cells, autoimmunity was induced also by the A4Y-VLPs, and the immunized mice developed diabetes or a statistically significant elevation of blood glucose levels compared to mice immunized with WT-VLPs in the presence of anti-CD25 antibodies (Figure 5A).

Histological analysis revealed infiltration of the pancreas in mice immunized with A4Y-VLPs in the presence or absence of CD25⁺ regulatory T cells. However, the frequency of the infiltrated islets was strongly increased in the anti-CD25 treated mice ($p < 0.05$) (Figure 5B). Moreover, in A4Y-VLP immunized mice, infiltrating cells failed to enter the islets and remained at their periphery (Figure 5C). This only caused a transient insulinitis and failed to trigger increased blood glucose levels (Figure 4 and 5A). In contrast, upon depletion of CD25⁺ regulatory T cells, inflammatory cells were able to enter the islets (Figure 5C) causing increased blood glucose levels. Thus, APLs may be able to cause disease; however, only in hosts with a deregulated immune system.

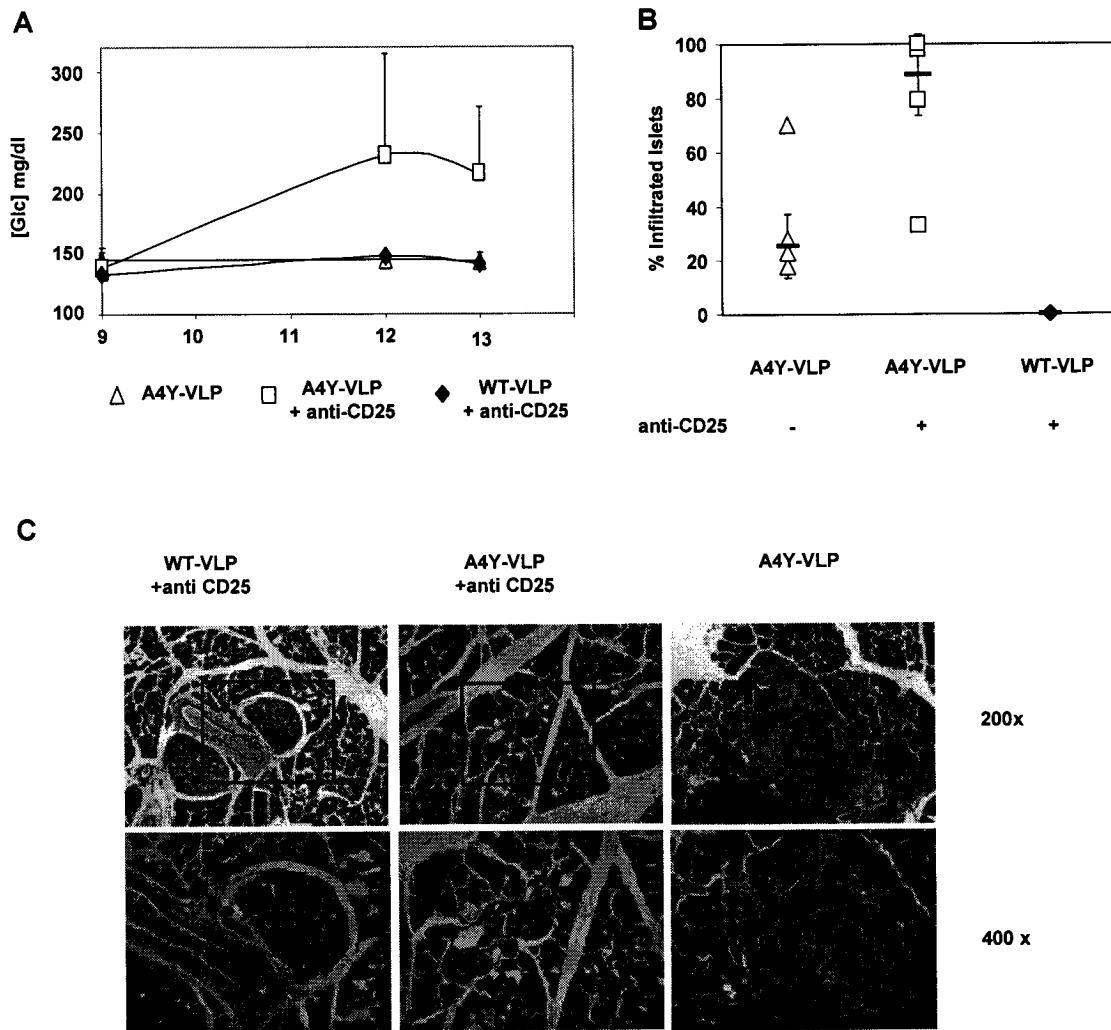


Figure 5(26). Inflammation of the pancreas upon immunization with variant peptides in the absence of regulatory T cells: RIP-GP mice were immunized either with 100 μ g of A4Y- or WT-VLPs and 100 μ g anti-CD40 antibodies. In order to deplete regulatory T cells, groups of mice were additionally treated with 1 mg of anti-CD25 antibodies four days after immunization. Four mice were used per group. **(A)** Blood glucose levels were measured at different intervals starting 9 days after immunisation. A4Y-VLPs in the presence of anti-CD25 antibodies induced either diabetes (1/4) or statistically significant increase of blood glucose levels in the remaining mice. **(B)** The pancreas of immunized mice was removed and sections were stained with H&E and the frequency of infiltrated islets was determined. **(C)** Pancreas sections of the immunised mice. The treatments are indicated above the respective panels and the magnification is shown to the right of the respective panels. The lower panels show a higher magnification of the area indicated with a black box in the top panel.

5.5. Discussion

The biology of APLs has mostly been studied *in vitro*. Here we assessed the ability of variant peptides to mediate anti-viral protection and autoimmunity *in vivo*. We found that T cell responses directed against even strongly cross-reactive peptides had a limited but distinct ability to halt viral replication. Moreover, induction of autoimmunity by variant peptides in a normal host was weak and transient unless the natural homeostasis was disturbed by depletion of CD25+ regulatory T cells.

Molecular mimicry has been proposed to be a major cause for autoimmunity. Indeed, amongst others, causal associations between B4 Coxsackievirus or Chlamydia infection and myocarditis and borrelia burgdorferi and arthritis have been reported [475-478]. These epidemiological correlations have been supported by the demonstration of cross-reactive T cells, either in patients or experimental animal models, which recognize both the infecting agent and a self peptide. Overall, the surprisingly extensive degeneracy of the T cell repertoire is indeed compatible with molecular mimicry as an important cause for autoimmunity [479]. Nevertheless, most studies on T cell reactivity and recognition of APLs have been performed *in vitro* [446]. Such experiments, as also shown here, may reveal more extensive cross-reactivity than *in vivo* experiments [480]. Indeed, *in vivo* lysis of splenocytes coated with APLs, as well as induction of protective anti-viral immunity was limited in the normal host. Thus, even extensive *in vitro* cross-reactivity may not necessarily translate into *in vivo* effector functions. Nevertheless, cross-reactive T cells may protect from low dose viral infections, indicating that affinity/avidity requirements for anti-viral protection are not absolute but vary with the pathogen and its dose.

Two additional parameters to the extent of T cell cross-reactivity will dictate *in vivo* effector function of cross-reactive T cells. On the one hand, the immunogenicity of the agent inducing T cell responses will be of key importance. We have used VLPs in combination with potent activators of DCs, CpGs or anti-CD40 antibodies, for priming of T cells. We have shown previously that this induces very strong CTL responses, as exemplified by the fact that recombinant vaccinia virus expressing LCMV-GP fails to induce diabetes in RIP-GP transgenic mice [367] while p33-VLPs

in combination with anti-CD40 antibodies were able to induce early onset of diabetes. Nevertheless, still more immunogenic agents, such as LCMV itself, may be able to induce even stronger T cell responses that are more cross-reactive *in vivo*.

A second parameter that may be crucial for the extent of damage caused by cross-reactive T cells is the presence of regulatory T cells. Indeed, as shown here, in a normal host, A4Y-specific T cells only caused a transient insulinitis in transgenic mice expressing LCMV-GP in the pancreas. In contrast, p33-specific T cells caused extensive damage, if they were triggered by activated DCs. Interestingly, A4Y-specific T cells were able to inflame the pancreatic tissue but failed to enter the islets. This indicates that local damage caused by the inflammatory T cells is critical to precipitate the disease and allow further destruction of the tissue. If the T cells are only slightly less effective than normal, such as A4Y-induced T cells cross-reacting with p33, the local damage is not sufficient to allow for infiltration of the islet itself. The present study points to regulatory T cells as an essential component of this safety mechanism that limits local damage by activated T cells. Specifically, in the absence of regulatory T cells, also cross-reactive T cells may be able to enter the islet tissue and cause damage, leading to increased blood glucose levels and diabetes.

Thus, induction of autoimmunity is controlled at several check-points: As also shown here, high affinity ligands fail to induce autoimmunity unless presented by activated DCs, indicating that autoimmunity may only be caused if the innate (activation of DCs) and the adaptive (specific T cells) immune systems are triggered simultaneously. Variant peptides have to overcome one hurdle more that is regulatory T cells. Thus, variant peptides may only cause autoimmunity if innate and adaptive immunity are stimulated and if the natural immunological balance has been disturbed. These considerations may explain why disease caused by molecular mimicry is rare and may only occur, if at all, in a small fraction of infected individuals.

6. ORIGINAL ANTIGENIC SIN FOR T CELLS: ANALYZED IN A VIRAL INDEPENDENT MODEL SYSTEM

Patrick Bättig¹, Philippe Saudan¹ and Martin F. Bachmann¹

¹Cytos Biotechnology AG, Wagistrasse 25, 8952 Zürich-Schlieren, Switzerland

Address correspondence to: martin.bachmann@cytos.com

Keywords: CTL, altered peptide ligands, original antigenic sin

6.1. Abstract

The phenomenon of original antigenic sin was first described for antibody responses to influenza virus. After an initial infection, re-infection with a drifted strain of the virus boosted the response against the initial virus. Although the antibodies cross-reacted with the new strain, they showed higher affinity for the old strain. Later, the same observation was made for cytotoxic T cell responses against viruses, indicating that original antigenic sin may interfere with antibody and T cell responses. P33 peptide derived from the glycoprotein of lymphocytic choriomeningitis virus is a well studied model antigen for CTL responses. Moreover, a set of altered peptide ligands has been described that act as weak and partial agonist or antagonist on p33-specific TCR transgenic T cells. In order to study the influence of pre-existing T cells specific for variant peptides on the development of protective immunity, we fused three different APLs and peptide p33 to VLPs and used these VLPs for immunization of mice. Using this model system, we could demonstrate that i) boosting with peptide p33 primarily elevates frequencies of APL-specific T cells and ii) that this pre-existing APL-specific T cells impairs antiviral immunity. Thus the concept of original antigenic sin could be confirmed in a virus-independent model system, where the mutation is restricted to one amino acid in the dominant antigen peptide.

6.2. Introduction

The term original antigenic sin was first used to describe a particular feature of antibody responses against influenza virus. After an initial infection, re-infection with a new strain of the virus boosted mainly the antibody response against the initial virus. The antibody response induced showed some specificity for the new strain, but the affinity for the old strain was higher. Moreover, antibodies against the old strain would not react with the new strain [481,482]. Stimulated by studies with HIV infected patients, where CTL responses against immunodominant viral strains of HIV were found, with weak or even no responses against other immunogenic variants simultaneously present in the host [483,484], Klenerman et al studied in the LCMV model whether original antigenic sin may also occur for T cells [453]. In this study the authors found, that mutated viral strains induced asymmetrical CTL responses. Mice infected with wild-type LCMV showed only weak cross-reactivity against mutant strains, whereas mice infected with mutant LCMV strains crossreacted equally to wild-type and mutant strains. In addition, infection of LCMV-immune mice with viral variants primarily enhanced the response to the wild-type virus. These findings indeed were similar to the earlier observations for the influenza virus specific B cell response. Original antigenic sin for T cells is of particular interest for the generation of CTL vaccines against such rapidly mutating pathogens like HIV, because the induction of original antigenic sin in vaccinated individuals would not only impair vaccine efficacy, but may even reduce the build up of protective immunity against variant strains. The *in vivo* relevance of the concept of original antigenic sin was further supported by a study on the pathogenesis of dengue hemorrhagic fever [485]. In this report, the authors found in acutely infected patients many dengue virus-specific T cells of low affinity, showing higher affinity for other, probably previously encountered strains.

In the present study we wanted to test the concept of original antigenic sin in a defined system without viral replication. As model system, we used peptide p33 derived from the LCMV and variant peptides derived thereof [74,459]. To mimic viral antigen presentation and to induce strong immune responses against the peptides, we fused the epitopes genetically to virus-like particles derived from the hepatitis B core antigen (HBcAg). We have previously shown that such VLPs, if combined with

bacterial DNA rich in non-methylated CG motifs (CpGs) or anti-CD40 antibodies, induce potent CTL responses that surpass responses induced by recombinant vaccinia virus [460,486]. The APLs (A4Y, V4Y or S4Y) used differ only at the position four of the p33 sequence [73], a position particularly important for TCR interaction [487]. Using these tools we mimicked with the APL-HBcAg the initial infection with the wild-type virus and simulated by immunization with p33 coupled to a different VLP (Q β) the challenge with an escape mutant virus. By final infection with Vacc-GP the induced protection against the p33 "mutant" strain was tested. We found good induction of APL-specific CD8⁺ T cells after the initial immunization, measured by APL-loaded tetramers. The immunization with p33-VLP also induced a specific response which was even enhanced by final infection with vaccinia-p33. However, as predicted by the concept of original antigenic sin, a clearly reduced protection against Vacc-GP could be detected in mice previously immunized with APL-HBcAg followed by boost with p33-Q β compared to mice only immunized with p33-Q β . This was especially surprising as the amount of p33-specific CD8⁺ T cells after Vacc-GP infection reached equal levels in both groups of mice.

