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IDENTIFICATION AND CHARACTERISATION OF DIFFERENTIALLY EXPRESSED GENES IN NAIVE AND ACTIVATED CD8$^+$ T CELLS

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To the reader

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SUMMARY

T cells play a crucial role in many diseases and their appropriate activation has to be accomplished for the defence against viruses and tumours. On the other hand, proper silencing of activated T cells has to occur to maintain T cell homeostasis and to hinder the development of autoimmune diseases. Tightly regulated programs of differential gene expression mediate T cell activation, differentiation and apoptosis. Insight in these distinct patterns of gene expression is not only essential for the understanding of complex biological processes, it can also lead to the identification of new objectives for the therapy of various diseases.

The search for novel genes involved in these processes was conducted through analysis of differential gene expression in naive and in effector and memory $CD8^+$ T cells generated in vivo. The recently developed technique of mRNA differential display was used, which allowed comparing, identifying and isolating differentially expressed transcripts. Furthermore, the use of an adoptive transfer model with $CD8^+$ T cells from transgenic mice expressing a lymphocytic choriomeningitis virus (LCMV)-specific T cell receptor allowed the characterisation and isolation of defined effector and memory $CD8^+$ T cell populations at different points in time after viral infection.

Using this approach, the LGALS1 (lectin-galactoside-binding, soluble) gene was found to be strongly up-regulated in $CD8^+$ effector T cells. The protein coded by the LGALS1 gene is a β-galactoside-binding protein (βGBP), which is released by cells as a monomeric negative growth factor but which can also associate into homodimers (galectin-1) with lectin properties. Northern blot analysis revealed that ex vivo isolated $CD8^+$ effector T cells induced by a viral infection expressed high amounts of LGALS1 mRNA whereas LGALS1 expression was almost absent in resting $CD8^+$ T cells. LGALS1 expression was also detected in virus specific $CD8^+$ memory T cells, though at a lower level when compared to $CD8^+$ effector T cells. LGALS1 expression could be induced in $CD4^+$ and $CD8^+$ T cells upon activation with the cognate peptide antigen in vivo and in vitro. Moreover, comparison of LGALS1 expression by naive and memory cells stimulated with the cognate peptide antigen in vitro revealed that LGALS1 expression occurred 12 hours earlier in memory cells compared to naive cells. This finding suggests a cytokine-like role for the LGALS1 gene product. High levels of LGALS1 expression were also found in Concanavalin A-activated T cells, but not in LPS-activated B cells. Accordingly, LGALS1 was detected in various tumour cell lines of T cell but not of B cell origin. Gel filtration and Western blot analysis revealed that after in vitro stimulation with the cognate peptide antigen only monomeric βGBP was

released by activated $CD8^+$ T cells. In vitro experiments further demonstrated that recombinant βGBP was able to inhibit antigen-induced proliferation of naive and antigen-experienced $CD8^+$ T cells. Mice with a disrupted LGALS1 gene ($Lect14^{o/o}$ mice), when compared to normal mice, showed elevated numbers of $CD8^+$ T cells several weeks after LCMV infection. This data indicates a role for βGBP as an autocrine negative growth factor for $CD8^+$ T cells with cytokine-like functions.

The second gene isolated by differential display of naive and effector $CD8^+$ T cells was the mouse homologue of the rat mast cell function-associated antigen (MAFA). MAFA belongs to the family of C-type lectins and is an inhibitory receptor that was originally defined on the cell surface of the rat mucosal mast cell line, RBL-2H3. This thesis describes the identification and cloning of the mouse homologue of the rat MAFA gene. Sequence comparison revealed a high degree of conservation (86% homology) suggesting that also in the mouse the MAFA molecule represents a putative inhibitory receptor. Northern blot analysis demonstrated that mouse MAFA (mMAFA) gene expression was strongly induced in effector $CD8^+$ T cells and lymphokine-activated NK cells, but was absent in $CD4^+$ effector cells and in bone marrow derived mast cells. Moreover, mMAFA gene expression was only found in $CD8^+$ effector T cells, which had been primed in vivo with life virus because in vitro activated $CD8^+$ T cells did not express mMAFA. mMAFA expression was not restricted to LCMV-infection and infection with vesicular stomatitis virus and vaccinia virus also induced expression. mMAFA surface expression could be detected by an anti-mMAFA monoclonal antibody on 70% of LCMV-specific $CD8^+$ effector T cells but not on any other cell type. Only infection with a low dose of LCMV induced mMAFA expression by $CD8^+$ effector T cells whereas infection with a high dose failed to do so despite the induction of potent cytolytic activity in these cells. Concerning the inhibitory function of mMAFA, some results in this thesis indicate that mMAFA could serve as an inhibitory receptor on T cells. However, a decisive conclusion of the inhibitory function of mMAFA could not be drawn. In analogy to the inhibitory effect of rat MAFA on mast cell degranulation it could be presumed that mMAFA has a similar function and serves as an inhibitory receptor on $CD8^+$ effector cells and NK cells. However, whether cytolytic activities, cytokine release, differentiation, activation or silencing of $CD8^+$ T cells are regulated by mMAFA remains elusive and further studies will have to clarify the functional role of mMAFA expressed by virus-induced cytotoxic T lymphocytes.

ZUSAMMENFASSUNG

T Zellen spielen eine zentrale Rolle bei vielen Krankheiten und ihre ausreichende Aktivierung sowie auch Deaktivierung ist unumgänglich für ein Funktionieren des Immunsystems. Die Aktivierung, Differenzierung sowie auch die Apoptose der T Zellen werden durch streng regulierte Prozesse der differentiellen Genexpression gesteuert. Die Charakterisierung dieser unterschiedlichen Prozesse ist nicht nur für das generelle Verständnis von komplexen biologischen Prozessen notwendig, sondern kann auch zur Entdeckung neuer Strategien für die Therapie verschiedener Krankheiten führen.

Die Analyse der differentiellen Genexpression von naiven $CD8^+$ T Zellen im Vergleich zu Effektor- und Gedächtnis-T-Zellen sollte zur Identifizierung neuer Gene führen, welche im Prozess der T-Zellaktivierung und Deaktivierung von Bedeutung sind. Zu diesem Zweck wurde die kürzlich entwickelte Technik des sogenannten „mRNA Differential Display" verwendet, welche den Vergleich, die Identifizierung und die Isolation von differentiell exprimierten Genen erlaubt. Ein adoptives Transfermodel mit $CD8^+$ T Zellen von transgenen Mäusen mit einem für das Lymphozytäre Choriomeningitis Virus (LCMV)-spezifischen T-Zell-Rezeptor ermöglichte die Charakterisierung und Isolation einer definierten Effektor- oder Gedächtnis-T-Zellpopulation.

Auf diese Weise wurde das LGALS1 (lectin-galactoside-binding, soluble) Gen identifiziert, welches von $CD8^+$ Effektorzellen exprimiert wird. Das von dem LGALS1 Gen codierte Protein ist ein β-Galaktose bindendes Protein (βGBP für β-galactoside-binding protein). βGBP wird von den Zellen als monomeres Molekül sekretiert, welches als autokriner, negativer Wachstumsfaktor wirkt. Es kann aber auch als Homodimer (galectin-1) vorliegen und weist dann Eigenschaften von Lektinen auf. Mittels Northern Blot Analyse wurde festgestellt, dass $CD8^+$ Effektorzellen hohe Mengen an LGALS1 RNA produzierten, während dies bei naiven $CD8^+$ T Zellen nicht der Fall war. Auch $CD8^+$ Gedächtnis-T-Zellen exprimierten LGALS1, wenn auch in viel kleineren Mengen als Effektorzellen. Nach erneuter Stimulation mit Peptidantigen in vitro zeigten sie jedoch eine 12 Stunden frühere Expression von LGALS1 RNA im Vergleich zu naiven T Zellen. Dieses Resultat weist auf eine mögliche Zytokin-ähnliche Rolle des LGALS1 Genproduktes hin. Die Aktivierung von $CD8^+$ wie auch von $CD4^+$ T Zellen mit dem passenden Peptidantigen in vivo oder in vitro führte zu starker LGALS1 Expression. Im Weiteren wurde eine starke LGALS1 Expression in Concanavalin A-aktivierten T

Zellen aber nicht in LPS-aktivierten B Zellen gefunden. In Analogie dazu exprimierten verschiedene T-Zellinien grosse Mengen an LGALS1 RNA während B-Zellinien keine Expression zeigten. Mittels Gelfiltration und Western Blot konnte gezeigt werden, dass aktivierte $CD8^+$ T Zellen nur monomeres βGBP freisetzten. Weitere in vitro Experimente konnten zeigen, dass rekombinantes βGBP die antigen-induzierte Proliferation von naiven und antigenerfahrenen $CD8^+$ T Zellen verhindern konnte. In LGALS1 defizienten Mäusen ($Lect14^{o/o}$ Mäuse) war über mehrere Wochen nach LCMV-Infektion eine erhöhte Zahl von $CD8^+$ T Zellen nachweisbar. Die oben erwähnten Daten lassen eine Rolle für βGBP als autokrinen negativen Wachstumsfaktor für $CD8^+$ T Zellen mit Zytokin-ähnlichen Funktionen erkennen.

Das zweite Gen, welches mittels „mRNA Differential Display" von naiven und Effektor $CD8^+$ T Zellen isoliert wurde war das Maus-Homolog des Ratten „mast cell function-associated antigen" MAFA. MAFA gehört zur Familie der C-Typ Lektine. MAFA ist ein inhibitorischer Rezeptor, welcher ursprünglich auf der Zelloberfläche der Ratten Mast-Zellinie RBL-2H3 gefunden wurde. Die Ähnlichkeit der beiden Gene in Ratte und Maus ist sehr hoch (86% Homologie) und lässt vermuten, dass auch das MAFA in der Maus ein inhibitorischer Rezeptor ist. Mittels Northern Blot Analyse konnte gezeigt werden, dass die Expression des Maus-MAFA (mMAFA) Gens in $CD8^+$ Effektor-T-Zellen und in IL-2-aktivierten NK Zellen stark induziert wurde. Im Gegensatz dazu konnte keine Expression des mMAFA Gens in $CD4^+$ Effektor-T-Zellen sowie in Mastzellen nachgewiesen werden. Die Genexpression von mMAFA konnte nur in $CD8^+$ Effektor-T-Zellen beobachtet werden, welche in vivo durch eine virale Infektion aktiviert worden waren. $CD8^+$ T Zellen welche in vitro aktiviert wurden, zeigten keinerlei Expression des mMAFA Gens. Mit Hilfe eines anti-mMAFA monoklonalen Antikörpers konnte die mMAFA Oberflächenexpression auf 70% der LCMV-spezifischen Effektor-T-Zellen nachgewiesen werden. Eine gute Expression des mMAFA Gens konnte jedoch nur durch eine Infektion mit einer kleinen LCMV-Dosis induziert werden, während eine Infektion mit einer hohen Dosis an LCMV zu keinerlei mMAFA Expression führte. Die vorliegende Arbeit gibt Anhaltspunkte für eine inhibitorische Rolle des mMAFA, kann aber keine endgültige Aussage darüber machen, dass mMAFA eine Funktion als inhibitorischer Rezeptor hat. In Analogie zur inhibitorischen Rolle des Ratten-MAFA in Bezug auf die Mastzell-Degranulation kann aber eine ähnliche Funktion des mMAFA bei $CD8^+$ T Zellen und bei NK Zellen in Betracht gezogen werden.

1. INTRODUCTION

The immune system is a remarkable defence system. Found in its most advanced form in higher vertebrates. It provides the organism with the means to develop rapid, highly specific, and mostly very protective responses against a myriad of potentially pathogenic microorganisms that inhabit the world in which it lives.

A wide range of different cell types define the immune system. Lymphocytes play a central role because they mediate the specificity of an immune response and their activity determines the outcome of an effective immune response.

Two main classes of lymphocytes can be defined. B lymphocytes are the precursors of antibody secreting plasma cells. T lymphocytes have important regulatory functions as $CD4^+$ T helper cells or are involved in direct lysis of pathogen-infected cells or certain tumour cells as $CD8^+$ cytotoxic T lymphocytes (CTLs). B cells recognise with their membrane-bound B cell receptor native antigens that are localised extracellularly. In contrast to B cells, T cells recognise with their membrane-bound T cell receptor (TCR) not antigen alone but antigen that is presented on the surface of an antigen presenting cell in association with major histocompatibility complex (MHC) molecules. The antigen can not be recognised in its native form but needs to be processed intracellularly into short fragments called peptides that are then loaded onto MHC molecules (Townsend et al., 1986). Two classes of MHC molecules can be recognised, the MHC class I and the MHC class II molecules. The $CD4^+$ T cells recognise antigen only in association with MHC class II molecules whereas $CD8^+$ T cells recognise antigen in association with MHC class I molecules. This phenomenon has been described as MHC-restriction (Zinkernagel and Doherty, 1974). The emphasis of this thesis will be on $CD8^+$ T cells that function as an essential element in antiviral defence.

The immune system is anatomically and functionally divided into two distinct compartments. In the primary lymphoid organs, such as bone marrow and thymus, lymphocytes are continuously produced in enormous numbers and the antigen specificity of their receptors is determined. Secondary lymphatic organs such as lymph nodes and spleen are specialised in offering a suitable microenvironment for efficient interactions between lymphocytes and antigens. In these organs antigens are locally concentrated, processed and presented to T lymphocytes. The activated cells then mainly exert their effector functions in extralymphoid tissues.

An elaborate system of continuous lymphocyte recirculation has evolved to connect the two types of lymphoid tissue. After release from the thymus, each T lymphocyte can freely migrate between the blood and different lymphatic tissues. This maximises the probability that a lymphocyte with a distinct antigenic specificity encounters its cognate

antigen that invaded anywhere the body (Butcher and Picker, 1996). These phenotypically and functionally naive T lymphocytes are able to survive for long periods in the periphery in the absence of antigen encounters (Sprent and Tough, 1994). Recent data suggested that the presence of MHC molecules is crucial for T cell survival in the periphery (Tanchot et al., 1997; Kirberg et al., 1997).

Mechanisms involved in T cell activation

Naive T lymphocytes must encounter antigen and be properly activated for an immune response to occur. The outcome of lymphocyte activation is determined mainly by the strength and quality of the antigenic signal and the cellular environment present when the lymphocyte is confronted with antigen. For successful T cell activation, antigenic fragments of viral proteins must be presented to naive $CD8^+$ T cells by professional antigen-presenting cells (APCs). APCs provide the necessary costimulatory signals as discussed below. Once activated, the $CD8^+$ T cells undergo extensive proliferation and acquire their specific effector functions resulting in the ability to directly lyse virus-infected cells. Efficient CTL responses follow virus kinetics by a few days and become measurable 6-8 days after systemic infection with lymphocytic choriomeningitis virus (LCMV). At the time when CTL activity can be measured, viral titers decrease and fall below detection level 8-10 days after infection. By this time, CTL activity starts to decline. After several weeks, the number of $CD8^+$ T cells has reached normal levels and the specific immune response is terminated. A state of long-lived memory follows successful virus elimination. This memory status is characterised by the presence of virus-specific memory T cells that are able to mount a faster and more efficient immune response after antigen encounter leading to rejection of the particular pathogen. Numerous events contribute to the activation, differentiation and homeostatic regulation of $CD8^+$ T cells, some of which will be discussed below. Figure 1 schematically summarises stages in the development and homeostasis of an immune response (b) and kinetics of virus and T lymphocytes (a).

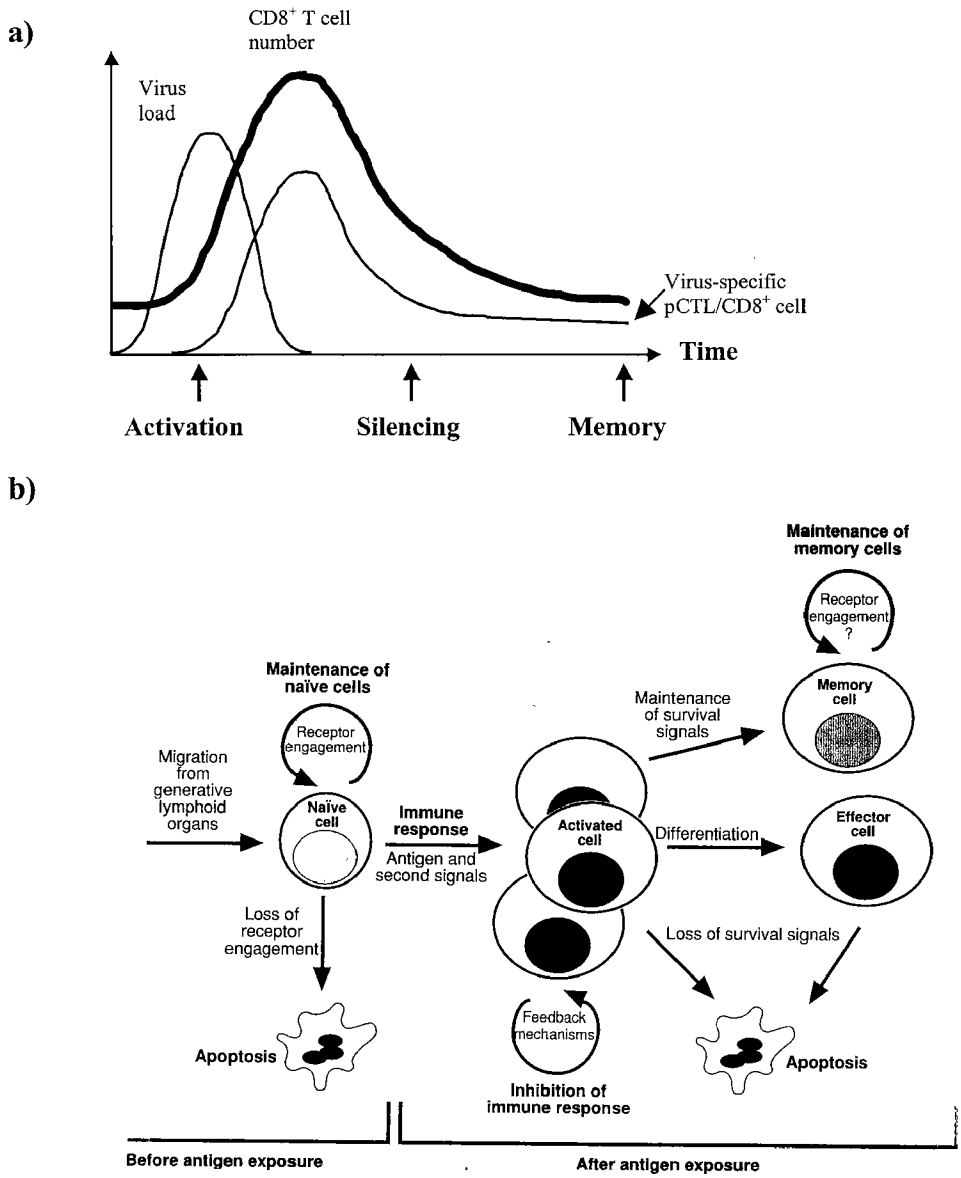


Figure 1. a) Idealised kinetics of virus load and induced CD8$^+$ T lymphocytes.
b) Stages in the development and homeostasis of an immune response. The signals that maintain lymphocyte populations and induce their proliferation and differentiation are acting together with signals that terminate immune responses and maintain homeostasis. From L. Van Parijs and A. Abbas, Science, 1998, 280:243-248.

Initiation of T cell activation

Efficient T cell activation requires two stimuli for most T cells. According to the two-signal hypothesis, T cells can only be successfully activated if two types of stimuli simultaneously act on the T cell (Matzinger, 1994; Schwartz, 1990). The first signal is provided by interaction of the specific TCR on the surface of T cells with its ligand, a MHC-peptide complex on the surface of APCs (Townsend et al., 1986). The second signal includes costimulatory molecules on the cell surface as well as cytokines such as IL-2 that promote clonal expansion of the specific T cells and their differentiation into

effector cells. If the T cells encounter an antigen stimulus below a certain threshold in the absence of the second signal they remain indifferent e.g. neither activated nor inactivated (Ohashi et al., 1991). In contrast, a stronger antigen engagement in the absence of the second signal may tolerise the T cells by functional inactivation (anergy) or deletion (Matzinger, 1994; Schwartz, 1990). The best characterised costimulatory molecules for T cells are the two members of the B7 family, B7-1 and B7-2 which are induced on APCs by microbes and cytokines produced during innate immune reactions (Linsley et al., 1991). B7 molecules are recognised by the CD28 receptor on T cells that delivers activating signals resulting in IL-2 production (Fraser et al., 1991).
Adhesion molecules such as the counterparts ICAM-1/LFA-1 and LFA-3/CD2 can also strengthen the interaction between APCs and T cells and might therefore act as a second signal by raising the overall avidity of the APC-T cell interaction without generating intracellular signals (Shaw et al., 1986; Springer, 1990).
Recently another system for T cell costimulation has become apparent. Generally, the in vivo activation of CTLs requires the participation of $CD4^+$ T helper cells. These provide cytokines and appropriate activation of APCs to enhance antigen presentation. It became clear that ligation of CD40 on the surface of APCs with its ligand CD40L on activated T helper cells increased the APCs antigen-presentation and costimulatory capacity and made it competent to directly prime CTLs (Schoenberger et al., 1998; Bennett et al., 1998). CD40/CD40L interaction is required for in vivo activation of $CD4^+$ and $CD8^+$ T in several experimental models (Yang and Wilson, 1996; Grewal et al., 1996). Nevertheless, generation of CTLs and antigen-specific T helper cell responses were completely normal in CD40L-deficient mice after infection with LCMV, Pichinde virus or vesicular stomatitis virus (VSV) (Oxenius et al., 1996; Borrow et al., 1996). This finding suggests that the need for costimulation is strongly dependent on the strength and quality of the antigenic signal. Viral infections have the characteristic capacity to challenge the immune system with a qualitatively strong antigenic stimulus. This stimulus is sufficient to induce cytokines during innate immune responses that can directly activate APCs, thereby initiating proper T cell responses without the need for further costimulation.

Successful engagement of the TCR with MHC molecules on APCs initiates multiple intracellular signaling events. The outcome of this process is proliferation and the acquisition of effector functions. Figure 2 highlights schematically the very first steps of TCR engagement.

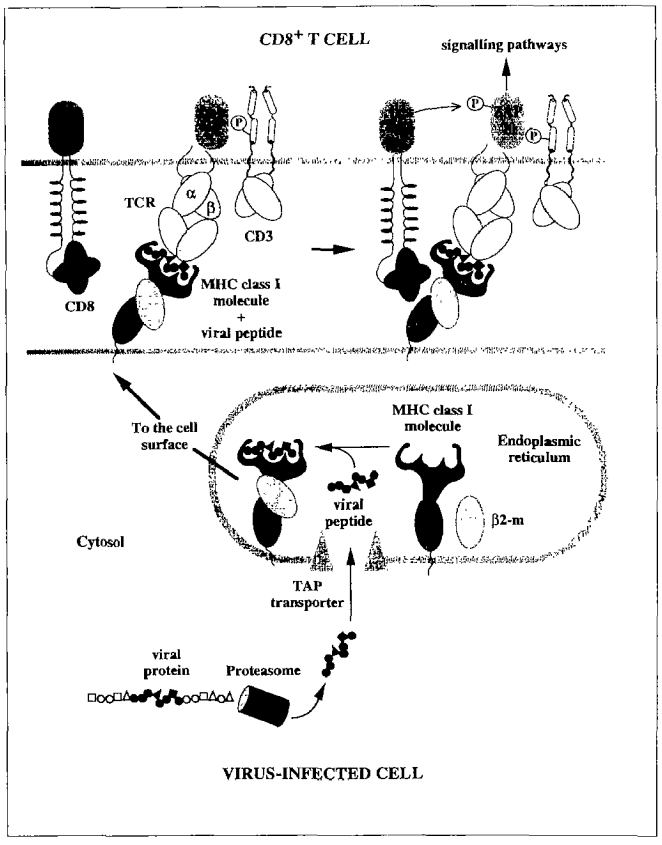


Figure 2. Model of antigen recognition by $CD8^+$ T lymphocytes. Peptides generated by the proteasome, an intracellular protease complex, are actively fed into the lumen of the endoplasmatic reticulum (ER) by a peptide transporter made of the two subunits TAP-1 and TAP-2. In the ER lumen peptides are loaded on newly synthesised MHC class I molecules which are subsequently transported to the cell surface via the Golgi complex. Once at the surface, the MHC molecules with their loaded peptides persist long enough for T cell recognition to occur. This specific recognition and the following transduction into intracellular signals are mediated by the multi-subunit transmembrane complex known as TCR-CD3 complex. The cytoplasmic domains of the various CD3 subunits are responsible for coupling the TCR αβ heterodimer to intracellular signaling pathways and contain one or multiple immunoreceptor tyrosine-based activation motifs (ITAMs, shown as cylinders) that fully account for their transducing capacity.

Once phosphorylated the tyrosine residues found within the ITAMs constitute high-affinity binding sites for the SH2-domain containing ZAP-70 protein kinase. From P. Bongrand and B. Malissen, BioEssays 20: 412-422, 1998.

The TCR is a complex, composed of 6 different polypeptide chains thought to be organised into an eight-chain structure (Orloff et al., 1990; Blumberg et al., 1990). The heterodimer αβ or γδ are required for ligand binding whereas the non-polymorphic chains CD3ε, CD3γ, CD3δ and TCRζ are responsible for receptor assembly, cell surface expression and signaling (Ashwell, 1990). Critical regions of the cytoplasmic domains of these chains are the immunoreceptor tyrosine-based activation motifs (ITAMs) which were primarily identified on the cytoplasmic chains of the B cell receptor (Reth, 1989; Cambier, 1995). Upon TCR engagement, the tyrosine residues within the ITAMs become phosphorylated by the protein tyrosine kinases (PTKs) lck and fyn of the Src-family (van Oers et al., 1996). The TCR-CD3 complex requires the help of $CD8^+$ molecules, which are constitutively associated with lck. Phosphorylation of the ITAMs allows the recruitment of src-homlogy 2 (SH2) containing proteins such as ZAP-70, which can bind with its SH2-domain specifically to phospho-tyrosine

containing polypeptides. ZAP-70 itself can act again as an enzyme responsible for protein-tyrosine phosphorylation and as an adaptor protein which can recruit many other signaling molecules (Qian and Weiss, 1997). The outcome of these complex signaling events is manifested in the expression of a specific gene related to the activation status of the cell.

Gene expression and T cell activation

The various cell types of a multicellular organism become different from each other because they synthesise and accumulate different sets of RNA and protein molecules. These changes are related to different transcriptional events (e.g. RNA synthesis) and normally occur without alteration of the DNA sequence that is preserved during cell differentiation. From the 10'000-20'000 proteins a cell is able to synthesise, 2000 represent the most abundant proteins in quantities of 50'000 or more copies per cell. These proteins are common to all cells and include structural components of the cytoskeleton and chromosomes, essential proteins for the endoplasmatic reticulum and the Golgi membranes as well as various enzymes involved in the central reactions of metabolism. Some proteins are abundant in specialised cells and can only be found there like hemoglobin in red blood cells. It is assumed that most of the proteins with very low copy numbers per cell are common to all cells and that only a small number of proteins (e.g. 1%) differ between cell types. This small number of differentially produced proteins is responsible for large differences in cell morphology and cell function, which is the basis for tissue differentiation and development (Derman et al., 1981). In addition, most of the specialised cells in a multicellular organism are capable of altering their pattern of gene expression in response to external stimuli. These induced changes of gene expression are related to many different levels of regulation such as alteration of transcriptional rate, RNA processing, RNA stability and others (Alberts et al., 1994).

In T cells, a whole set of specifically responsive genes becomes transcriptionally active as result of the TCR-mediated signaling events after antigen encounter. These transcriptional events are responsible for T cell proliferation and differentiation leading to specific effector functions. It has become evident that the changes leading to proliferation and differentiation are not the result of a single event of gene activation but that they rather result from a tightly regulated cascade of sequential gene activation events. The products of the first event would contribute to the transcriptional activation of the second set of targeted genes and so on.

During the process of T cell activation one does therefore differentiate between "immediate early activation genes" that are transcriptionally active within minutes after activation, "early activation genes" that are active within hours and "late activation

genes" that are active within several days after activation. Many transcription factors like NF-AT (nuclear factor of activated T cells) (Shaw et al., 1988) and NF-κB (nuclear factor κB) (Cross et al., 1989) belong to the immediate early activation genes. Cytokines produced by activated T cells such as IFN-γ, IL-2 and IL-4 as well as the later discussed surface molecules CD69, IL-2 receptor (IL-2R) and anti-apoptotic protein Bcl-2 are expressed within hours and belong to the group of early activation genes. Expression of granzymes A and B, the inhibitory molecule CTLA-4 as well as the surface molecule VLA-4 occurs a few days after activation and they represent members of the group of the late activation genes (Ullmann et al., 1990).

T cell effector phase

As mentioned above, successful activation is manifested in proliferation of T cells. Especially viral infections are characterised by substantial expansion of $CD8^+$ T cells. This is seen in humans during acute phase of infection with viruses that replicate systemically, such as human immunodeficiency virus (HIV), Epstein-Barr virus (EBV), measles and cytomegalovirus. Expansion of $CD8^+$ T cells is also seen in experimental models such as infection of mice with vaccinia virus (VV) and vesicular stomatitis virus (VSV). It is most pronounced after infection with LCMV, the animal model the present work is based on.

The proliferative capacity of LCMV-specific cells within the $CD8^+$ T cell compartment was demonstrated by adoptive transfer experiments using LCMV-specific TCR transgenic T cells (Zimmermann et al., 1996a). Transferred into normal immunocompetent hosts, these TCR transgenic T cells expanded vigorously upon LCMV-infection and multiplied their number by a factor of 10^3 to 10^4. Today the use of soluble tetramers of MHC-class I molecules presenting viral peptides to specifically detect antigen-specific T cells, obviates the need to use TCR transgenic cells (Altmann et al., 1996). By applying this novel technique, it became evident that 50-70% of the activated $CD8^+$ T cells were LCMV-specific 8 days after infection (Murali-Krishna et al., 1998). Similar expansions of CTLs have been observed in acute human EBV infection where up to 44% of peripheral $CD8^+$ T cells were found to be EBV-specific (Callan et al., 1998).

During this proliferation phase, the $CD8^+$ cells undergo drastic changes in their phenotype. They no longer patrol through different lymphatic tissues but instead specifically extravasate to those sites of inflammation where virus antigen is located. These changes in migration capacity are induced by surface expression of defined molecules that are involved in cell adhesion. L-Selectin (CD62L) the first molecule

described which mediated tissue-specific lymphocyte homing (Gallatin et al., 1983), is down-regulated on $CD8^+$ T cells after viral infection (Zimmermann et al., 1996a), thereby abrogating entry of effector T cells into the lymphnodes by high endothelial venules. Other adhesion molecules like VLA-4, LFA-1 and ICAM-1 are upregulated on activated $CD8^+$ T cells (Andersson et al., 1994). These molecules are thought to interact with counterparts on endothelial cells and extracellular matrix molecules thus promoting effector T cell homing to sites of inflammation. The surface molecules CD11b (Mac-I), CD49d, CD44, CD69 and IL-2R are also up-regulated, indicating a state of cellular activation (Zimmermann et al., 1996a; Andersson et al., 1994). In addition, the cells gain a blasted appearance, start to secrete various cytokines such as IL-2 and IFN-γ and accumulate perforin/granzyme-loaded granules, a prerequisite for their cytolytic effector function.

As effector cells, $CD8^+$ T cells eliminate viral antigen by inducing the death of virus-infected target cells using at least three distinct mechanisms: secretion of cytokines and calcium-dependent or calcium-independent cytotoxicity. Among the cytokines, TNF-α and TNF-β are the most important ones that can promote cell death (Paul and Ruddle, 1988; Old, 1985). The calcium-dependent pathway relies on the secretion of cytotoxic granules containing perforin and granzymes whereas the calcium-independent pathway causes target cell death via the interaction of Fas ligand on the CTL with the Fas receptor on the target cell (see below). Perforin, granzymes and other granule proteins are stored in cytotoxic granules that are released during interaction of target cell and CTL. Released perforin polymerises under the influence of free calcium ions to form channels in the target membrane. Through these channels granzymes may then pass into the target cell and exert their function on specific substrates involved with the ultimate death of the cell via apoptosis (Henkart, 1985; Podack, 1985).
The in vivo relevance of perforin and granzymes has been addressed by analysis of perforin and granzyme A and B deficient mice. Perforin-deficient mice failed to lyse Fas negative antigen presenting target cells and are not able to clear LCMV (Kägi et al., 1994a). Similarly, granzyme A and B double knock out mice had defective granule mediated apoptosis (Simon et al., 1997).

Mechanisms involved in T cell silencing

Once antigen is cleared it is important to maintain T cell homeostasis and preserve the primary repertoire, thus keeping responsiveness to new pathogens. Antigen-clearance after infection with LCMV is followed by a drastic loss of LCMV specific effector T cells between day 8 and 20, characterised by programmed cell death (apoptosis) of 90-95% of virus-specific $CD8^+$ T cells (Zimmermann et al., 1996a; Murali-Krishna et al., 1998). This event could in general be explained by two mechanisms: (1) the absence or loss of stimuli (i.e. antigen, cytokines) that provide necessary survival and growth signals to T cells leads to functional inactivation and apoptosis; (2) lymphocyte activation itself triggers mechanisms to control lymphocyte proliferation and differentiation.

(1) T cell silencing by loss of stimuli

It was suggested that members of the Bcl-2 family might play critical roles in the regulation of apoptosis, T cell survival and memory T cell generation (Akbar and Salmon, 1997). Bcl-2 family members have divers activities and regulate apoptosis in several ways. Some inhibit apoptosis such as Bcl-2 and Bcl-x_L, whereas others such as Bax, Bad and Bid promote apoptosis (Adams and Cory, 1998). Activated lymphocytes that are deprived of survival stimuli such as IL-2 in vitro lost expression of the anti-apoptotic proteins Bcl-2, Bcl-x_L and underwent apoptosis (Akbar et al., 1996; Broome et al., 1995). The addition of IL-2 could prevent apoptosis by upregulation of Bcl-2 protein expression. In addition, expression of the bcl-2 as a transgene inhibits IL-2 deprivation-induced apoptosis in cycling cells (Strasser et al., 1991). The analysis of bcl-x_L and bcl-2 transgenic mice to address the role of these two proteins in vivo revealed some controversy. The introduction of a bcl-x_L or bcl-2 transgene into P14 LCMV TCR-transgenic mice revealed that the constitutive expression of Bcl-x_L and Bcl-2 protein inhibited indeed LCMV peptide antigen-induced peripheral deletion of mature $CD8^+$ T cells, as well as death of activated $CD8^+$ effector cells induced by growth deprivation in vitro. However, neither Bcl-x_L nor Bcl-2 influenced clonal down-regulation of $CD8^+$ LCMV-specific T cells during the silencing phase of an antiviral immune response (Petschner et al., 1998).

(2) T cell silencing by regulatory mechanisms

Four regulatory feedback mechanisms triggered by activated T lymphocytes themselves have so far been identified.

- CTLA-4, the second receptor for B7 molecules binds B7 with a 10-20 fold higher affinity than CD28 (Linsley et al., 1994). In contrast to CD28, CTLA-4 is not expressed on resting T cells, but is induced following T cell activation (Freeman et al., 1992). CTLA-4 was shown to shut off T cell activation by transducing signals that inhibit IL-2 transcription and cell cycle progression (Thompson and Allison, 1997). CTLA-4 deficient mice are characterised by a severe lymphoproliferative disease (Tivol et al., 1995; Waterhouse et al., 1995) and T cells of these mice reveal hyperphosphorylation of various proteins including TCRζ, the protein tyrosine kinases lck and fyn and ZAP-70 (Marengere et al., 1996). Surprisingly, if mice lacking CTLA-4 were crossed into $CD8^+$ TCR transgenic mice expressing an LCMV specific TCR, they exhibited normal viability, decreased lymphoproliferation (Waterhouse et al., 1997), normal T cell activation and downregulation of ongoing T cell responses after elimination of antigen (Bachmann et al., 1998). It is possible that the lymphoproliferative disorder in mice lacking CTLA-4 is mainly due to $CD4^+$ T cells that are strongly reduced in number in $CD8^+$ TCR transgenic mice.
- The Fas/Fas ligand (FasL)-system comprises another regulatory pathway. Fas (CD95) belongs to the tumour necrosis factor (TNF)/nerve growth factor (NGF) receptor family and is expressed on a variety of cell types including hepatocytes and activated lymphocytes (Watanabe-Fukunaga et al., 1992a). Its ligand FasL (CD90) is found on activated T cells and on nonlymphoid tissue in immunologically privileged sites (Griffith et al., 1995). It was suggested that Fas-mediated cell death, induced by the interaction of Fas and FasL on activated T cells provides a mechanism for silencing activated T cells in vivo (Crispe, 1994; Nagata and Golstein, 1995). In T cells, the pathway of Fas apoptosis is thought to be induced by repetitive activation and is probably most important for eliminating cells that repeatedly encounter persistent antigen, such as self-antigens (Kurts et al., 1998). This hypothesis is supported by the observation that mice bearing mutated Fas or FasL genes develop lymphadenopathy with accumulation of abnormal $CD4^-CD8^-$ $B220^+$ T cells (Watanabe-Fukunaga et al., 1992b). They do not exhibit abnormally prolonged responses to viruses (Zimmermann et al., 1996b; Lohman et al., 1996) or to immunisation with foreign antigens (van Parijs et al., 1998). On the other hand, the Fas/FasL system also plays an important role in CTL-mediated cytotoxicity. The remaining CTL activities in perforin deficient mice were mediated by FasL on the CTLs and were totally abolished towards Fas negative target cells (Kägi et al., 1994b; Lowin et al., 1994).
- The TNF-TNF receptor pathway displays many similarities to the Fas pathway. The two different receptors mediating TNF induced signaling belong to the TNF/NGF

receptor family and are both expressed upon activation of the T cell (Scheurich et al., 1987). The TNF receptor type I (TNFR1, p75) expresses like Fas a death domain in its cytoplasmic domain and mediates apoptosis upon triggering (Zheng et al., 1995). Binding of TNF to the TNF receptor type 2 (TNFR2, p55) can also trigger apoptosis, even though TNFR2 does not bear a death domain (Rothe et al., 1995). Several reports described that TNF, TNFR1 and TNFR2 are involved in peripheral T cell deletion in vivo (Sytwu et al., 1996; Speiser et al., 1996). TNF was thereby mediating the death of most $CD8^+$ T cells whereas FasL mediated the death of most $CD4^+$ T cells (Zheng et al., 1995), indicating that both pathways contribute by two distinct molecular mechanisms to the autoregulatory apoptosis of mature T cells.

- A fourth regulatory feedback mechanism has been proposed. Recent research suggested that the TCR-mediated activating pathways are subject to negative regulation. The best characterised receptor that mediates inhibitory signals is the low-affinity receptor for IgG FcγRIIB which is expressed on murine B cells, T cells, mast cells and many other cell types. A 13 amino acid sequence has been described in the intracytoplasmic domain of FcγRIIB as being necessary for this receptor to inhibit B cell activation (Amigorena et al., 1992). This sequence contains the motif V/IxYxxL/V, termed ITIM for immunoreceptor tyrosine-based inhibition motif in analogy to the immunoreceptor tyrosine-based activation motif (ITAM) (Daeron, 1996). Following coaggregation with the ITAM-containing B cell receptor (BCR), the tyrosine residue within the ITIM of FcγRIIB is phosphorylated and the receptor becomes a potential ligand for SH2 containing proteins. Three protein tyrosine phosphatases SHP-1, SHP-2 and SHIP are recruited by the phosphorylated FcγRIIB (D´Ambrosio et al., 1995; Ono et al., 1996). These phosphatases are able to dephosphorylate the ITAMs in the Igα and Igβ chain of the BCR thereby blocking the activation signal. FcγRIIB mediated negative regulation was extended to other receptors whose cell-triggering ability also depends on ITAMs. FcγRIIB was then shown to inhibit Fcε receptor I (FcεRI)-dependent mast cell activation as well as TCR-dependent T cell activation (Daeron et al., 1995).

  A second class of ITIM-bearing coreceptors has recently been discovered to control natural killer (NK) cell activation. NK cells express several killer cell inhibitory receptors (IRs) that transduce a negative signal if ligated to their ligands, MHC class I molecules on target cells (Moretta and Moretta, 1997). This interaction leads to the inhibition of natural cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC). Killer cell inhibitory receptors belong to two distinct superfamilies of molecules: the immunoglobulin superfamily that includes the human killer cell inhibitory receptors (KIRs) and gp49B1 in the mouse and the C-type lectin superfamily that includes the murine inhibitory receptors and CD94/NKG2A in

human. IRs express also ITIMs in their intracytoplasmic domains that become tyrosine-phosphorylated and recruit SH2-domain-containing phosphatases (Olcese et al., 1996). Again, coaggregation between an ITAM-bearing activating receptor and a KIR is required for the inhibition of cell activation.

At the time of the discovery of inhibitory receptors on NK cells (Moretta and Moretta, 1997; Wang and Yokoyama, 1998), it was also reported that a small subset of peripheral T cells expressed these IRs (Lanier and Phillips, 1996; Mingari et al., 1998b). Different groups demonstrated that IRs could inhibit TCR-induced T cell functions, including cytolytic activity and cytokine production (Phillips et al., 1995; Mingari et al., 1995; D`Andrea et al., 1996). In healthy subjects, inhibitory receptors are expressed by 1-2% of predominantly $CD8^+$ T cells in peripheral blood, lymph nodes, spleen and tonsils but not in thymus or cord blood. Surface marker analysis revealed that these $CD8^+$ T cells always expressed an activated or memory phenotype suggesting that the expression of inhibitory receptors is a consequence of antigen-driven stimulation e.g. self-antigen or viral infection (Mingari et al., 1997; Mingari et al., 1998b). Essentially all IRs, whether belonging to the Ig superfamily or to the C-type lectin family are expressed by the T cells (Mingari et al., 1997). It should be stressed that unlike the above-mentioned regulatory pathways, IRs have not yet been reported to be directly coupled to apoptosis inducing pathways. Yet inhibitory receptors could deprive T cells of stimulation either by abolishing TCR mediated activating signals or by influencing cytokine production.

A scheme summarising the four main regulatory pathways that are induced by activated T cells and supposed to be actively involved in T cell silencing is displayed in Figure 3.

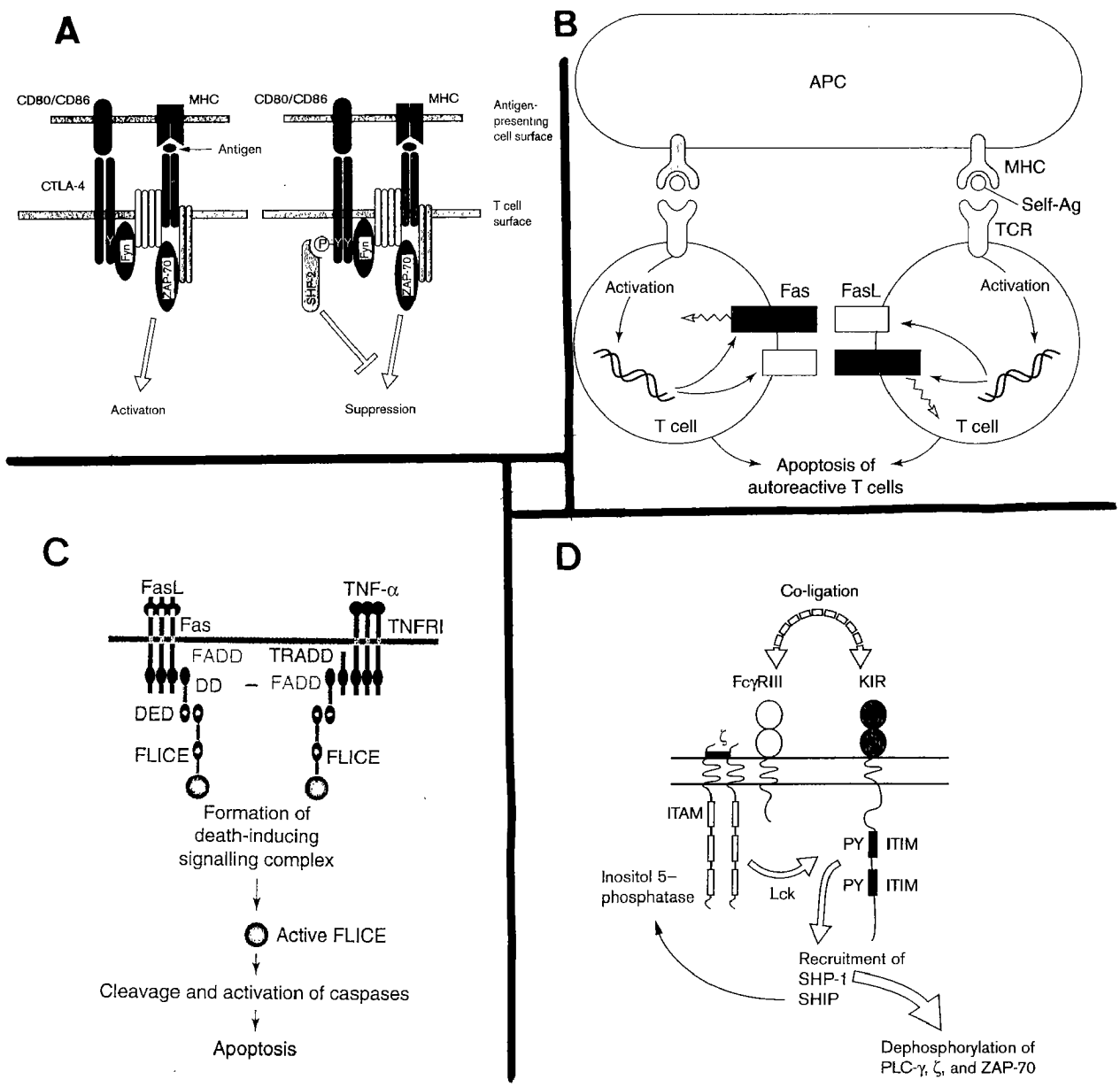
Figure 3A. A model for the dynamic regulation of expression and signaling of CTLA-4 by Src-family kinases associated with the TCR complex. Src-family kinases such as fyn are able to phosphorylate CTLA-4 within its cytoplasmic tail. Phosphorylated CTLA-4 is resistant to endocytosis and remains on the cell surface where it can recruit SH2-containing tyrosine phosphatases, which may dephosphorylate TCR-associated kinases (Marengere et al., 1996). From T. Saito, Curr. Op. Immunol., 1998, 10:313-321.

3B. A model for Fas-mediated clonal deletion of activated T cells in the periphery. Presentation of antigen by APCs leads to activation of antigenspecific T cells that express Fas and FasL upon activation. Subsequent interaction between T cells expressing Fas and FasL results in Fas-mediated apoptosis (zigzagged arrow) and clonal deletion. From S. Nagata and T. Suda, Immunology Today, 1995, 16:39-43.

3C. Apoptotic signaling by death receptors. Binding of FasL to Fas or TNF-α to TNFR1 leads to recruitment of the adaptor molecule Fas-associated death domain (FADD). While FADD binds directly to Fas, the recruitment of FADD to TNFR1 is mediated by another adaptor molecule TNFR1-associated death domain (TRADD). The interactions are due to homophilic association of death domains (DD) in the cytoplasmic domains. FADD then recruits the interleukin-1β-converting enzyme (ICE) family protease

(FLICE) to form a death-inducing signaling complex (DISC) (Kischkel et al., 1995; Muzio et al., 1996). FLICE is activated by autocatalytic cleavage and presumably activates a cascade of downstream proteases, which then initiate DNA fragmentation and the ultimate cell death (Enari et al., 1998). From E. Meinl et al., Immunology today, 1998, 19:474-479.

3D. The potential mechanisms by which an ITIM-bearing inhibitory receptor (KIR) might interfere with activating signals produced by an ITAM-bearing activating receptor. Coligation of the KIR which binds MHC class I molecules and the activating receptor FcγRIII results in phosphorylation of the KIRs ITIM by lck, creating a docking site for the phosphatases SHP-1 and SHIP. SHP-1 subsequently dephosphorylates ZAP-70 and the FcγRζ-chain, whereas SHIP leads to the inhibition of external Ca^{2+} influx. In NK cells, SHP-1 plays the dominant role. From J. Unkeless and J. Jin, Curr. Op. Immunol., 1997, 9:338-343.



Mechanisms involved in the generation of T cell memory

Eliminating all effector cells participating in the primary response by the previously mentioned mechanisms would be counterproductive and lead to tolerance rather than immunity. Memory cell generation therefore reflects a highly complex process whereby a small fraction of cells stimulated in the primary response somehow evades apoptosis and survives for prolonged periods (Zinkernagel et al., 1996; Ahmed and Gray, 1996). Very recent data showed that the TCR repertoire of a primary antiviral $CD8^+$ T cell response was both structurally and functionally similar to that of the memory pool and the secondary $CD8^+$ T cell effectors. These results suggest a stochastic selection of memory cells from the pool of $CD8^+$ T cells activated during primary infection (Sourdive et al., 1998). Yet, how memory cells escape apoptosis after primary infection is poorly understood. Some in vitro evidence suggests that memory cells remain resistant to apoptosis through upregulation of Bcl-2 and Bcl-x_L following antigen contact (Müller et al., 1996). In the context of activation induced cell death it was reported that T cells that escaped apoptosis did express high levels of IL-4 and IL-10 that may induce rescue signals in these cells (Zhang et al., 1996).

The requirements of memory T cells

The molecular basis of $CD8^+$ T cell memory is still a controversial issue. It is not clear whether memory responses depend exclusively on an increased frequency of antigen-experienced cells (Cerottini and MacDonald, 1989; Allouche et al., 1982) or if memory T cells with a novel biological capacity are generated (Bruno et al., 1995). Some groups reported that memory responses are dependent on continuous antigen stimulation (Gray and Matzinger, 1991; Oehen et al., 1992), whereas others have observed the persistence of increased frequencies of antigen-specific $CD8^+$ T cells in the absence of antigen (Hou et al., 1994; Lau et al., 1994). However, in the absence of antigen, memory T cells seem to require some interaction with self-MHC molecules that do not necessarily represent the selecting H-2-restricting element (Tanchot et al., 1997; Markiewicz et al., 1998). For the generation of antiviral $CD8^+$ T cell memory, interaction with $CD4^+$ T cells and also B cells seems to be crucial. The absence of $CD4^+$ T cells leads to impaired $CD8^+$ T cell memory, to virus spread and exhaustion of the virus-specific CTLs (Matloubian et al., 1994; von Herrath et al., 1996; Thomsen et al., 1996). This occurs despite the fact that $CD4^+$ T cells are not necessary for the induction of a primary CTL response against LCMV (Rahemtulla et al., 1991; Ahmed et al., 1988). Whether $CD4^+$ helper cells are required for the initial generation of $CD8^+$ memory cells or for long-term survival of these cells is not clear. Experiments in B cell deficient mice demonstrated similar results

(Thomsen et al., 1996). However, these results are subject to controversy and other groups described normal $CD8^+$ effector functions and long-lasting $CD8^+$ T cell memory in B cell and class II deficient mice (Asano and Ahmed, 1996; Brundler et al., 1996; Di Rosa and Matzinger, 1996). Recently it was shown that the lack of CD40L had a direct effect on antiviral $CD8^+$ cells. Rapid impairment of CTL responsiveness and failure to permanently control virus replication despite normal primary activation of the $CD8^+$ T cells was observed in CD40L deficient mice (Thomsen et al., 1998). This data suggests that the requirement for cell-cell communication and costimulation in long term memory is complex and probably crucially dependent on the antigenic stimulus used.

Characterisation of memory T cells

Determination of the turnover rate of memory cells using bromodeoxyuridine (BrdU) as readout demonstrated that the $CD8^+$ memory cells exist as a phenotypically heterogeneous population comprising slowly cycling cells (Zimmermann et al., 1996a). The rate of memory T cell division can be enhanced in an antigen independent way by the administration of growth factors such as IL-15 and IFN-α (Tough and Sprent, 1994; Tough et al., 1996). This suggests that the properties of memory cells are not solely dependent on TCR engagement but that memory cells might also be stimulated by lymphokine receptors. The capacity of infectious agents to induce non-antigen-specific stimulation of T cells may therefore play a role in boosting the memory cells and thereby ensuring long-term survival.

The expression of specific "memory markers" on the surface of $CD8^+$ T cells is still a controversial issue. T cell activation leads to CD62L downregulation and CD44, ICAM-1, LFA-1, CD49d and CD11b upregulation. In parallel to the gradual transition from activated cells into resting cells, the phenotype of long-lived memory cells shows a partial reversion towards a naive phenotype, i.e. $CD62L^{hi}$ and $CD11b^{lo}$. However, some memory cells remained in a $CD62L^{lo}$, $CD44^{int}$ and $CD49^{int}$ phenotype suggesting that the cells had encountered antigen after viral elimination (Zimmermann et al., 1996a). Thus, non of the markers analysed so far was useful for the description of $CD8^+$ memory T cells. Some reports described the upregulation of Ly6C (Walunas et al., 1995) and CD11b (McFarland et al., 1992) on activated T cells and postulated that these molecules could serve as useful markers for memory T cells.

The qualitative differences between naive T cells and memory T cells have only recently been addressed in vivo using an adoptive transfer system. Studies with $CD4^+$ memory cells generated in vitro suggested that memory cells have less stringent requirements for activation than naive cells and secrete more complex patterns of cytokines (Croft et al., 1994; Sagerström et al., 1993). The analysis of $CD8^+$ naive and memory T cells generated in vivo revealed that naive and memory cells had similar antigen thresholds, comparable proliferation rates and similar kinetics of surface marker up- or downregulation. But memory cells were able to acquire cytolytic activity and secrete cytokines more rapidly than naive T cells and were more efficient than naive cells in controlling growth of tumour cells expressing a defined tumour associated antigen (Zimmermann et al., 1999; Bachmann et al., 1999).

Differential display

Differential expression of a common set of inherited genes is essential for the development of specialised functions in multicellular systems.
The mouse genome encodes 70'000 genes from which only about 15'000 distinct RNA transcripts are expressed. The most abundant transcripts can be expressed at a level up to 10^5 copies/cell and constitute 10-20% of the total mRNA population. However, 90-99% are rarely expressed and do not exceed 300-500 copies/cell (Alberts et al., 1994).

A broad repertoire of novel techniques has been established to identify and isolate differentially expressed mRNAs. Subtractive hybridisation was the original method of choice because prior knowledge of the gene function was not required. In 1992, a new approach to differential screening was proposed, which is today generally known as "differential display"(DD) (Liang and Pardee, 1992; Welsh et al., 1992). The basis of DD is as follows:

1. Pools of cDNA fragments are produced from the total RNA of the samples being compared.
2. cDNA is subjected to randomly primed PCR and PCR products are resolved side by side on a polyacrylamide gel, generating a specific banding pattern called fingerprint.
3. Fragments (defined by length) that are present in only one sample or much more abundant in one than in the other, are excised from the gel and investigated.

Figure 4 gives a detailed overview about the method and the parameters used for this thesis.

The main advantage of this approach compared with subtractive hybridisation is the ability to compare more than two RNA samples of different source at once and to perform DD with very small amounts of total RNA. Also weakly expressed transcripts with expression differences of only several-fold between the cDNA populations analysed can be detected with DD whereas they would be under-represented or totally lost in subtractive hybridisation (Wan et al., 1996). On the other hand, in DD it may be difficult to isolate a strongly expressed transcript because differences in fragment abundance of 50:1 and 1000:1 could have the same appearance on a polyacrylamide gel. The success of certain product amplifications also depends on the arbitrary primer match to the corresponding cDNA sequence. In principle, even the rarest transcript could be represented on a fingerprint as an intensive band if the arbitrary primer match to this sequence is perfect. In contrary abundant transcripts could be represented as a very weak band due to a poor primer match. This was the case for the band obtained for the LGALS1 gene described in this work.

One of the main disadvantages of classical DD is the high proportion of false positive cDNA fragments e.g. differentially appearing fragments that do not correspond to differentially expressed transcripts. Several improvements of the technique have been made in the years after its discovery. The most obvious cause of false positives is the use of short (10-12 bases) oligos as proposed in the original publications. Such PCR is very sensitive to cycling conditions leading to unreproducible banding patterns due to minor temperature variations. The use of longer oligos (more than 20 bases) made effective arbitrary priming at low annealing temperature (40°C) possible in the first PCR cycles. Amplification of the generated products was then carried out under high stringency conditions (60°C for annealing) in the following cycles. This modified method significantly improved the reproducibility and sensitivity of DD while still keeping the characteristics of the original method (Zhao et al., 1995).

Until today, DD has been successfully used for the isolation of several hundreds of genes expressed in numerous cell types. However, based on personal communication with other scientists in the field one may expect that much more approaches have been performed which were not successful. Analysis of lymphopoietic cells led to the identification of several genes involved in lymphocyte development and activation and provided some insights into the pathogenesis and treatment of diseases. Similarly to lymphopoiesis, tumourigenesis is accompanied by changes in the pattern of gene

expression and serves as a convenient target for the application of DD. Several oncogenes and tumour supressor genes have been identified that way (Hess et al., 1998). Furthermore, many other physiological and pathological conditions including cardiovascular disease, HIV-infection and nutrient deficiencies have been subject of DD.

Figure 4. Flow chart of the cDNA synthesis and PCR fingerprint reaction in the DD protocol. Single strand cDNA is synthesised from total RNA using an oligo $(dT)_9$ primer. Subsequent PCR reactions are carried out by pair-wise combination of a modified oligo(dT) primer ($CATTATGCTGAGTGATATC(T)_9XX$) and an arbitrary primer ($ATTAACCCTCACTAAA(X)_9$). During 3 low-stringency cycles (annealing at 40°C) the arbitrary primer has the chance to bind sites in many cDNAs with imperfect or incomplete matches. The products of these early cycles are then amplified during 22-25 high-stringency PCR cycles (annealing 60°C). Analysed by polyacrylamide gelelectrophoresis these PCR products produce characteristic fingerprints of the starting RNA with approximately 40-80 bands. A fingerprint example for naive (N), effector (E) and memory (M) RNA is shown in original. Bands that appear to be consistently different (arrow) are isolated from the gel, reamplified and subjected to Northern blot analysis. Confirmed PCR products are cloned, sequenced and further investigated.

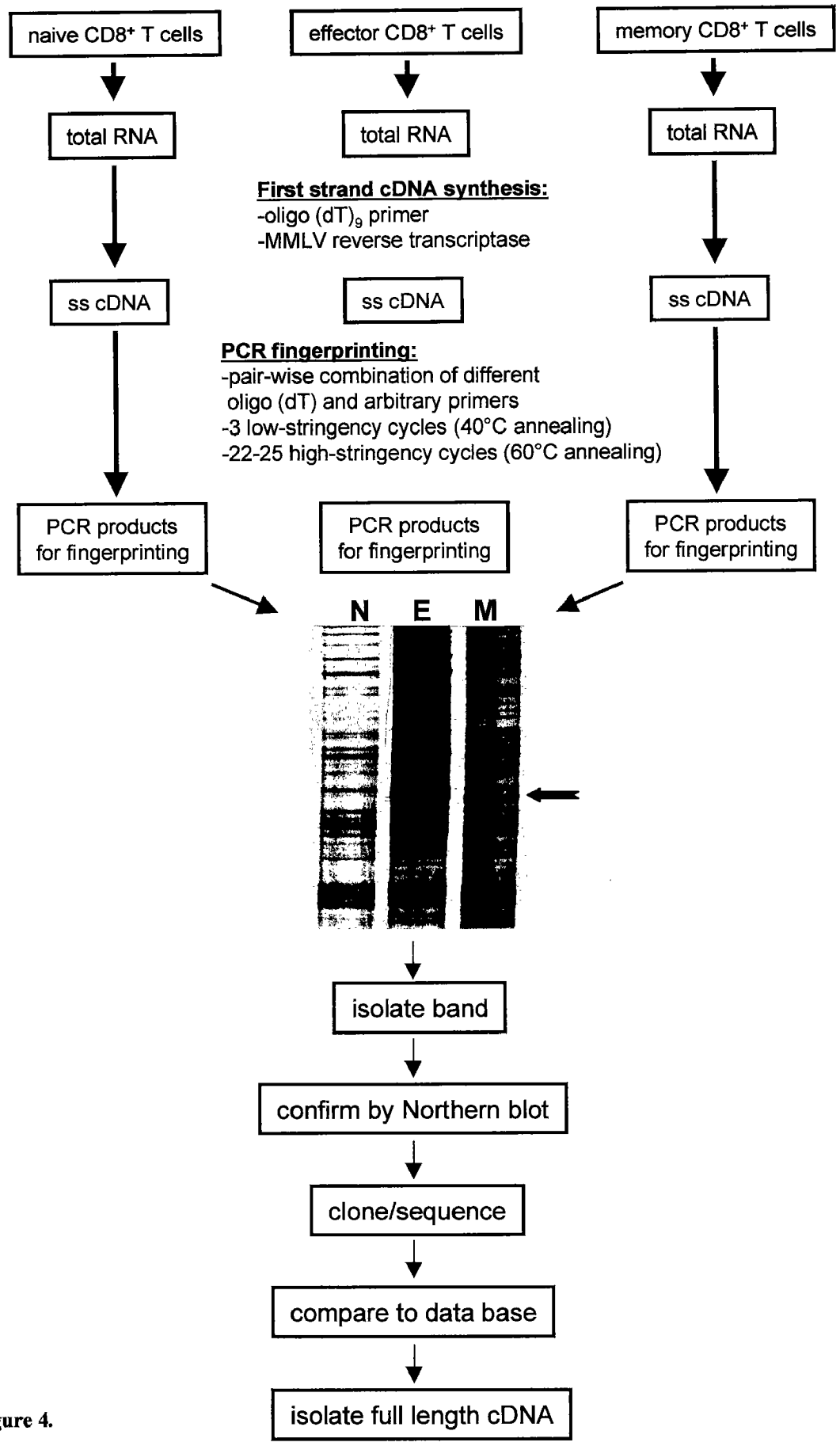

naive CD8+ T cells
effector CD8+ T cells
memory CD8+ T cells
total RNA
total RNA
total RNA
First strand cDNA synthesis:
-oligo (dT)9 primer
-MMLV reverse transcriptase
ss cDNA
ss cDNA
ss cDNA
PCR fingerprinting:
-pair-wise combination of different
oligo (dT) and arbitrary primers
-3 low-stringency cycles (40°C annealing)
-22-25 high-stringency cycles (60°C annealing)
PCR products for fingerprinting
PCR products for fingerprinting
PCR products for fingerprinting
N
E
M
isolate band
confirm by Northern blot
clone/sequence
compare to data base
isolate full length cDNA

Figure 4.

Aims of the study

Numerous processes involved in T cell activation, silencing and the generation of protective T cell memory determine in a complex pattern the outcome of an immune response. Despite today's particular knowledge, the overall control of an immune response remains a puzzling event. A detailed understanding of mechanisms underlying T cell activation is indispensable for the development of new strategies against tumourigenic or virus-infected cells. Despite many advances made in the treatment and control of infectious diseases, chronic viral infections such as infection by hepatitis B and C virus as well as by HIV remain a significant cause of mortality. In addition, the control of tumour development is still one of the major goals in basic as well as in applied immunology. Efficient silencing of activated T cells has to be achieved for intervention into ongoing autoimmune diseases such as autoimmune diabetes type I, rheumatoid arthritis and multiple sclerosis, and for suppression of graft rejection. The T cell system provides a unique opportunity for targeted therapeutic approaches because signals from the same receptor (TCR) can result in apoptosis or T cell activation.

The aim of this thesis was to identify and characterise novel genes involved in processes of $CD8^+$ T cell activation and regulation. The use of an adoptive transfer system with LCMV-specific TCR-transgenic T cells allowed the generation of $CD8^+$ effector and memory cells in vivo. Transferred into normal C57BL/6 recipients the transferred TCR transgenic T cells expressing a TCR specific for the glycoprotein peptide aa33-41 of LCMV undergo extensive proliferation upon LCMV-infection. These LCMV-specific T cells can be detected in the host by monoclonal antibodies against the LCMV-specific TCR for more than a year after infection. This system provided for the first time a tool to follow and isolate a defined population of antigen-specific $CD8^+$ effector and memory T cells ex vivo. Phenotypic characterisation of naive, effector and memory $CD8^+$ T by analysis of several surface proteins was limited to the availability of corresponding antibodies. The newly developed technique of the "mRNA differential display" allowed a comparison of these different T cell phenotypes by directly comparing gene expression patterns on RNA level. This had the advantage, that a prior knowledge of the gene or its function was not necessary and that the comparison of the different cell types was not restricted to surface molecules. Thus, a combination of the adoptive transfer system together with the differential display technique promised to be an ideal tool to identify novel genes relevant for T cell activation and regulation in vivo.