6.3. Materials and Methods

6.3.1. Mice viruses and cell lines

C57BL/6 mice were purchased from Harlan (Horst, The Netherlands) at the age of 8–14 wk. 327 TCR-transgenic mice expressing a TCR specific for peptide p33 [461] were kept under specific pathogen-free conditions at Cytos Biotechnology AG.

Recombinant vaccinia virus expressing LCMV-GP was grown and titrated on BSC40 cells as described [462]. To examine antiviral immunity, vaccinated females C57BL/6 were infected i.p. with 1.5×10^6 PFU Vacc-GP. Five days later ovaries were collected, and the vaccinia titers were determined.

6.3.2. Peptides, oligonucleotides, peptide-loaded tetramers and VLPs

LCMV glycoprotein peptide p33–41 (KAVYNFATC) and the subvariants A4Y (KAVANFATC), V4Y (KAVVNFATC) and S4Y (KAVSNFATC) were synthesized by a solid phase method and were purchased from Eurogentec (Hestel, Belgium). Phosphorothioate-modified CpG-ODN was synthesized by Microsynth (Balgach, Switzerland). The following oligonucleotide sequence was used: 1668pt (5'-TCC ATG ACG TTC CTG AAT AAT-3') [460]. APL-loaded tetramers were purchased from Proimmune Limited (Oxford) and used according to the manufacturer's recommendations. Briefly, blood samples from peptide-VLP immunized C57BL/6 mice were stained with an anti-CD8 antibody and APL-loaded tetramers and analyzed by FACS. The p33-VLP was generated as described in [460] using standard PCR methods, but instead of KAVYNFATM, KAVYNFATC was added. The p33 variants were fused the same way to the HBcAg (aa 1-183). The VLPs were analyzed with SDS-PAGE and on a 1% agarose gel. Production, purification, and characterization of Q β p33-VLPs were previously described in detail [488,489].

6.4. Results and discussion

In order to be able to induce strong CTL responses in the absence of viral replication, peptide p33 and three APLs derived thereof were fused to the HBcAg as described [460]. As APLs the two partial agonistic peptides A4Y and V4Y and the antagonistic peptide S4Y were used. The agonistic and antagonistic capacity has previously been determined using TCR transgenic T cells [73]. These APL-VLPs were then used to simulate immunization with different viral variants of one virus strain. Thus, the APL-HBcAg were used to mimic the initial infection with a wild-type virus, whereas the boost with p33-Q β was used to simulate the subsequent infection with a mutant strain. Four mice per group were immunized with APL-HBcAg or p33-HBcAg. To enhance CTL response and in order to reach similar frequencies as observed after immunization with recombinant vaccinia virus, CpGs were added as adjuvant [489]. The immune response induced by the first "infection" was monitored at the peak of the response around day eight by measuring the frequencies of antigen-specific CD8⁺ T cells using APL- or p33-loaded tetramers. All immunized mice showed good induction of antigen-specific CTLs. Peptide p33, A4Y, V4Y or S4Y all induced high frequencies of specific T cells, ranging from 3.1% \pm 1.0% for p33 to 11.0% \pm 1.8% for V4Y (Figure 1A, Table 1). This result shows that in wild-type mice the APLs are equivalent to p33 for induction of T cell responses and are higher than the frequencies usually obtained with recombinant vaccinia virus. By day 30 the frequency of specific T cells was clearly reduced to about 1-2% for all groups (Figure 1B, Table 1). The cross-reactivity of APL-specific CTLs to p33 was also measured on day 8 after immunization. Only weak cross-reactivity could be found, actually reduced to two mice of the V4Y-VLP group which showed 1.1% and 1.9% p33-crossspecific T cells (data not shown). The p33 immunized mice showed no cross-reactivity at all against the APLs (data not shown).

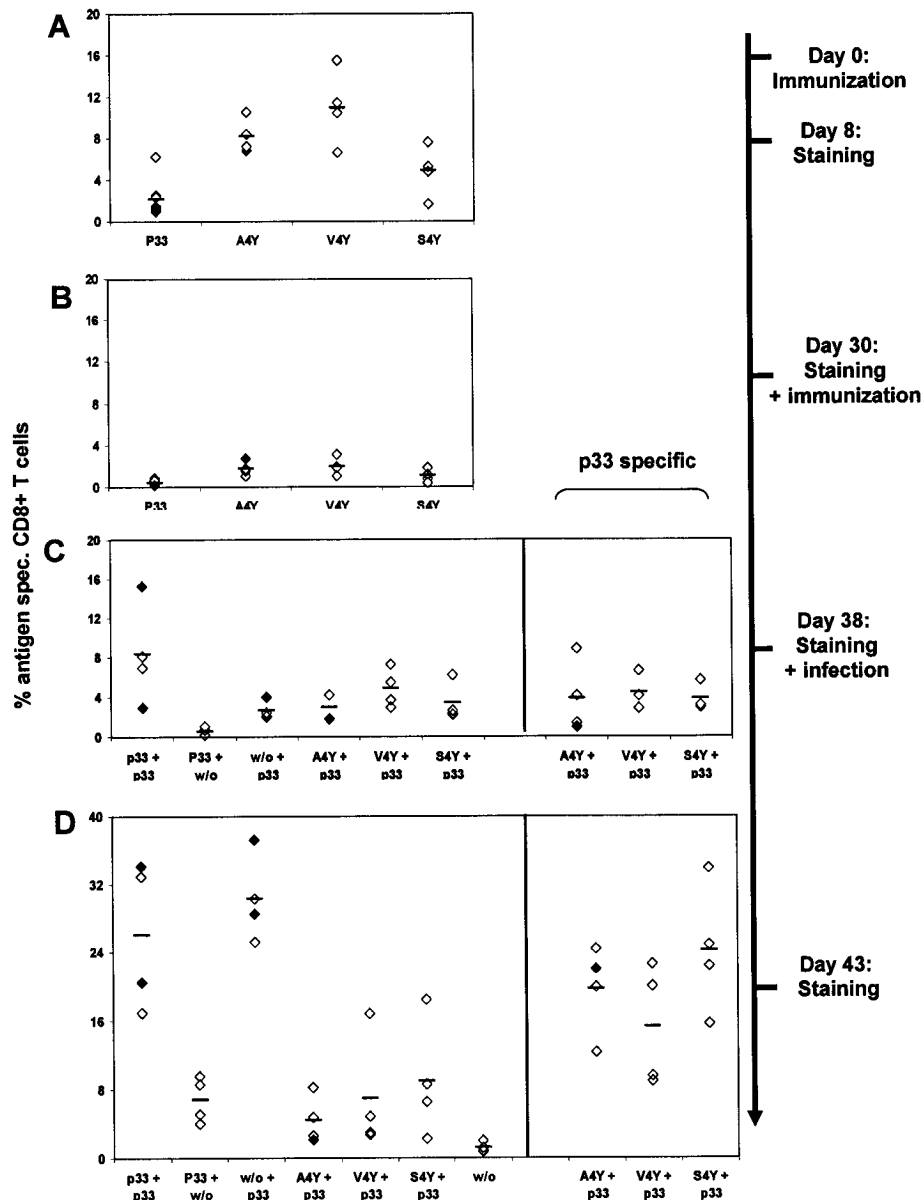


Figure 1(27). Tetramer staining of blood samples from mice immunized with p33- or/and APL-VLP: C57BL/6 mice were immunized s.c. with either 30 μ g of p33-, A4Y-, V4Y- or S4Y-VLP (HBcAg) with 20 nmol CpGs. As a control, mice were injected with PBS and 20 nmol CpGs. On day 8 (**A**) and day 30 (**B**) blood from the immunized mice was stained with anti-CD8 and p33-, A4Y-, V4Y- or S4Y-loaded tetramers and analyzed by FACS. **C.** On day 30 the mice were immunized with p33-VLP (Q β) without CpG and on day 38 their blood was analyzed on specificity for p33, A4Y, V4Y and S4Y. In addition to the originally immunized APL, also p33 specificity was measured (on the right side). **D.** Then the mice were infected with Vacc-GP (2×10^6 PFU) and on day 43 the blood was analyzed once more. The peptides used for the first and the second immunization are indicated on the x-axes. If not otherwise indicated the specificity for the APL used in the first immunization was measured. The percentage of tetramer and CD8 double positive T-cells are indicated on the y axes. Each square

represents one mouse and lines show average specific CD8⁺ T cells in each group. Black squares indicate mice which were protected from vaccinia infection.

For the boost with p33 on day 30 simulating infection with a viral variant, p33 coupled to second VLP, Q β , was used. The change of the VLP was made to avoid a potentially disturbing antibody response covering the carrier due to antibodies against HBcAg and to focus the T cell response on a single, short epitope. Since Q β has a higher intrinsic immunogenicity than p33-HBcA-, p33-Q β was injected without CpG. On day 35 (data not shown) and 38 (Figure 1C, Table 1) the responses against p33 and against the APLs were determined. As expected, the mice immunized and boosted with p33 showed a high frequency of p33 specific CD8⁺ T cells around 8.3% \pm 2.6%. The mice immunized on day 30 with p33 without pre-immunization with APL showed specific CD8⁺ T cell frequencies of 2.7% \pm 0.5%. Upon boost of the APL-immunized mice with p33-Q β , the response against the APLs was also enhanced. A4Y, V4Y and S4Y immunized mice showed frequencies of 3.0% \pm 0.7%, 4.8% \pm 1.0% and 3.4% \pm 0.9% of APL-specific CD8⁺ T cells (Figure 1C, left side, Table 1). At the same time these mice also showed a response against p33 which was similar or even better than for the mice immunized against p33 without APL-preimmunization. The frequencies observed were 3.8% \pm 1.8%, 4.4% \pm 0.8% or 3.4% \pm 0.6% for A4Y, V4Y and S4Y immunized mice (Figure 1C, right side, Table 1). In mice immunized on day 0 with p33-HbcAg without boosting, frequencies of specific T cells had almost dropped to background. Thus the pre-immunization with APLs did not impair the p33 specific response as revealed by tetramer staining, since the frequencies of p33 specific CD8⁺ T cells reached similar levels. Furthermore, as predicted by the concept of original antigenic sin, APL specific CD8⁺ T cells were boosted by p33-Q β , although essentially no cross-reactivity was apparent at the peak of the primary response. For the group immunized and boosted with p33, a memory effect could be observed, as the frequencies of specific CD8⁺ T cells was higher than in all other groups.

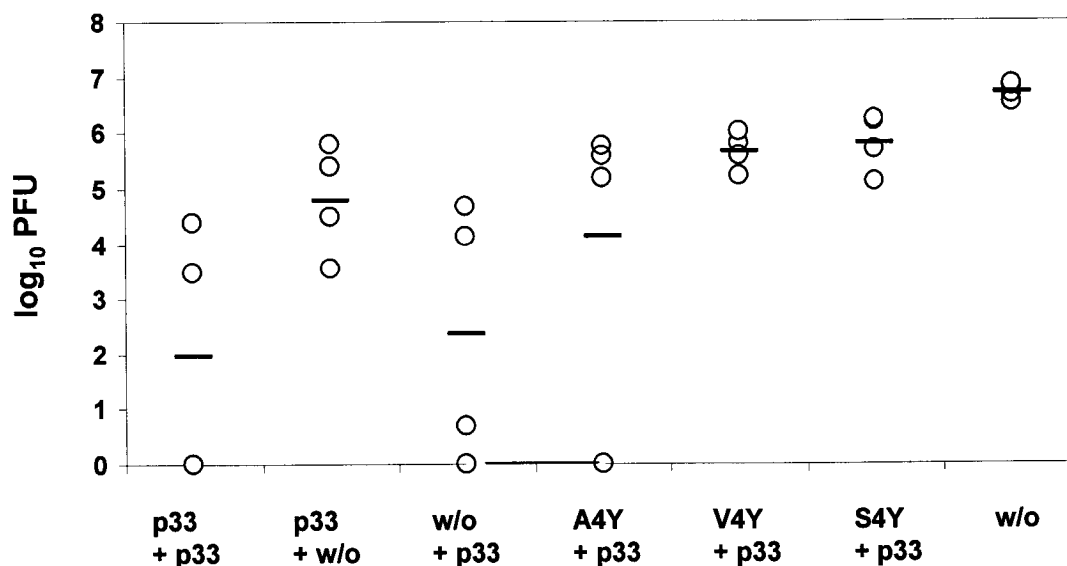


Figure 2(28). Immunization with APLs disturbs p33 mediated viral protection: Groups of four C57BL/6 mice were immunized s.c. with either 30 μ g of p33-, A4Y-, V4Y- or S4Y-VLP (Hbcore) and 20 nmol CpGs. Control mice received PBS and 20 nmol CpGs. 30 days after priming mice were immunized s.c. with p33-VLP (Qb) and were challenged eight days later with 1.5×10^8 PFU Vacc-GP. Five days later ovaries were isolated, and viral titers in the organs were determined. Circles represent individual mice and lines show average viral titers in each group.