2. RESULTS I

β-GALACTOSIDE-BINDING PROTEIN (βGBP) SECRETED BY ACTIVATED T CELLS INHIBITS ANTIGEN-INDUCED PROLIFERATION OF T CELLS

LGALS1 mRNA is expressed in $CD8^+$ effector and memory T cells isolated ex vivo. mRNA differential display PCR was performed to identify genes differentially expressed in naive, effector and memory $CD8^+$ T cells. Effector and memory cells were generated in vivo using an adoptive transfer system with transgenic T cells expressing a TCR specific for the glycoprotein peptide aa33-41 from LCMV. On the differential display fingerprint gel a band could be identified that appeared only in the cDNA from effector and memory T cells but not in the cDNA from naive T cells (data not shown). DNA eluted from this band was amplified by PCR with original primers and sequenced. The sequence revealed 100% homology with a gene originally designated LGALS1 (lectin, galactoside-binding, soluble) (Wells and Mallucci, 1991). The LGALS1 gene product can either be released by the cells as a monomer (beta-galactoside-binding protein, βGBP) (Wells and Mallucci, 1991) or it can associate into a homodimer (galectin-1) with lectin properties (Barondes et al., 1994a).

To confirm the results of the differential display analysis, Northern blot analysis of the RNA from naive, effector and memory cells was performed using a full length LGALS1 cDNA probe. As shown in Fig. 1, naive $CD8^+$ T cells expressed low levels of LGALS1 mRNA (lane 1), while ex vivo isolated $CD8^+$ effector T cells expressed very high levels (lane 2). LGALS1 mRNA was still expressed in $CD8^+$ memory T cells (lane 3) but to a lower extent than in effector T cells.

To test whether LGALS1 expression was also induced in non-transgenic $CD8^+$ T cells during viral infection, Northern blot analysis of mRNA from spleen cells of acutely LCMV-infected C57BL/6 mice was performed. As shown in Fig. 1, lane 4, spleen cells from day 8 LCMV-immune mice expressed LGALS1 at levels comparable to those of purified TCR-transgenic effector T cells (lane 2). To demonstrate that increased LGALS1 expression was exhibited only by $CD8^+$ effector T cells, RNA was isolated from day 8 LCMV-immune spleen cells that were depleted of $CD8^+$ T cells by negative selection with Dynabeads in vitro. As shown in lane 5, immune spleen cells depleted of $CD8^+$ T cells expressed only a small amount of LGALS1 mRNA close to that of the naive $CD8^+$ T cells (lane 1). This result confirms that the high level of LGALS1 expression can be attributed to $CD8^+$ effector T cells and is not due to contamination of the cell preparations.

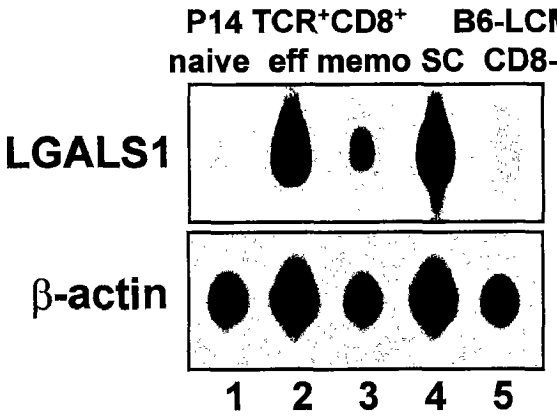


Figure 1. Northern blot analysis of LGALS1 expression in different T cell populations isolated ex vivo. Lane 1: naive $CD8^+$ T cells isolated from uninfected P14 TCR transgenic mice. Lane 2: $CD8^+$ P14 TCR transgenic effector T cells isolated 8 days after adoptive transfer into C57BL/6 recipient mice, followed by LCMV-infection with 200pfu i.v.. Lane 3: $CD8^+$ P14 TCR transgenic memory T cells isolated 4 weeks after adoptive transfer. Lane 4: spleen cells (SC) from day 8 LCMV-immune C57BL/6 mice. Lane 5: $CD8^+$ T cell depleted spleen cells (SC) from day 8 LCMV-immune C57BL/6 mice. For each lane 5μg of total RNA was loaded. Hybridisation with a β-actin probe shows equivalent sample loading.

$CD4^+$ and $CD8^+$ T cells, but not B cells, express high levels of LGALS1 mRNA upon stimulation in vitro.

To assess LGALS1 expression after in vitro stimulation, $CD8^+$ T cells from P14 TCR transgenic mice were stimulated in vitro with the LCMV glycoprotein peptide GP33. Three and 8 days after peptide stimulation the cultures were harvested, RNA was isolated and Northern blot analysis was performed. As shown in Fig. 2, lanes 2 and 3, in vitro activation of $CD8^+$ T cells induced high levels of LGALS1 mRNA. Naive spleen cells (lane 1) showed only marginal expression of LGALS1.

To examine whether activated $CD4^+$ T cells also express LGALS1 mRNA, spleen cells from 2B4 TCR transgenic mice expressing a TCR specific for the moth cytochrome c (MCC) peptide 88-103 were stimulated in vitro with the cognate MCC peptide. Five days after peptide stimulation $CD4^+$ T cells were purified and RNA was isolated. Lane 4 in Fig. 2 shows that peptide antigen activated $CD4^+$ T cells also expressed LGALS1 mRNA. Furthermore, LGALS1 expression in activated $CD4^+$ T cells displaying a Th1 or Th2 phenotype was determined. $CD4^+$ T cells from DO11.10 $CD4^+$ TCR transgenic mice, expressing a TCR specific for the Ovalbumin peptide OVA323-339 (Murphy et al., 1990), were stimulated with the cognate peptide antigen in the presence of 200

units/ml IL-4 for Th2 cells or 10 units/ml IL-12 for Th1 cells. T cells were restimulated with Phorbolmyristateacetate (PMA)/Ionomycine after 1 week and RNA for Northern blot analysis was isolated 24 and 48 hours after restimulation. In addition, RNAs from the $CD4^+$ T cell clones L1/1 and B10BI were also analysed by Northern blotting. L1/1 is a Th2 cell clone specific for Leishmania major (Lohoff et al., 1988) and B10BI is a Th1 cell clone specific for bovine insulin (Lohoff et al., 1990). Primary Th1 and Th2 cultures as well as the Th1 and Th2 cell lines all exhibited strong LGALS1 expression (data not shown). Similarly, Concanavalin A (ConA) activated $CD8^+$ as well as $CD4^+$ T cells both expressed high LGALS1 mRNA levels (Fig. 2 lanes 5 and 6). Interestingly, however, lipopolysaccharide (LPS)-activated B cells expressed LGALS1 mRNA only at low levels close to those of the naive spleen cells (Fig. 2, lanes 7 and 1). Thus, in contrast to antigen- or mitogen-activated $CD4^+$ and $CD8^+$ T cells, LPS-activated B cells showed only weak LGALS1 expression. LGALS1 was equally expressed in Th1 or Th2 $CD4^+$ T cell subsets.

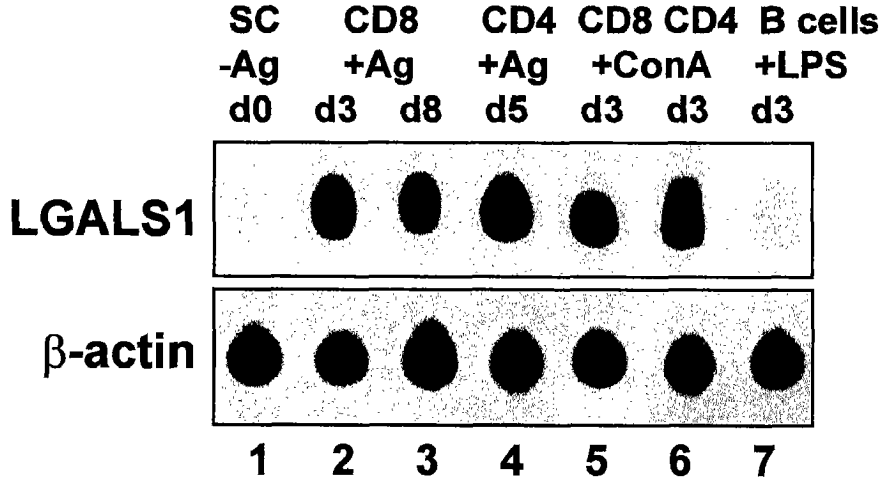


Figure 2. Northern blot analysis of LGALS1 expression in $CD8^+$ and $CD4^+$ T cells and B cells activated in vitro. Lane 1: Spleen cells (SC) from naive C57BL/6 mice. Lanes 2 and 3: $CD8^+$ T cells from P14 TCR transgenic mice 3 and 8 days after GP33 peptide stimulation. Lane 4: $CD4^+$ T cells from 2B4 TCR transgenic mice 5 days after MCC peptide stimulation. Lanes 5 and 6: ConA activated $CD8^+$ and $CD4^+$ T cells from C57BL/6 mice 3 days after mitogen stimulation. Lane 7: LPS-activated B cells from C57BL/6 mice 3 days after mitogen stimulation. 5µg of total RNA was loaded per lane. Hybridisation with a β-actin probe shows equivalent sample loading.

Kinetics of LGALS1 expression induced after peptide antigen stimulation in vivo and in vitro.

Injection of LCMV GP33 peptide into P14 TCR transgenic mice leads to transient activation of TCR transgenic T cells (Kyburz et al., 1993). To test whether stimulation with a soluble peptide antigen also induces LGALS1 mRNA expression, $CD8^+$ T cells were isolated from P14 TCR transgenic mice that had been treated once intraperitoneally (i.p.) with 500μg of the LCMV GP33 peptide. Fig. 3a shows Northern blot analysis of $CD8^+$ T cells isolated ex vivo at various points in time after peptide application. $CD8^+$ T cells from untreated animals (Fig. 3a, lane 1) expressed low levels of LGALS1 mRNA. Twenty-four hours after peptide treatment, LGALS1 expression was induced (lane 3) and further increased at 48 hours after peptide treatment (lane 4) reaching levels comparable to those seen in effector T cells (Fig. 1, lanes 2 and 4). This result indicates that in $CD8^+$ T cells LGALS1 expression is induced within 24 hours and further enhanced until 48 hours after stimulation with the cognate peptide antigen in the absence of a viral infection.

Next, the kinetics of LGALS1 expression in naive and LCMV-immune memory T cells was compared after stimulation with the cognate peptide antigen in vitro. LCMV-specific memory T cells were generated using the adoptive transfer system with P14 TCR transgenic T cells as described in Materials and Methods. TCR transgenic T cells from naive or LCMV-immune mice were stimulated with GP33 peptide-loaded spleen cells in vitro and RNA was isolated at the indicated points in time after peptide activation. As shown in Fig. 3b, lanes 1-6, kinetics of LGALS1 expression in naive T cells stimulated with the cognate peptide antigen in vitro was identical to that obtained in vivo (Fig. 3a) and LGALS1 expression was strongly induced within 48 hours. LGALS1 expression in restimulated memory cells (lanes 7-12) was induced already within 24 hours and occurred 12 hours earlier than in naive cells.

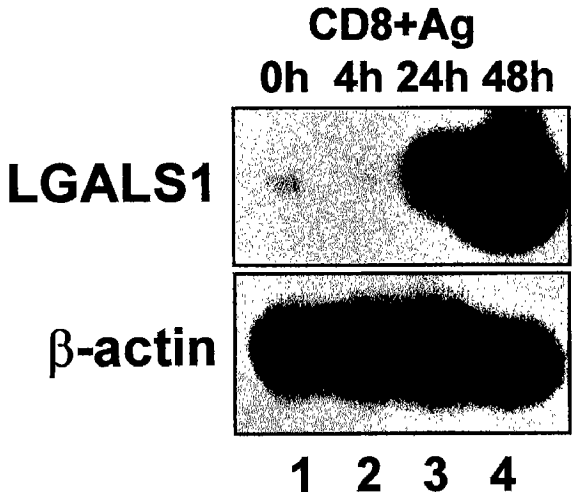


Figure 3a. Northern blot analysis of LGALS1 expression in CD8⁺ T cells after peptide antigen treatment in vivo. P14 TCR transgenic mice were injected once with 500μg LCMV glycoprotein peptide 33-41 i.p.. Mice were killed at the indicated points in time after peptide injection. The blot shows LGALS1 expression in CD8⁺ T cells isolated from untreated animals (lane 1) and from peptide antigen-treated mice 4h, 24h and 48h after peptide injection (lanes 2, 3 and 4 respectively).

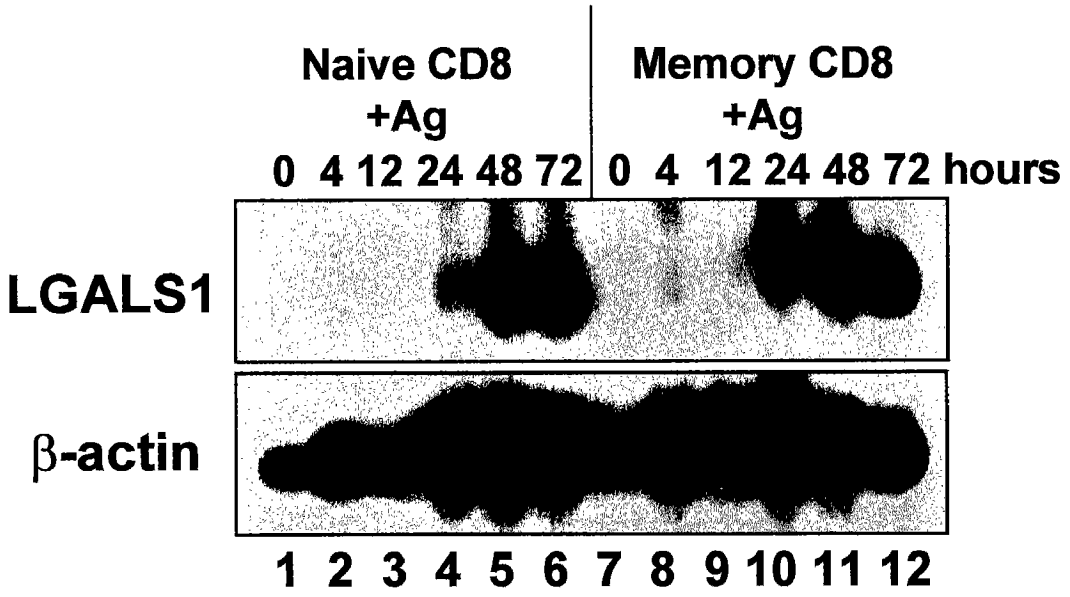


Figure 3b. Northern blot analysis of LGALS1 expression in naive and memory CD8⁺ T cells after GP33 peptide activation in vitro. Lane 1: CD8⁺ T cells from naive P14 TCR transgenic mice. Lanes 2-6: CD8⁺ T cells from naive P14 TCR transgenic mice indicated points in time after peptide stimulation in vitro. Lane 7: CD8⁺ TCR transgenic memory T cells isolated from C57BL/6 recipient mice several weeks after adoptive transfer. Lanes 8-12: CD8⁺ TCR transgenic memory T cells after peptide activation in vitro for indicated periods. 10μg of total RNA was loaded per lane and equivalent sample loading was assured by hybridisation with a β-actin probe.

LGALS1 mRNA expression in mouse and human cell lines of lymphoid and nonlymphoid origin.

LGALS1 expression was analysed in a number of mouse and human T and B cell lines by Northern blotting. As shown in Fig. 4a, LGALS1 mRNA was expressed in all mouse cell lines of T-cell origin tested (Fig. 4a, lanes 1-7) including EL-4, RMA-S and BW 5147 lymphoma cells as well as cloned CTL lines (CTLL-2, Hy-Ad9) and T-cell hybridomas (BWLy2-3, IT H6/A11). In contrast, J558 and P3-X63Ag8 myeloma cells (lanes 8 and 9) and three B cell hybridomas tested (lanes 10-12) did not express LGALS1 mRNA at detectable level. These results agree well with the findings above, namely that high LGALS1 mRNA expression is a characteristic of activated T cells but not of B cells.

LGALS1 was also expressed in all nonlymphoid cell lines tested (Fig. 4a, lanes 13-16) including mouse fibrosarcoma MCA and MC57, mastocytoma P815 and melanoma B16 cell lines. This data is well in line with several reports describing the expression of galectin-1 (the homodimeric LGALS1 gene product) on various tumours and tumour cell lines (Skrincosky et al., 1993; Xu et al., 1995).

Analysis of LGALS1 expression in human cell lines did not show a clear segregation between T and B cells (Fig. 4b). LGALS1 was expressed in the cell lines of T cell origin MT-2, MT-4, CB15, KAD and CEM (Fig. 4b, lanes 3, 4, 7, 8 and 9), whereas it was not expressed in Molt-4 (lane 6) and Jurkat (lane 5), both cell lines of T cell origin as well. Furthermore, LGALS1 was expressed in the macrophage cell line U937 (lane 10) as well as in the carcinoma cell line HeLa (lane 11). LGALS1 expression levels varied considerably among the cell lines tested.

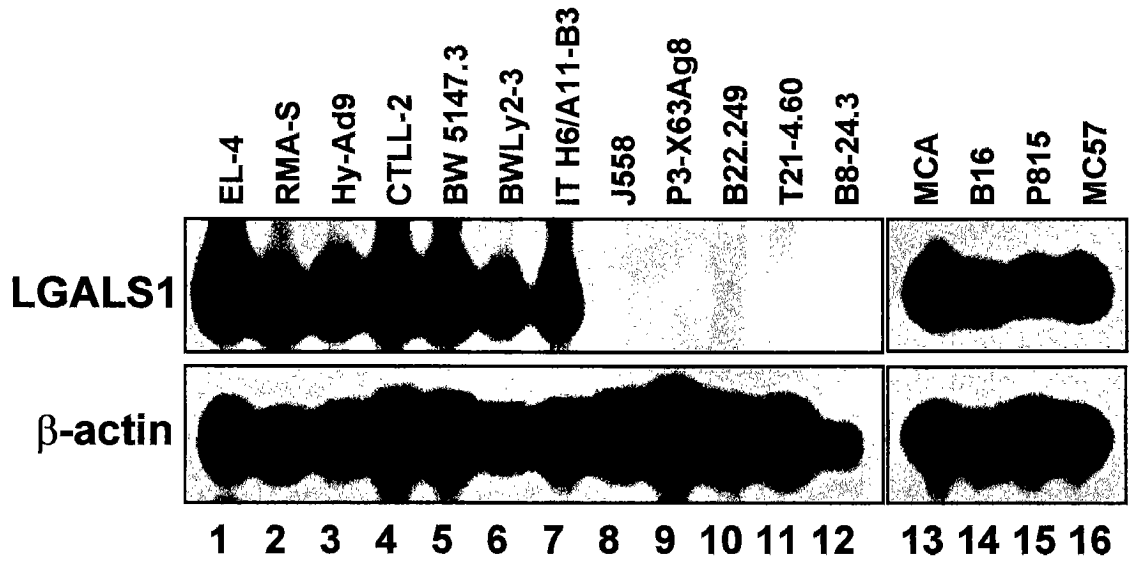


Figure 4a. **Northern blot analysis of LGALS1 expression in mouse cell lines.** 5μg of total RNA isolated from lymphoma (EL-4, RMA-S, BW 5147), cloned CTL (CTLL-2, Hy-Ad9), T cell hybridoma (BWLy2-3, IT H6/A11), myeloma (J558, P3-X63Ag8), B cell hybridoma (B22.249, T21-4.60, B8-24-3) and non-lymphoid cell lines (MCA, B16, P815, MC57) was loaded per lane. Equivalent sample loading was assured by hybridisation with a β-actin probe.

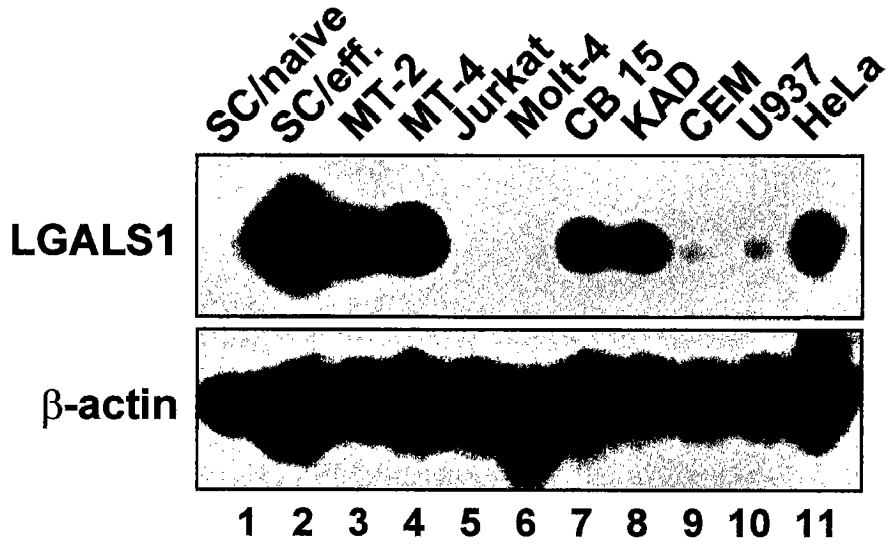


Figure 4b. **Northern blot analysis of LGALS1 expression in human cell lines.** Total RNA was isolated from T cell leukaemia (Jurkat, Molt-4, CEM), transformed T cell lines (MT-2, MT-4, CB 15, KAD), carcinoma- (HeLa) and macrophage (U937) cell lines. As control, RNA from naive (lane 1) and effector (lane 2) mouse T cells was loaded. The mouse LGALS1 probe does cross-hybridise with human LGALS1 RNA.

LGALS1 expression in thymocytes.

As LGALS1 was expressed in peripheral T cells after activation, its expression in mature and immature thymocytes was analysed. As depicted in Fig. 5, lane 3, thymocytes from naive C57BL/6 mice expressed the same level of LGALS1 RNA as spleen cells from day 8 LCMV-immune C57BL/6 mice (lane 2). To deplete double positive $CD4^+CD8^+$ thymocytes in vivo, mice were treated with Cortisone 2 days before thymus removal. As shown in lane 4, Cortisone-treated thymocytes expressed LGALS1 RNA at levels comparable to naive spleen cells, suggesting that the remaining single positive (SP) $CD8^+CD4^-$ and $CD8^-CD4^+$ and double negative (DN) $CD8^-CD4^-$ thymocytes did not express LGALS1. However, when $CD8^+CD4^-$, $CD8^-CD4^+$ SP and $CD8^-CD4^-$ DN thymocytes were analysed separately after positive and negative selection with Dynabeads in vitro, as described in Materials and Methods, all subpopulations expressed LGALS1 at levels comparable to effector T cells (lanes 6-8). It is not clear at the moment why different results emerged depending on the method to enrich SP and DN thymocytes. One explanation could be that Cortisone-treatment somehow influenced LGALS1 expression in thymocytes that are not killed by Cortisone. Also thymocytes from $TCR\alpha^{o/o}$ mice which lack $CD8^+CD4^-$ and $CD8^-CD4^+$ SP thymocytes do express LGALS1 (Fig. 5, lane 5). Thus, it seems likely that $CD8^+CD4^-$, $CD8^-CD4^+$ SP and $CD8^-CD4^-$ DN thymocytes express LGALS1 at levels comparable to effector T cells.

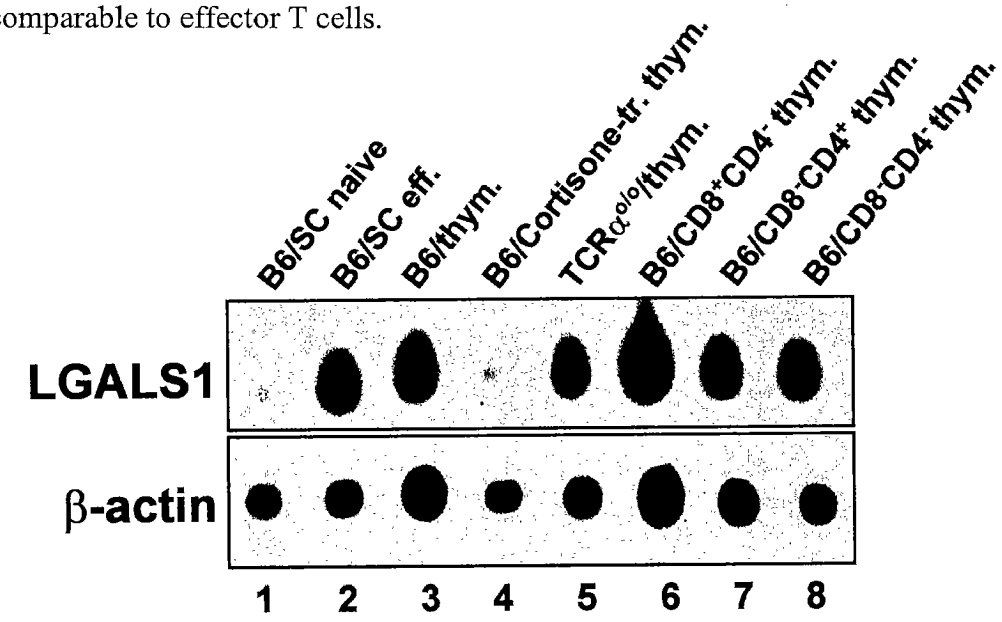


Figure 5. Northern blot analysis of LGALS1 expression in thymocytes. Total RNA was prepared form untreated thymi of C57BL/6 mice (lanes 3, 6, 7 and 8) or $TCR\alpha^{o/o}$ mice (lane 5) and from thymi of C57BL/6 mice treated with Cortisone in vivo (lane 4). $CD8^+CD4^-$ (lane 6), $CD8^-CD4^+$ (lane 7) and $CD8^-CD4^-$ (lane 8) thymocytes were purified by negative selection in vitro with Dynabeads as described in Materials and Methods. Hybridisation with a β-actin probe shows equivalent sample loading.

LGALS1 mRNA expression analysed by in situ hybridisation.

LGALS1 expression in the spleens of uninfected and LCMV-infected C57BL/6 mice at day 8 was analysed by in situ hybridisation using a radiolabelled LGALS1 antisense RNA probe. A strong signal was observed in LCMV-infected mice (Fig. 6 B) but not in the uninfected controls (Fig. 6 A). The clustered distribution of the silver grains is compatible with the idea, that activated splenocytes, presumably $CD8^+$ T cells, produced LGALS1 RNA. Most of the LGALS1 positive cells were localised in the red pulp. A similar pattern was found when the splenic sections were analysed by in situ hybridisation, using a radiolabelled perforin antisense RNA probe which specifically detects $CD8^+$ effector CTLs (Fig. 6 C, D). The signal obtained for LGALS1 was much stronger than the one for perforin.

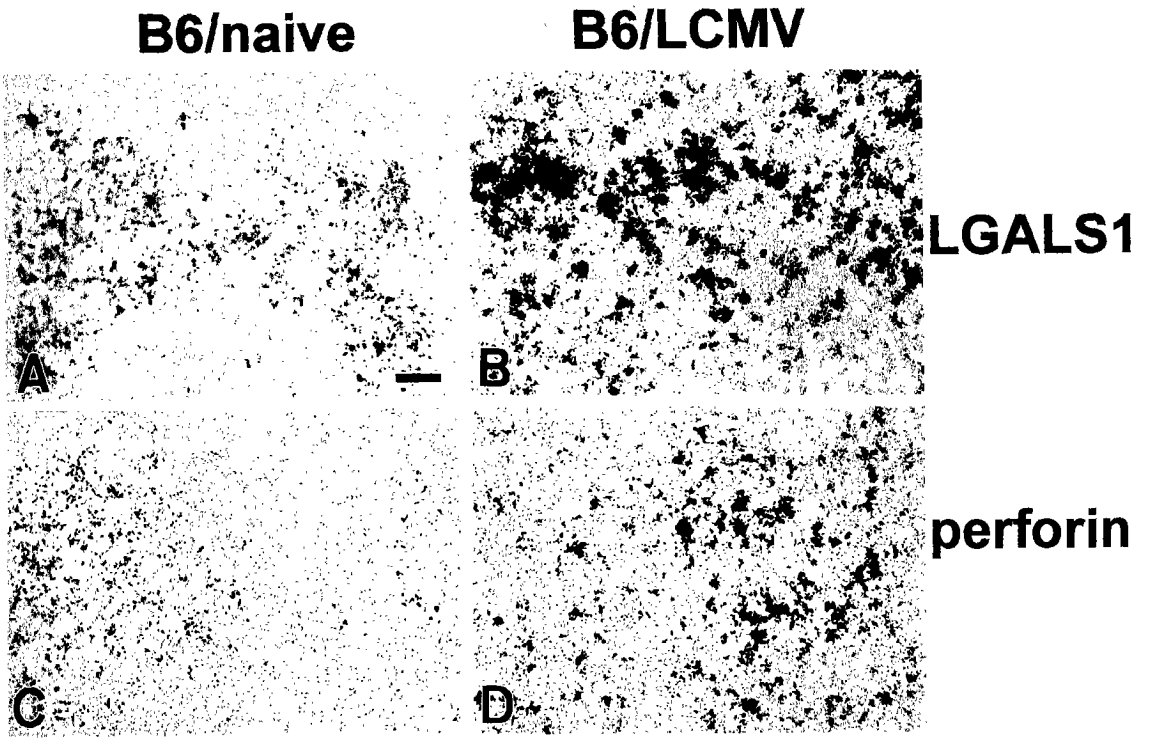


Figure 6. In situ hybridisation of LGALS1 and perforin mRNA. Spleen sections from uninfected C57BL/6 mice (A and C) and from day 8 LCMV-immune mice (B and D) were hybridised with a ^{35}S-labelled RNA antisense probe for the LGALS1 gene (A and B) or the perforin gene (C and D). Hybridisation was specific since labelling with corresponding LGALS1/perforin sense probe did not yield significant staining (not shown). Bar in A = 64μm.

βGBP protein release by $CD8^+$ T cells after peptide stimulation in vitro.

Supernatants of $CD8^+$ spleen cells from P14 TCR transgenic mice, which had been stimulated in vitro with the LCMV GP33 peptide for different time periods, were analysed by Western blotting. A marked expression of monomeric βGBP in its 15,000 Mr form and a feint expression of monomeric βGBP in its glycan linked 18,000 Mr form was found (Wells and Mallucci, 1992). No evidence of release of the protein in its dimeric galectin-1 form was found as examined by gelfiltration (data not shown). βGBP released in the cell free supernatant by activated $CD8^+$ T cells was detected on day 1 (Fig. 7, lane 2) with a further strong increase on day 2 (lane 3) and 3 (lane 4) after antigen stimulation. $CD8^+$ T cells which had been stimulated for only 4 hours did not show any detectable release of βGBP (lane 1). These results are in full accordance with the expression of LGALS1 mRNA after in vivo and in vitro T cell stimulation as shown in Fig. 3a and 3b. Densitometric analysis using recombinant βGBP as a standard revealed that the amount of βGBP released corresponded to 31.2ng per ml (2nM), a 7-8 fold increase over the basal level of the naive cells. It is noteworthy that this amount of βGBP secreted by the activated T cells is about 100 to 1000 fold higher than that constitutively released by mouse embryonic fibroblasts (L. Mallucci, personal communication).

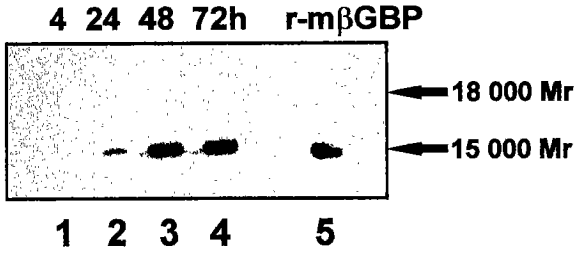


Figure 7. Western blot analysis of βGBP release. Spleen cells of P14 TCR transgenic mice were incubated with 10^{-6} M GP33 peptide in vitro and cell free supernatant was collected at 4h (lane 1), 1 day (lane 2), 2 days (lane 3) and 3 days (lane 4) after peptide stimulation. Supernatants were concentrated100-times by affinity chromatography. A 15,000 Mr and a 18,000 Mr band comigrate with the corresponding bands of recombinant murine βGBP (200ng, lane 5).