In order to test protective immunity, the mice were challenged on day 38 by infection with Vacc-GP. Five days after viral challenge, the frequencies of specific CD8⁺ T cells and viral titers in ovaries were determined (Figure 1D, Table 1). Viral infection boosted the p33 response in all groups of mice. For the mice immunized and boosted with p33-VLP and the mice immunized only once with p33-Q β on day 30, the best responses were reached with 26.4% \pm 4.4% and 30.4% \pm 2.6%, respectively. The mice immunized with p33-HBcAg on day 0 without re-immunization also showed some memory effect as the frequency of p33 specific CD8⁺ T cells was higher than in untreated mice, 6.8% \pm 1.3% versus 1.2% \pm 0.3%. By infection with Vacc-GP also the frequency of APL specific CD8⁺ T cells increased. In the A4Y, V4Y and S4Y group, 4.4% \pm 1.4%, 6.9% \pm 3.3% and 9.0% \pm 3.4% of APL specific CD8⁺ T cells could be measured (Figure 1D, left side, table 1). These numbers were clearly higher than those found prior viral infection. Most interestingly the frequencies of p33-specific CD8⁺ T cells found in these mice were almost as high as those found in mice primed and boosted with p33 (Figure 1D, right side, Table 1). These numbers

suggest that the mice should be protected from Vacc-GP infection. Nevertheless, and in accordance with the concept of original antigenic sin, these mice were not protected from infection with Vacc-GP. In fact, viral titers were even higher than those observed in mice that were immunized with p33-Q β alone (Figure 2, Table 1).

Table 1(2). Tetramer staining and protection in p33/APL-immunized mice.

Immunization	day8	day30	day35	day38	day43	protection	Average LOG(PFU)
p33/p33	2.1	0.4	10.5	8.3	26.1	2/4	2.0
p33/-	2.1	0.4	0.4	0.5	6.8	0/4	4.8
A4Y/p33	8.2	1.8/0.3	2.1/3.8	3.0/3.8	4.4/19.7	1/4	4.1
V4Y/p33	11.0	2.0/0.9	3.9/4.5	4.8/4.4	6.9/15.3	0/4	5.6
S4Y/p33	4.9	1.1/0.3	1.8/2.5	3.4/3.7	9.0/24.2	0/4	5.8
-/p33	n.d.	n.d.	2.6	2.6	30.4	2/4	2.4
-/-	n.d.	n.d.	n.d.	n.d.	1.2	0/4	6.7

The mice either immunized with p33-HBcAg on day 0 and the non-immunized mice showed no protection. In conclusion these results indicate that pre-existing T cells specific for APLs indeed impair the build-up of protective immunity against a variant virus. Despite the successful induction of p33 specific CD8⁺ T cells in these mice, and although similar frequencies of p33-specific T cells were reached as in the protected mice, no viral protection resulted. This may suggest that the CD8⁺ T cells generated in the APL-pre-immunized mice were less efficient killers than the CD8⁺ T cells generated in the protected mice. Indeed, despite a low affinity for p33, APL-specific memory CTLs may be restimulated by p33 [453,490]. Since these APL-specific low affinity memory CTLs are present at elevated frequencies, they may compete with the high affinity T cells, present at low frequency, inhibiting their activation and expansion. As a consequence, the cell population has a low affinity overall for p33, leading to impaired protection.

7. ENHANCED APOPTOTIC ACTIVITY OF A STRUCTURALLY OPTIMIZED FORM OF GALECTIN-1

*Patrick Bättig, *Philippe Saudan, †Tea Gunde and *Martin F. Bachmann

*Cytos Biotechnology AG, Wagistrasse 25, 8952 Schlieren-Zürich, Switzerland

† ESBATech AG, Wagistrasse 21, 8952 Schlieren-Zürich, Switzerland

Address correspondence to: martin.bachmann@cytos.com

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Autoimmunity

7.1. Abstract

Galectin-1 is a homodimeric protein with potent anti-inflammatory properties due to its ability to induce apoptosis in thymocytes and T cells. The galectin-1 subunits are not covalently linked but the monomers are in a dynamic equilibrium with the dimeric form. Since the affinity of the monomers for each other is rather low (in the range of 10^{-5} M), the *in vivo* efficacy of galectin-1 is limited because the equilibrium is shifted towards the inactive monomeric form at lower concentrations. In order to overcome this problem, we designed a covalently linked form of the dimer based on the galectin-1 crystal structure. Here we show that this irreversibly dimeric form of galectin-1 is a potent inducer of apoptosis in murine thymocytes as well as murine mature T cells at concentrations 10-fold lower than wild-type galectin-1. This structurally optimized form of galectin-1 may therefore be a potentially powerful tool to treat chronic inflammatory diseases.

7.2. Introduction

Galectins, also referred to as S-type lectins, are a conserved family of proteins defined by the presence of at least one characteristic carbohydrate recognition domain with an affinity for β -galactoside [491-493]. Galectin-1 was the first mammalian galectin discovered and is the most extensively characterized [494-496]. It was shown to be expressed in a variety of different tissues including muscle [497,498], spleen [499,500], thymus [501], lung [497,502] and the olfactory system [503,504]. In accordance with its wide tissue distribution, galectin-1 has been reported to be involved in many biological processes including cell adhesion [498,501,505,506], cell growth [502,507,508], neoplastic transformation [509], migration [510,511], T cell maturation [501,506] and the induction of apoptosis in T cells and thymocytes [501,506,512]. This latter property has been harnessed for therapeutic use. Specifically, administration of galectin-1 was shown to suppress the development of collagen-induced arthritis in rodent models [513,514] and to exert protective effects on concanavalin A-induced hepatitis in mice [515]. Thus, galectin-1 exhibits potent anti-inflammatory properties in these model systems. Nevertheless, in these experiments, relatively high concentrations of galectin-1 had to be administered in order to see an effect. In fact, 4 mg/kg/day were administered, precluding use in humans [513]. Thus, in order to be therapeutically useful, a more active form of galectin-1 is required.

As galectin-1 binds to lactosamine, a component of both N- and O-linked glycans, many possible counter-receptors for galectin-1 arise [492,516,517]. On T cells CD2, CD3, CD4, CD7, CD43 and CD45 have been discussed as possible glycoprotein counter-receptors for galectin-1 [518-520]. CD45 was shown to modulate galectin-1 induced apoptosis in a manner dependent on its glycosylation state, as oligosaccharide-mediated clustering of CD45 was found to facilitate galectin-1 induced cell death [521]. However CD45 does not seem to directly trigger apoptosis, since cells lacking CD45 are also susceptible to galectin-1 induced cell death [521,522].

In contrast, CD7 was shown to be essential for galectin-1-mediated death, since CD7 negative cells are no longer susceptible to galectin-1 induced apoptosis [523].

Galectin-1 lacks a N-terminal signal peptide and is transported through the cell membrane by an unknown secretory pathway [524,525]. After secretion galectin-1 stays associated with the cell membrane. This is crucial for preserving the activity of the protein, since the binding of galectin-1 to sugar residues on membrane proteins prevents it from being oxidized by the cell environment [526,527].

For most activities the physiologically active form of galectin-1 has been reported to be a homodimer with a subunit molecular mass of 14.5 kDa [506,526,527]. The monomeric and the dimeric form are found in a reversible equilibrium with a K_D of $\approx 7 \mu\text{M}$ [526,527]. Based on this low affinity, high amounts of the protein are needed to reach the critical concentration of the active dimer required for biological efficacy as a therapeutic agent. Hence, we hypothesised that the generation of a covalent dimer should improve the dose response curve for the pharmacological effects of galectin-1 and thereby help to develop galectin-1 as a drug to treat inflammatory diseases.

Based on crystallographic data [528,529] and a report which mapped the dimerization domain of galectin-1 by mutational analysis [530], we decided to fuse two galectin-1 moieties (C to N) via a two-glycine linker. We reasoned this should allow for proper interaction of the N- and C-terminal residues of the two subunits in order to form a dimer with the correct tertiary structure.

We found that this novel dimeric form of galectin-1 was still efficiently secreted. Moreover, the covalently dimerized galectin-1 was 3-10 times more potent at inducing apoptosis in cultivated primary cells. Thus the covalent dimer exhibits improved pharmacological properties and may be a new tool to treat chronic inflammatory diseases.

7.3. Experimental procedures

7.3.1. Construction of eukaryotic expression plasmids containing the sequence of the dimeric and wild-type galectin-1

The human galectin-1 cDNA subcloned into puc19 was a kind gift of L. Mallucci [531]. The wild-type galectin-1 sequence encoding the complete open reading frame of human galectin-1 was amplified by PCR using forward primer1 (5'-agtgtgaagtgctctagaccacc ATG GCT TGT GGT CTG GTC-3') and reverse primer1 (5'-agtagtataccgctcgagtca TCA AAG GCC ACA C-3') with galectin-1 cDNA cloned into puc19 as a template. The resulting PCR product was subcloned into the eukaryotic expression vector pCEP-Pu [532]. For the generation of the covalent dimeric form of galectin-1, the sequence encoding galectin-1 was amplified by a first PCR using forward primer 1 and reverse primer 2 (5'-accacaagccat ACC GCC GTC AAA GGC CAC ACA TTT G -3') and a second PCR using forward primer 2 (5'-gtggcctttgac GGC GGT ATG GCT TGT GGT CTG GTC-3') and reverse primer 1. Finally, the two PCR products were mixed and amplified with forward primer 1 and reverse primer 1 leading to a cDNA encoding two galectin-1 monomers genetically linked by a two glycine linker. This cDNA was then subcloned into pCEP-Pu. Both the wild-type galectin-1 and the covalent dimeric form were also made as N-terminal HA tagged (MSYPYDVPDYSPDLLQTT-3) versions using standard PCR protocols.

7.3.2. Expression and purification of the recombinant proteins

293-EBNA cells were seeded at a density of 4×10^6 cells/ 90mm plate in DMEM (GIBCO BRL) containing 10% FCS (GIBCO BRL) 1 day prior to transfection. The cells were then transfected with lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation. One day after transfection cells were passed under puromycin selection. One to two days later all non-transfected cells had detached. The resistant population was then further expanded. Recombinant protein was either purified from supernatants or from cell lysates of transfected cells. Briefly, serum-free supernatants were collected and centrifuged at 450xg for 5 min to discard cell debris. Then 4 mM β -mercaptoethanol (SIGMA) was added and the supernatants were stored at 4°C until affinity purification. Cells were collected in PBS by scraping with

rubber policeman and pelleted at 340xg for 5 min. The pellet was then resuspended in PBS containing 4 mM β -mercaptoethanol and 150 mM lactose. After the addition of phenylmethylsulfonyl fluoride (SIGMA), the cells were physically broken by sonication (Bandelin Sonopuls). The homogenate was centrifuged for 30 min at 50xg to remove cell debris and aggregates, and then for one hour at 48'000xg to discard precipitates. After a dialysis step to remove the lactose, an affinity purification with lactose-sepharose was performed. The lactose-sepharose was prepared as described previously [533]. After a second dialysis step, the proteins were stored in PBS with 4 mM β -mercaptoethanol. The protein concentrations were determined by Lowry (SIGMA).

7.3.3. Nondenaturing gel electrophoresis

Based on a calculated pI of 4.8 for wild-type galectin-1 and 5.0 for the covalent dimer, a 100 mM sodium phosphate nondenaturing 12.5% polyacrylamide gel of pH7 was cast. The two proteins are negatively charged under these conditions and run to the anode, resulting in a separation by size and charge. The gel was run at 30 mA for 12 hours to get a proper separation of galectin-1 monomers and dimers.

7.3.4. Reduction by acetylation and oxidation of the recombinant proteins

Covalent reduction of galectin-1 was achieved by alkylation with iodoacetamide (SIGMA). Purified galectin-1 was diluted 1:2 with an alkylating buffer (0.02 M Na phosphate buffer pH7.8, 100 mM Tris/HCl, 140 mM NaCl, 5 mM β -mercaptoethanol, 50 mM iodoacetamide). The samples were then incubated at 4°C for 3 hours. Purified wild-type galectin-1 and the covalent dimer were oxidized by an air oxidation method using CuSO₄ as a catalyst as described previously [534].

7.3.5. Hemagglutination assay

The lectin activity of wild-type galectin-1 and the covalent dimer was determined by a hemagglutination assay using mouse erythrocytes. Briefly, the assay was performed in 96-well microtiter plates with serial two-fold dilutions (50 μ g/ml-0.8 μ g/ml) of the purified untreated, reduced or oxidized proteins in PBS in the absence or presence of 4 mM β -mercaptoethanol. Samples (50 μ l) were mixed with 50 μ l 1%

(v/v) suspension of mouse erythrocytes and incubated at room temperature for two hours. Agglutination activity was determined by the sedimentation state of the erythrocytes. As a positive control the lectin concanavalin A was used.