To assess whether neutralisation of secreted βGBP, using a βGBP neutralising antibody, influences the cytolytic activity of activated T cells, P14 TCR transgenic T cells were stimulated in vitro with the LCMV GP33 peptide in the presence or absence of 4.6μg/ml neutralising anti-βGBP antibody (clone B2). 2 and 3 days after stimulation, GP33-specific cytolytic activity was assessed in a ^{51}Cr-release assay using GP33-loaded EL-4 cells as target cells. Anti-βGBP antibody treatment did neither enhance nor reduce the cytolytic activity of GP33-specific T cells in comparison to control cell cultures (data not shown).

Recombinant βGBP (r-mβGBP) inhibits antigen-induced proliferation of naive and primed $CD8^+$ T cells.

To address the functional role of βGBP secretion by activated T cells, the growth inhibitory activity of βGBP on T cells was analysed. P14 TCR transgenic T cells were stimulated in vitro with the LCMV GP33 peptide in the presence of varying concentrations of recombinant βGBP (r-mβGBP) (Wells and Mallucci, 1991). As depicted in Fig. 8A, TCR transgenic T cells proliferated vigorously during in vitro culture with the cognate peptide antigen. The proliferative response of the TCR transgenic T cells was inhibited in a dose dependent manner after addition of r-mβGBP. Addition of 350ng/ml r-mβGBP (25nM) reduced the antigen-specific proliferation to 50% and 1400ng/ml r-mβGBP (100nM) effectively abolished proliferation.

To examine the role of apoptosis regulating genes, such as fas/CD95 and bcl-2, in the context of the growth inhibitory activity of r-mβGBP, spleen cells from Fas-deficient P14 TCR/lpr mice and from P14 TCR/bcl-2 doubly transgenic mice overexpressing the anti-apoptotic protein Bcl-2, were stimulated with LCMV GP33 peptide in vitro in the presence of r-mβGBP. As shown in Fig. 8 B and C, spleen cells from TCR/lpr and TCR/bcl-2 mice were equally susceptible to the growth inhibitory activity of r-mβGBP as the normal TCR transgenic spleen cells (Fig. 8 A). These results argue against a crucial role of these two genes in the βGBP-induced inhibitory signaling pathway.

Finally, it was tested whether βGBP also affected antigen-induced proliferation of antigen-experienced T cells. As shown in Fig. 8 D, r-mβGBP inhibited LCMV GP33 peptide-induced proliferation of a $CD8^+$ T cell line derived from P14 TCR transgenic mice in a dose dependent manner.

To rule out that addition of r-mβGBP to the cultures affected cell viability and early steps in T cell activation, flow cytometric analysis of several T cell activation markers was performed at various points in time after activation. As shown in Fig. 9, CD69 (A) and CD25 (IL-2Rα-chain) (C) upregulation as well as TCR downregulation (B) were identical after activation with GP33 in the presence or absence of r-mβGBP. In (C), T cells activated with GP33 only showed less $CD25^+$ cells due to variations in the cell preparations.

Taken together, this data demonstrated that nM amounts of r-mβGBP were able to block antigen-induced proliferation of naive and primed $CD8^+$ T cells and that the growth inhibitory activity of r-mβGBP was independent of Fas/CD95 and Bcl-2 expression. r-mβGBP did as well not affect early steps of T cell activation as up to 24 hours after addition of r-mβGBP treated T cells showed normal signs of activation.

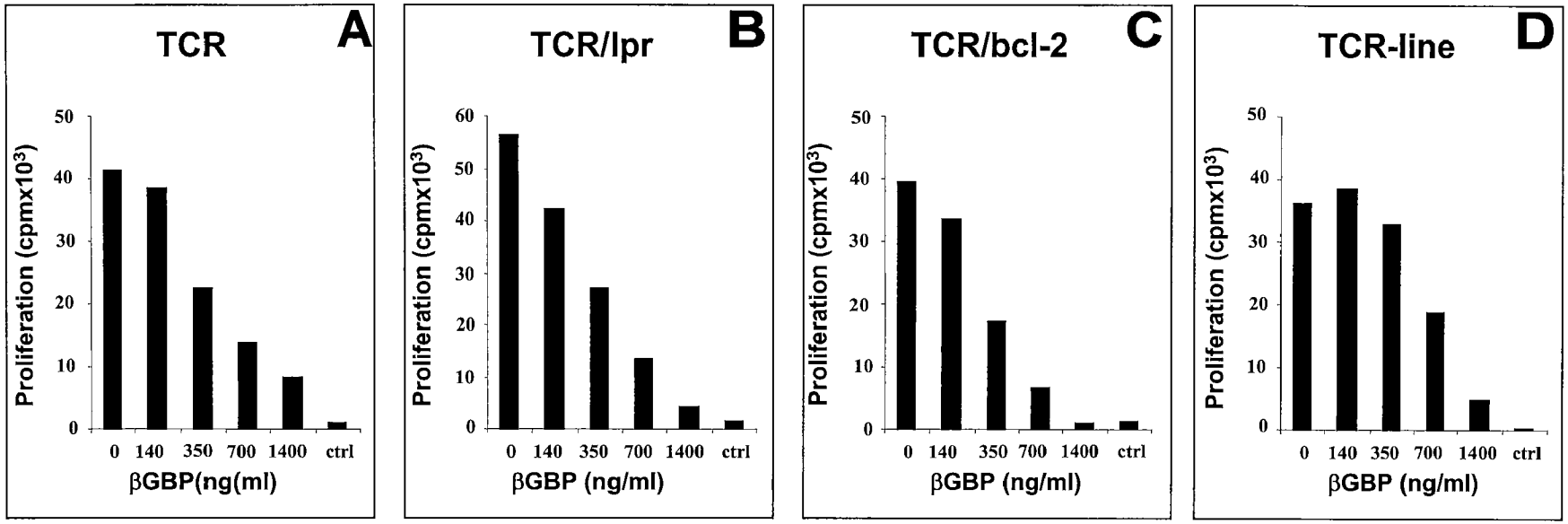


Figure 8. Inhibition of LCMV GP33 peptide-induced T cell proliferation by r-mβGBP. Spleen cells from P14 TCR (A), P14 TCR/lpr (B), P14 TCR/bcl-2 transgenic mice (C) and an antigen-experienced $CD8^+$ T cell line derived from P14 TCR transgenic mice (D) were stimulated with LCMV GP33 peptide (A-C) or GP33 peptide loaded C57BL/6 spleen cells (D) in the presence of the indicated concentrations of r-mβGBP. Proliferation was measured after 2 days by (^{3}H)-thymidine incorporation. As a control (ctrl), proliferation of cells without GP33 peptide and without r-mβGBP is shown. Shown is one representative experiment of two.

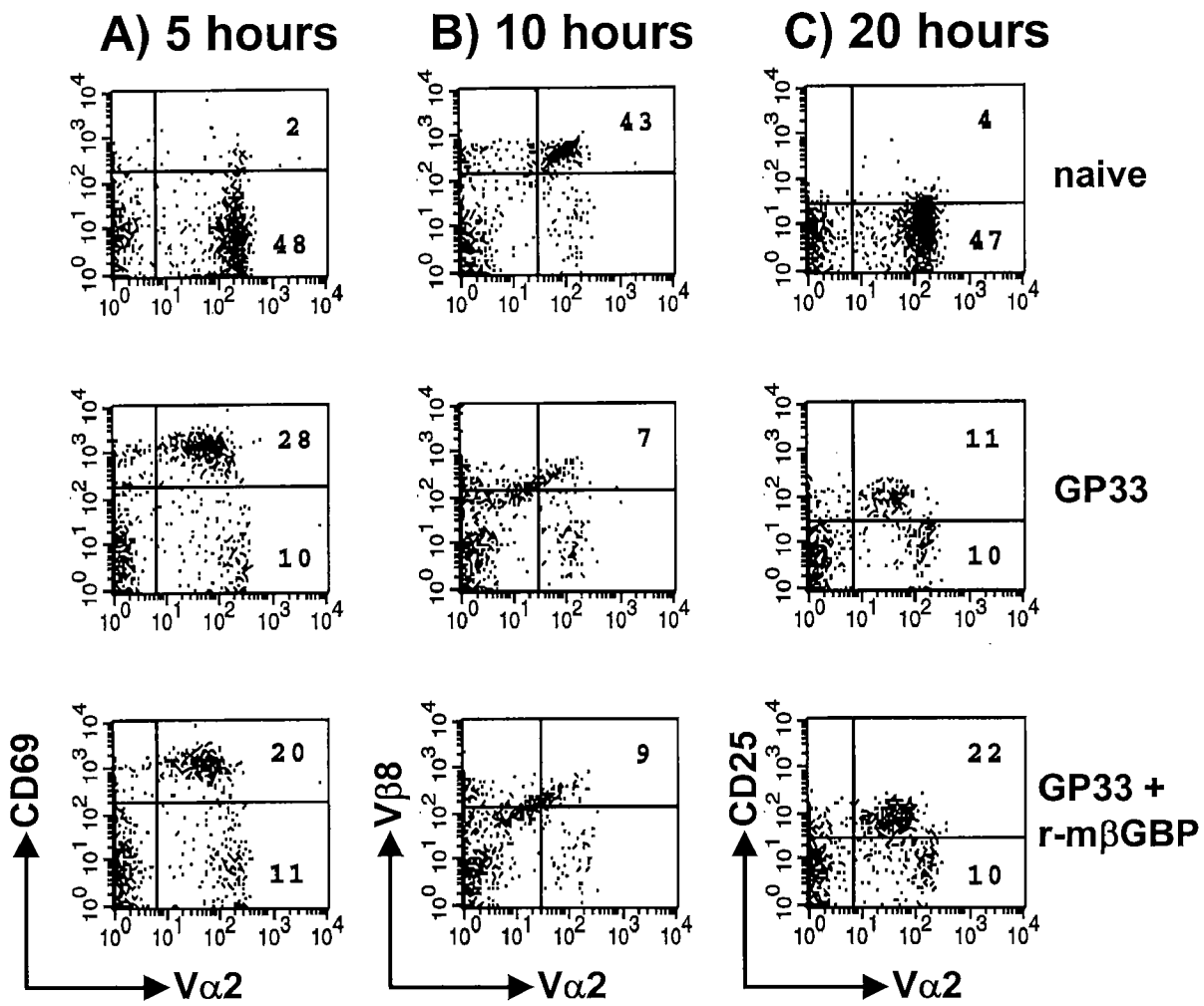


Figure 9. Surface phenotype of P14 TCR transgenic T cells activated in vitro with the LCMV peptide GP33 in the presence of r-mβGBP. Spleen cells from P14 TCR transgenic mice were cultured in vitro without antigen or with GP33 in the presence or absence of r-mβGBP. Cells were analysed 5, 10 and 20h after culture start by flow cytometry. Cells were stained after 5h with antibodies specific for CD8, Vα2 and CD69 (A), after 10 hours with anti-CD8, anti-Vα2 and anti-Vβ8 antibodies (B) and after 20h with anti-CD8, anti-Vα2 and anti-CD25 antibodies (C). The dot plots shown are of gated $CD8^+$ cells and the numbers in the quadrants indicate the percentage of $CD8^+$ cells.

Lect14$^{o/o}$ (=LGALS1 deficient) mice are indistinguishable from wild-type mice with respect to lymphocyte distribution and CTL activity after LCMV infection.

LGALS1 gene deficient mice have recently been generated to analyse the role of galectin-1 in the development of the olfactory system (Poirier and Robertson, 1993). They were termed Lect14$^{o/o}$ mice, were found anatomically indistinguishable from wild-type littermates and were fully viable and fertile. The availability of these mice (provided by F. Poirier) made it possible to analyse in vivo the effect of LGALS1 deficiency in the context of a viral infection.

First, the distribution pattern of different lymphocyte subsets in different organs was assessed by flow cytometric analysis of thymus, spleen, lymphnode and peripheral blood. In Fig. 10 the percentages of CD8$^+$ (A) and CD4$^+$ (B) T cells and B cells (C) in the indicated organs are summarised. No significant differences between wild-type mice (strain 129/SV) and Lect14$^{o/o}$ mice were obtained, suggesting that lymphocyte development and homeostasis in the periphery occurs normally.

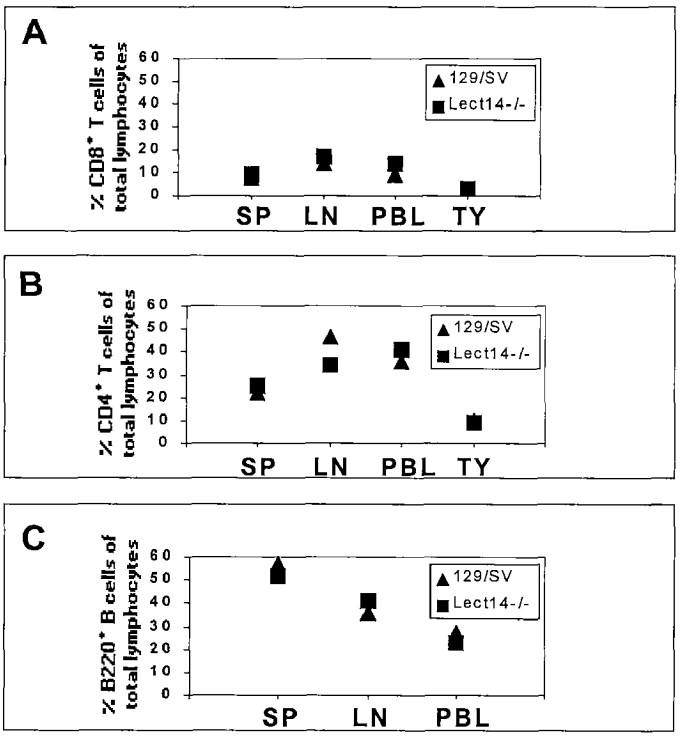


Figure 10. Cytofluorimetric analysis of thymus and secondary lymphatic organs of Lect14$^{o/o}$ mice and 129/SV littermates. Cells from spleen, lymphnode, blood and thymus were stained with antibodies specific for CD8, CD4 and B220. Shown is the percentage of CD8$^+$ (A) and CD4$^+$ (B) T cells and B220$^+$ B cells (C) in the indicated organs.

To examine whether the lack of βGBP in vivo alters the cytolytic activity of LCMV-specific T cells, Lect14$^{o/o}$ mice and 129/SV wild-type littermates were infected intravenously (i.v.) with 200 pfu LCMV-WE. Eight days after infection the cytolytic activity of LCMV-specific spleen cells was determined in a ^{51}Cr-release assay. All mice tested were able to mount strong GP33-specific CTL responses (data not shown). This data is in line with data where naturally produced βGBP was neutralised with an anti-βGBP antibody that did not influence the cytolytic activity of the activated T cells. This suggests that βGBP does not directly influence the cytolytic activity of CD8$^+$ T cells.

Visualisation of LCMV-specific $CD8^+$ T cells with tetrameric MHC-I-peptide complexes after LCMV infection in $Lect14^{o/o}$ mice.

To address whether the lack of βGBP has an effect on the proliferative capacity and kinetics of $CD8^+$ T cells after LCVM infection, $Lect14^{o/o}$ mice and 129/SV negative littermates were infected with 200 pfu LCMV i.v. and flow cytometry of peripheral blood lymphocytes (PBL) was then performed using anti-CD8 and anti-CD62L mAbs. Fig. 11a displays the percentage of $CD8^+$ T cells before and several weeks after infection. A small but reproducible increase of the $CD8^+$ T cell number in $Lect14^{o/o}$ mice (squares) in comparison to 129/SV littermates (triangles) was observed (Fig. 11a). Furthermore, the $CD8^+$ T cells in $Lect14^{o/o}$ mice displayed a more activated phenotype for several weeks after infection as measured by CD62L downregulation (Fig. 11b, squares).

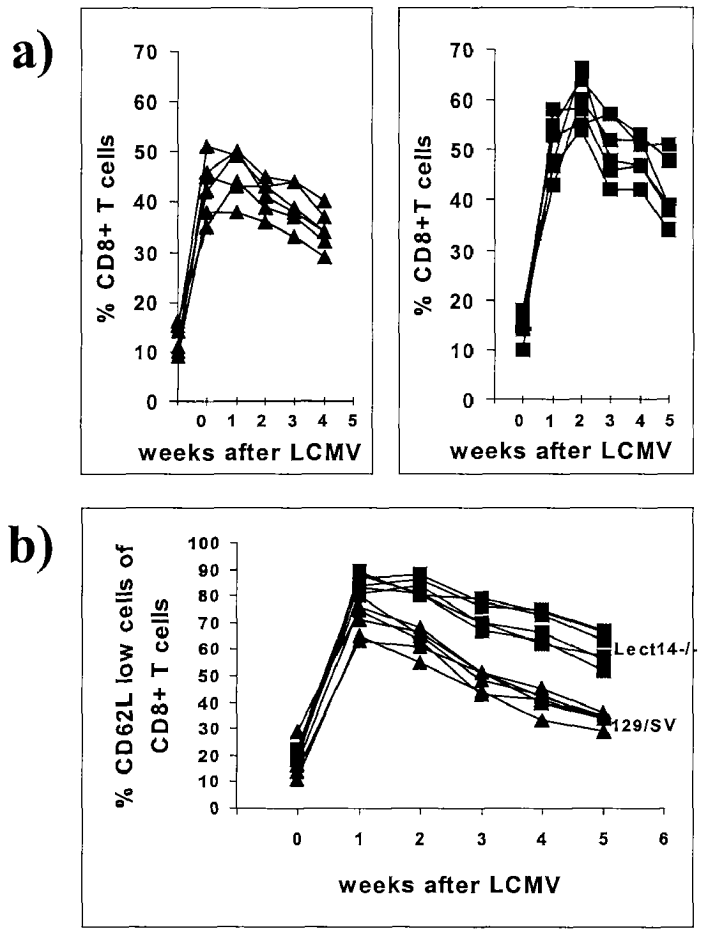


Figure 11. Kinetics of $CD8^+$ T cells in $Lect14^{o/o}$ mice and 129/SV negative littermates following LCMV infection with 200pfu i.v..
PBL were stained once a week after infection with antibodies specific for CD8 and CD62L.
In Fig. 11a, the percentage of $CD8^+$ T cells from total PBL is shown from 129/SV negative littermates (left, triangles) and $Lect14^{o/o}$ mice (right, squares) several weeks after infection. Week 0 indicates the percentage of $CD8^+$ T cells in uninfected mice.
In Fig. 11b, the percentage of CD62L low expressing cells is shown among the $CD8^+$ T cells. Each curve represents one individual mouse.

The recently developed technique of soluble peptide-MHC tetrameric complexes made it possible to follow the fate of virus-specific T cells in vivo (Altmann et al., 1996). To address the question of T cell homeostasis in Lect14$^{o/o}$ mice after LCMV-infection, PBL of LCMV-infected Lect14$^{o/o}$ mice or 129/SV negative littermates were analysed weekly by flow cytometry. PBL were stained with MHC-tetramers complexed to the peptides GP33-41 and NP 396-404 of the glyco- and nucleoprotein of LCMV (obtained from R. Ahmed).

As displayed in Fig. 12, the number of CD8$^+$ T cells (A), as well as of GP33-specific CD8$^+$ cells (B) was increased in Lect14$^{o/o}$ mice (squares) compared to 129/SV control mice (triangles). In contrast, the number of NP396-specific CD8$^+$ cells was similar in both mouse lines. It is possible that the two epitopes do not activate the T cells in the same way. It has recently been proposed that indeed the fate of T cells activated by GP33 or by NP396 can be differentially regulated (Zajac et al., 1998).

Taken together this data demonstrated that βGBP indeed had an inhibitory effect on activated CD8$^+$ T cells in vivo and that the lack of βGBP resulted in slightly increased numbers of antigen-specific CD8$^+$ T cells displaying an activated phenotype for several weeks after viral infection. That the animals did no show a lymphoproliferative disease in older age could have several reasons. It is likely that other compensatory mechanisms play a role or that even other members of the galectin family could take over some of the functions mediated by the LGALS1 gene product and enable a normal lymphocyte homeostasis in the animal.

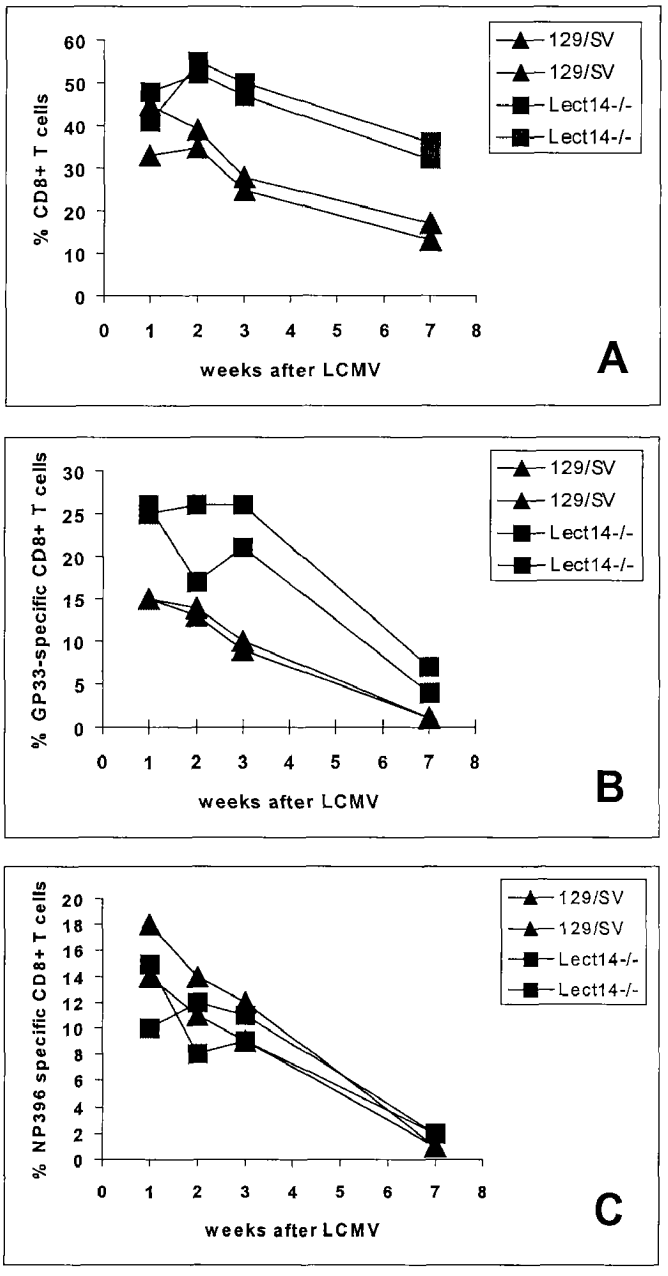


Figure 12. Kinetics of LCMV-specific $CD8^+$ T cells in $Lect14^{o/o}$ mice and 129/SV negative littermates following i.v. infection with 200 pfu LCMV. PBL were stained once a week with anti-CD8 mAb and tetrameric MHC complexes containing the GP33-41 peptide and the NP396-404 peptide from LCMV. The percentage of $CD8^+$ T cells (A), and of GP33- (B) and NP396-specific T cells (C) is shown. Each curve represents one individual mouse.

3. RESULTS II

VIRUS-ACTIVATED CD8$^+$ T CELLS AND LYMPHOKINE-ACTIVATED NK CELLS EXPRESS THE MAST CELL FUNCTION-ASSOCIATED ANTIGEN (MAFA), AN INHIBITORY C-TYPE LECTIN

Identification of the mouse MAFA (mMAFA).

mRNA differential display PCR was performed to identify genes induced in CD8$^+$ T cells upon activation and a PCR product which revealed significant sequence homology to the mast cell function-associated antigen (MAFA) in the rat (Guthmann et al., 1995) was isolated. Full-length cDNA was obtained by RT-PCR with mRNA from CD8$^+$ effector T cells generated in vivo using the adoptive transfer model and the full primary sequence was determined (both described in Materials and Methods). The primary nucleotide sequence was aligned with that of the rat MAFA (rMAFA) and sequence comparison revealed 86% homology of the mouse to the rat sequence (Fig. 1). This result suggested that the mouse homologue of the rMAFA gene has been isolated.

```
mouse    ATGGCTGACAGCTCTATCTATTCAACACTAGAGCTGCCGGAGGCACCTCAAGTCCAAGAT 60
rat      ATGGCCGACAACTCTATCTACTCAACATTAGAGCTGCCTGCTGCACCTCGAGTCCAAGAT

mouse    GAGTCCAGATGGAAGCTCAAAGCTGTCTTACACCGGCCCCATCTTTCCCGCTTTGCAATG 120
rat      GACTCCAGATGGAAGGTCAAAGCTGTCTTACACCGACCCTGTGTTTCCTACCTTGTGATG

mouse    GTGGCTTTGGGGCTTTTGACTGTGATTCTCATGAGTCTACTGATGTATCAACGGATCCTG 180
rat      GTGGCTTTGGGGCTTTTGACTGTGATTCTCATGAGTCTACTGTTGTACCAACGGACTCTG

mouse    TGCTGCGGCTCCAAGGACTCTACATGTTCCCACTGCCCCAGCTGCCCCATCCTCTGGACG 240
rat      TGCTGTGGCTCCAAGGGCTTTATGTGTTCCCAGTGCTCCAGGTGCCCCAACCTCTGGATG

mouse    AGGAATGGTAGCCACTGTTACTATTTTTCAATGGAGAAAAAGGACTGGAATTCTAGTCTG 300
rat      AGGAACGGGAGCCACTGTTACTACTTCTCAATGGAGAAAAGGGACTGGAACTCTAGTCTG

mouse    AAATTCTGTGCAGACAAAGGCTCACATCTCCTTACATTTCCGGACAACCAGGGAGTGAAG 360
rat      AAGTTCTGTGCAGACAAAGGCTCGCATCTCCTTACATTTCCGGACAACCAGGGAGTGAAC

mouse    CTGTTTGGAGAGTACCTGGGTCAGGACTTTTACTGGATCGGCTTGAGGAACATTGATGGC 420
rat      CTGTTCCAGGAGTATGTGGGCGAGGACTTTTACTGGATTGGCTTGAGGGACATCGATGGC

mouse    TGGAGGTGGGAAGGCGGCCCAGCGCTCAGCTTGAGGATTCTTACCAACAGCTTGATACAG 480
rat      TGGAGGTGGGAAGATGGCCCAGCTCTCAGCTTAAGCATTCTCTCTAACAGCGTGGTACAG

mouse    AGGTGCGGTGCCATTCACAGAAATGGCCTCCAAGCCTCCAGTTGTGAAGTTGCTTTGCAG 540
rat      AAGTGTGGCACCATCCACAGGTGTGGCCTCCACGCCTCCAGTTGTGAGGTTGCTTTGCAG

mouse    TGGATCTGTAAGAAGGTCCTATACTGA 567
rat      TGGATCTGTGAGAAGGTCCTGCCCTGA
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Figure 1. Nucleotide sequence alignment of mouse and rat MAFA coding sequence. Sequence numbering starts at the start codon ATG and stops at position 564 with the TGA stop codon. The full-length nucleotide sequences are available from EMBL GenBank under accession number AJ010751 (mMAFA) and X79812 (rMAFA).

```
       1                                  30
Mouse  MADSSIYSTL ELPEAPQVQD ESRWKLKAVL
Rat    ...N...... ...A..R... D....V....
Human  .T..V...M. ...T.T.A.N DYGPQQ.SSS

       31                                 60
Mouse  HRPHLSRFAM VALGLLTVIL MSLLMYQRIL
Rat    ...CV.YLV. .......... ....L...T.
Human  SK.SC.CLVA IT.....AV. L.V.L..W..

       61                                 90
Mouse  CCGSKDSTCS HCPSCPILWT RNGSHCYYFS
Rat    .....GFM.. Q.SR..N..M ..........
Human  .Q..NY...A S.....DR.M KY.N......

       91                                120
Mouse  MEKKDWNSSL KFCADKGSHL LTFPDNQGVK
Rat    ...R...... .......... .........N
Human  V.E....... E..LARD... .VIT...EMS

       121                               150
Mouse  LFGEYLGQDF YWIGLRNIDG WRWEGG-PAL
Rat    ..Q..V.E.. ......D... ....D.-...
Human  .LQVF.SEA. C......NS. ....D S.LN

       151                               180
Mouse  SLRILTNSLIQ RCGAIHRNGL QASSCEVAL
Rat    ..S..S..VV. K..T...C.. H........
Human  FS..SS..FV. T....NK... .......P.

       181    189
Mouse  QWICKKVLY
Rat    ....E...P
Human  HGV....RL
```

Figure 2. Amino-acid sequence alignment of mouse, rat and human MAFA. Sequence numbering starts with the N-terminus that is located in the cytoplasma. The putative immunoreceptor tyrosine-based inhibition motif (ITIM) is shown in bold type, the putative transmembrane region is underlined and the conserved motifs CYYF and WIGL are doubly underlined. The complete nucleotide sequences are available from the EMBL database under accession numbers AJ010751 for mMAFA, X79812 for rMAFA and AF034952 for hMAFA.

In Fig. 2, the comparison of deduced mouse, rat and human MAFA (hMAFA) amino acid sequences is displayed.

rMAFA has been identified as an inhibitory receptor on the rat mast cell line RBL-2H3 and belongs to the family of C-type lectins. Like rMAFA, mMAFA is predicted to be a type II transmembrane protein. Both share a very similar N-terminal cytoplasmic domain of 34 amino acids harbouring the consensus sequence **SIYSTL**. This motif is similar to that of the functional immunoreceptor tyrosine-based inhibition motif (ITIM) (V/IxYxxL/V) found in the cytoplasmic tail of inhibitory receptors expressed by natural killer cells and other lymphocytes. The motifs CYYF and WIGL in the 133 amino acid extracellular region are shared by many other lectin-like receptors on lymphocytes like CD94, CD69, NKG2A/B, NKR-P1 and Ly49 family members (Weis et al., 1998). The extracellular region includes a conserved carbohydrate recognition domain shared by many lectins. Recently the human MAFA has been cloned which showed similar characteristics and revealed 75% homology to the mouse MAFA (Lamers et al, 1998; Butcher et al, 1998). Like many other members of the C-type lectin family, the human MAFA is encoded in the NK cell gene complex.

mMAFA is encoded by a single gene.

To assess the copy number of the mMAFA gene, genomic DNA isolated from C57BL/6 kidneys was digested with several restriction enzymes and hybridised with a full length mMAFA cDNA probe. The resultant pattern suggested that mMAFA is a single copy gene in the mouse (Fig. 3). The presence of only a single band hybridising to the EcoRI-digested genomic DNA further strengthened this suggestion. The same conclusion has been drawn for the MAFA gene in the rat (Bocek et al., 1997), as well as for the mMAFA gene, which had been identified simultaneously and independently by another group (Hanke et al., 1998).

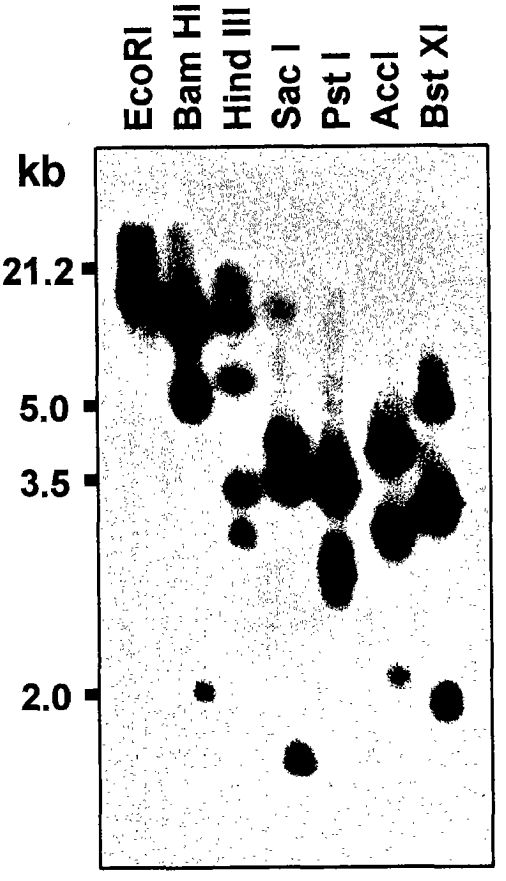

Figure 3. Southern blot analysis of the mouse MAFA gene. 10μg genomic DNA isolated from C57BL/6 mice was digested with the enzymes indicated and the blotted membrane was hybridised with the full length mMAFA cDNA. The DNA molecular size markers are indicated on the left.

mMAFA expression in different mouse tissues.

Total RNA was prepared from several organs and the presence of mMAFA RNA was evaluated by Northern blot analysis using the full length mMAFA cDNA as a probe. As shown in Fig. 4, lanes 3-12, mMAFA expression was not detectable in any of the tested organs including spleen, lymphnode and lung. The transcript obtained for $CD8^+$ effector cells (lane 2) was approximately 1.4 kb in size. In the rat, evidence has been presented for three different transcripts detected by RT-PCR (Bocek et al., 1997): a full length transcript spanning exon 1 to 5 and two smaller transcripts lacking exon 2 or exon 2 and 3, respectively. Northern blot analysis, however, was not sensitive enough to detect different transcripts in the mouse and only one signal was obtained for effector T cells (Fig. 4, lane 2). When highly sensitive RT-PCR of $CD8^+$ effector T cell cDNA was performed using primers specific for the mMAFA coding region, three different bands were obtained corresponding to the full length mMAFA cDNA: one with 567 bp and two smaller fragments with 398 bp and 293 bp lacking either exon 3 or exon 2 and 3 respectively (D. Vöhringer, unpublished). In man, other alternatively spliced variants have been reported, representing exon 3 or exon 3 and 4 negative transcripts (Lamers et al., 1998). The absence of mMAFA expression in different tissues suggests that mMAFA is not expressed significantly in resting lymphocytes nor in most non-hematopoietic cells.

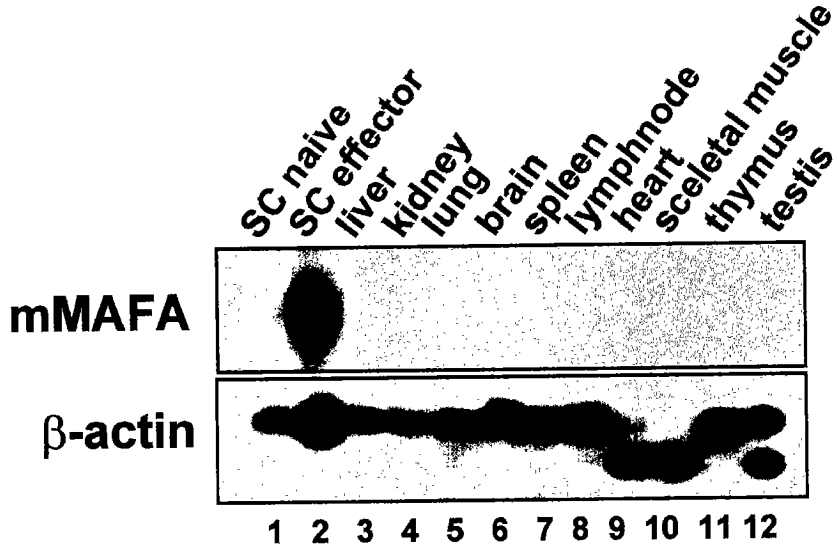

Figure 4. mMAFA expression in different mouse tissues analysed by Northern blotting. RNA was isolated from spleen cells from naive (lane 1) and day 8 LCMV-immune C57BL/6 mice (lane 2) and from the indicated tissues (lanes 3-12) of naive C57BL/6 mice. For each lane, 10μg of total RNA was loaded. Equivalent sample loading was assured by hybridisation with a β-actin probe. The smaller fragment obtained in heart, skeletal muscle and testis represents α-actin, the abundant actin-form in the muscle which does cross-hybridise with the β-actin probe.

mMAFA is induced in $CD8^+$ but not in $CD4^+$ T cells after LCMV infection.

Northern blot analysis using the mMAFA cDNA as a probe demonstrated that $CD8^+$ T cells from P14 TCR transgenic mice isolated on both day 8 and day 14 after adoptive transfer expressed high levels of mMAFA mRNA (Fig. 5a, lanes 2 and 3). In contrast, naive $CD8^+$ T cells from these mice did not express mMAFA at detectable levels (Fig. 5a, lane 1). In $CD8^+$ memory T cells mMAFA gene expression was still detectable 3-4 weeks after infection, but to a lower extent (Fig. 5a, lanes 4 and 5). Importantly, mMAFA gene expression was also induced in $CD8^+$ effector T cells from normal C57BL/6 mice isolated 8 days after LCMV infection (Fig. 5b, lane 2). To test whether mMAFA is exclusively expressed by $CD8^+$ effector T cells, spleen cells from C57BL/6 mice taken on day 8 after LCMV infection were depleted of $CD8^+$ T cells by negative selection with Dynabeads in vitro. As shown in Fig. 5b, lane 3, $CD8^+$ depleted LCMV-immune spleen cells did not express mMAFA at significant levels indicating that mMAFA was virtually exclusively expressed in activated $CD8^+$ T cells. To directly compare mMAFA expression in activated $CD4^+$ and $CD8^+$ T cells, effector T cells were generated using SMARTA and P14 TCR transgenic mice which both express an antigen receptor specific for the LCMV glycoprotein (GP). T cells from P14 TCR transgenic mice are specific for LCMV GP aa33-41 in the context of the MHC molecule $H\text{-}2D^b$ (Pircher et al., 1989) whereas SMARTA TCR transgenic mice express an antigen receptor specific for LCMV GP aa61-80 together with the MHC molecule $I\text{-}A^b$ (Oxenius et al., 1998). $CD4^+$ and $CD8^+$ T cells from both TCR transgenic lines were activated in vivo using an adoptive transfer system as described in Materials and Methods, which allows the same vigorous expansion of the transferred transgenic T cells (Zimmermann et al., 1996a; Oxenius et al., 1998). As shown in Fig. 5b, lane 5, $CD4^+$ effector cells from SMARTA TCR transgenic mice expressed mMAFA at low levels when compared to effector cells from P14 TCR transgenic mice (lane 4). Thus, these results indicated that mMAFA is exclusively expressed in $CD8^+$ effector T cells.

a)

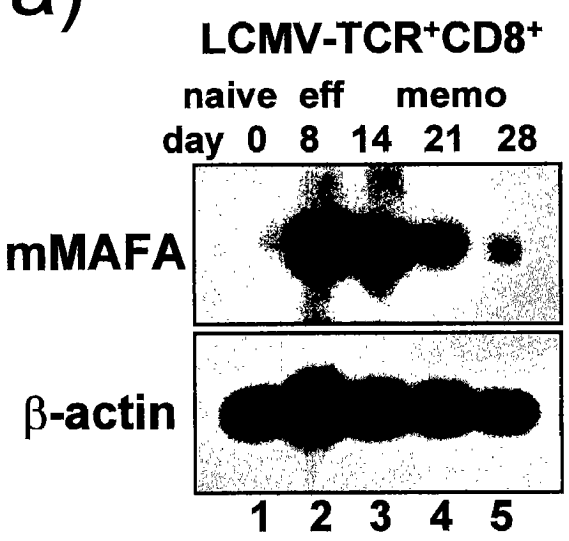

Figure 5a. Northern blot analysis of mMAFA expression in CD8+ T cells from P14 TCR transgenic mice isolated at the indicated points in time after LCMV infection. Lane 1: CD8+ T cells from naive P14 TCR transgenic mice. Lanes 2-5: Effector/memory CD8+ TCR transgenic T cells isolated on day 8 (lane 2), day 14 (lane 3), day 21 (lane 4) and day 28 (lane 5) after adoptive transfer into C57BL/6 recipient mice. Mice were infected with 200 pfu LCMV i.v..

b)

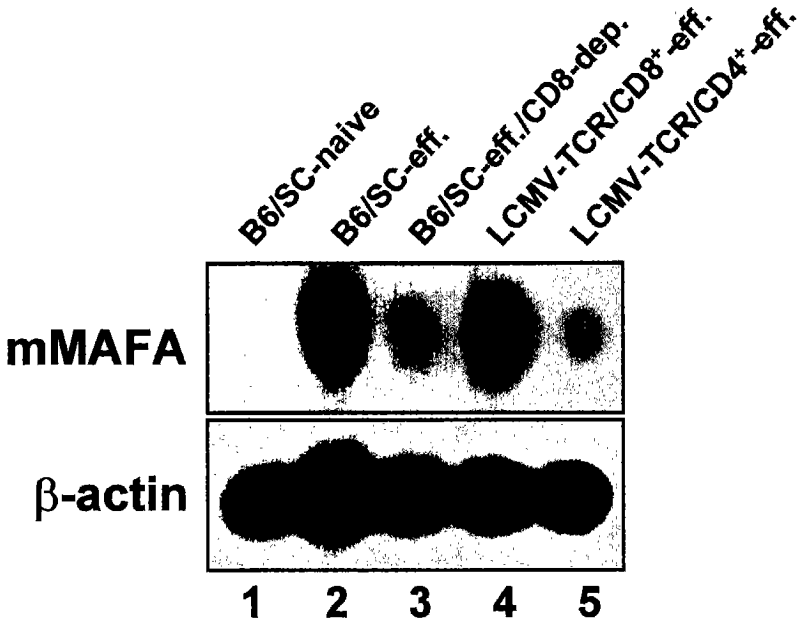

Figure 5b. Northern blot analysis of mMAFA expression in C57BL/6 spleen cells and in SMARTA and P14 TCR transgenic T cells after LCMV infection. Lane 1: Spleen cells (SC) from naive C57BL/6 mice. Lane 2: SC from day 8 LCMV-immune C57BL/6 mice. Lane 3: CD8+ T cell-depleted SC from day 8 LCMV-immune C57BL/6 mice. Lane 4: CD8+ P14 TCR transgenic effector cells isolated 8 days after adoptive transfer into C57BL/6 recipient mice. Lane 5: CD4+ SMARTA TCR transgenic effector cells isolated 8 days after adoptive transfer into C57BL/6 recipient mice. For each lane 10 μg of total RNA was loaded. Equivalent sample loading was assured by hybridisation with a β-actin probe.

The availability of the anti-mouse MAFA monoclonal antibody 2F1 (obtained from T. Hanke) enabled the analysis of mMAFA surface expression on $CD8^+$ T cells by flow cytometry. $CD8^+$ effector T cells where generated using an adoptive transfer system with P14 TCR transgenic T cells that are Thy1.1 positive. 10^5 of these P14 TCR-Thy1.1 transgenic cells were transferred in C57BL/6 recipient mice that carry the Thy1.2 allele. Both transfer systems, whether transferring $Thy1.2^+$ P14 TCR transgenic T cells into $Thy1.1^+$ recipients as it was done for all before mentioned experiments, or the other way around, give rise to the same progeny of effector and memory T cells. Spleen cells and peripheral blood lymphocytes (PBL) were analysed 8 days (effector) or 4 weeks and 8 months (memory) after LCMV-infection. Representative data obtained for PBL are depicted in Fig. 6a. A small proportion of $mMAFA^+$ T cells was detected in mice that had not undergone deliberate immunisation (A). These T cells were most likely activated by endogenous stimuli. After LCMV-infection, a segregation of mMAFA positive (40%) and mMAFA negative (15%) $Thy1.1^+$ cells could be observed (B). A population of $Thy1.1^-$ $mMAFA^+$ cells represented activated, endogenous $Thy1.2^+$ T cells. Four weeks after LCMV-infection 50% of the transferred virus-specific $CD8^+$ cells were still mMAFA positive (C), but mMAFA expression clearly declined with time and 8 months after LCMV-infection (D) mMAFA expression was comparable to that observed in naive T cells (A).

Taken together, this data demonstrated, that mMAFA was highly expressed on the surface of about 70% of LCMV-specific $CD8^+$ effector T cells 8 days after infection and that mMAFA expression declined with time, reaching levels comparable to that observed in naive $CD8^+$ T cells. It is noteworthy, that the expression data obtained by Northern blot analysis (Fig. 5a) could be confirmed by flow cytometry. Yet, 4 weeks after infection mMAFA expression on RNA level seemed to be lower than on protein level.

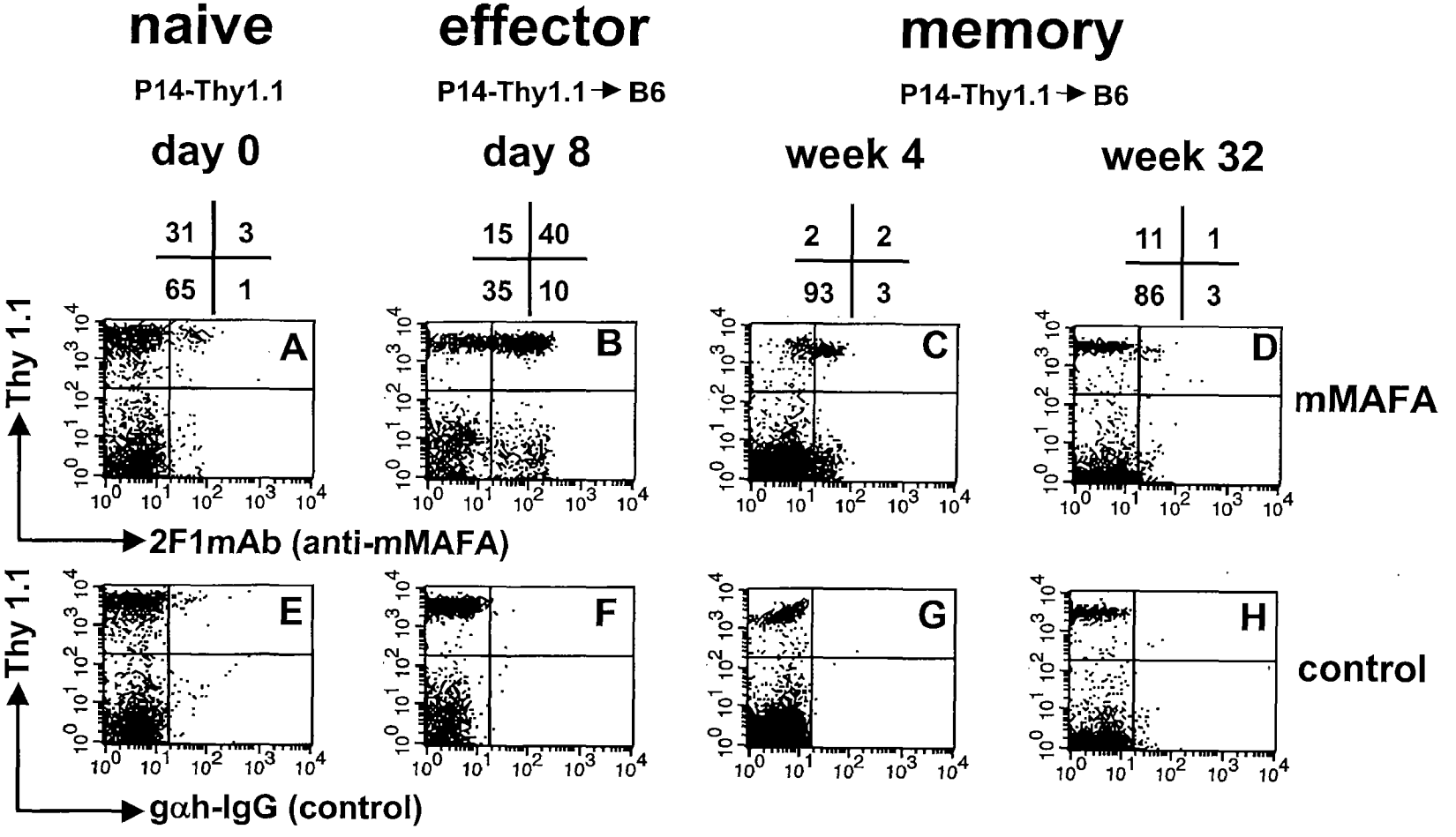

Figure 6a. **Flow cytometric detection of mMAFA expressing CD8⁺ T cells after LCMV infection.** PBL of naive P14 TCR-Thy1.1 mice (A and E) and of C57BL/6 recipient mice after adoptive transfer of 10^5 P14 TCR transgenic T cells 8 days (B and F), 4 weeks (C and G) or 8 months (D and H) after LCMV infection were stained with antibodies specific for Thy1.1 and mMAFA. Plots E-H show control stainings of cells stained with goat anti-hamster IgG only. The numbers in the quadrants above the dot plots indicate the percentage of the corresponding cell population of total lymphocytes for plots A-D. Identical results were obtained when spleen cells were analysed (data not shown). Note that in A and E, CD4⁺ and CD8⁺ cells are stained by Thy1.1 antibody, whereas in B, C, D, F, G and H only expanded TCR transgenic CD8⁺ T cells are stained. Due to two different transfer experiments, the percentage of Thy1.1⁺ cells is lower in C than in D.

Similar to mMAFA, other cell surface molecules are also only expressed after T cell activation. Mac-1 (CD11b) for example is expressed on virus-specific CTLs only after LCMV-infection in vivo and was correlated with cell activation (McFarland et al., 1992; Zimmermann et al., 1996a). Mac-1 (CD11b) is a member of the β2-integrin family of adhesion molecules, which include LFA-1 (CD11a), Mac-1 (CD11b) and p150.95 (CD11c). Surface expression of Mac-1 together with mMAFA on naive and activated $CD8^+$ T cells was analysed by flow cytometry. As depicted in Fig. 6b (C) 76% of the $Mac\text{-}1^+/CD8^+$ T cells were also mMAFA positive 8 days after LCMV-infection. Similar results were obtained for adoptively transferred P14 TCR transgenic T cells 8 days after LCMV-infection where 80% of the $Mac\text{-}1^+/CD8^+$ T cells were $mMAFA^+$ (B). Three smaller populations were detectable, representing $CD8^+/Mac1^+/mMAFA^-$, $CD8^+/Mac\text{-}1^-/mMAFA^+$ and $CD8^+/Mac\text{-}1^-/mMAFA^-$ cells. Only very few naive T cells did express mMAFA or Mac-1 (A). Thus, the majority of the mMAFA expressing $CD8^+$ T cells coexpressed Mac-1.

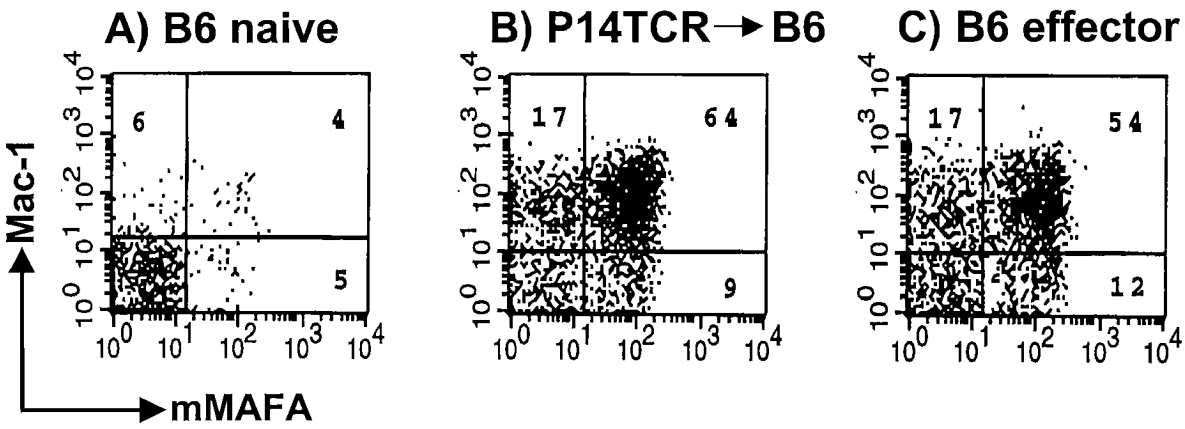

Figure 6b. Coexpression of mMAFA and Mac-1 (CD11b) on the surface of activated $CD8^+$ T cells measured by flow cytometry. PBL were stained with antibodies specific for CD8, Mac-1 (CD11b) and mMAFA. The dot plots show expression of mMAFA and Mac-1 gated for $CD8^+$ T cells. PBL were isolated from naive C57BL/6 mice (A), from day 8 LCMV-immune C57BL/6 mice (C) and from C57BL/6 recipient mice 8 days after adoptive transfer of P14 TCR transgenic T cells (B). The numbers in the quadrants indicate the percentage of the corresponding cell population of $CD8^+$ T cells. Similar results were obtained when spleen cells were analysed (data not shown).

Induction of mMAFA expression in $CD8^+$ T cells is not restricted to LCMV infection but requires in vivo priming with virus.

To examine whether viruses other than LCMV were also capable of inducing mMAFA expression, spleen cells from C57BL/6 mice acutely infected with LCMV, vaccinia virus (VV) or vesicular stomatitis virus (VSV) were examined. Fig. 7a shows that mMAFA expression levels were comparable in splenocytes from mice infected with LCMV, VSV or VV. It is noteworthy that mMAFA expression was lower in splenocytes from mice infected with a high dose (10^6 pfu) of LCMV-DOCILE, a virus strain known to induce clonal exhaustion of $CD8^+$ effector T cells (Moskophidis et al., 1993). To test if after infection with VV mMAFA is exclusively expressed in $CD8^+$ effector T cells, spleen cells from C57BL/6 mice isolated on day 6 after VV infection were depleted of $CD8^+$ T cells, $CD4^+$ T cells or B cells by negative selection with Dynabeads. As shown in Fig. 7b, mMAFA expression after VV infection was restricted to $CD8^+$ T cells as spleen cells depleted of $CD8^+$ T cells alone (lane 2) or of $CD4^+$ and $CD8^+$ T cells (lane 4) did only express mMAFA at levels comparable to naive T cells (Fig. 7a, lane 1). In contrast, spleen cells depleted of $CD4^+$ T cells (lane 3) or B cells (lane 5) still expressed significant amounts of mMAFA. Thus, after infection with VV which is not causing such a massive expansion of $CD8^+$ T cells as LCMV, mMAFA was also exclusively expressed in $CD8^+$ T cells.

To test whether peptide-antigen stimulation in vitro could induce mMAFA expression, $CD8^+$ T cells from P14 TCR transgenic mice were stimulated with LCMV-infected APCs. Antigen stimulation of $CD8^+$ T cells from P14 TCR transgenic mice in vitro is known to lead to vigorous proliferation and induction of CTL activity (Kyburz et al., 1993). However, these in vitro conditions were not sufficient to induce significant mMAFA expression (Fig. 7c, lanes 2-5). Only after repeated restimulation and prolonged in vitro culture (4-5 weeks) low level mMAFA expression became apparent in these cultures (Fig. 7c, lane 6). In striking contrast, high levels of mMAFA expression were induced in memory $CD8^+$ T cells within 3 days of in vitro restimulation (Fig. 7c, lane 7) - these memory $CD8^+$ T cells had been primed in vivo with LCMV.
Peptide-antigen stimulation in vivo also failed to induce mMAFA expression, as $CD8^+$ T cells isolated from P14 TCR transgenic mice that had been treated once with 500µg of the LCMV peptide GP33 i.p. did not express mMAFA at significant levels 48 hours after peptide application (data not shown).

a)

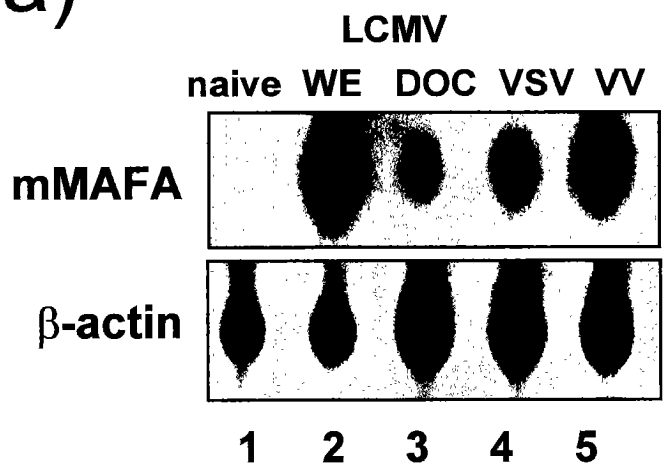

Figure 7a. Northern blot analysis of mMAFA expression in the spleen of C57BL/6 mice after infection with different viruses. Lane 1: SC from naive C57BL/6 mice. Lane 2: SC from C57BL/6 mice infected i.v. with 200 pfu LCMV-WE 8 days after infection. Lane 3: SC from C57BL/6 mice infected i.v. with 10^6 pfu LCMV-DOCILE 8 days after infection. Lane 4: SC from day 6 VSV-immune mice infected with $2x10^6$ pfu VSV i.v.. Lane 5: SC from day 6 VV-immune mice infected with $2x10^6$ pfu VV i.v..

b)

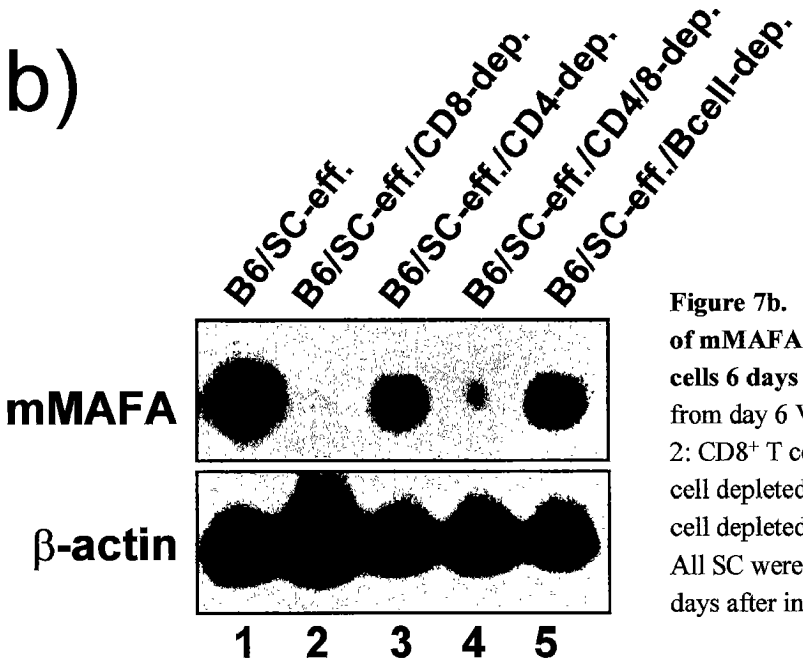

Figure 7b. Northern blot analysis of mMAFA expression in C57BL/6 spleen cells 6 days after VV infection. Lane 1: SC from day 6 VV-immune C57BL/6 mice. Lane 2: $CD8^+$ T cell depleted SC. Lane 3: $CD4^+$ T cell depleted SC. Lane 4: $CD4^+$ and $CD8^+$ T cell depleted SC. Lane 5: B cell depleted SC. All SC were isolated from C57BL/6 mice 6 days after infection with $2x10^6$ pfu VV.

c)

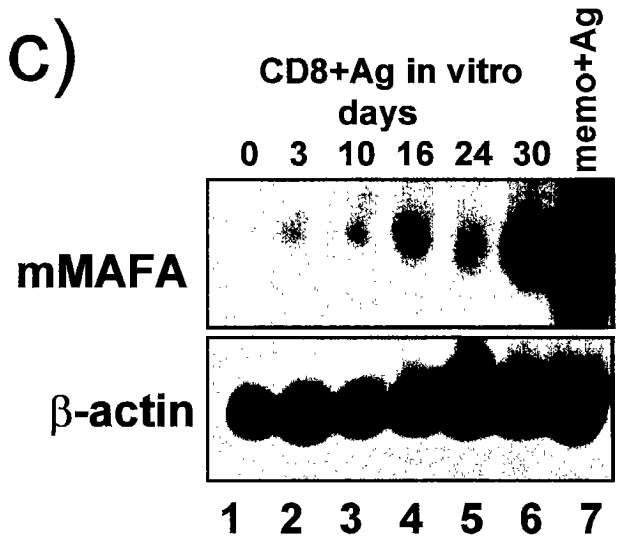

Figure 7c. Northern blot analysis of mMAFA expression in $CD8^+$ T cells from P14 TCR transgenic mice stimulated in vitro. Naive $CD8^+$ T cells from P14 TCR transgenic mice (lane 1) were stimulated once a week with LCMV-infected macrophages in vitro and $CD8^+$ T cells were harvested from the culture on the days indicated (lanes 2-6). Lane 7: $CD8^+$ T cells from LCMV-immune (memo) mice were restimulated for 3 days in vitro with LCMV-infected macrophages. For each lane 10 μg of total RNA was loaded; hybridisation with a β-actin probe shows constant sample loading.

mMAFA expression occurs in NK cells and in the Hy-Ad9 T cell line but not in mast cells.

mMAFA expression was analysed in a number of T and B cell lines by Northern blotting. As depicted in Fig. 8a, mMAFA was not expressed in EL-4, RMA-S, BW 5147 lymphoma cells or in $CD4^+$ and $CD8^+$ T-cell hybridomas (BWLy2-3, IT H6/A11). Similarly, J558 and P3-X63Ag8 myeloma cells and B cell hybridomas did not express mMAFA. However, the CTL clone Hy-Ad9 (Fig. 8a, lane 3) which exhibits NK-like activity (Acha-Orbea et al., 1983) expressed mMAFA RNA as well as protein (not shown) at levels comparable to $CD8^+$ effector T cells. mMAFA expression was therefore examined in lymphokine activated $NK1.1^+$ killer cells (LAK). As shown in Fig. 8b, lane 3, lymphokine activated $NK1.1^+$ cells (95% purity), generated by culturing T and B cell depleted spleen cells in the presence of IL-2, expressed high levels of mMAFA mRNA.

The MAFA gene was originally identified in the rat mucosal mast cell line RBL-2H3 and MAFA gene expression in the rat has been reported to be mast cell specific (Bocek et al., 1997). Surprisingly, mMAFA gene expression could not be observed in IL-3/IL-4 induced bone marrow-derived mast cell cultures, which are thought to represent an in vitro analogue of mucosal mast cells (Huels et al., 1995) (Fig. 8c lane 2). In addition, stimulation of these cells with Ionomycine did not lead to mMAFA gene expression (Fig. 8c, lane 3). This data is well in line with a very recent, independent report about mMAFA expression on NK cells where even highly sensitive RT-PCR failed to detect mMAFA in murine BMMC (Hanke et al., 1998). However, rat MAFA was detected on lung-derived mast cells (Bocek et al., 1997) and human MAFA cDNA could be obtained from mast cell-enriched human lung cells (Lamers et al., 1998). This suggests that lung-derived mast cells might have a slightly different phenotype than bone-marrow-derived mast cells or that the situation in the mouse is different from that in rat and human.

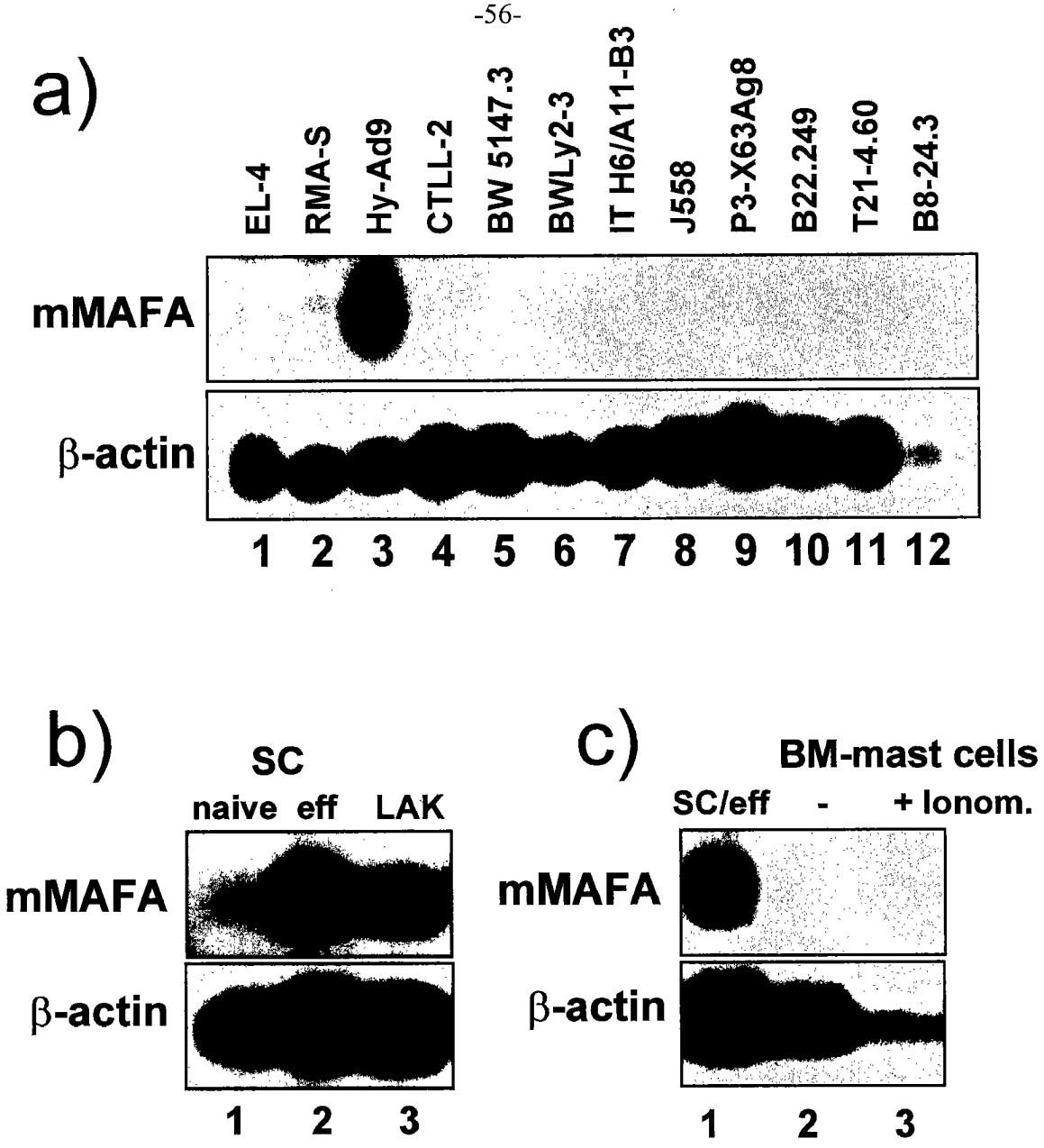

Figure 8. Northern blot analysis of mMAFA expression in different cell types.

a) Expression of mMAFA in lymphomas (EL-4, RMA-S, BW 5147), cloned CTLs (CTLL-2, Hy-Ad9), T cell hybridomas (BWLy2-3, IT H6/A11), myelomas (J558, P3-X63Ag8) and B cell hybridomas (B22.249, T21-4.60, B8-24-3).

b) mMAFA expression in lymphokine-activated $NK1.1^+$ cells (LAK). Lane 1: Spleen cells (SC) from naive C57BL/6 mice. Lane 2: SC from day 8 LCMV-immune C57BL/6 mice. Lane 3: NK 1.1^+ lymphokine-activated killer cells (LAK) from C57BL/6 mice.

c) mMAFA expression in bone marrow derived mast cells (BM-mast cells). Lane 1: Spleen cells (SC) from day 8 LCMV-immune C57BL/6 mice. Lane 2: BM-mast cell culture isolated from C57BL/6 mice. Lane 3: Ionomycine-treated BM-mast cell culture. For each lane 10 μg of total RNA was loaded; constant sample loading was controlled by hybridisation with a β-actin probe.

mMAFA expression in mice carrying different null mutations.

The striking contrast of mMAFA expression by in vivo versus in vitro activated $CD8^+$ T cells encouraged investigation of mMAFA expression after LCMV infection in several mouse strains lacking specific effector molecules. pko (perforin deficient), $Tnfr1^{o/o}$ (TNF receptor 1 deficient), $A_\beta^{o/o}$ (MHC class II deficient), $IFN\gamma R^{o/o}$ (IFN-γ receptor deficient), $IFN\gamma^{o/o}$ (IFN-γ deficient) and IL-12 $p40^{o/o}$ (IL-12 p40 deficient) mice including wild-type C57BL/6 and 129/SV controls, were infected with 200 pfu LCMV-WE. Eight days after infection spleens were harvested, total RNA was isolated and Northern blot analysis was performed. Representative data are summarised in Table 1. As described before, mMAFA was highly expressed in day 8 LCMV-immune C57BL/6 spleen cells. The same was observed for day 8 LCMV-immune 129/SV spleen cells that served as wild-type controls for $IFN\gamma R^{o/o}$ mice. mMAFA expression in pko, $Tnfr1^{o/o}$ and $A_\beta^{o/o}$ was slightly reduced compared to that of C57BL/6 mice whereas IL-12 $p40^{o/o}$ mice expressed mMAFA at the same level. Surprisingly, spleen cells from both $IFN\gamma^{o/o}$ and $IFN\gamma R^{o/o}$ mice were negative for mMAFA expression 8 days after infection. The $CD8^+$ T cell number 8 days after infection was normal in $IFN\gamma^{o/o}$ mice (30% of total lymphocytes) whereas in $IFN\gamma R^{o/o}$ only 3% $CD8^+$ T cells could be detected. Despite this very low $CD8^+$ T cell number, the killing activity of these remaining $CD8^+$ T cells was comparable to that of normal C57BL/6 or 129/SV effector T cells (data not shown). In our facilities, $IFN\gamma R^{o/o}$ as well as $IFN\gamma^{o/o}$ mice both died at day 12 to 15 after LCMV-infection probably due to immunopathology. The lack of IFN-γ or its receptor may lead to a change in virus-kinetics and may facilitate virus spread resulting in immunopathology.

To investigate whether the lack of IFN-γ and its receptor or the different virus-kinetics resulted in diminished mMAFA expression, spleen cells of $IFN\gamma R^{o/o}$ and $IFN\gamma^{o/o}$ mice were isolated on day 12 after infection and restimulated in vitro with LCMV-infected peritoneal macrophages as APCs. To exclude the presence of IFN-γ or its receptor in the cultures peritoneal macrophages from $IFN\gamma R^{o/o}$ or $IFN\gamma^{o/o}$ mice were used as APCs. As shown in Table 1b, in vitro restimulation resulted in strong mMAFA expression in the indicated spleen cells ruling out that mMAFA expression is directly controlled by interaction of IFN-γ with its receptor. However, it is difficult to understand why spleen cells of $IFN\gamma^{o/o}$ and $IFN\gamma R^{o/o}$ mice did not express mMAFA after LCMV-infection in vivo. One explanation could be that the T cells are so extremely activated in reaction to the high virus load in the periphery that they shut down the expression of several genes, including mMAFA, to be able to produce large quantities of perforin, granzymes and cytokines necessary for their effector function.