7.3.6. Measurement of apoptosis by Annexin-Cy5 staining

Thymi or spleens were removed from C57Bl/6, TCR318 or TCR327 mice, smashed over a metal grid to obtain single cell suspensions. The cell suspensions were then washed with PBS and the erythrocytes were eliminated via a density gradient centrifugation step using Lympholite®-M from Cedarlane® Laboratories Ltd. according to the manufacturer's recommendations. EL-4 thymoma cells, thymocytes, naive splenocytes from C57Bl/6 mice or LCMV p33 peptide activated splenocytes (from TCR318 or TCR327 TCR transgenic mice expressing a TCR specific for peptide p33) [461] at concentration of 10^6 cells/ml were incubated at 37°C in medium with serial dilutions of wild-type galectin-1 or covalent dimer in the presence of 1 mM β -mercaptoethanol for five or eight hours. Samples were adjusted to 0.1 M lactose for ten minutes to dissociate galectin-1-agglutinated cell clumps and washed with PBS before flow cytometry. Induced cell death was measured using the AnnexinV-Cy5 Apoptosis Detection Kit from Medical and Biological Labs, Co, Ltd using a FACScan Calibur (Becton Dickinson). The data was analyzed using WinMDI software version 2.8. The percentage of induced death was calculated relative to the percentage of live cells obtained in the unstimulated culture using the following equation: induced death = $100 \times [1 - (\% \text{ treated live cells} / \% \text{ untreated live cells})]$

7.4. Results

7.4.1. Dimerized galectin-1 is still efficiently secreted via the unknown non-classical pathway and does not form tetramers

Galectin-1 exists in a reversible monomer-dimer equilibrium with a K_D of 7 μM [526,527]. The crystal structure of galectin-1 suggests that dimer formation is based on hydrophobic interactions between residues within the N- and the C-termini at the subunit interface (Figure 1A) [528,529]. As the active form of galectin-1 is thought to be the dimeric form [506,526,527], we reasoned that a covalently dimerized galectin-1 would show improved activity compared to the wild-type galectin-1 protein. To test this we created a covalent dimeric form by linking two galectin-1 monomers genetically by a two glycine linker, as shown in Figure 1A.

Galectin-1 is secreted via a non-classical pathway not involving the ER [525,526]. We therefore wondered whether the dimeric form was secreted in the same manner as the wild-type galectin-1. N-terminally HA tagged versions of the proteins were generated. These different constructs were then transfected into 293-EBNA cells and stable cell populations containing the plasmids were generated. Supernatants from cell populations expressing the HA-tagged monomer or covalent dimer were then analyzed by SDS-PAGE and subsequent western-blot analysis with an antibody against the HA tag (1B). As shown in Figure 1B comparable amounts of wild-type protein and covalent dimer were found in supernatants of transfected cells. Thus the covalent dimer is still efficiently produced and secreted by the cells.

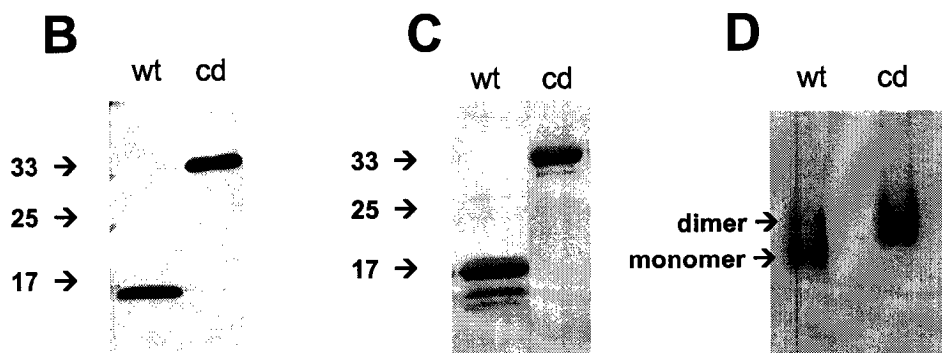
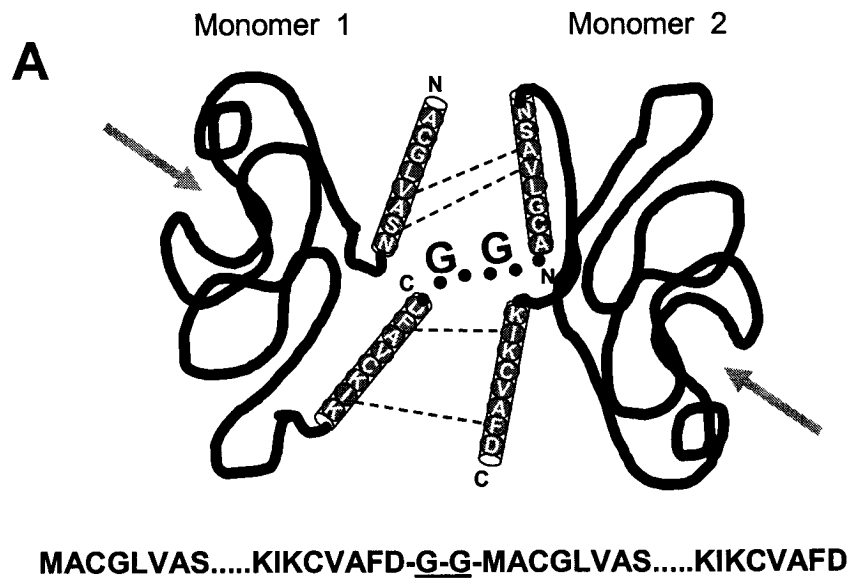


Figure 1(29). Generation and expression of dimerized galectin-1. **A**, A model for the galectin-1 dimer based on crystallographic data is shown and possible hydrophobic interactions between the first β -strand (residues 4-7) in the N-terminus and the last β -strand (residues 126-132) in the C-terminus of the respective monomer subunits 1 and 2. The interactions between Val-5 of monomer 1 and Ala-6 of monomer 2; Ser-7 of monomer 1 and Val-5 of monomer 2; Ile-128 of monomer 1 and Phe-133 of monomer 2; and Phe-133 of monomer 1 and Ile-128 of monomer 2 are indicated by broken lines. The sugar-binding grooves are indicated by arrows. The glycine linker which connects the two monomers in the covalent dimer is indicated by a pointed line. Below the picture the amino acid sequence of the linker region is shown. **B**, Immunoblot analysis of recombinant HA tagged versions of wild-type galectin-1 and covalent dimers. The proteins were produced in 293 cells and analyzed by SDS-PAGE. The two galectin-1 forms were detected with a HA-tag specific antibody. **C**, Coomassie-stained SDS-PAGE gel of affinity purified recombinant proteins. **D**, Coomassie-stained native gel of affinity purified recombinant proteins. In B, C and D wt corresponds to wild-type galectin-1 and cd to the covalent dimer. Molecular mass markers are indicated in kDa by arrows at the right.

In order to study the biological activity of our new constructs, the recombinant proteins were purified over lactose-sepharose columns. For this purpose the untagged versions of wild-type galectin-1 and the covalent dimer were used. As shown in Figure 1C both recombinant proteins could be purified to a high degree using this affinity purification procedure. The fact that the covalent dimer is still efficiently purified by this method, which is based on the lectin properties of galectin-1, demonstrates that our engineered galectin-1 is active as a lectin and efficiently binds to lactose.

Since galectin-1 forms dimers, we wondered whether the covalent dimer would dimerize to form galectin-1 tetramers. To test this, purified wild-type galectin-1 and the covalent dimer were loaded on a native gel which allows the separation of native proteins according to their size and charge. As shown in Figure 1D wild-type galectin-1 migrates as two bands. Whereas the dominant band corresponds to the monomer, the slower migrating band corresponds to galectin-1 dimers. The distribution of these two bands reflects the equilibrium situation between monomer and dimer. In contrast the covalent dimer migrates as a single band running at the height of the wild-type dimer. Hence the covalent dimer does not itself dimerize to form galectin-1 tetramers.

7.4.2. Covalent dimerization of galectin-1 strongly increases the agglutination activity of the protein.

Induction of apoptosis by galectin-1 is dependent on its divalent sugar binding ability [506,527]. We therefore tested whether the covalent dimer would show an improved sugar binding activity compared to wild-type galectin-1. To do this we performed a hemagglutination assay, which shows the lectin activity of galectin-1. In this assay the ability of a molecule to bind carbohydrates on two separate cells leads to the formation of erythrocyte-aggregates, which have a lower density than single cells and therefore do not sediment. Mouse erythrocytes were incubated with serial dilutions of purified wild-type galectin-1 and covalent dimer and the hemagglutination activity was monitored. As shown in Figure 2, wild-type galectin-1 exhibits hemagglutination activity at concentrations as low as 25 $\mu\text{g/ml}$. In contrast the covalent dimer induced strong agglutination at 12.5 $\mu\text{g/ml}$, and was therefore four times more potent in respect to its molarity than wild-type galectin-1.

To exhibit sugar binding activity the galectin-1 molecule has to be in a reduced state. To ensure complete reduction of the wild-type and the covalent dimer form of the protein, we alkylated the SH groups of the cysteines, which leads to an irreversible reduction of the protein. By this treatment the activity of the wild-type protein was increased by a factor of two and the activity of the covalent dimer was increased four-fold (Figure 2).

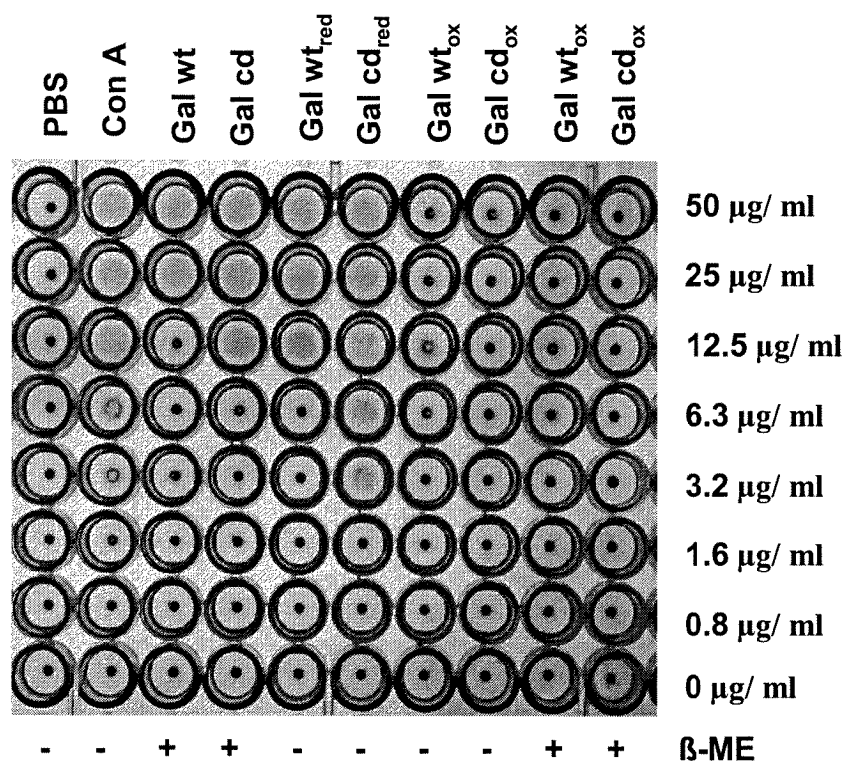


Figure 2(30). Effect of covalent dimerization on hemagglutinating activity of galectin-1. 1% v/v mouse erythrocytes in PBS were mixed with different concentrations of wild-type galectin-1 or covalent dimer in a round bottom 96 well plate and incubated for one hour at 37°C. The protein concentrations are shown to the right of the respective row. In the absence of agglutinating activity the erythrocytes form a tight pellet at the bottom of the well as seen in the PBS lane, whereas agglutination of the erythrocytes lead to a cloudy appearance of the wells. Purified wild-type galectin-1 or covalent dimer was used as untreated, covalently reduced (red) and oxidized (ox) variants, and the assay was performed in the presence or absence of β-mercaptoethanol (indicated below the respective columns). The preparations of the proteins are indicated above the respective columns. wt corresponds to wild-type galectin-1 and cd to the covalent dimer. Concanavalin A (Con A) was used as a positive control.

The greater increase in activity observed upon alkylation for the covalent dimer compared to the monomer suggests that the dimer is more susceptible to oxidation. Probably because the dimer contains the double amount of SH groups. Moreover one oxidation event in a dimer molecule inactivates two galectin-1 subunits, whereas an oxidation event in the monomer molecule just inactivates one subunit. However as shown above, the protein can be maintained in a fully active state by covalent reduction. We decided to further investigate the effect of oxidation on the hemagglutinating activity of the proteins. Oxidation of the proteins with CuSO_4 leads to complete inactivation of the two forms of the protein. As expected from previous reports, the oxidized inactive protein could not be reactivated by reducing agents such as β -mercaptoethanol (Figure 2). Thus, the engineered covalent dimer and the wild-type protein reacted both similarly to reduction and oxidation.

In conclusion if the protein preparations are irreversibly reduced the covalent dimer shows a 10-fold increased hemagglutination activity. These results demonstrate that the covalent dimer is fully functional and that shifting the monomer-dimer equilibrium towards the dimer by genetic linkage of two monomers drastically increases the activity of the molecule.

7.4.3. Covalent galectin-1 dimers are active and kill thymocytes ten times more efficiently than the wild-type form.

Wild-type galectin-1 has previously been shown to kill thymocytes in as little as five hours by the induction of apoptosis. This activity of galectin-1 has been postulated to be involved in the negative selection of thymocytes [512]. A high concentration of 10^{-5} M (0.14 mg/ml) galectin-1 has been reported to be required for the efficient induction of apoptosis. Since the monomer-dimer equilibrium has a dissociation constant in a similar range, it is expected that the presence of the active dimer becomes limiting under these conditions. Thus, the covalent dimers of galectin-1 should show an apoptotic activity at lower concentrations than the wild-type protein. To test the apoptotic potency of the galectin-1 produced in this study, we first treated EL-4 thymoma cells with three-fold dilutions of wild-type galectin-1 or covalent dimer starting at a concentration of 10^{-5} M. Induced cell death was measured after eight hours treatment. The covalent dimer was three-fold more potent in respect to its apoptotic activity than wild-type galectin-1 (Figure 3).