Taken together, this data demonstrated that expression of mMAFA was not directly controlled by the activity of IL-12, IFN-γ and TNF and that the lack of MHC-II

molecules also did not influence mMAFA expression. Furthermore the ability to display strong cytolytic activity seems not to be the only prerequisite for mMAFA expression.

Table 1a. mMAFA expression by day 8 LCMV-immune spleen cells (SC) of mice carrying different null mutations.

Mouse strain	mMAFA expression in SC 8 days after LCMV infection
C57BL/6	+++
129/SV	+++
pko	++
$Tnfr1^{o/o}$	++
$A_{\beta}^{o/o}$	++
$IFN\gamma R^{o/o}$	-
$IFN\gamma^{o/o}$	-
IL-12 $p40^{o/o}$	+++

Table 1b. mMAFA expression by $CD8^+$ T cells isolated from day 12 LCMV-immune C57BL/6, 129/SV, $IFN\gamma^{o/o}$ and $IFN\gamma R^{o/o}$ after antigen-restimulation in vitro.

Mouse strain	mMAFA expression in $CD8^+$ T cells after antigen-restimulation in vitro
C57BL/6	+++
129/SV	+++
$IFN\gamma R^{o/o}$	+++
$IFN\gamma^{o/o}$	+++

Table 1. Northern blot analysis of mMAFA expression in spleen cells of mice carrying different null mutations after LCMV infection in vivo.

Table 1a. The indicated mice were infected with 200 pfu LCMV-WE i.v., spleen cells were isolated 8 days after infection and total RNA was isolated.

For Table 1b, spleen cells of the indicated mice were isolated 12 days after LCMV infection and kept in vitro for 4 additional days in IMDM containing 10% FCS and 20U/ml IL-2. Cells were restimulated in the presence of LCMV-infected peritoneal macrophages as APCs derived from C57BL/6, 129/SV, $IFN\gamma^{o/o}$ or $IFN\gamma R^{o/o}$ mice and RNA was isolated. mMAFA expression was strong in all restimulated cultures independent of the APCs used. "+++" indicates the maximal mMAFA expression obtained for day 8 LCMV-immune C57BL/6 spleen cells.

mMAFA expression by $CD8^+$ T cells after infection with a high dose of LCMV.

The data obtained for $IFN\gamma R^{o/o}$ and $IFN\gamma^{o/o}$ mice (Table 1) indicated that T cell activation leading to normal cytolytic activity did not necessarily induce mMAFA expression in $CD8^+$ T cells. This implied that the T cells need to be in a very specific status to enable them to express mMAFA. To further characterise the parameters that may induce mMAFA expression, P14 TCR transgenic mice were infected either with 200 pfu (low-dose) or with 10^6 pfu (high-dose) LCMV-WE. After a high-dose virus infection, a strong transgenic CTL response is starting on day 3 after infection (Moskophidis et al., 1992). Spleen cells were analysed by flow cytometry 4 days (high-dose) or 8 days (low-dose) after infection (Fig. 9). Spleen cells from naive P14 TCR transgenic mice (A) and from C57BL/6 mice, 8 days after adoptive transfer of 10^5 P14 TCR transgenic T cells followed by infection with 200 pfu (low-dose) LCMV (B) are shown as a control (see also Fig. 6a). P14 TCR transgenic mice infected with a low-dose of LCMV eliminated virus in the spleen very quickly and were able to mount LCMV-specific CTL activities 8 days after infection (data not shown). Comparable to spleen cells from naive mice (A), mMAFA was expressed only by very few cells in these mice (C). It was not determined in this experiment whether the TCR transgenic T cells or the endogenous $CD8^+$ T cells expressed mMAFA and exhibited cytolytic activity. After infection of P14 TCR transgenic mice with a high-dose of virus, the $CD8^+$ T cells did not express mMAFA at all (D) despite a considerable expansion of the $CD8^+$ T cell population. Similar results were obtained when $5x10^5$ P14 TCR transgenic spleen cells (5 times more than for the normal transfer protocol) were transferred into C57BL/6 recipients that received a high-dose of virus (10^6 pfu) (E). Spleen cells of these mice exhibited strong cytolytic activity 4 days after infection and the transferred TCR transgenic spleen cells underwent extensive proliferation (500-fold). Surprisingly, also these virus-specific CTLs did not express any mMAFA. The same results were obtained by analysing spleen cells of the above-mentioned mice by Northern blot analysis (data not shown).

This experiment made clear that extensive proliferation and high cytolytic activities, two hallmarks of successful activation, did not necessarily induce mMAFA expression in $CD8^+$ effector T cells.

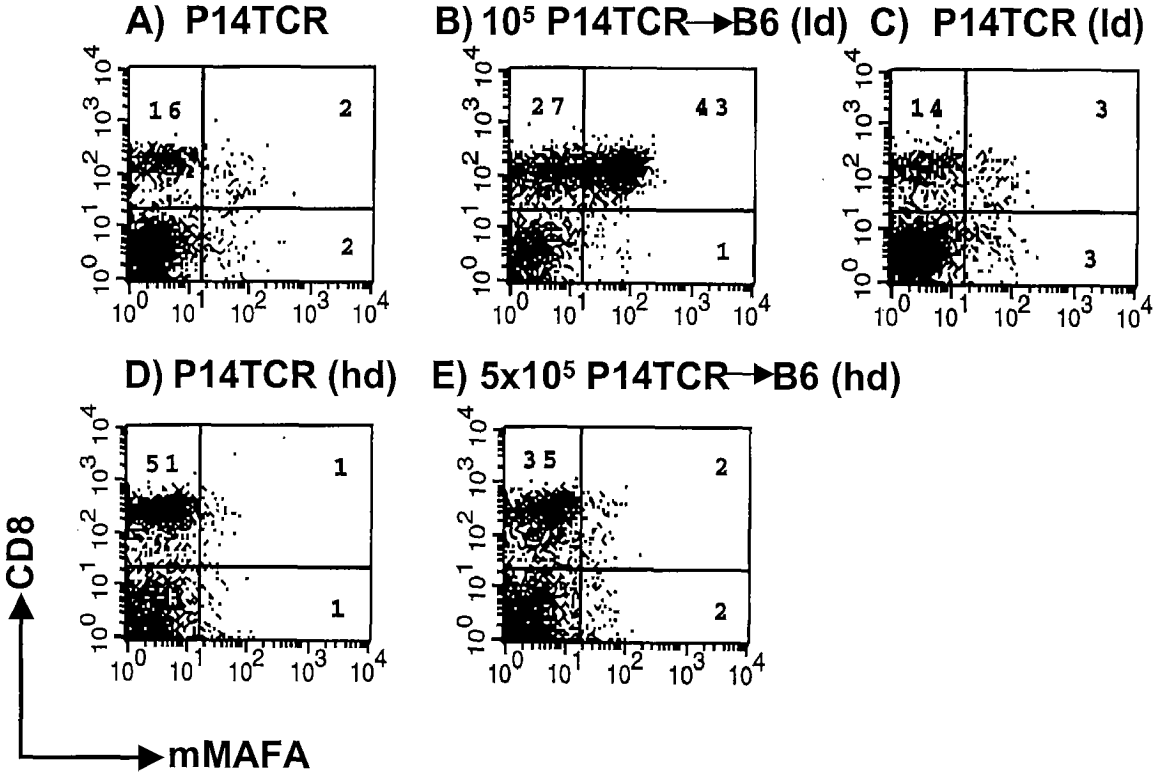

Figure 9. Flow cytometric detection of mMAFA expressing T cells after infection with a high-dose and a low-dose of LCMV. Spleen cells from naive P14 TCR transgenic mice (A), from C57BL/6 recipient mice after adoptive transfer of 10^5 P14 TCR transgenic T cells (B) and from P14 TCR transgenic mice (C) were analysed 8 days after i.v. infection of the animals with 200 pfu of LCMV (low-dose (ld)). Spleen cells from P14 TCR transgenic mice (D) and C57BL/6 recipient mice after adoptive transfer of $5x10^5$ P14 TCR transgenic T cells (E) were analysed 4 days after i.v. infection of the animals with 10^6 pfu LCMV (high-dose (hd)) . Spleen cells were stained with antibodies specific for CD8 and mMAFA. The numbers in the quadrants indicate the percentage of the corresponding cell population of total lymphocytes. After adoptive transfer of TCR transgenic T cells into C57BL/6 recipient mice (B and E), 90% of the $CD8^+$ T cells were TCR $V\alpha2/V\beta8^+$ (not shown).

rMAFA expression by rat spleen cells after infection with a high dose of vaccinia virus (VV).

As described by the group of I. Pecht, rMAFA expression in vivo was restricted to lung-derived mast cells and was not detected by RT-PCR in any other organ (Bocek et al., 1997). The failure to detect rat MAFA expression by other cells than lung-derived mast cells could be due to the fact that virus-activated rat T cells were never analysed.

It was described that acute infection of adult Lewis rats with a high dose (10^7 pfu) of VV into the footpad led to primary footpad swelling, increased numbers of lymphocytes in the corresponding lymphnodes and the ability of these lymphocytes to lyse virus-infected target cells (Zinkernagel et al., 1977). Furthermore, after peripheral infection of rats with 10^7 pfu VV, ex vivo cytolytic activities can be detected in the spleen (L. Stitz, Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen, Germany, personal communication). Therefore, rMAFA expression by $CD8^+$ rat spleen cells after i.v. infection with a high-dose of VV was determined. Fig. 10a summarises data obtained by flow cytometry for rat spleen cells stained with specific antibodies for CD8, CD62L (L-Selectin) and rMAFA (mAb G63, obtained from I. Pecht). Spleens from naive rats contained about 15% $CD8^+$ T cells and most of them were $CD62L^{hi}$ (A). After infection with VV the $CD8^+$ T cells did not expand but some of them became $CD62L^{lo}$, a sign for cell activation (C). $CD8^+$ spleen cells of naive and infected rats both did not express rMAFA on their surface as analysed by the anti-rat MAFA antibody G63 (B and D).

To confirm this data obtained by flow cytometry, Northern blot analysis of rat spleen cells before and after VV infection was performed. As shown in Fig. 10b, spleen cells from VV infected rats did express low levels of rMAFA (lanes 4 and 5) whereas no rMAFA expression could be detected in spleen cells from naive rats (lanes 2 and 3). Compared to rMAFA expression in RBL-2H3 cells (lane 1), rat spleen cells expressed very low amounts of rMAFA. The low level of rMAFA mRNA expression could be explained by the fact that VV infection did not induce extensive proliferation and activation of $CD8^+$ T cells. The amount of rMAFA expression detected by Northern blot analysis was probably too low to be detected by flow cytometry. The $CD8^-mMAFA^+$ population represents B cells that are stained by the goat-anti-mouse IgG antibody.

This experiment demonstrated that rMAFA expression is not restricted to lung-derived mast cells as it was reported before but that also rat spleen cells induced by a viral infection in vivo express rMAFA.

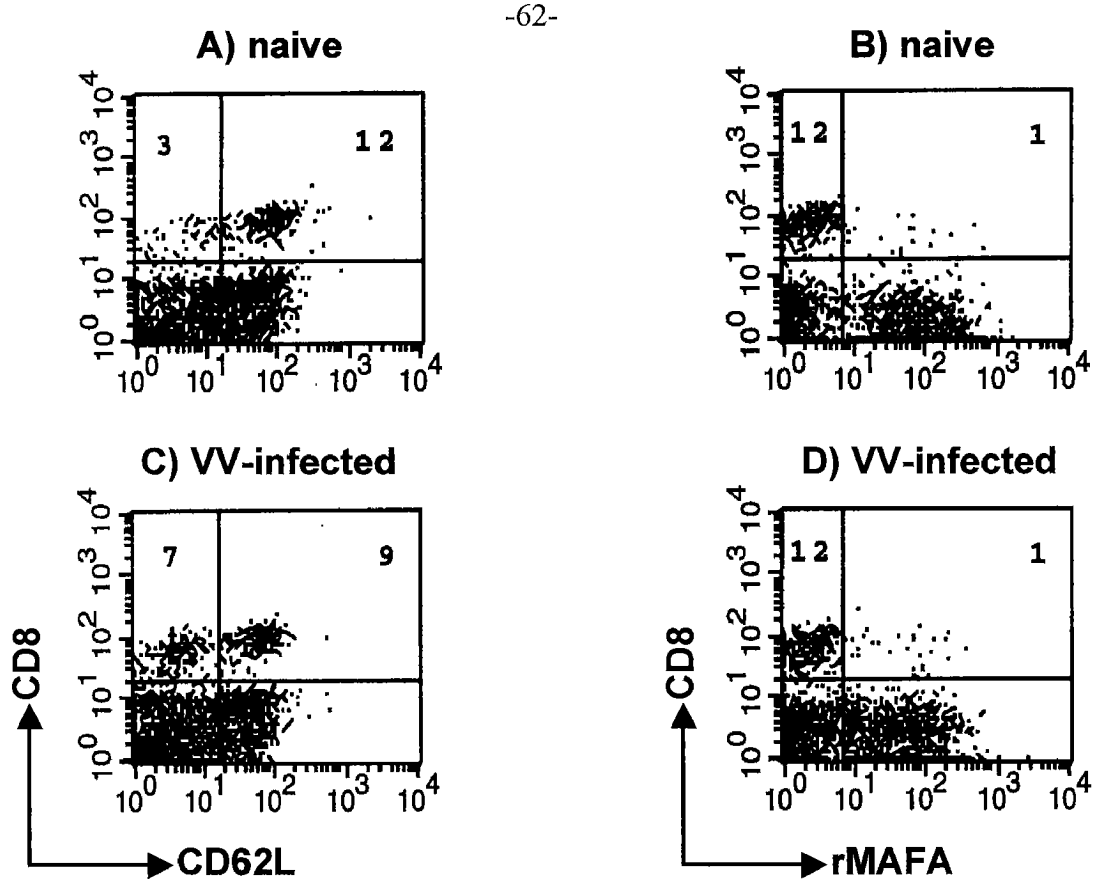

Figure 10a. Cytofluorimetric analysis of rat spleen cells after i.v. infection of Lewis rats with 10^7 pfu VV. Spleen cells of naive and day 6 VV-immune rats were stained with antibodies specific for CD8, CD62L (L-Selectin) and rMAFA (mAb G63). (A) and (B) represent data obtained for naive rat spleen cells whereas in (C) and (D) data for spleen cells from VV-infected rats are displayed. The numbers in the quadrants indicate the percentage of the corresponding cell population of total lymphocytes.

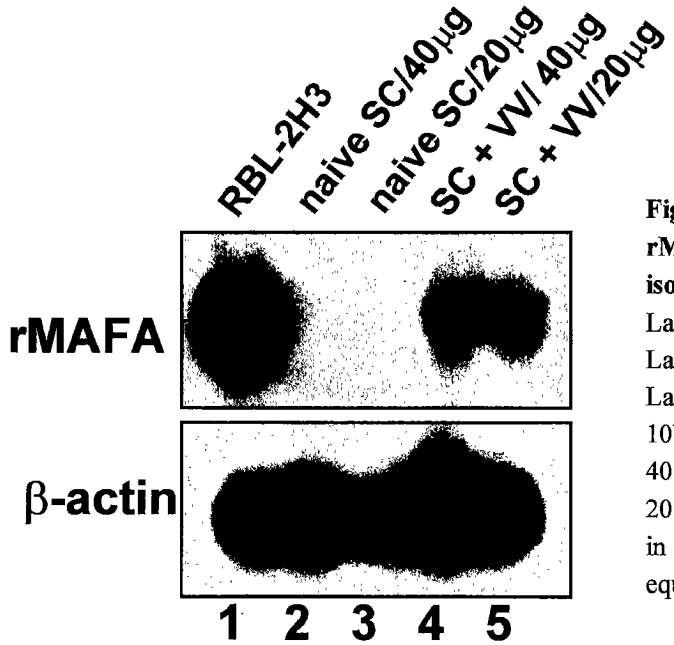

Figure 10b. Northern blot analysis of rMAFA expression in rat spleen cells (SC) isolated 6 days after VV infection
Lane 1: RBL-2H3 rat mast cell line.
Lanes 2 and 3: SC from naive Lewis rats.
Lanes 4 and 5: SC from rats infected i.v. with 10^7 pfu VV 6 days after infection.
40µg total RNA is loaded in lanes 2 and 4, 20µg is loaded in lanes 3 and 5 and 10µg in lane 1. Hybridisation with a β-actin probe shows equivalent sample loading.

Generation of a chimeric mouse-rat MAFA molecule to study the functional activity of the cytoplasmic tail of mMAFA.

For rMAFA it has been reported that clustering by monoclonal antibodies induces a dose-dependent inhibition of the RBL-2H3 mast cell lines response to immunological stimuli provided by the type 1 Fcε receptor (Fcε RI) (Soto and Pecht, 1988). This effect was mediated by a decrease in the cytoplasmic concentration of free Ca^{2+} ions, known to be one of the biochemical signals involved in the secretion of inflammatory mediators in this mast cell line. So far it is not clear whether this inhibitory effect is mediated by the immunoreceptor tyrosine-based inhibition motif (ITIM) in the cytoplasmic tail of rMAFA. However, in analogy to other inhibitory receptors like the low affinity FcγRIIB (Fong et al., 1996) a similar scenario could be envisaged for the rMAFA.

The question, whether the ITIM in the cytoplasmic tail of the mouse MAFA would be able to exert inhibitory activity in T cells was addressed with the following experimental approach. Due to the lack of anti-mMAFA antibodies at the time when the experiments were carried out, a chimeric molecule with the cytoplasmic tail of the mMAFA and the extracellular domain of the rMAFA was constructed. The schematic organisation of the chimeric mouse-rat MAFA is shown in Fig. 11.

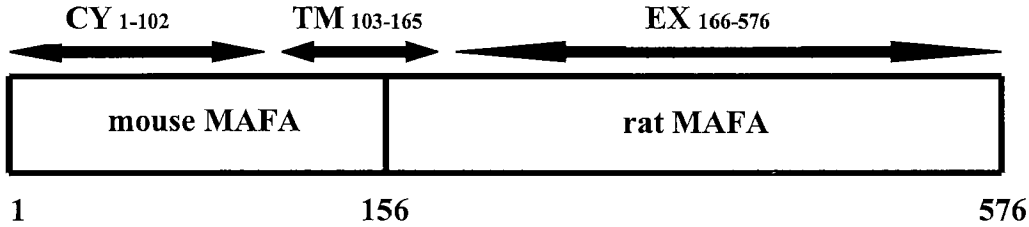

Figure 11. Schematic organisation of the chimeric mouse-rat MAFA. Mouse and rat sequences were obtained from full length cDNA by PCR with specific primers. Numbering starts with the start codon ATG from the mouse sequence. The first 156 bp from the mMAFA sequence containing the cytoplasmic domain as well as the major part of the transmembrane domain were combined with the extracellular domain of rMAFA (bp 166-576) and a small part of the transmembrane domain (bp 157-165). PCR products were cloned, sequenced and the chimeric molecule was inserted after several cloning steps into the retroviral vector pLXSN.

This chimeric mouse-rat MAFA construct was introduced into the T cell line A5 by retroviral transfection using the retroviral vector pLXSN. The T cell line A5 was used because it expresses the green fluorescent protein (GFP) under the NF-AT promoter (kindly provided by E. Palmer and K. Karjaleinen). Activation of these cells by TCR-

mediated crosslinking leads to GFP expression, which provides a convenient readout to follow the activation state of the cells.

Successfully transfected A5 cells were designated A5MAFA and were analysed by flow cytometry for MAFA surface expression using anti-rat MAFA mAb G63. A histogram displaying MAFA surface expression of A5MAFA cells is depicted in Fig. 12 A. A5MAFA cells were expressing the chimeric mouse-rat MAFA (A, thick line) at levels comparable to the expression of rMAFA by the mast cell line RBL-2H3 (B, thick line).

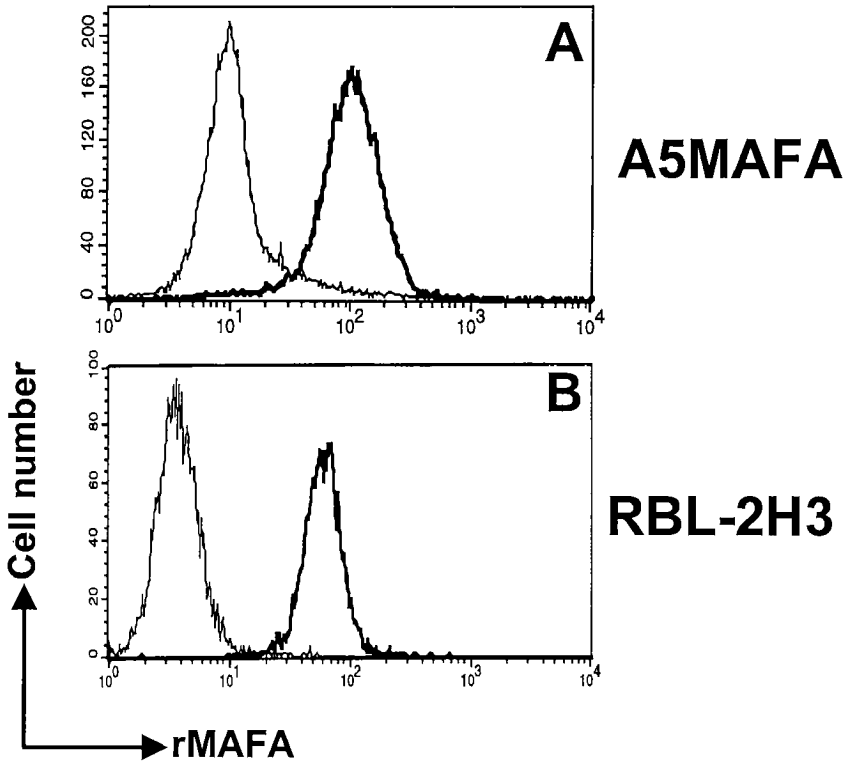

Figure 12. Cytofluorimetric analysis of rMAFA surface expression by the T cell line A5 transfected with a chimeric mouse-rat MAFA molecule. Transfected A5MAFA cells (A) and RBL-2H3 rat mast cells (B) were stained with anti-rat MAFA mAb G63 (thick line). The thin line represents untransfected A5 cells stained with G63, and A5MAFA or RBL-2H3 cells stained with an isotype control.

TCR-mediated activation of A5MAFA cells leads to reduced GFP expression compared to expression in parental A5 cells.

Expression of GFP upon TCR-mediated activation of A5 and A5MAFA cells represented a nice readout to analyse MAFA-mediated activity as long as this activity would emerge upstream of NF-AT responsive elements. Parental A5 and transfected A5MAFA cells were activated by Vβ8-crosslinking in the presence or absence of additional rMAFA-crosslinking by mAb G63. As shown in Fig. 13, 80% of both A5 and A5MAFA cells equally expressed GFP if an optimal concentration of the crosslinking goat anti-mouse IgG mAb was used (1μg/ml). Yet, providing a weaker stimulus by diminishing the amounts of crosslinking goat anti-mouse IgG (i.e. 0.6 and 0.4 μg/ml) led to substantially reduced GFP expression in A5MAFA cells (squares) compared to A5 cells (diamonds).

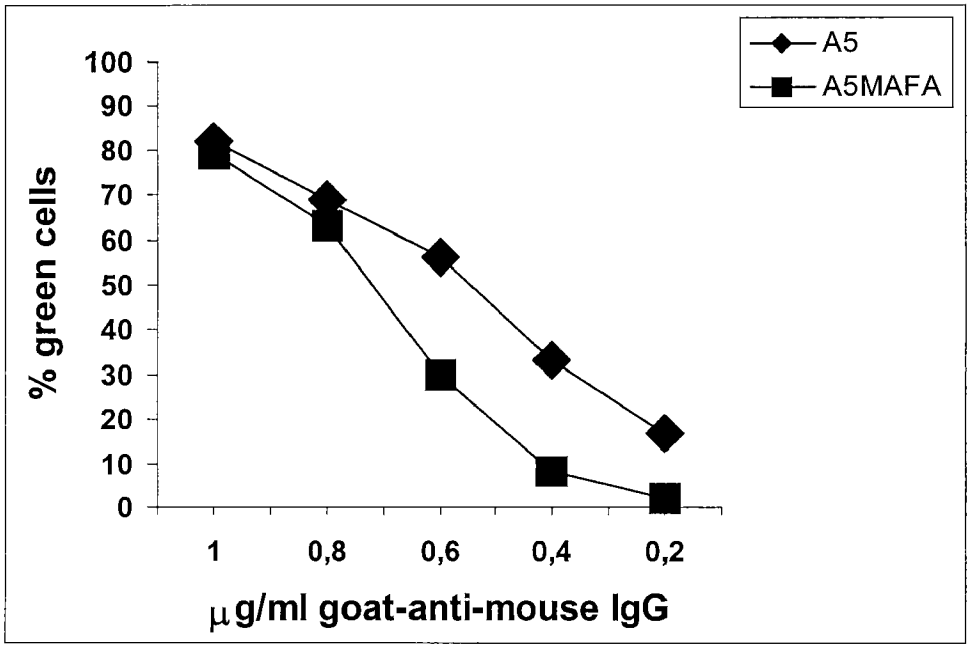

Figure 13. Flow cytomeric analysis of GFP-expressing A5 and A5MAFA cells after TCR-mediated activation. A5 (diamonds) and A5MAFA cells (squares) were coated with anti-Vβ8 mAb alone or together with anti-rMAFA mAb G63. Both antibodies were subsequently crosslinked by different concentrations of plate-bound goat anti-mouse IgG (1, 0.8, 0.6, 0.4 and 0.2 μg/ml) for 8h at 37°C. The percentage of GFP expressing cells was analysed by flow cytometry. The data obtained with additional MAFA crosslinking are very similar (not shown).

Unfortunately, crosslinking of chimeric mouse-rat MAFA itself did not further diminish GFP expression and the same data was obtained with or without additional crosslinking of MAFA. This finding was unexpected, as in analogy to the rMAFA one might have expected that also mMAFA exerts its activity upon clustering or crosslinking by anti-MAFA mAb. Two conclusions can be drawn from this experiment. Firstly, mouse-rat

MAFA might interact with a ligand present on the cell surface of neighbouring cells or with other MAFA molecules in a homodimeric interaction. This could lead to the activation of mouse-rat MAFA and diminished GFP expression in the transfected cells. Secondly, retroviral transfection followed by selection with G418 could have resulted in the isolation of low GFP-expressing cells. The generation of a chimeric mouse-rat MAFA with a mutation in the ITIM would be necessary to finally answer the question of the inhibitory activity of the mMAFA ITIM.

NK-like killing by Hy-Ad9 cells is not influenced by mMAFA crosslinking.

Natural expression of mMAFA by the CTL line Hy-Ad9 (see Fig. 8a) provided an ideal system to further characterise mMAFA activity. In this experiment the influence of mMAFA crosslinking on NK-like killing activity of Hy-Ad9 cells towards NK-sensitive target cells YAC1 was assessed. As shown in Fig. 14, Hy-Ad9 cells were able to lyse YAC1 target cells very efficiently, independently of pre-treatment with anti-mMAFA mAb 2F1 (circles). Thus, crosslinking of mMAFA on Hy-Ad9 cells did not inhibit the NK-like killing activity of these cells.

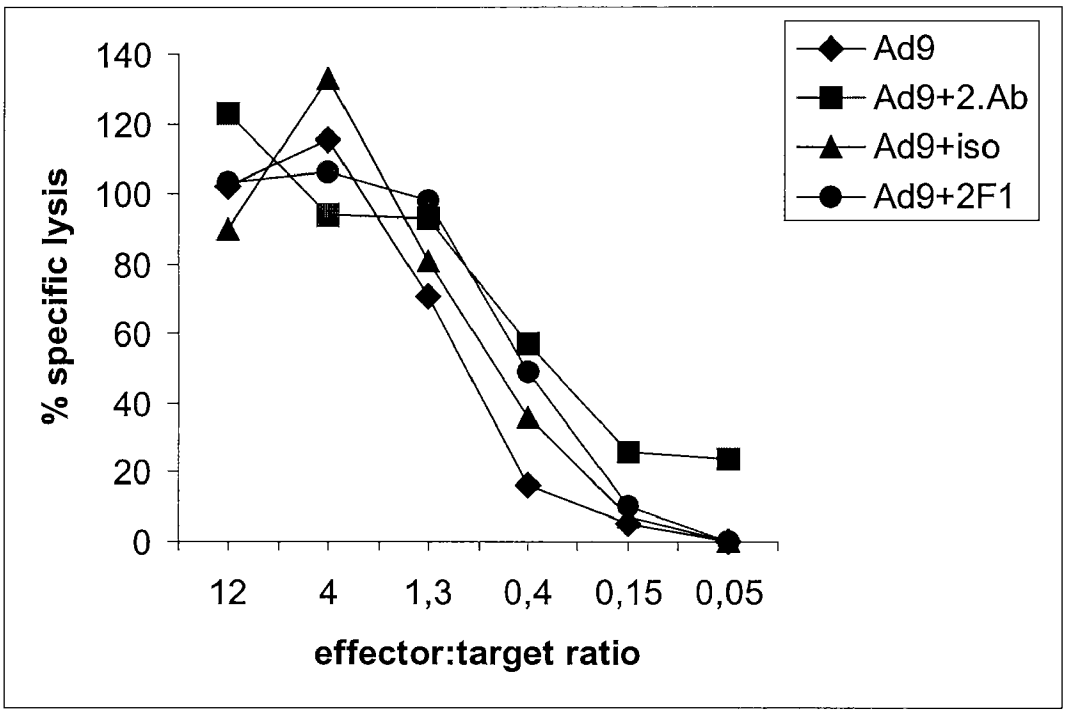

Figure 14. Crosslinking of mMAFA did not directly influence NK-like killing activity of Hy-Ad9 cells. Hy-Ad9 cells that naturally express high levels of mMAFA, were used untreated (diamonds), coated with 2. Ab goat anti-hamster IgG alone (squares), coated with anti-mouse MAFA mAb 2F1 (circles) and isotype control (triangles) followed by crosslinking with goat anti-hamster IgG. After antibody treatment cells were incubated for 15h with ^{51}Cr labelled YAC1 target cells and cytolytic activities were determined. Spontaneous release values were 37%.

mMAFA$^+$ and mMAFA$^-$ CD8$^+$ effector T cells exhibit both high cytolytic activities 8 days after LCMV infection.

Since Fig. 6a shows a segregation of mMAFA$^+$ and mMAFA$^-$ cells, mMAFA$^+$ and mMAFA$^-$ CD8$^+$ effector T cells were sorted 8 days after LCMV infection using a FACSort. The cytolytic activity of both populations was determined in a ^{51}Cr-release assay with GP33-loaded target cells. In Fig. 15a, dot plots of spleen cells stained with antibodies specific for CD8 and mMAFA before sorting (A) and after sorting (B and C) are displayed. In Fig. 15b cytolytic activities of unsorted CD8$^+$mMAFA$^{+/-}$ cells and sorted CD8$^+$mMAFA$^+$ and CD8$^+$mMAFA$^-$ cells are displayed. mMAFA$^+$ (diamonds) as well as mMAFA$^-$ (squares) T cell populations were equally able to lyse GP33-loaded target cells and lysis was identical to that obtained with unsorted CD8$^+$mMAFA$^{+/-}$ cells (triangles).

This data was well in line with those obtained for the T cell line Hy-Ad9 and suggested that surface expression of mMAFA did not directly influence cytolytic activities of effector T cells.

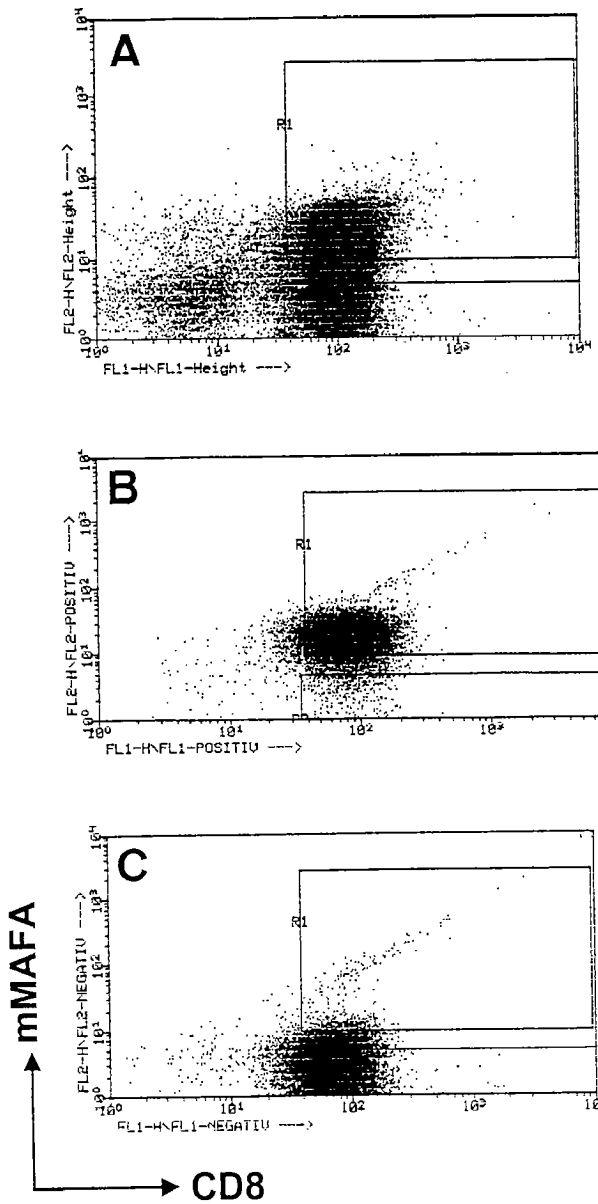

Figure 15a. Sorting of mMAFA$^+$ and mMAFA$^-$ T cells using flow cytometry. CD8$^+$ P14 TCR transgenic effector T cells were isolated 8 days after adoptive transfer and were sorted after staining with anti-CD8 and anti-mMAFA mAbs into a mMAFA$^+$ and mMAFA$^-$ cell population using a FACSsort. The dot plots are shown from unsorted cells (A), mMAFA$^+$ (B) and mMAFA$^-$ cells (C).

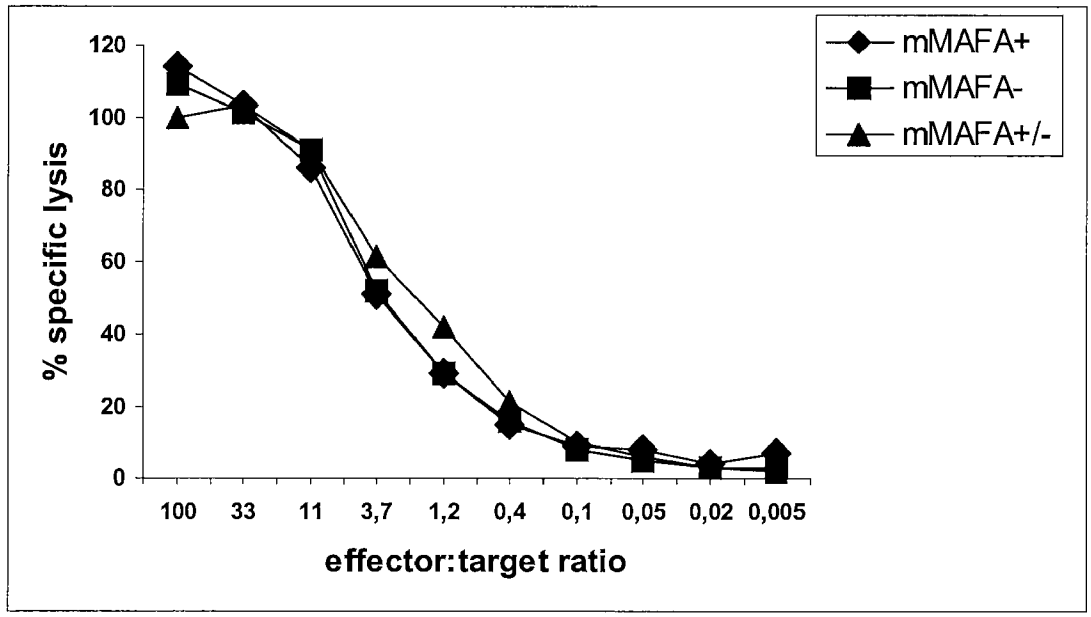

Figure 15b. Cytotolytic activity of mMAFA$^+$ and mMAFA$^-$ CD8$^+$ T cells 8 days after LCMV infection. 10^5 TCR transgenic T cells from P14 TCR-Thy1.1 transgenic mice were transferred into C57BL/6 mice and 8 days after LCMV infection, TCR transgenic CD8$^+$ T cells were purified by depletion of red blood cells, Thy1.2$^+$ host cells and B cells. Remaining cells were stained with mAb for CD8 and mMAFA (2F1) and sorted using a FACSort. Ex vivo CTL activities of unsorted mMAFA$^{+/-}$ and sorted mMAFA$^+$ and mMAFA$^-$ CD8$^+$ T cells were assayed in a ^{51}Cr-release assay for 15h. B16 melanoma target cells were coated with 10^{-6}M GP 33 peptide. Spontaneous release values were 20%.

4. DISCUSSION

The differential display (DD) technique has mostly been applied to culture cell lines or primary cell cultures in vitro, neglecting the fact that changes in gene expression patterns could be strictly dependent on environmental factors and direct cell-cell interactions in vivo. The goal of this thesis was the identification of genes involved in differentiation, activation and silencing of $CD8^+$ T cells. The adoptive transfer system using $CD8^+$ transgenic T cells from P14 TCR transgenic mice allowed the generation and characterisation of $CD8^+$ effector and memory T cells in vivo (Zimmermann et al., 1996a). Thus naive, effector and memory T cells could be isolated directly ex vivo and subjected to DD analysis. There was no report in the literature describing DD analysis from ex vivo isolated cells before. Successful DD did result in the identification of two genes involved in T cell activation and/or silencing that are discussed below.

BETA-GALACTOSIDE-BINDING PROTEIN (βGBP)

The product of the LGALS1 gene can be expressed in two molecular forms, namely as a non-covalently associated homodimer (galectin-1) with lectin properties (Barondes et al., 1994a; Barondes et al., 1994b; Cho and Cummings, 1995) and as a monomer (βGBP) with negative cell growth regulatory properties (Wells and Mallucci, 1991; Wells and Mallucci, 1992; Cho and Cummings, 1995). The present work demonstrates

i) the expression of LGALS1 upon antigen-specific T cell activation,
ii) the release of monomeric βGBP by activated T cells,
iii) the growth inhibitory properties of recombinant βGBP on antigen-activated T cells and
iv) increased numbers of $CD8^+$ T cells in LGALS1 deficient mice (=$Lect14^{o/o}$ mice) after LCMV infection.

Although only monomeric βGBP was found in the cell free supernatant of activated T cells and no monomeric or dimeric protein was detected in the cell lysate (data not shown), it can not be ruled out that some protein might be produced and secreted by these cells as a dimeric lectin (galectin-1) under in vivo conditions. Indeed, the LGALS1 gene product has been isolated from various tissues including spleen as a homodimer (Ahmed et al., 1996).

Different scenarios relating to the dimeric and monomeric form of the LGALS1 gene product in immunomodulation need to be discussed. In the murine system, the monomer

βGBP was initially isolated and cloned because of its property to function as an autocrine negative growth factor in embryonic fibroblasts (Wells and Mallucci, 1991). Secreted by the cells, monomeric βGBP binds with high affinity to about $5x10^4$ receptor sites per cell through molecular domains other than those that link saccharide determinants. Monomeric, recombinant βGBP inhibits proliferation of fibroblasts by arresting the cells in G_0 or at S/G_2 traverse. The negative growth factor activity on fibroblasts (Wells and Mallucci, 1991) and other cell types (Novelli et al., 1999) including leukaemia cell lines is evident already at nM concentrations. The growth inhibitory effect is independent of the carbohydrate binding site, since i) addition of lactose does not block the inhibitor activity and ii) the 18,000 Mr form of βGBP with the carbohydrate binding site blocked by a glycan complex, has full growth inhibitor activity (Wells and Mallucci, 1992).

In this context, a role of βGBP during the course of a T cell immune response could be possible. Expression of βGBP by activated T cells could result in self-induced inhibition of their own proliferation after a clonal expansion phase. Cessation of cell proliferation may be necessary for differentiation into effector cells that secrete cytokines or destroy virus-infected target cells. A physiological role of βGBP as a cytokine-like immunomodulator is supported by current evidence demonstrating that βGBP upregulates the α– and β chain of the IFN-γ receptor in human T cells, rendering activated T cells sensitive to IFN-γ induced apoptosis (Allione et al., 1998). A cytokine-like role is also supported by the findings that memory cells expressed LGALS1 RNA 12 hours earlier upon stimulation when compared to naive T cells. Similar results were obtained for the kinetics of IL-2 and IFN-γ expression by $CD8^+$ memory cells (Zimmermann et al., 1999; Bachmann et al., 1999). In a recent report, a monomeric tumour-derived galectin-1 has been shown to exhibit transforming growth factor (TGF)-like activity (Yamaoka et al., 1996). Despite the fact that the protein is a monomer, it is named galectin-1 in that report. The mitogenic activity of the monomeric galectin-1 was only visible under non-reducing conditions whereas under reducing conditions the protein lost its mitogenic activity but gained sugar-binding properties. This indicates that the LGALS1 gene product might not only exhibit different functions in its monomeric or dimeric form but also that, depending upon the experimental system analysed, as a monomer it can promote opposing effects such as growth inhibitory and mitogenic activities.