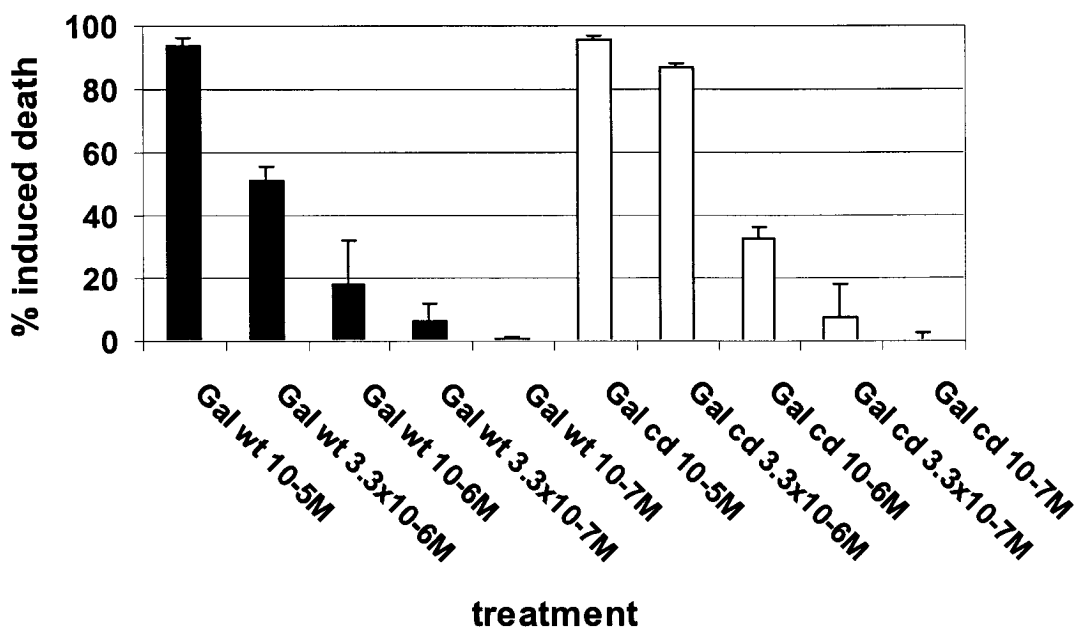


Figure 3(31). Dimeric galectin-1 is more potent in apoptosis induction in EL-4 cells compared to the wild-type protein. EL-4 Thymoma cells were incubated for eight hours with wild-type galectin-1 or covalent dimer at different concentrations ranging from 10^{-5} M to 10^{-7} M. Induction of apoptosis was measured by AnnexinV-Cy5 staining and analyzed by flow cytometry. Percent of induced cell death was calculated relative to live cells in unstimulated controls. The percentage induced death with five different concentrations of recombinant wild-type galectin-1 and covalent dimer compared to mock treated cells is shown on the Y-axis. The treatment of the thymocytes is shown on the X-axis. The error bars represent the standard deviation of triplicates.

We further set out to test the different forms of galectin-1 for their ability to induce apoptosis *ex vivo*. Thymocytes were prepared and incubated with serial dilutions of the wild-type or covalent dimer form of galectin-1. Five hours later the induction of apoptosis was monitored by AnnexinV-Cy5 staining. As shown in Figure 4, the covalent dimer still showed strong induction of apoptosis with a ten-fold lower concentration than the wild-type protein. Surprisingly for the highest concentration of 10^{-5} M, the galectin-1 monomer induced more apoptosis than the covalent dimer.

This may be explained by the observed loss of apoptotic cells by massive agglutination upon treatment with high concentrations of covalent galectin-1 dimers (see also discussion).

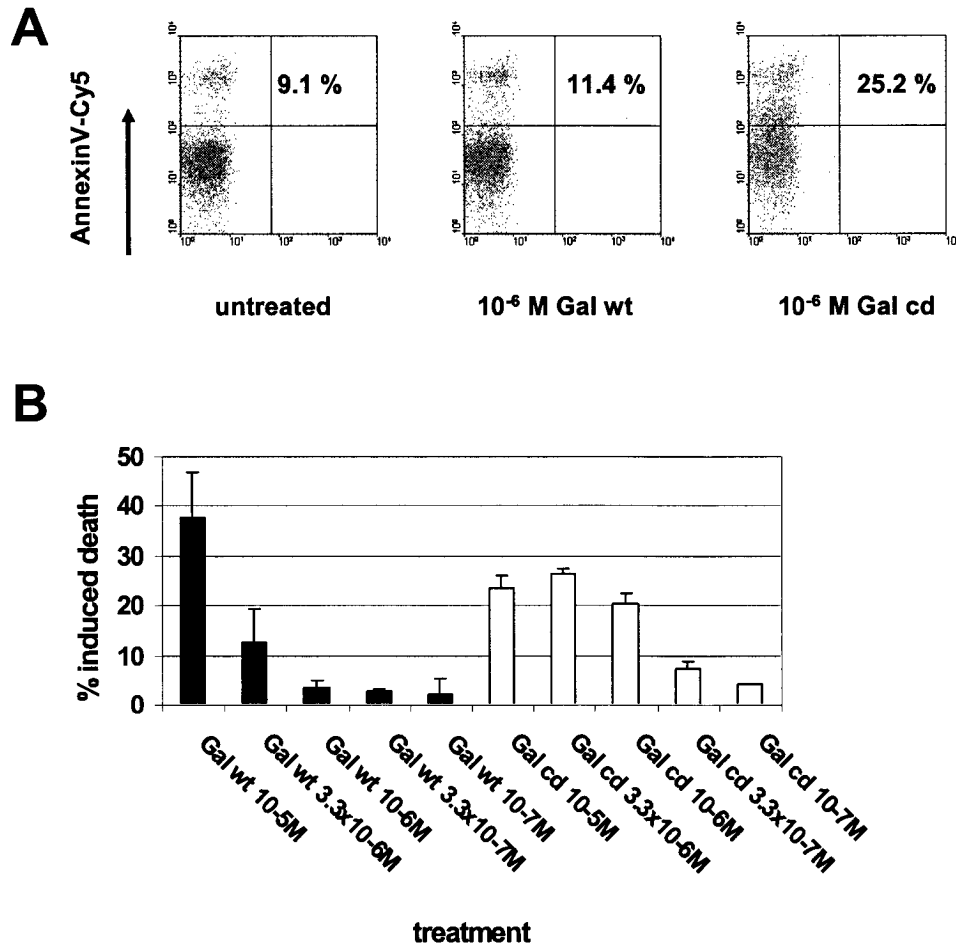


Figure 4(32). Effect of galectin-1 dimerization on apoptotic activity in thymocytes. Thymocytes from C57Bl/6 mice were incubated for five hours with wild-type galectin-1 or covalent dimer at different concentrations ranging from 10^{-5} M to 10^{-7} M. Induction of apoptosis was measured by AnnexinV-Cy5 staining and analyzed by flow cytometry. Percent of induced cell death was calculated relative to live cells in unstimulated controls. Panel A shows a representative dot plot of control cells and cells treated with the highest concentrations of wild-type galectin-1 and covalent dimer, respectively. AnnexinV-Cy5 staining is detected on the Y-axis. Panel B shows the induced death with five different concentrations of recombinant wild-type galectin-1 and covalent dimer compared to mock treated cells. The percentage induced death is shown on the Y-axis. The treatment of the thymocytes is shown on the X-axis. The error bars represent the standard deviation of three independent experiments. Different mouse donors and two different galectin-1 preparations were used in each replicate.

Induction of apoptosis by both forms of galectin-1 could be neutralised by pre-incubation of the proteins with 100 mM lactose (Data not shown). Hence the apoptotic activity of the covalent dimer is also dependent on the lectin properties of the protein, as previously shown for the monomer [506]. These findings further emphasize that the amount of dimer at low concentration is limiting for galectin-1 to exert its apoptotic activity and that this can be circumvented by the covalent dimerization of the protein.

7.4.4. Covalent galectin-1 dimers kill activated splenocytes more efficiently than the wild-type protein

Thymocytes are the precursors of mature T cells and are efficiently killed by the covalent dimer of galectin-1 as shown in the previous section. However in the case of chronic inflammation, peripheral T cells are the most important targets for a galectin-1 therapeutic. We therefore tested the activity of the covalent dimer on activated T cells. As a source of activated T cells, we used splenocytes from transgenic mice expressing a MHC class I restricted TCR specific for the p33 peptide from LCMV [461], since these cells can be quantitatively activated with the specific peptide *in vitro*. Because the transgenic TCR is MHC class I restricted, only CD8+ T cells are activated by the peptide. T cells were activated for three days and then incubated with serial dilutions of wild-type and covalent dimers of galectin-1. As shown in Figure 5C, apoptosis was induced by the addition of both the wild-type and the covalent dimer form of galectin-1. However the covalent dimer is much more effective in inducing apoptosis compared to the wild-type protein. We further investigated which cell types are most susceptible to galectin-1 mediated cell killing. Strong induction of apoptosis was observed mainly in the activated CD8+ T cells (Figure 5E).

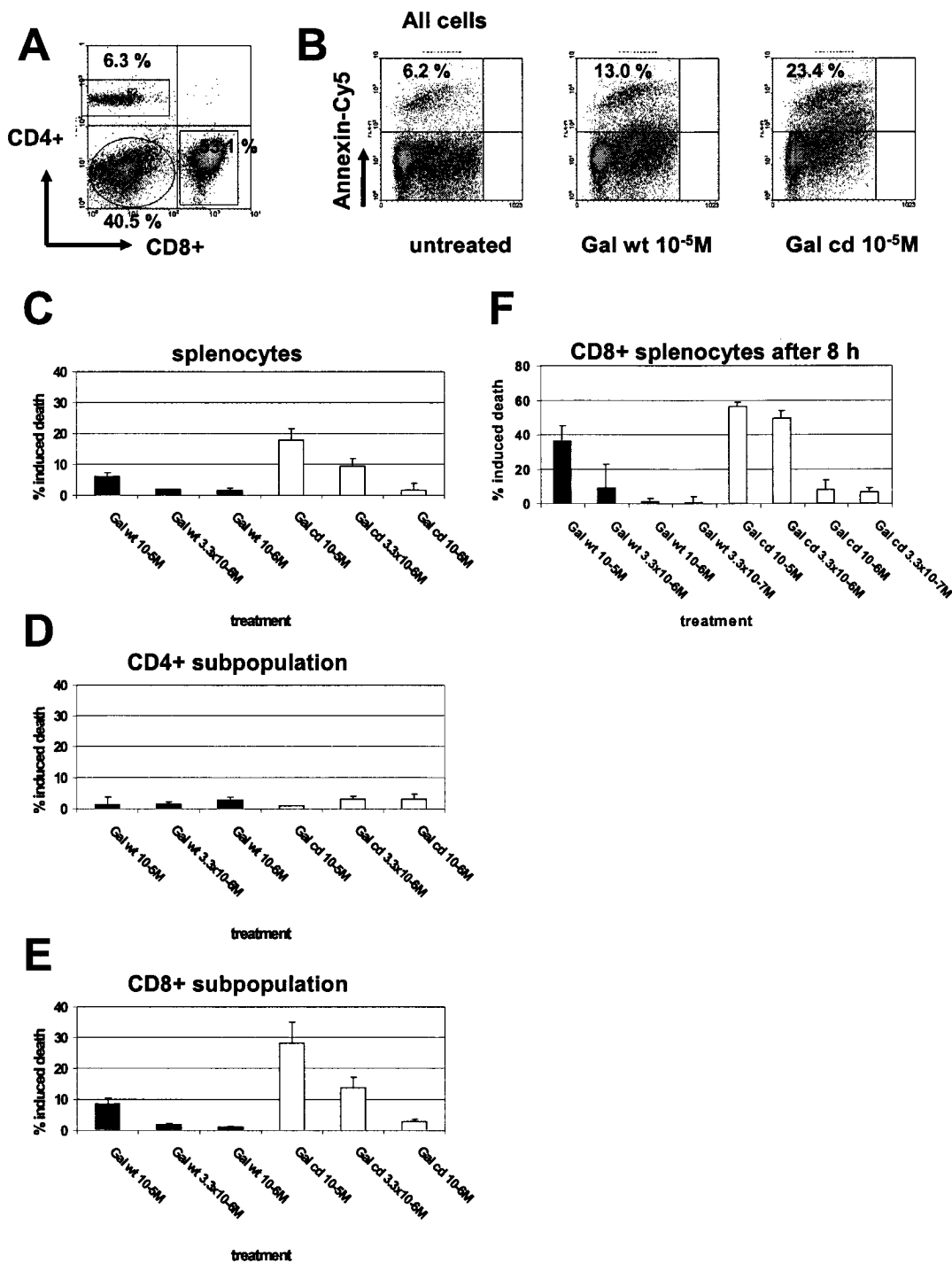


Figure 5(33). Effect of dimerization on apoptotic activity of galectin-1 measured for activated splenocytes and subpopulations, especially CD8+ cells. TCR 318 or TCR 327 mouse splenocytes, activated for two days with the MHC class I restricted p33 LCMV peptide, were incubated for five or eight hours with wild-type galectin-1 or covalent dimer at different concentrations ranging from 10⁻⁵ M to 3.3x10⁻⁷ M. Apoptosis was measured by Annexin-Cy5 staining and subsequent flow cytometry analysis. **A**, The distribution of CD4+, CD8+ and CD4-CD8- cells in the *in vitro* activated splenocytes is shown. The gates used for subsequent analysis of CD4+ cells (R2) and for CD8+ cells (R3) are indicated in the dot blot. **B**, Representative dot plots of control cells and cells treated with the

highest concentrations of wild-type galectin-1 (Gal wt) and covalent dimer (Gal cd), respectively are shown. The treatment is indicated below the respective panels. AnnexinV-Cy5 staining is detected on the Y-axis, the X-axis shows the side scatter. **C-E**, Induction of apoptosis in subpopulations of activated TCR 318 splenocytes of serial dilutions of wild-type galectin-1 (Gal wt) and covalent dimer (Gal cd) treated cells. C shows cell death induced in the ungated population, whereas D and E show the induced death in the gated CD4+, and CD8+ cells, respectively. F shows cell death induced in activated TCR 327 splenocytes after eight hours of galectin-1 treatment. Percent of induced cell death was calculated relative to live cells in mock treated activated splenocytes. The concentrations and proteins used are shown on the X-axis and the % induced cell death on the Y-axis. Different mouse donors and two different galectin-1 preparations were used. The error bars represent the standard deviation of three independent experiments (C-E) or triplicates (F).