Perillo et al. have recently demonstrated that the dimer galectin-1 can induce apoptosis of immature thymocytes and activated, but not naive, peripheral T cells (Perillo et al., 1995; Perillo et al., 1997). It is important to stress that in contrast to the growth inhibitory effect of monomeric βGBP which requires nM concentrations, the apoptotic

effect of recombinant galectin-1 on activated T cells requires 20μM. In these experiments apoptosis was induced in peripheral blood mononuclear cells and several T cell lines with dimeric recombinant galectin-1 in vitro. The apoptotic effect could be inhibited by addition of lactose due to the lectin properties of the molecule. As apoptosis was induced in T cells that were given an activating stimulus, it is possible that galectin-1 acted via crosslinking of glycoproteins on the T cell surface, an event known to render activated T cells apoptotic by mechanisms of activation-induced cell death (Russell et al., 1991). Galectin-1 was therefore proposed to be involved in the modulation of immune responses.

In line with this data, other reports demonstrated the apoptotic role of galectin-1 like proteins. CLL-1, a chicken lactose lectin-1 (Rabinovich et al., 1997) as well as a galectin-1 like protein recently isolated from activated rat macrophages (Rabinovich et al., 1998) were shown to induce a dose-dependent growth-inhibition of ConA-stimulated rat spleen cells. In contrast to the data demonstrated in this thesis where growth inhibition of antigen-induced T cells was achieved with nM concentrations of recombinant βGBP, the effect of CLL-1 and macrophage derived galectin-1 was only visible in μM concentrations. Similar to galectin-1, the carbohydrate recognition domain appeared to be involved in the inhibitory properties of CLL-1 suggesting that the molecule exerts its effect as a dimer. Apoptosis of the target cells was one of the molecular mechanisms underlying the growth regulatory properties of CLL-1 and the target cells predominantly affected were activated T cells and not B cells. In this context one could speculate that effector T cells kill each other or themselves, by secreting galectin-1, which would ensure that the immune response mounted would decline appropriately after antigen is cleared. This mechanism could support other down-regulating pathways such as CD95- and/or growth factor deprivation-mediated apoptosis.

Interestingly, there is increasing evidence that apoptosis occurs by mechanisms that have been conserved throughout evolution (Vaux et al., 1994; Raff, 1992). Since β-galactoside binding proteins are among the most highly conserved proteins (Barondes et al., 1994a; Barondes et al., 1994b) one may speculate that they could affect the timing when an individual cell starts its apoptotic program. A relationship between the apoptotic pathway and a member of the β-galactoside-binding protein family was indeed demonstrated by the fact that galectin-3 binds Bcl-2 in a carbohydrate-dependent manner (Yang et al., 1996). In contrast to galectin-1 galectin-3 does not promote apoptosis but participates in cell death inhibition pathways. The opposite effect of galectin-1 and galectin-3 is reminiscent of that found for members of the Bcl-2 family -

despite sequence similarities some members inhibit apoptosis whereas others promote it (Adams and Cory, 1998).

Galectin-1 was reported to be expressed by thymic epithelial cells and was postulated to participate in thymocyte-thymic epithelial cell interactions (Baum et al., 1995). Thymocytes were able to bind recombinant galectin-1 in a carbohydrate-dependent manner and the degree of galectin-binding to thymocytes correlated with the maturation of the cells, as immature thymocytes bound more galectin-1 than mature thymocytes did. According to the data presented in this thesis, LGALS1 RNA was expressed by thymocytes in all maturation states. Whether a downregulation of LGALS1 expression occurred in the thymus in very mature thymocytes, or already in the periphery in recent thymic emigrants is not clear so far. Understanding the role of LGALS1 expression and galectin-1 binding to thymocytes during T cell development requires further research.

Due to its lectin properties, galectin-1 has a high affinity for poly-N-acetyllactosamine glycans and binds strongly to laminin and fibronectin in the extracellular matrix (ECM), where it could promote cell adhesion (Zhou and Cummings, 1993). Galectin-1 was also reported to be present on the extracellular surface of endothelial cells, mediating tumour cell adhesion (Lotan et al., 1994; Woynarowska et al., 1994; Van den Brule et al., 1995). Furthermore, increased expression of galectin-1 by tumour cells themselves has been observed to correlate with a more poorly differentiated and metastatic phenotype (Skrincosky et al., 1993).
In the context of cell adhesion mediated by galectin-1, activated T cells may modulate their environment by secreting dimeric galectin-1 into the extracellular space where it could bind to the endothelial cell surface or to ECM molecules. Following binding to glycoproteins on the T cell surface, the dimeric lectin may then help T cells to attach to endothelial surfaces or ECM molecules, allowing them to enter target tissues and exert their effector functions. In contrast to this scenario, it has been shown that secretion of galectin-1 induced a loss of myoblast-laminin adhesion, suggesting that galectin-1 modulates myoblast detachment during differentiation and fusion into tubular myofibers (Cooper et al., 1991). This idea suggests that the secretion of galectin-1 might promote detachment of the T cells from endothelial surfaces or ECM molecules by weakening overall adhesion to the endothelium and thereby allowing the cell to migrate. It is not clear so far which of the two mechanisms is dominant, but it is likely that a β-galactoside-binding protein secreted by activated T cells could influence their migration pattern during an immune response.
A similar role, as an extracellular adaptor molecule, was proposed for a galectin-1 like protein that was found to be associated via its carbohydrate domain with the

complement receptor 3 (CD11b, Mac-1) on macrophages (Avni et al., 1998). In analogy, an association of galectin-1 with CD11b on activated T cells (Zimmermann et al., 1996a; Andersson et al., 1994) would therefore have been expected. Yet, by flow cytometry using a βGBP-specific mAb (data not shown) no galectin-1/βGBP could be detected on the surface of activated T cells.

Activation of T cells leads to a sequential induction of several genes which can be divided into immediate (< 12h), early (1-2 days) and late (> 3 days) genes (Ullmann et al., 1990). The present work demonstrates that LGALS1 expression was high in $CD8^+$ T cells 8 days after virus infection and expression was still visible in memory T cells 4 weeks after infection. After peptide stimulation, in vivo and in vitro LGALS1 expression reached its peak in $CD8^+$ T cells 2 to 3 days after antigen exposure. Thus, compared to other T cell activation markers, LGALS1 expression appears rather late in the process of activation. Its gene product may therefore be involved in effector cell differentiation and migration, or be involved even in the silencing phase of the T cell immune response.

This thesis highlights a new strategy of activated T cells to actively influence their environment. Monomeric βGBP released by activated T cells, inhibits as an autocrine effector molecule the growth of antigen-induced T cells. Whether in vivo released βGBP could also influence the migration pattern of different cell types or even exert mitogenic activity are questions beyond this thesis. The analysis of LGALS1 deficient mice revealed that these mice have increased $CD8^+$ T cell numbers after LCMV infection compared to normal mice and that their T cells are in a more activated status. This suggests that galectin-1/βGBP indeed could have an inhibitory effect on activated $CD8^+$ T cells in vivo and could contribute to down-regulating pathways during an immune response. The recent identification of several new members of the galectin family indicates that additional roles for galectins will soon be known. Understanding the cell-type specific effects of different galectins will allow the therapeutic modulation of these activities in the treatment of human diseases.

MAST CELL FUNCTION-ASSOCIATED ANTIGEN (MAFA)

The mast cell secretory response is triggered via the clustering of Fcε receptors (FcεR) by IgE immune complexes. A cell surface protein has been identified on the rat mucosal mast cell line RBL-2H3 that inhibits FcεR-mediated degranulation and cytokine release after aggregation by a specific mAb (Soto and Pecht, 1988). This molecule has been named mast cell function-associated antigen (MAFA). Molecular analysis revealed that MAFA is a type II transmembrane protein that belongs to the C-type lectin superfamily (Guthmann et al., 1995). MAFA exists both as a monomer and a disulfide-linked homodimer. Its intracellular domain contains a putative immunoreceptor tyrosine-based inhibition motif (ITIM) that was found to be constitutively phosphorylated. These characteristics reveal a close relationship of MAFA with other members of the C-type lectin superfamily that function as inhibitory receptors (e.g. Ly49 and CD94/NKG2) in NK cells and in T cells.

This thesis describes the identification of the mouse homologue of the rat MAFA gene in the context of T cell activation. It demonstrates that murine MAFA (mMAFA) expression was strongly induced in $CD8^+$ T cells, which had been activated by a viral infection in vivo. Besides these anti-viral effector T cells, lymphokine activated murine NK cells expressed mMAFA. In contrast, activated murine $CD4^+$ T cells did not express mMAFA at significant levels. The observed expression pattern of mMAFA is reminiscent of inhibitory receptors (IRs) on both human NK cells (Lanier, 1997; Moretta and Moretta, 1997) and T cells (Phillips et al., 1995; Mingari et al., 1995; De Maria et al., 1997). IRs either are immunoglobulin-related molecules or belong to the family of C-type lectins. Similar to mMAFA, IRs are expressed on NK cells and on those $CD8^+$ T cells, which exhibit an effector/memory phenotype (Mingari et al., 1998b). The TCR Vβ repertoire of KIR^+ T cells was found to be skewed suggesting an oligoclonal or monoclonal expansion (Mingari et al., 1996). The dramatic increase of KIR^+ $CD8^+$ T cells in patients undergoing HLA-incompatible bone marrow transplantation and high proportions of inhibitor receptor bearing T cells identified in HIV-infected patients, further supports the notion that expression of inhibitory receptors on T cells is inducible in vivo (De Maria et al., 1997; Albi et al., 1996).

Attempts to induce IR expression on T cells by in vitro stimulation were unsuccessful (Mingari et al., 1998b; D`Andrea and Lanier, 1998). Similarly, in vitro antigen stimulation of $CD8^+$ T cells from P14 TCR transgenic mice induced vigorous proliferation and high CTL activity but did not induce significant mMAFA expression.

This data support the concept that the expression of inhibitory receptors is a consequence of antigen-driven stimulation requiring unique microenvironmental conditions - possibly a particular set of cytokines or specific interaction with antigen presenting cells. It has recently been shown that indeed in the presence of IL-15 or TGF-β, T cells activated by superantigen or allogeneic cells expressed de novo the CD94/NKG2A receptor molecules belonging to the C-type lectin family (Mingari et al., 1998a; Bertone et al., 1999). IL-15 is produced by a wide variety of cells including macrophages during an innate immune response and stimulates the proliferation of T, B and NK cells (Grabstein et al., 1994). TGF-β is produced by macrophages, T and B cells, and can have immunosuppressive as well as proinflammatory effects (Roberts et al., 1990). In line with these findings, IL-12 was reported to upregulate NKRP1A, a human C-type lectin, on NK cells (Poggi et al., 1998). In the experimental system analysed in this work, addition of IL-15 to P14 TCR transgenic T cells activated in vitro with the cognate peptide antigen did not induce mMAFA expression (data not shown). Unexpectedly, activation of P14 TCR transgenic T cells with a high dose of LCMV in vivo did not induce mMAFA expression despite the fact that the transgenic T cells proliferated significantly (about 500-fold) and displayed normal cytolytic activities. It is therefore obvious to speculate that the induction of mMAFA depends tightly on several parameters defined by the virus itself such as dose of infection, virus spread, tissue distribution and virus kinetics. It will be important to define the stimuli leading to the expression of mMAFA on murine $CD8^+$ T cells.

In contrast to activated $CD8^+$ T cells and NK cells in the mouse, expression of the mMAFA gene was not detected in murine bone marrow-derived, IL-3/IL-4 induced, mast cells cultures, which are thought to represent an in vitro analogue for mucosal mast cells (Huels et al., 1995). Similarly, mouse P815 mastocytoma cells did not express mMAFA (data not shown). The level of mMAFA gene expression in murine effector $CD8^+$ T cells isolated ex vivo was similar to levels seen in the rat mast cell line RBL-2H3 (not shown). In the rat, MAFA gene expression appeared to be mast cell specific since rMAFA transcripts were obtained from lung tissue but not from other organs, including spleen and lymph nodes when the sensitive RT-PCR technique was used (Bocek et al., 1997). Results of the present thesis indicate that MAFA gene expression in the mouse is not mast cell specific and may not even be expressed on mucosal mast cells in this species. The same conclusion has been drawn by Hanke et al. in an independent report about mMAFA expression on NK cells (Hanke et al., 1998). Furthermore, rat MAFA (rMAFA) is not exclusively expressed by mast cells as reported, because low expression of rMAFA was obtained in rat spleen cells after VV infection in vivo. In addition, NK cells in the rat were found to express MAFA (L.

Lanier, personal communication) suggesting that the negative results from the original RT-PCR experiment described by Bocek et al. (Bocek et al., 1997) were due to the lack of sensitivity. The human homologue of MAFA has also been described and human MAFA expression was found in human basophils, lung-derived mast cells, NK cells and in organs such as spleen, lymphnodes and peripheral blood (Butcher et al., 1998; Lamers et al., 1998). It is likely that the expression pattern of MAFA is identical in the three species and it will be interesting to analyse MAFA gene expression in human $CD8^+$ T cells activated by infection with EBV, HIV or cytomegalovirus.

Both activated $CD8^+$ T cells and IgE-loaded mast cells are effector cells. Antigen stimulation through the TCR or via IgE/FcεRI complexes, respectively, results in degranulation and secretion of inflammatory mediators. The two receptors share close similarities and the cytoplasmic tail of the FcεRIγ-chain expresses the same consensus motif involved in signal transduction as the TCRζ-chain (Keegan and Paul, 1992). FcεRIγ- and TCRζ-chains are also interchangeable in their ability to mediate T cell development and function (Shores et al., 1997). Similar to mast cells, T cells contain granules in which effector molecules such as perforin and granzymes are stored (Metcalfe et al., 1981; Podack et al., 1991). In analogy to the inhibitory effect of rMAFA on mast cell degranulation it could be assumed that the mouse homologue has a similar function and serves as an inhibitory molecule on $CD8^+$ effector T cells and on NK cells.

Direct evidence that mMAFA could inhibit T cell degranulation could not be demonstrated. mMAFA positive as well as mMAFA negative $CD8^+$ T cells exhibited the same cytolytic activities suggesting that expression of mMAFA alone has no effect on T cell degranulation. In addition, crosslinking of mMAFA expressed by the CTL line Hy-Ad9 by anti-mMAFA mAb 2F1 did not inhibit NK-like cytolytic activity of these cells. Using the same 2F1 mAb, Hanke et al. also did not succeed in demonstrating an inhibitory function of mMAFA on NK cells (Hanke et al., 1998). It remains possible that crosslinking by the anti-mMAFA mAb 2F1 does not release an appropriate signal to the T cells or that simultaneously other signals are required to render mMAFA fully active. It has also proven difficult to demonstrate inhibition caused by antibodies against known inhibitory receptors including various Ly49 receptors. Inhibition of effector functions could often only be achieved when the proper ligand on the target cell was present (T. Hanke, D. Speiser, personal communication). It is however important to stress that both mouse and rat MAFA contain the same ITIM in the cytoplasmic tail of the molecule and that the inhibitory function of the rMAFA molecule has been clearly established (Soto and Pecht, 1988). Experiments with the chimeric mouse-rat MAFA did not allow a conclusive answer concerning the inhibitory role of mMAFA - yet they

suggest that the intracytoplasmic tail of the mMAFA could have inhibitory activity. In Figure 16, a possible scenario of the interaction of mMAFA with the TCR is demonstrated in analogy to the reported events occurring in rMAFA expressing mast cells.

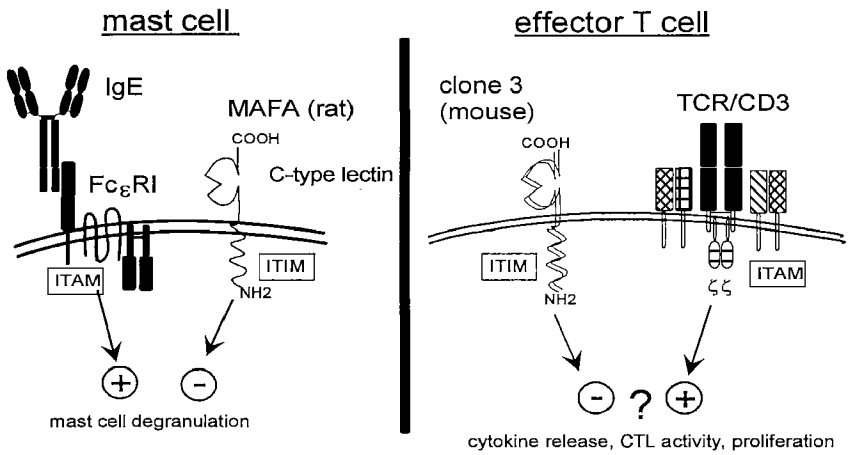

Figure 16. Hypothetical model for the interaction of mMAFA with the TCR. The mast cell secretory response is induced via crosslinking of the FcεRI with IgE leading to phosphorylation of the activation motif (ITAM) in the cytoplasmic tail of the FcεRI (left). Crosslinking of rMAFA with a monoclonal antibody simultaneously to the activating stimulus may lead to the recruitment of the phosphatases SHIP and SHP-1 by the inhibitory motif (ITIM) in the cytoplasmic tail of rMAFA (Daeron, 1997). The phosphatases could then dephosphorylate the ITAM of the FcεRI and thereby abrogate activating events. A similar scenario could be envisaged for the T cell (right). T cell activation via TCR-engagement leads to cytolytic activity mediated by the release of cytotoxic granules. The first step of TCR-engagement is the phosphorylation of the ITAMs in the TCRζ chains. Crosslinking of mMAFA could induce the recruitment of phosphatases via its ITIM leading to the dephosphorylation of the ITAMs in the TCRζ chains. This could influence downstream signaling events resulting in alteration of cytokine release, cytolytic activity or T cell proliferation.

Functional analysis of other inhibitory receptors on T cells revealed that crosslinking of these receptors leads to inhibition of TCR-induced T cell functions, including cytolytic activity and production of cytokines like IFN-γ and TNF (Le Drean et al., 1998; D`Andrea et al., 1996). Inhibitory receptors expressed by $CD8^+$ T cells of HIV-infected patients could inhibit lysis of autologous lymphoblastoid cells expressing HIV antigens. In addition, blocking of these receptors resulted in de novo appearance or in significant increase of HIV-specific target cell lysis. This indicates that the HIV-specific CTLs were not anergic but just inhibited by their function because of interaction of their inhibitory receptor with MHC class I molecules (De Maria et al., 1997).

The introduction of an inhibitory receptor (KIR3DL1) into a melanoma-specific $CD8^+$ CTL line led to transmission of negative signals upon engagement with MHC class I molecules (Bakker et al., 1998). Thereby, the activating signal generated via TCR-MHC class I-peptide interaction was effectively suppressed.
Furthermore, peptide-specific T cell lines specific for a protein expressed by normal melanocytes all expressed CD94, an inhibitory C-type lectin. Only 50% of them expressed a functional inhibitory receptor composed of the heterodimeric complex of CD94/NKG2 (Noppen et al., 1998). Interestingly the modulatory activity of CD94/NKG2 did depend on the quantity of the antigenic epitope expressed on target cells. Blocking of CD94 with monoclonal antibodies in the presence of peptide pulsed APCs did only induce significant enhancement of cytolytic activity if suboptimal concentrations (i.e. 10-100ng/ml) of peptide were used for pulsing. This finding could support the hypothesis that inhibitory receptors might prevent the activation of T cells by APCs presenting relatively low amounts of potentially antigenic epitopes - a situation that might well occur during clearance of viral antigen by a vigorous CTL response.

In the context of above-mentioned findings it appears quite obvious that mMAFA functions as an important regulatory receptor for virus-induced CTLs. In contrast to rat MAFA which is constitutively expressed in the rat mast cell line RBL-2H3, mouse MAFA was induced in $CD8^+$ T cells upon viral infection and was still detectable in memory T cells 4 weeks after infection. Thus, compared to other T cell activation markers mMAFA appears rather late in the course of a CTL response. This late kinetic of mMAFA expression fits in well with the postulated inhibitory role of this molecule for $CD8^+$ effector T cells. Further studies, perhaps involving mMAFA gene knockout mice, will be required to determine the functional role of mMAFA. Also the characterisation of its ligand will be interesting and demanding research.

5. GENERAL CONCLUSIONS

This thesis describes the identification of the two proteins βGBP and mMAFA; both produced by virus-induced CTLs at the peak of the immune response. Very interestingly, though somewhat unexpected, they both show clear characteristics of inhibitory molecules.

It has been mentioned before that the down-regulation in lymphocyte number after viral infection is associated with high levels of apoptosis. The factors that drive lymphocyte apoptosis in viral infections are still poorly understood and probably involve a number of mechanisms. The fact that in LCMV-infected bcl-2-transgenic mice or Fas-deficient lpr mice apoptosis of lymphocytes proceeds normally (Petschner et al., 1998; Zimmermann et al., 1996b), suggests that in vivo apoptosis must be driven by other stimuli than those. A very common idea is that the amount of antigen itself controls the T cell number. Effector T cells die due to their limited lifespan and as soon as antigen is cleared from the periphery the generation of new effector cells is stopped, leading automatically to the down-sizing of the antigen-specific CTL population. This obvious scenario however does not answer the question why virus-specific CTLs would produce molecules such as βGBP or MAFA that turn them off.

Different possible scenarios could describe the functional relevance of βGBP and MAFA expression.

- It seems likely that secretion of βGPB acts in an autocrine manner influencing directly the T cells that secrete it. Interaction with its still unidentified receptor can directly induce growth inhibition of activated T cells, and apoptosis of tumour cells by activation of caspase-involving signaling pathways (L. Mallucci, personal communication). It will be an interesting task to define the receptor of βGBP and to demonstrate whether its expression is also modulated by the activation state of the T cell.