As expected the non-activated T cells (CD4+) were hardly affected by the two forms of galectin-1 (Figure 5D). We additionally tested a second TCR transgenic mouse line (strain 327) which also has a TCR specific for the p33 peptide of LCMV, but in this case cells were exposed for a longer time to galectin-1. Splenocytes were activated with the p33 peptide and after 2 days the cells were incubated for eight hours with wild-type or covalent dimer of galectin-1. Treatment with p33 peptide again only activated the CD8+ T cells, which were clearly the major population induced to undergo apoptosis. This prolonged treatment with galectin-1 drastically increased the proportion of cells undergoing apoptosis. The induced death after treatment with the galectin-1 wild-type protein was 40% and after treatment with the covalent dimer was 60%. This in contrast to only 10% induced death with the monomer and 30% death with the dimer measured after 5h treatment (Figure 5E (after 5h) and 5F (after 8h)). These observations with both forms of galectin-1 are in accordance with the beneficial role reported for galectin-1 in inflammatory diseases due to induced apoptosis in peripheral T cells.

7.5. DISCUSSION

In the present study, we demonstrate that galectin-1 can be genetically engineered to improve the activity of the protein. Upon covalent dimerization, the protein displays two CRDs and becomes independent of the monomer-dimer equilibrium. This is particularly important at a protein concentration below 10^{-5} M, since the concentration of the active dimeric form becomes limiting under these conditions. These covalent dimers were produced in eukaryotic cells and we could show that this engineered protein is still efficiently secreted. On a non-reducing native gel wild-type galectin-1 protein was observed in the typical monomer-dimer equilibrium as shown by the appearance of two bands. In contrast the engineered protein migrated as single band with the size of a galectin-1 dimer (Figure 1). Since the glycine linker is connecting the two dimerization surfaces in the correct orientation, no further dimerization was expected, proper folding assumed. Therefore the absence of tetramers suggests that the engineered galectin-1 molecule shows a correct tertiary structure. This was further emphasised by the observation that the protein was fully biologically active, since it efficiently lead to the agglutination of erythrocytes.

The galectin-1 proteins produced for this study were additionally treated with oxidizing or reducing agents to measure the influence of oxidation status on protein activity. Interestingly, the covalent dimer was more susceptible to oxidation. This could be explained by the fact that the covalent dimer can not change its binding partner due to the covalent linkage and therefore a single oxidation in the dimer molecule will be sufficient to inactivate the whole molecule. This model is consistent with the four-fold enhanced activity of the covalent dimer compared to an only two-fold increased activity observed for the wild-type protein upon covalent reduction.

Most importantly, the engineered galectin-1 was found to be much more potent at both hemagglutination and apoptosis induction compared to the wild-type protein. Thus the engineered variant of the protein is a powerful tool for the induction of apoptosis. In addition, the increased potency of galectin-1 was most obvious at lower concentrations. This was expected from the physico-chemistry of the reversible

monomer-dimer equilibrium. Based on the monomer-dimer equilibrium with a K_D of $7 \mu\text{M}$ the concentration of dimer should be in the range of $3 \times 10^{-6} \text{M}$ at a wild-type galectin-1 concentration of 10^{-5}M (see Figure 6A).

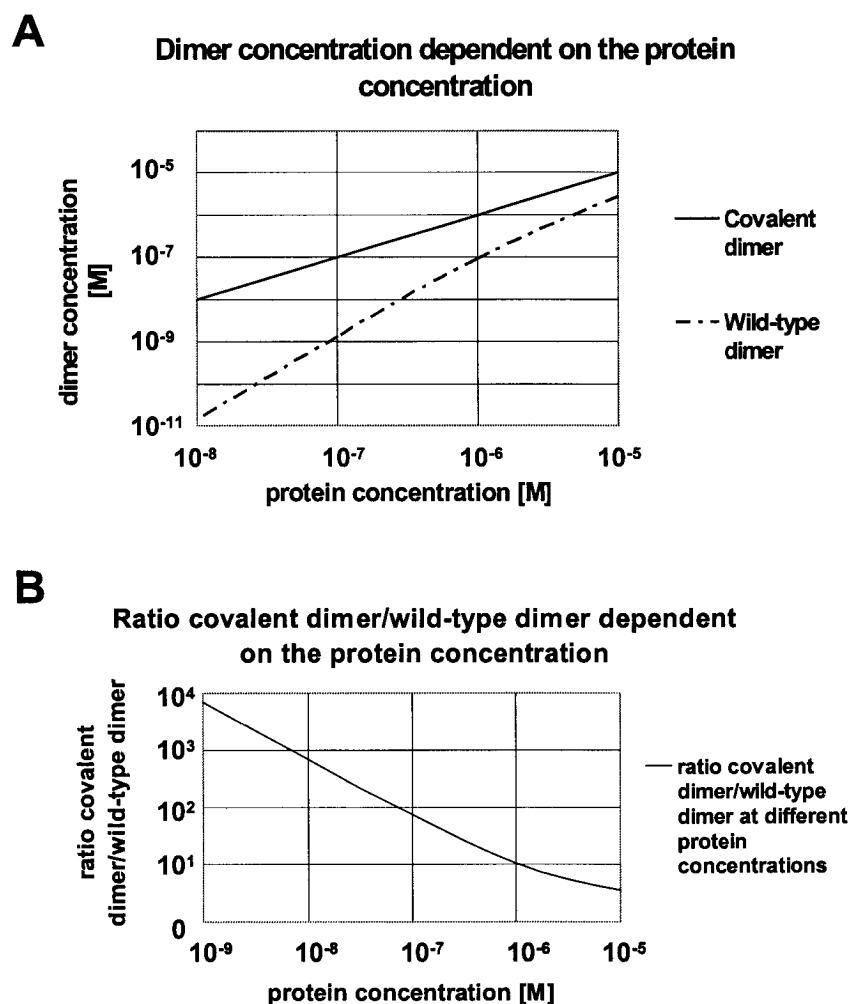


Figure 6(34). Galectin-1 monomer/dimer equilibrium at different concentrations. With the dissociation constant of $7 \times 10^{-6} \text{M}$, one galectin-1 dimer disaggregates to two monomers. Different monomer and dimer concentrations were calculated for different protein concentrations ranging from 10^{-9}M to 10^{-5}M . **A.** Depicts the theoretical concentration of the wild-type dimer and the covalent dimer at different total protein concentrations. The total protein concentration is shown on the X-axis and the dimer concentration on the Y-axis. **B.** Ratio of covalent dimer/wild-type dimer is dependent on the protein concentration. The total protein concentration is shown on the X-axis and the ratio between covalent dimer and dimerized wild-type galectin-1 is shown on the Y-axis.

Therefore, if our covalent dimer is fully functional, we should observe a similar effect if we use roughly 3 times less protein. This is indeed the case as observed with

thymocytes and activated T cells. Hence our engineered version behaves as predicted from the mathematical model.

Moreover, the concentration of wild-type galectin-1 required to treat induced autoimmune diseases in animals was between 10^{-6} M to 3×10^{-6} M. At these concentrations, wild-type galectin-1 shows very little effect on cells cultured *ex vivo*. Following the mathematical model, we predict that the covalent dimer is even ten times more potent in this concentration range.

Upon treatment of the cells with galectin-1 we always observed massive agglutination of the cells. For correct flow cytometry analysis this effect had to be reversed, as the cell multimers are otherwise lost from the analysis. These multimers have contacted galectin-1 protein and are therefore candidates for an apoptotic reaction. To separate such multimers the cells were treated with 100 mM lactose before analysis. Although this treatment led to a clear reduction in the number of these multimers, we still observed a substantial fraction of them during analysis by flow cytometry, especially for the highest concentrations of the covalent dimers of galectin-1. This may explain why the induction of apoptosis is not higher for the highest covalent dimer concentration than for the wild-type protein. Importantly induction of apoptosis is much higher at lower concentrations for the covalent dimer as compared to the wild-type protein.

Since the induction of apoptosis in activated T cells is more important for the treatment of inflammatory diseases, we also analyzed the effect of the covalent dimers on activated T cells which have previously been reported to be killed by galectin-1 [506]. We compared the covalent dimer and the wild-type protein with respect to induction of apoptosis in activated T cells and thymocytes. The picture was similar for both cell types. However the percentage of cells undergoing apoptosis declined faster at lower concentrations for the activated T cells compared to thymocytes. This may be explained by a smaller fraction of cells susceptible to galectin-1. For the thymocytes it is mainly the CD4+CD8+ cells which die and they represent over 70% of the cells in the thymus [535]. In the spleen the situation is different. There, T cells (both CD4+ and CD8+) compose about 20% of the total cells. After stimulation and rapid proliferation it increases to 60%, but remains lower than in the thymus, which could explain the weaker effect observed at lower concentrations of galectin-1.

Indeed, if the analysis was focused on activated CD8⁺ T cells (as used in our mouse models), the percentage of cells which underwent apoptosis was higher. In addition, levels of CD45 and other molecules involved in apoptosis induction by galectin-1 may be different for thymocytes and activated T cells. The glycosylation pattern varies depending on the splice form and the expression of glycosyltransferases and may therefore explain the altered susceptibility to galectin-1 induced apoptosis found for naive and activated T cells [506,512,517,521].

In conclusion, the data presented here demonstrate the increased apoptotic activity of a covalent galectin-1 dimer. This structurally optimized effector molecule may be an interesting and powerful tool for the treatment of inflammatory diseases like arthritis. Furthermore we have shown that the covalent dimerization of a protein is a useful strategy for improving a protein that is biologically active as a dimer. This approach could be envisaged for other proteins with therapeutic potential that act as dimers.

Acknowledgments

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GENERAL DISCUSSION

8. DENDRITIC CELLS AND AUTOIMMUNITY

8.1. Tolerance versus immunity: DCs provide the key

Autoimmunity describes the pathogenic state in which the adaptive immune system attacks the own body. The main mediators are auto-reactive B and T cells. Through their respective antigen-specific receptors, the BCR and the TCR, they recognize self molecules and initiate tissue destruction. For this to occur B and T cells need to be activated by antigen presentation in order to expand and to execute their effector functions.

The activation of the adaptive immune system is initiated by APCs, which process self- and nonself-antigens and present them to T cells. DCs are the most potent APCs in the immune system [182]. In contrast to other APCs, they can be in an immature or mature state. Through the different characteristics of these two different maturation states, they on one hand regulate the activation of the immune system and on the other hand prevent autoimmunity through induction of peripheral tolerance. In the immature state DCs build a network of sentinel cells that capture antigens, process and present them on their MHC molecules. After viral or bacterial infection DCs get activated by danger signals mediated by ligation of TLRs or by cytokines released after infection. These signals lead to maturation of DCs [536], characterized by the upregulation of co-stimulatory molecules and pro-inflammatory cytokines. Hence mature DCs are able to prime immune responses provided they encounter a T cell with a proper TCR. In the absence of danger signals, DCs remain in an immature state and therefore rather induce tolerance.

One of the mechanism by which imDCs induce tolerance is the generation of T_{regs} . Jonuleit et al reported that repeated stimulation of T cells with imDCs results in the generation of IL-10 producing T_{regs} , which inhibit immune reactions [178]. Other reports using hemagglutinin or ovalbumin as model self-antigens, revealed that presentation of auto-antigens via DCs are a prerequisite for T cell tolerance [329,537]. This notion was further corroborated with a Cre/LoxP-based inducible

antigen presentation system for DCs *in vivo* [407]. These experiments revealed that presentation of LCMV-derived CTL epitopes by resting DCs resulted in antigen-specific tolerance, whereas antigen presentation by activated DCs led to the expansion of endogenous CTLs mediating protection from viral challenge. Additional experiments support the idea that antigen presented by immature DCs inhibit antigen specific immunity and induce regulatory T cells [179,538]. Taken together these data strongly support the central role played by imDCs as inducer of tolerance and regulators of immune responses. Hence, the immune system can be controlled through the maturation state of DCs. This is used in vaccines by the co-administration of DC-activating adjuvant substances enhancing immune responses. Moreover prevention of DC maturation could be used as a therapeutic approach to promote peripheral tolerance or to inhibit inflammatory reactions. Given the central role played by DC maturation factors, further characterization of such molecules might lead to the identification of yet more potent adjuvants. And furthermore specific targeting of such factors may be beneficial for the treatment of autoimmune diseases.

8.2. Role of DCs in the induction of autoimmunity

DCs do not discriminate between self and nonself and therefore present self-along with pathogen-derived antigens on their cell surface. Most of the self-antigens presented by DCs arise from apoptotic cells. In contrast to necrosis, apoptosis is a physiological process and is considered to promote tolerogenicity and therefore prevent autoimmunity. In fact necrotic cells were found to elicit other responses in DCs than apoptotic cells [539]. DCs exposed to necrotic cells promote stimulatory responses from T cells, whereas DCs that have had contact to apoptotic cells do not. The uptake and ingestion of apoptotic cells by phagocytes plays an important role in cellular immunity and resolution of inflammation [540]. It allows the clearance of dying cells in a way which prevents autoimmune disease induction. However under some circumstances apoptosis can still lead to autoimmunity. Especially in tissues where massive apoptosis is ongoing, it may be that phagocytic cells like neutrophils, macrophages or DCs are not sufficient to clear all the accumulating apoptotic cells. Such uncleared dying cells can lead to subsequent necrosis, which could induce maturation of DCs, leading to the induction of self-reactive immune responses.

A special feature during the apoptotic process is that many proteins and other cell constituents are uniquely modified and also cryptic epitopes can be presented [541]. Furthermore it is important to note that exposure to high numbers of apoptotic cells leads to DC maturation even in the absence of further inflammatory signals [542]. In line with this finding, increased apoptotic rates have been reported in tissue-specific as well systemic autoimmune disorders. Furthermore if acute infection is ongoing, DCs presenting self-antigens may be activated in an unspecific bystander manner, leading to the presentation of self-antigens in an immunogenic way. Diverse infectious agents were shown to be associated with autoimmune diseases like: Streptococci in rheumatic fever, Coxsackie virus in myocarditis and Rubella in type 1 diabetes [458].

8.3. DCs in various autoimmune disorders

DCs as the most potent APCs are crucial for the induction of immune and therefore also autoimmune reactions. For several autoimmune diseases, effects on DCs were shown to be important. Hyperactive DCs and accumulation of DCs in affected tissues are observed and may be responsible for disease induction.

SLE is one of the prototypic systemic autoimmune diseases characterized by B cell hyperactivity, renal immune complex deposition leading to nephritis and anti-dsDNA auto-antibodies. Recent studies revealed a connection between CD40/CD40L signaling and enhanced migration of DCs to the spleen. The concentrations of CD40L in the serum of patients were found to be correlated with disease severity [543]. In addition to the influence of CD40L on DCs, it was shown to be also important in B cell activation and essential for isotype switching [544]. Moreover in murine models for SLE, dysregulated DC homeostasis led to disease and CD40/CD40L signaling was found to enhance DC migration from periphery to the spleen [545].

Similarly, in a murine model for type I diabetes, DCs were found to be crucial for the pathogenesis of islet autoimmunity. Transgenic mice, which overexpress TNF- α in the β -cells of the Langerhans islets, were shown to develop autoimmunity which has triggered DCs and macrophages [546]. This could be explained by the pro-inflammatory and DC maturing activity of TNF- α .

Moreover high numbers of DCs were found in the serum and synovial fluids of RA patients [547]. These DCs were found to be mature and to extensively interact with T cells [548]. The fact that DCs and T cells accumulate at sites of inflammation suggests a causative role of DC accumulation and progression of disease.

Finally in MS patients high levels of activated DCs are found, which secrete proinflammatory cytokines thereby contributing to the pathogenesis of the disease [549]. Taken together these observations in various diseases suggest that DCs play a fundamental role in the development of autoimmunity.

Additional evidence supports the central role played by DCs in autoimmunity. Women are more affected by autoimmune diseases than men. SLE, hashimoto's thyroiditis and scleroderma are known to predominantly affect woman, since over 80% of the patients suffering from these diseases are female [550]. Under the patients suffering from RA, MS and myasthenia gravis, 60 to 70% are female. The difference in the susceptibility for autoimmune disorders of man and woman may be explained by differences in sex hormones. In fact high doses of prolactin were shown to induce DC maturation [551]. Similarly estrogen was shown to promote the differentiation of functional DCs from murine bone marrow precursor cells [552]. Hence different observations point to DC maturation activities of female hormones, a finding which may provide an explanation for the higher prevalence of autoimmune diseases in woman.

8.4. Therapeutic outlooks

DC maturation is definitely an important step during the development of autoimmune diseases. Hence preventing DC maturation may offer new therapeutic approaches to inhibit immune reactions, which could allow the treatment of autoimmunity. Several factors are known to induce DC maturation and some of them are currently being investigated as potential targets for the development of immune regulatory drugs. Considering the importance of the CD40/CD40L ligation for DC and T cell interaction, selective blocking of this signaling pathway provides one possible target. In animal studies, functional disruption of CD40/CD40L signaling by neutralizing antibodies lead to enhanced renal function and reduced nephritis in an animal model of SLE [553]. Similarly human studies also have revealed beneficial effects in patients with lupus [554].

Other molecules known to inhibit DC maturation are glucocorticoids. These substances are also successfully used to treat autoimmune diseases [555]. Another molecule is Vitamin D, which has immunosuppressive effects resulting in part from the inhibitory effect on DC maturation [556].

However although DC maturation is one of the most important features for the induction of immune responses, other activities of DCs like antigen capturing or presentation or migration to the lymphatic tissues may provide possible therapeutic approaches. The migration to the lymphatic tissues is guided mainly by the CCR7/CCL19/CCL21 chemokine/chemokine receptor system. Interestingly CCL19, a known inducer of migration, was found here to be a potent maturation factor for DCs. Targeting of CCL19 would allow therefore the killing of two birds with one stone. On one hand the maturation of DCs may be affected and additionally, the migration of DCs to the lymphatic organs would be reduced or even abolished, preventing the DCs to present antigen in an immunogenic form to T and B cells. For the molecular twin of CCL19, CCL21, an effect on the proliferation of CD4⁺ T cells and progression of autoimmunity was observed [557], highlighting the importance of the CCR7 ligands in the development of autoimmunity. Depletion of CCL19 by passive or active immunisation using monoclonal antibodies or vaccination technologies may prevent DC maturation and migration. Furthermore CCL19 could be used as adjuvant for immunization protocols. Added to the injection mixture of vaccine formulations it may boost the response against the administered antigen. In fact CCL19 and CCL21 were successfully used as adjuvant to induce protective immune responses against herpes simplex virus [558]. In this study, plasmid DNA encoding CCL19 and CCL21 was administered together with vaccinia virus encoding herpes simplex virus glycoprotein. The vaccinated mice showed increased antibody titres, increased number of virus specific CTLs and were protected against viral infection. Another study in which CCL19 was injected intratumorally showed significant systemic reduction in tumor volume in the treated mice [559]. In both studies the rationale to use CCL19 as an adjuvant was its capacity to induce DC and T cell migration. However the ability to induce DC maturation may also have played an additional role for the beneficial effects found. The fact that CCL19 acts as both maturation and migration factor of DCs makes it to a versatile molecule, which offers new opportunities for the successful controlling of immune responses.

9. MOLECULAR MIMICRY AND AUTOIMMUNITY

9.1. APLs as model for molecular mimicry

Different observations suggest that acute infections can lead to the development of autoimmune disease. And several mechanisms have been proposed to explain these observations. One proposed mechanism has been termed molecular mimicry. This model proposes that due to the homology of pathogen-derived antigens with host antigens an immune response is mounted against self molecules. The connection between pathogen-derived antigen and the development of autoimmune disease has extensively been studied for several infectious agents:

First, Coxsackievirus B3 has been identified from patients with cardiovascular disease like myocarditis [560]. Furthermore infection of mice with this strain can reproduce human clinical heart disease in rodents [561]. Secondly, it is widely accepted that rheumatic fever occurs following an overactive immune response by a genetically susceptible host to oropharyngeal infection with group A β -hemolytic Streptococci [562]. Third, heart tissue destruction in chronic Chagas' disease cardiomyopathy, occurring in 30% of individuals chronically infected by the protozoan parasite *Trypanosoma cruzi*, may be caused by autoimmune recognition of patients' heart tissue by a T cell rich inflammatory infiltrate. And antigenic mimicry by *Trypanosoma cruzi* antigens that share epitopes with mammalian tissues may drive these auto-reactive B- or T-cell clones to expand and cause autoimmune pathogenesis [563].

And finally, Lyme arthritis was shown to result from infection with *Borrelia burgdorferi* [564]. Lyme arthritis is an inflammatory joint disorder resembling RA. An epitope mimicry between the outer surface protein A of the bacteria and the human LFA-1 protein was found to be the cause for induction of autoimmunity [565].

Although molecular mimicry may be one of the leading causes for the induction of autoimmune diseases, other mechanisms like bystander activation or pathogen

caused tissue damage leading to the activation of immunity against self molecules may be responsible for the development of autoimmunity.

The T cell response to peptides with amino acid substitutions at residues interacting with the TCR, called altered peptide ligands, were first examined in T cell clones. It was found that APLs have different effects on T cell responses [445]. Whereas some APLs were shown to have agonistic effects, others were shown to inhibit the proliferation of T cell clones. This inhibitory effect is TCR specific and has been termed TCR antagonism [566]. The use of TCR antagonist peptides has been proposed as a potential therapy for T cell mediated autoimmune disorders [567]. This concept was successfully tested in an EAE model [568]. Using a specific peptide of myelin basic protein, two APLs were identified that act as TCR antagonists on transgenic T cells and which inhibit EAE induction in a murine model. However the clinical trials in MS patients with such antagonistic APLs failed [569,570]. In fact the trials had to be stopped because in some of the patients an aggravation of the disease was observed. These patients displayed an expansion of both wild-type and APL specific T cells. Hence this highlighted the difficulties using variant peptide therapy due to the differences between polyclonal T cell responses generated *in vivo* and the situation found for T cell clones. A specific T cell clone reacts reproducibly to agonist or antagonist peptides but the reaction of patients with different TCR repertoires is unpredictable.

Based on the clinical results obtained, APLs are no longer considered for the treatment of autoimmune diseases. However they still offer a good model system to study the concept of molecular mimicry.

One of the best studied APL systems is derived from the LCMV glycoprotein peptide p33 [73,74]. Using this system in an experimental model for type I diabetes (RIP-GP mice expressing p33 in β -islet cells of the pancreas), the efficacy of molecular mimicry to induce autoimmunity was investigated. Using VLPs as carriers, the delivery of agonists/antagonists of p33 was not sufficient to induce autoimmunity. The immune system has control mechanisms to avoid the induction of autoimmunity that seem to interfere. Specifically T_{regs} , previously shown to play a role in the control of immune responses, turned out to be important in the diabetes model. While the wild-type peptide p33 was able to cause diabetes in normal RIP-GP mice, APLs were able to trigger autoimmunity only in absence of T_{regs} . This indicates that molecular

mimicry alone is not sufficient to induce autoimmunity. Additional prerequisites have to be fulfilled, like the disturbance of peripheral tolerance as shown in our experiments where T_{regs} have been depleted.

9.2. Regulatory T cells in autoimmunity prevention

Regulatory T cells are important for the control of immune responses. They were found to be especially important to down-regulate the immune system after the peak of response or for the maintenance of self-tolerance. These immunoregulatory functions make them potential candidates for autoimmune disease therapies. But the main problem handling regulatory T cells is to recognize them. Characterization of T_{regs} was only possible on the basis of their function. Looking for surface markers, the T_{regs} turned out to be quite diverse, nevertheless they can be classified in two main types, the natural and the adaptive (antigen induced) T_{regs} [571]. Both groups were shown to be essential for the maintenance of tolerance. For example natural T_{regs} were found to be important in the control of autoimmune diabetes [128]. Adaptive T_{regs} can be divided into several subgroups including T_{H1} , T_{H2} , T_{H3} , T_{r1} , CD8+ T or $\gamma\delta$ T cells. CD4+ and CD8+ regulatory T cells were shown to protect from EAE [142]. Similarly T_{H2} cells considered by some to have regulatory functions, inhibit diabetes induction [143]. Furthermore it could be shown that tolerance could be transferred by the transfer of T_{regs} as shown by the transfer of cyclosporine induced T_{regs} into rats to prevent allograft rejection [572]. Mice, in which the T_{regs} have been eradicated by thymectomy between day 3 and 5 after birth, develop a polyautoimmune syndrome. This disease can be reversed by the application of CD4+ T cells from normal mice [573]. Furthermore similar to our experiments others showed that depletion of regulatory T cells led to the induction of autoimmunity in a collagen-induced arthritis model [574] and in an autoimmune gastritis model [575].

All these results highlight the importance of T_{regs} in the maintenance of self tolerance and indicate that disturbance of the regulation system may enhance autoimmunity induction.

9.3. Molecular mimicry in autoimmune disease as function of signal strength and immune regulation

In spite of antigen presentation in a VLP context and co-administration of anti-CD40 Ab or CpGs, molecular mimicry by APLs turned out to be not sufficient for the induction of autoimmunity. In contrast delivering of the p33 peptide in the same context was sufficient to induce autoimmunity, demonstrating the effectiveness of the delivering system. However, using APLs, perturbation of the immune regulation by depletion of T_{regs} is crucial for molecular mimicry to work. VLPs are non-replicating and it may be that the situation may be different if the APL was given in a viral context. Viral infection disturbs the immune system and may have also an impact on the T_{regs} , similar to the situation created in our experiments. Additionally to the delivery of antigen, replicating virus leads to tissue trauma and may induce danger signals. In our experiments anti-CD40 Ab was used as DC maturation factor, simulating an endogenous signal. CpG which is used alternatively is simulating an exogenous signal. Viral infection is inducing endogenous and exogenous signals telling the immune system that danger is approaching. It may be that the self-tolerance is such a vital function that circumvention of T_{regs} requires the additive effect of intrinsic and extrinsic signaling, triggering at the same time different pathways and receptors. The immune system has to balance between immunity and autoimmunity. Not responding to a pathogen may mean to die from infection. Therefore a response also arises to pathogen antigens mimicking self-antigens. Who wants to decide which of the two is the smaller evil is in this case? However the immune system has to.

9.4. Original antigenic sin: right or wrong, who decides?

The concept of original antigenic sin was first postulated by Davenport in 1953 based on work with influenza virus [576]. They described a phenomenon in which the antibody response elicited in an individual after a secondary viral infection reacted more strongly to the viral variant that originally infected the individual. Later on, a similar phenomenon was postulated for T cell responses [453]. In both cases, it is thought that the dominance of the original response impairs protection from infection with the new strain. However, the concept has rarely been tested directly. Nevertheless escape mutants arise during viral infections and several indications

cater the concept of original antigenic sin further on [485,577,578]. The observations are particularly important for the development of vaccines against viruses such as human immunodeficiency virus and hepatitis C virus, in which myriad viral variants are present throughout the human population. In order to circumvent the effects of original antigenic sin for viruses showing strong genetic variance, vaccination with multiple peptide variants has been proposed [579].

Using the APL system fused to VLP as antigen carrier the concept of original antigenic sin was tested directly leading to surprisingly clear results. In these experiments animals were immunized first with an APL, boosted with the p33 peptide and then challenged with a p33 carrying virus. Albeit immunization with p33-VLP induced good specific T cell response, pre-immunization with APL-VLP kept the specific CTL activity focused on the APLs leaving the mice unprotected from viral infection. The difference between the APLs used and p33 was limited to one amino acid at the position four of the peptide. And the outcome was the same for agonistic and antagonistic APLs. How can these results be explained? Since original antigenic sin has also been observed with CTL responses, it was speculated that it could be explained by very strong CTL memory responses to viral infection. Viruses like LCMV induce massive CTL responses with percentages of virus specific CD8⁺ T cells up to 25-50% [580,581]. It has been postulated that a weakly cross-reacting new virus might reactivate these CTL memory cells, more readily than the less abundant naïve CTL precursors.

However this high amount of specific CD8⁺ T cells was not reached by APL-VLP immunization, which weakens this explanation. Furthermore it was speculated that memory CTLs could remove APCs, thereby aborting the primary CTL response to the new viral epitope. However this seems unlikely, since high amounts of memory CTLs would be required. Yet another explanation would be deactivation of APCs by memory CTLs through IFN- γ secretion [582], or T cell receptor antagonism [583], in which wild-type virus would antagonize the response against the mutant viral strain. Finally an additional possibility would be that the originally primed CTLs are anergized in the second challenge and develop to antigen specific regulatory T cells inhibiting further response against the APL, thereby allowing mutants to escape. Anergized antigen specific T cells have been proposed previously to have negative regulatory activities [584]. And this phenomenon may turn out to be responsible for

the original antigenic sin. To further test this hypothesis, it would be worthwhile to deplete the T_{regs} after APL and p33 immunization and prior viral challenge to evaluate their influence on protection induction.

10. T CELLS AND AUTOIMMUNITY

10.1. Killing the killers

T cells are known to be main mediators in most known autoimmune diseases. They are involved in organ-specific and systemic autoimmune diseases. In the organ-specific ones, they are normally directly involved in the destruction of the tissue by killing of cells. In the systemic autoimmune diseases, they are mediating help to self-specific B cells and thereby allow the production of self-specific antibodies which are involved in the tissue destruction. Furthermore the T_{H} but also the CTLs activate the immune system by the secretion of cytokines. Therefore, it would be optimal to have the possibility to prevent the expansion of self-reactive T cells. Galectin-1 was found to induce apoptosis in activated and proliferating T cells [506]. However the problem with galectin-1 as a therapeutic inducer of apoptosis is that relative high amounts of protein are needed to see an effect. This is due to the fact that galectin-1 exerts its activity as a dimer and that the monomeric subunits have only a low affinity for each other. To circumvent this problem, a genetically linked dimer was constructed. This covalent dimer was shown to increase galectin-1 apoptotic activity. However although the activity of galectin-1 was increased about 4 -10 times, the amount necessary to treat autoimmune diseases may still be too high since about 0.4-1 mg/kg/day should be administered. Yet dimerization of proteins to increase activity proved to be a potential approach to generate "better proteins".

11. GENERAL OUTLOOK

Autoimmune diseases are one of the most troubling health problems of humans. In Europe and the USA the frequency of people suffering from autoimmune diseases are 1 in 20. In the United States about 50 million people are treated after autoimmunity diagnosis, and over 80 different autoimmune diseases are known.

Among the most important are SLE, Type 1 diabetes, scleroderma, MS, Crohn's disease, chronic acute hepatitis, RA and myasthenia gravis. The etiology of autoimmune diseases is mostly unknown. They seem to be a consequence of the interplay of different factors. The genetic background is definitely important since many people suffering from an autoimmune disease have relatives suffering from the same or another autoimmune disease. Furthermore the environment and there especially infections with viruses or bacteria appear to be implicated. The concept of molecular mimicry offers an explanation for this link of infections and autoimmunity. But the exact interplays and the individual relevance is still under debate. The damage to the body is caused by the own immune system, and therefore many arising therapeutic strategies try to affect it. Most of the drugs used thus far are immunosuppressive unspecific agents, all of them bearing considerable toxicity.

RA, a systemic disorder, in which immune cells attack the joints, affects about 2.1 millions Americans. To date is treated mainly by anti-inflammatory or immunosuppressive medications like aspirin [585], cyclosporine, cortisone [586], methotrexate [587] or gold-injections and more recently, TNF- α antagonists [588].

MS, a disease, in which the immune system targets nerve tissue of the central nervous system, affects 300'000 people in the United states, and currently is treated by corticosteroids [589] or IFN- β [590].

Type 1 diabetes mellitus results from autoimmune destruction of the insulin-producing cells in the pancreas, occurring in 1 out of 800 people in the United States, is treated by special diet and the delivery of insulin. Most of the islet cells of the pancreas are destroyed before the patient develops symptoms of diabetes. Therefore early diagnosis, maybe already in the first months of life may raise prevention strategies.

SLE, a systemic disease where the body produces antibodies to certain molecules of the nucleus, affects half a million Americans and is mainly treated by anti-inflammatory medication like aspirin and steroids.

Until now there is no cure for autoimmune diseases, all the medications available treat the symptoms, try to ease the patient's situation or in the best case to inhibit progression of the disease. Therefore and because of the severe side effects of the drugs used, especially after long-time treatment, there is a quest for additional and

novel drugs. New drugs with higher specificity and smaller side effects are the goal of future autoimmune disease treatment. Key attack points for selective immunointervention have been identified: modulation of antigen recognition, co-stimulation blockade, induction of regulatory cells, neutralization of proinflammatory cytokines, induction or administration of anti-inflammatory cytokines or the modulation of leukocyte trafficking. Affecting these mechanisms, future immunosuppressive therapy can be directed to four cellular targets: APCs, auto-reactive T cells and T_{regs} with the common goal to selectively inhibit the activation of pathogenic $CD4^+$ T cells and additionally B cells. Blocking co-stimulation is the concept for administration of CTLA-4lg [591] or anti-CD40L monoclonal antibodies [554]. For B cells Rituximab, an anti-CD20 monoclonal mouse/human antibody, has been widely used under several autoimmune and rheumatic conditions, especially for arthritis therapy [592]. T_{regs} are known to be helpful in many animal models for autoimmune diseases [593]. An example for neutralization of proinflammatory cytokines may show experiments with IL-1. IL-1, a highly proinflammatory cytokine, is regulated by three types of inhibitors: IL-1 receptor antagonist, soluble IL-1 receptor and a non-signaling decoy receptor [301]. Soluble IL-1 receptor has proven efficacy in animal models [594]. The recombinant human IL-1 receptor antagonist anakinra was shown to be effective in patients with RA [595] and is on the market as a drug against RA [596]. Randomized, placebo-controlled trials have demonstrated that anakinra, alone and in combination with methotrexate, improves the symptoms of RA.

Additional proinflammatory cytokines, which represent possible targets are IL-2, IFN- γ , IL-12, IL-8, IL-15 or IL-5. As examples for the administration of anti-inflammatory cytokines IL-4, IL-10, IFN- β or TGF- β may be mentioned. IFN- β is successfully used for the treatment of MS. It could be shown that IFN- β treatment reduces attack frequencies significantly and that disease activity was reduced by over 80% [597]. The modulation of leukocyte trafficking has not been studied extensively, thus future work will show whether its interference could be used as treatment for autoimmune diseases.

This list of mechanisms and targets that are under investigation for the treatment of autoimmunity shows that concerted effort is made to solve the problem. Autoimmunity will stay one of the major challenges for the health care systems

around the world for this century. But the work will go on and once in the future autoimmunity may perhaps lose its horrible face.

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ABBREVIATIONS

Ab	antibody
AICD	activation-induced cell death
APC	antigen presenting cell
APL	Altered peptide ligand
BCR	B cell receptor
BM	bone marrow
CCR7	chemokine receptor 7
CD40L	CD40 ligand
CDR	complementary-determining region
COX-2	cyclooxygenase-2
CpGs	bacterial DNA rich in non-methylated CG motifs
CTL	cytotoxic T cell
CTLA-4	cytotoxic T-lymphocyte associated protein 4
DAG	diacylglycerol
DC	dendritic cell
DC-SIGN	DC-specific ICAM-3 grabbing non-integrin
DD	death domain
DN	double negative
DP	double positive
EAE	experimental autoimmune encephalomyelitis
ER	endoplasmatic reticulum
FACS	fluorescence activated cell sorter
GITR	glucocorticoid-induced TNF-receptor family related protein
GP	glycoprotein
Grb2	growth factor receptor-bound protein 2
HBc	Hepatitis B core
HBcAg	Hepatitis B core antigen
HEV	High endothelial venules
HIV	Human immunodeficiency virus
ICAM	molecules intracellular adhesion molecule
Ig	Immunoglobulin
IgG	Immunoglobulin G
IL-2	Interleukin-2
IFN- γ	Interferon- γ
imDC	immature DC
IP ₃	inositol trisphosphate
ITAM	immune receptor based activation motifs
kDa	kilo Dalton
KO	knock-out
LAT	linker for activated T cells
LCMV	lymphocytic choriomeningitis virus
LFA-1	Leukocyte Function-associated Antigen-1
LN	lymph node
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MHC II	Major histocompatibility complex
MS	multiple sclerosis
NF- κ B	nuclear factor- κ B

NF-AT	nuclear factor of activated T cells
NK	natural killer
PBS	phosphate buffer saline
PCR	poly chain reaction
PI-3K	phosphatidylinositol 3-kinase
PIP	phosphatidylinositol 3-phosphate
PIP ₂	phosphatidylinositol 4,5-kinase
PIP ₃	phosphatidylinositol 3,4,5-kinase
PKC	protein kinase C
PLC- γ	phospholipase C
RA	rheumatoid arthritis
RAG	Recombination Activating genes
SH2	Src homology region 2
SLE	systemic lupus erythematosus
STAT4	signal transducer and activator of transcription 4
T-bet	T-box expressed in T cells
TCR	T cell receptor
TGF- β	transforming growth factor- β
T _H	helperT cell
T _{H1}	helperT cell type 1
T _{H2}	helperT cell type 2
T _{reg}	regulatory T cell
TLR	Toll like receptor
TNF	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late activation antigen 4
VLP	virus like particle

PUBLICATIONS

Bättig P, Saudan P, Gunde T, Bachmann MF: **Enhanced apoptotic activity of a structurally optimized form of galectin-1.** *Mol Immunol* 2004, **41**:9-18.

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Bättig P, Bauer M, Eriksson U, Ruedl Christiane, Lässig U, Beerli R, Dietmeier K, Ivanova L, Pfister T, Vogt L, Kopf M, Saudan P, Bachmann MF: **Expression cloning identifies the CC-chemokine CCL19 as potent activator of dendritic cells.** In preparation.

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CURRICULUM VITAE

Patrick Bättig

Citizien of Hergiswil b. Willisau/LU, Switzerland

Born Mai 15, 1974, in Baden

- 1981-1987 Primary school in Fislisbach/AG and Winterberg/ZH
- 1987-1989 Secondary school in Grafstal/ZH
- 1989-1994 Gymnasium Kantonsschule im Lee in Winterthur/ZH
- 1994-1999 Study of biology at the Swiss Federal Institute of Technology (ETH) Zurich; final exams in microbiology, genetics, biochemistry, molecular biology and immunology; Diploma thesis at the institute of Microbiology in the lab of Prof. Dr. M. Aebi; Diploma: Dipl. Natw. ETH
- 2000-2004 PhD thesis at Cytos Biotechnology AG under the supervision of Dr. M.F. Bachmann, Cytos Biotechnology AG, Prof. Dr. H.M. Eppenberger, Institute of Cell Biology, ETH and Prof. Dr. S. Werner, Institute of Cell Biology, ETH