- The finding that inhibitory receptors might prevent the activation of T cells by APCs presenting relatively low amounts of antigenic epitopes (Noppen et al., 1998) opens a new way to look at inhibitory receptors. Their expression at the peak of an antiviral response when viral load declines could prevent further activation of naive or already triggered anti-viral T cells. Their reported expression in T cells after viral stimulus (e.g. LCMV, HIV) and their restriction to activated $CD8^+$ T cells would fit well in line with this concept. Figure 17 describes an envisaged scenario and the putative role of inhibitory receptors such as MAFA during an ongoing antiviral immune response. However, the contribution of inhibitory receptors would not

explain the observed drastic loss of virus-specific T cells after antigen-clearance, as they are so far not reported to render T cells apoptotic. One explanation could be that inhibitory receptors deprive T cells of stimulation either by abolishing TCR-mediated activating signals or by influencing cytokine production.

I believe that this thesis and the many reports summarised herein give good evidence that T cell silencing occurring after viral infection is much tighter regulated than often supposed. The role of βGBP, MAFA and many more identified and unidentified factors involved in that process need to be further clarified.

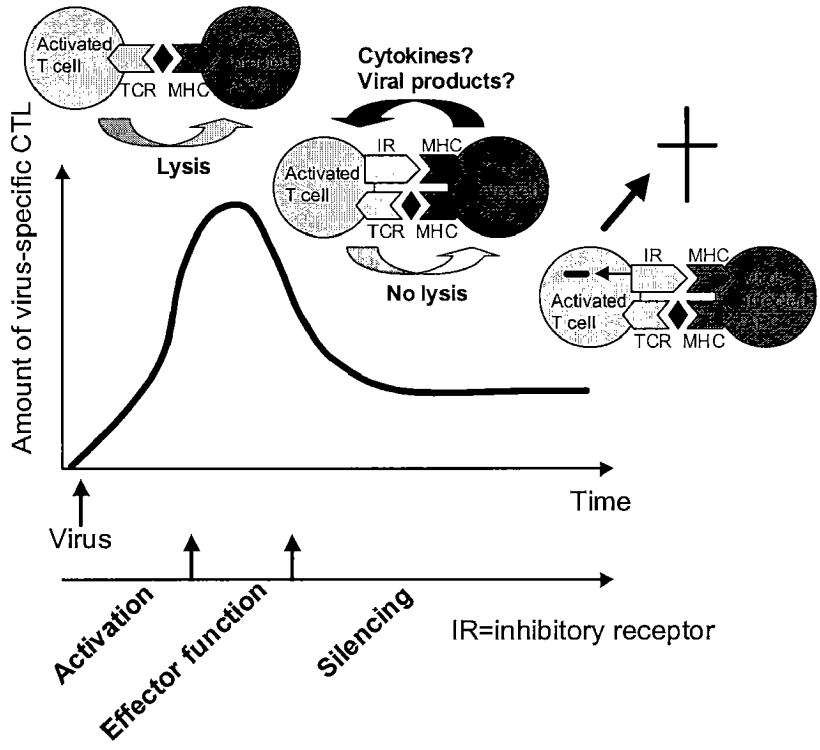

Figure 17. Hypothetical model of the possible contribution of inhibitory receptors (IR) in viral infections. After viral infection virus-specific T cells undergo extensive proliferation and differentiate into effector cells. During the ongoing anti-viral response virus-infected APCs could synthesise cytokines such as IL-15 capable of inducing inhibitory receptor expression on virus-specific CTLs. In addition, virus-encoded products released by infected cells may as well influence the expression of inhibitory receptors by CTLs. The progress of viral clearance by effector cells results in lower concentrations of antigen presented. As the activity of inhibitory receptors might be depended on the quantity of antigen presented, this state could enhance the activity of inhibitory receptors thereby preventing the activation of T cells through a negative signal released by inhibitory receptors.

6. MATERIALS AND METHODS

Animals. C57BL/6 (B6.Thy-1.2), BALB/c mice and Lewis rats were obtained from Harlan Winkelmann (Borchen, Germany) and B6.PL-Thy1^a (B6.Thy-1.1), 129/SV, IFNγR$^{o/o}$ (Huang et al., 1993), TCRα$^{o/o}$ (Philpott et al., 1992), IL-12 p40$^{o/o}$ (Magram et al., 1996) and 2B4 TCR transgenic mice expressing a TCR specific for amino acids (aa) 88-103 of the moth cytochrome c protein (MCC) in association with H-2 I-E^k or I-E^b (Berg et al., 1990) were a generous gift of H. Mossmann (Max-Planck Institute for Immunbiology, Freiburg, Germany). (B6.Thy-1.1 x B6.Thy-1.2) F1 mice were bred locally. P14 TCR transgenic mice (line 318 and 327) expressing a TCR (Vα2/Vβ8) specific for aa 33-41 of the LCMV glycoprotein in association with H-2 D^b (Pircher et al., 1989), TCR/bcl-2 doubly transgenic mice (Petschner et al., 1998), TCR/lpr (Zimmermann et al., 1996b) and P14 TCR-Thy1.1 mice were bred locally. SMARTA TCR transgenic mice expressing a TCR specific for aa 61-80 of the LCMV glycoprotein in association with I-A^b were a generous gift of A. Oxenius (Institute for Experimental Immunology, Zurich, Switzerland) and have been described previously (Oxenius et al., 1998). DO11.10 transgenic mice expressing a TCR specific for amino acids 323-339 from Ovalbumin (Murphy et al., 1990) and IFNγ$^{o/o}$ mice (Dalton et al., 1993) were generously provided by M. Kopf (Basel Institute for Immunology, Basel, Switzerland). Perforin $^{o/o}$ (pko) mice (Kägi et al., 1994a) were a gift of H. Hengartner (Institute for Experimental Immunology, Zurich, Switzerland), Tnfr1$^{o/o}$ mice (Rothe et al., 1993) were a gift of K. Pfeffer (Institute of Medical Microbiology, Munich, Germany) and Aβ$^{o/o}$ mice (Cosgrove et al., 1991) were provided by T. Brocker (Basel Institute for Immunology, Basel, Switzerland). Lect14$^{o/o}$ mice (Poirier and Robertson, 1993) were generously provided by F. Poirier (Unité INSERM 257, Paris, France).

Virus. LCMV-WE was propagated on L929 fibroblast cells (ATCC CRL-1) with a low multiplicity of infection. The virus titer was quantified in a virus plaque assay as described (Battegay et al., 1991). LCMV-DOCILE (a variant isolated from an LCMV-WE (UBC) carrier mouse) was grown on MDCK cells (ATCC CCL-34) with a low multiplicity of infection. Both virus strains were originally obtained from R. Zinkernagel (Institute for Experimental Immunology, Zurich, Switzerland). Vesicular stomatitis virus Indiana (VSV) (Mudd-Summers isolate) was originally obtained from D. Kolakofsky (University of Geneva, Geneva, Switzerland) and was grown with a low multiplicity of infection on BHK (ATCC CCL-49) cells. Vaccinia virus strain WR (VV) was produced by infecting BSC 40 cells. Mice were infected intravenously (i.v.) with

200 pfu of LCMV-WE, 10^6 pfu LCMV-DOCILE, $2x10^6$ pfu VSV and $2x10^6$ pfu VV. Lewis rats were infected i.v. with 10^7 pfu VV.

Peptides. The LCMV glycoprotein peptide 33-41 (GP33 peptide, KAVYNFATM) and the Ovalbumin peptide 323-339 (OVA peptide, ISQAVHAAHAEINEAGR) were purchased from Neosystem Laboratoire (Strasbourg, France). The moth cytochrome c peptide 88-103 (ANERADLIAYLKQATK) was kindly provided by K. Eichmann (Max-Planck Institute for Immunobiology, Freiburg, Germany) and the LCMV glycoprotein peptide 61-80 (P13, GLNGPDIYKGVYQFKSVEFD) was a gift of A. Oxenius (Institute of Experimental Immunology, Zurich, Switzerland).

Cell lines. Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL, Paisley, United Kingdom) supplemented with 10% FCS, Penicillin/Streptomycin, Glutamine and 20U/ml IL-2 (Pharmingen, San Diego, CA) if needed. The following cell lines were used: EL-4, RMA-S, CTLL-2, BW 5147.3, P3-X63Ag8, J558, CEM, Jurkat, Molt-4, U937, HeLa, MCA, B16, P815, MC57 (all obtained from ATCC, Manassas, VA), Hy-Ad9 (Acha-Orbea et al., 1983), BWLy2-3 (Burgert et al., 1989), IT H6/A11 (Kohler et al., 1997), T21-4.60 (Lemke et al., 1979), B8-24.3 (Köhler et al., 1981), B22.249 (Lemke et al., 1979), MT-2, MT-4 (Harada et al., 1985), CB 15 (Biesinger et al., 1992), KAD (kindly provided by A. Meyerhans, Institute for Med. Microbiol. and Hygiene, Freiburg, Germany), B10BI (Lohoff et al., 1990) and L1/1 (Lohoff et al., 1988).

Cell cultures. *CD8$^+$ T cells:* For stimulation of CD8$^+$ T cells, $5x10^5$ TCR transgenic spleen cells of P14 TCR transgenic mice were incubated in a well of 24-well plates for 3 days in IMDM supplemented with 10% FCS and 10^{-6}M of the LCMV GP33 peptide. For the proliferation assay with recombinant βGBP (r-mβGBP) in vitro, spleen cells of P14 TCR, P14 TCR/lpr or P14 TCR/bcl-2 mice containing $5x10^4$ TCR transgenic T cells were preincubated for 4h in 96-well plates with indicated concentrations of r-mβGBP. Afterwards, LCMV GP33 peptide (10^{-9}M) was added to the culture and after 2 days the cultures were pulsed with 1mCi of (^{3}H) thymidine per well for 8 hours before harvesting onto filter paper. For neutralisation of naturally produced βGBP during T cell activation, P14 TCR transgenic T cells were stimulated with GP33 peptide in the presence of 4.6μg/ml anti-βGBP mAb (clone B2)(Wells and Mallucci, 1991).

For long term cultures, T cells from P14 TCR transgenic mice were restimulated weekly with LCMV-infected, irradiated (2500 rad) C57BL/6 peritoneal macrophages in IMDM supplemented with 10% FCS and 20U/ml IL-2 (Pharmingen). CD8$^+$ memory T cells

isolated from LCMV-immune C57BL/6 mice 4-6 weeks after infection or from LCMV-immune 129/SV, IFNγR$^{o/o}$ and IFNγ$^{o/o}$ mice 12 days after infection were cultured and stimulated in the same manner either with LCMV-infected, irradiated (2500 rad) C57BL/6, 129/SV, IFNγR$^{o/o}$ or IFNγ$^{o/o}$ peritoneal macrophages. IFNγR$^{o/o}$ and IFNγ$^{o/o}$ mice died in our facilities at day 12-15 after LCMV infection probably due to the lack of virus clearance leading to immunopathology. They were therefore killed at day 12 for cell culture experiments.

CD4$^+$ T cells: CD4$^+$ T cells from 2B4 transgenic mice were stimulated with 10μg/ml moth cytochrome c (MCC) peptide. 5 days after stimulation, CD4$^+$ T cells were purified by removing CD8$^+$ T cells and B cells by negative selection in vitro with antibody coupled magnetic beads (Dynabeads, Deutsche Dynal GmbH, Hamburg, Germany).
To obtain CD4$^+$ T cells with a Th1 or Th2 phenotype, spleen cells from DO11.10 TCR transgenic mice were depleted from CD8$^+$ T cells and B cells by negative selection with Dynabeads. The remaining CD4$^+$ T cells were stimulated for one week with 0.3μM OVA peptide and irradiated (2500 rad) spleen cells from BALB/c mice as APCs in the presence of 200U/ml IL-4 (Pharmingen) or 10U/ml IL-12 (Pharmingen). Cells were harvested on day 6, washed and restimulated with Phorbolmyristateacetate (PMA, 50ng/ml) plus Ionomycine (500ng/ml) (Sigma-Aldrich, Deisenhofen, Germany) for 24 and 48h.

Various: To obtain mitogen activated CD8$^+$ and CD4$^+$ T cells, C57BL/6 spleen cells were incubated for 3 days with 5μg/ml Concanavalin A (ConA). CD4$^+$ or CD8$^+$ T cells were purified by negative selection with Dynabeads in vitro. To obtain activated B cells, spleen cells from C56BL/6 mice were incubated with 10μg/ml lipopolysaccharide (LPS). After 3 days the cells were depleted of CD4$^+$ and CD8$^+$ T cells by negative selection with Dynabeads. Bone marrow derived mast cells (BMMC) were isolated from C57BL/6 mice as described (Huels et al., 1995) and stimulated for 8 hours with 0.5μM Ionomycine. To generate lymphokine-activated-killer cells (LAK), C57BL/6 spleen cells were depleted after lysis of red blood cells from T and B cells by negative selection with Dynabeads. The remaining cells were cultured for 6 days in IMDM supplemented with 10% FCS and 1000U/ml IL-2. After 6 days, the cells (90-95% NK1.1$^+$) were harvested.

Generation of effector and memory T cells. Effector and memory CD8$^+$ and CD4$^+$ T cells were generated in vivo using an adoptive transfer system as described (Zimmermann et al., 1996a). Briefly, sex-matched spleen cells from naive P14 TCR transgenic mice or SMARTA TCR transgenic mice containing 10^5 CD8$^+$ TCR

$V\alpha2/V\beta8^+$ cells or $CD4^+$ TCR $V\alpha2/V\beta8.3^+$ cells respectively were injected i.v. into normal, nonirradiated (B6.Thy-1.1 x B6.Thy-1.2) F1 mice. 1 day after transfer, mice were infected with LCMV. Mice were killed 8 days after infection to isolate $CD8^+$ and $CD4^+$ effector T cells. $CD8^+$ memory T cells were isolated from mice killed four weeks after infection. Uninfected P14 TCR transgenic mice and SMARTA TCR transgenic mice were used as a source of naive $CD8^+$ and $CD4^+$ T cells. For experiments with the anti-mMAFA mAb 2F1, adoptive transfer was also carried out by transferring cells from P14 TCR-Thy1.1 transgenic mice into normal C57BL/6 recipients.

$CD8^+$ T cells were purified from spleen cells by a two step negative selection with Dynabeads. Firstly, red blood cells were lysed by hypotonic shock and B cells were removed using sheep anti-mouse IgG Dynabeads. To isolate naive $CD8^+$ T cells, $CD4^+$ cells were removed using anti-CD4 coupled Dynabeads. Afterwards, cells were coated with hybridoma supernatant (100 µl per 10^7 spleen cells) of the following mAb: anti-Mac-1 mAb (M1-70) (Sanchez-Madrid et al., 1983) and anti-FcγRII/III mAb (2.4G2, obtained from ATCC, Manassas, VA). To isolate $Thy1.2^+$ TCR^+ transgenic effector cells from the (B6.Thy-1.1 x B6.Thy-1.2) F1 recipient mice the following mAb were used: anti-FcγRII/III mAb (2.4G 2) and anti-Thy1.1 mAb (19E-12) (Lostrom et al., 1979). For the isolation of $Thy1.2^+$ TCR^+ transgenic memory cells, the following mAb were used: anti-Mac-1 mAb (M1-70), anti-FcγRII/III mAb (2.4G 2) and anti-Thy1.1 mAb (19E-12). Afterwards antibody coated cells were removed using sheep anti-rat and sheep anti-mouse IgG Dynabeads. After this two step negative selection procedure 90-95% of the remaining cells were $CD8^+$ T cells. $CD8^+$ T cell depletion of spleen cells was performed with anti-CD8 coupled Dynabeads. $CD4^+$ effector cells were purified by depletion of red blood cells and by depletion of $CD8^+$ T cells and B cells by negative selection with Dynabeads.

Isolation and purification of thymocytes. Single thymocyte cell suspensions from normal C57BL/6 and $TCR\alpha^{o/o}$ mice were prepared in IMDM containing 10% FCS. $CD8^+CD4^-$ and $CD8^-CD4^+$ single positive populations were obtained by removal of $CD4^+$ or $CD8^+$ thymocytes using complement lysis followed by positive selection with Dynabeads. Briefly, cells were coated with anti-CD4 mAb (RL1.172.4) (Ceredig et al., 1985) or anti-CD8 mAb (3.168.8.1) (Sarmiento et al., 1982) followed by complement lysis using low tox rabbit complement (Cedarlane, Ontario, Canada) and 100µg/ml DNase I (Boehringer Mannheim, Mannheim, Germany). After removal of dead cells in a Ficollgradient (Lympholyte M, Cedarlane), cells were positively selected on CD8 or CD4 using Dynabeads. The remaining cells were 99% $CD8^-CD4^-$ double negative cells.

For removal of $CD8^+CD4^+$ thymocytes with Hydrocortisone in vivo, C57BL/6 mice were injected i.p. with 6.25mg Hydrocortisoneacetate (Sigma-Aldrich) and thymi were isolated two days later.

Cytotoxicity assay. Ex vivo cytolytic activity of spleen cells was determined in a ^{51}Cr-release assay as described (Zinkernagel et al., 1985). Briefly, single spleen cell suspensions from mice infected with LCMV-WE 8 days prior to testing were prepared in IMDM containing 10% FCS. EL-4 lymphoma or B16 melanoma target cells were coated with LCMV peptide GP33 at a concentration of 1μM, and were labelled with 250μCi ^{51}Cr for 2 hours at 37°C on a rocking platform. NK-sensitive target cells YAC1 were only labelled with ^{51}Cr. Target cells were washed three times. 10^4 target cells were incubated in 96-well round bottom plates with spleen effector cells or Hy-Ad9 culture cells at the ratios indicated in the figures. After a 5-hour or 15-hour incubation period at 37°C, 70μl supernatants were harvested and analysed. Spontaneous release was 10% for 5-hours and 20% -40% for 15 hours.

Flow cytometry. For the detection of mMAFA on the surface of $CD8^+$ T cells, spleen cells and peripheral blood lymphocytes (PBL) were stained with anti-mMAFA mAb 2F1 (Hanke et al., 1998), followed by affinity-pure PE-labelled goat-anti-hamster IgG (Caltag, San Francisco, CA). Anti-mMAFA 2F1 mAb was a generous gift of T. Hanke (University of California, Berkely, CA). Free binding sites were blocked with 2μl rat- or mouse serum (depending on the next antibody) and cells were stained with FITC-labelled anti-CD8 mAb and biotinylated anti-CD11b (Mac-1), anti-CD8 or anti Thy1.1 mAb (all from Pharmingen, San Diego, CA) followed by Tricolor-streptavidin (Caltag). For surface staining of spleen cells, PBL or culture cells, cells were stained with FITC-labelled anti-CD8 mAb, PE-labelled anti-Vα2, anti-CD4 and anti-B220 and biotinylated anti-Vβ8, anti-CD69 or anti-CD25 (IL-2Rα chain) mAb (all from Pharmingen), followed by Tricolor-streptavidin (Caltag). Spleen cells or PBL of rats were stained with mouse anti-rat MAFA mAb G63 (Soto and Pecht, 1988) followed by affinity-pure PE-labelled goat-anti-mouse IgG (Caltag) and FITC-labelled anti-CD8 and anti-CD4 mAb and biotinylated anti-CD62L mAb (all from Pharmingen) followed by PE-streptavidin (Caltag). mAb G63 was a generous gift of I. Pecht (The Weizmann Institute of Science, Rehovot, Israel). Staining of PBL was performed in PBS containing 2% FCS, 0.1% NaN_3 and 10U/ml heparin (Liquemin; Roche). PBL were analysed after lysis of red blood cells using the FACS-Lysing Solution (Becton Dickinson &Co., San Jose, CA) according to the instructions of the manufacturer. Staining of spleen cells was carried out in PBS containing 2% FCS and 0.1% NaN_3. Cells were analysed on a

FACScan flow cytometer (Becton Dickinson & Co.). Data were collected from viable cells gated by a combination of forward light scatter and 90° side scatter.

For the detection of virus-specific T cells with tetrameric MHC class I-peptide complexes, PBL of LCMV-infected mice were stained with Allophycocyanin-labelled complexes containing LCMV peptides GP33-41 and NP396-404. Tetrameric complexes were generated in the laboratory of R. Ahmed (Emory University, Atlanta, GA) and generously provided by him.

For separation of mMAFA positive and mMAFA negative cells, P14 TCR-Thy1.1 transgenic spleen cells were isolated 8 days after adoptive transfer into C57BL/6 recipients and purified by negative selection with Dynabeads. The remaining cells were stained with antibodies specific for mMAFA and CD8 and sorted at a speed of 3000 cells per second in a FACSort (Becton Dickinson & Co.) into a $CD8^+$ $mMAFA^+$ and a $CD8^+$ $mMAFA^-$ T cell population.

Differential Display. Total RNA was isolated with a RNA Isolation Kit (Fluka Chemie AG, Buchs, Switzerland) according to the manufacturers protocol. For cDNA synthesis, total RNA was first treated with DNase to remove genomic DNA: 10-50µg total RNA was incubated in 50µl Diethylpyrocarbonate-treated water containing 50mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 20 units RNase free DNase and 20 000 units RNasin (both from Boehringer Mannheim, Mannheim, Germany) at 37°C for 20 minutes. 2µg of total RNA from naive, effector and memory T cells was used for first strand cDNA synthesis with the First Strand cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden). Synthesis was carried out in a volume of 10µl with an oligo-$(dT)_9$ primer according to the manufacturers instructions.

The product of each cDNA reaction was diluted 20-fold in H_2O and 1µl was used to perform the subsequent PCR reactions. The differential display PCR reactions were performed using the DELTA RNA Fingerprinting Kit (Clontech Laboratories, Inc. Palo Alto, CA), Advantage Klen Taq Polymerase Mix (Clontech, CA) and (α-^{33}P) dATP (Amersham International plc, Little Chalfont, United Kingdom) according to the manufacturers protocol. The use of the Advantage Klen Taq Polymerase Mix allows efficient and accurate amplification of cDNAs using a combination of a primary DNA polymerase and a minor amount of a proofreading polymerase in addition to a TaqStart Antibody to provide automatic hot start PCR. For PCR reactions, 1µl of the cDNA reactions was combined with 1µM of each differential display primer, 1xKlen Taq PCR reaction buffer, 50µM of each dNTP, 50nm of (α-^{33}P) dATP and 1xAdvantage Klen Taq Polymerase Mix. PCR´s were performed in a GeneAMP PCR system 9600 (Perkin Elmer, Foster City, CA) using the following conditions: incubation at 94°C for 5 min, 40°C for 5 min and 68°C for 5 min for one cycle, followed by 94°C for 2 min, 40°C for

5 min and 68°C for 5 min for two cycles, followed by 94°C for 1 min, 60°C for 1 min and 68°C for 2 min for 25 cycles. 4µl of the PCR reaction (total 20µl) were run on a 5% acrylamide (Long Ranger Gel Solution, Biozym, Hess. Oldendorf, Germany), 7.5M urea sequencing gel at 55W constant power. Gels were dried onto Whatman 3MM filter paper (Bender&Hobein GmbH, Bruchsal, Germany) and subjected to overnight autoradiography on Kodak BIOMAX^{TM}MR film (Amersham International plc, UK).
Bands that appeared to be consistently differential in multiple RNA preparations were excised from dried sequencing gels, rehydrated in 40µl water for 30 min at room temperature followed by heating for 15 min at 100°C to elute the DNA. Reamplification by PCR was performed with 7µl of DNA eluate in 50µl total volume using the appropriate differential display primers and performing 20 cycles of 94°C for 1 min, 60°C for 1 min and 68°C for 2 min. The PCR products were excised from a 1% agarose gel (Biozym, Hess. Oldendorf, Germany) and directly sequenced using the appropriate differential display primers and the Sequenase PCR Product Sequencing Kit (USB, Cleveland, OH) according to the manufacturers instructions. Sequences obtained were compared to the EMBL GenBank (Heidelberg, Germany) using the BLASTN algorithm.

Generation of full length cDNA clones. To obtain full length LGALS1 cDNA (EMBL database accession number M57470), RT-PCR of cDNA of the effector T cell population was performed using LGALS1 specific primers TTGAGGGATCCA-GGCTGGCTGGCTTCACTC (5′ primer) and CTTCGCTTAAGCTTCAATCATGGC-CTGTGG (3′ primer). The primer sequences have been modified to introduce BamH1 (3' primer) and HindIII (5' primer) restriction sites. The PCR product was cloned into the pGEM-T vector (Promega, Madison, WI) and the 450 basepair HindIII-BamH1 fragment was used for Northern blot analysis and in situ hybridisation.
Full length cDNA of mMAFA was obtained using long distance RT-PCR and the SMART PCR cDNA Synthesis Kit (Clontech) according to the manufacturers instructions. Briefly, ds cDNA of the effector T cell population was synthesised using an oligo $(dT)_{30}$ primer and the SMART-oligo which binds to the 5' end of the mRNA. For subsequent PCR reactions, ds cDNA was 5x diluted in H_2O and 5µl of the cDNA reaction was combined in 100µl total volume with 1x Klen Taq PCR buffer, 200mM of each dNTP, 1x Advantage Klen Taq Polymerase Mix, 200nM SMART PCR primer (binding to the SMART-oligo) and 200nM mMAFA specific primer CAGTGGCATCCATCAGTATAGGACC (binding to bp 626-650 of mMAFA). 24 cycles were performed with 95°C for 15 sec, 65°C for 30 sec and 68°C for 6 min. The obtained PCR product was reamplified using the same protocol as above and the product was cloned into the pGEM-T vector and sequenced. After reaching the 5' end

of mMAFA RNA by SMART PCR, RT-PCR of cDNA of the effector T cell population was performed with mMAFA specific primers CCCTCGAGCTGACAGCTCTATC (5' primer) and GGAGATCTTCAGTATAGGACCTTC (3' primer) to obtain full length cDNA. The cDNA sequence is available under the EMBL database accession number AJ010751.

Northern Blot Analysis. 5-10μg of total RNA were separated on a 1.2% agarose/ 1x MOPS/ 3.7% formaldehyde gel and transferred overnight to nylon membranes (GeneScreen Hybridisation Transfer Membrane, Du Pont (Europe), Brussels, Belgium). DNA probes were synthesised using the DECAprimeTM II Kit (Ambion, Inc., Austin, TX) and (α-^{32}P) dCTP (Amersham International plc, Little Chalfont, United Kingdom) according to the manufacturers protocol. Northern blots were hybridised at 42°C overnight and subsequently washed for 15 min in 2XSSPE at RT, 45 min in 2xSSPE/2% SDS at 65°C and 15 min in 0.1xSSPE at RT. Northern blots were exposed for 6-8 hours for LGALS1 and 1-2 days for mMAFA at -70°C to BIOMAX^{TM}MS film (Amersham International plc, Little Chalfont, United Kingdom). As probes the 400bp fragment of LGALS1 cDNA and the 600bp fragment of mMAFA cDNA, both representing the entire coding regions were used. The mouse skeletal β-actin probe was amplified from cDNA by PCR using 5' (ATGGATGACGATATCGCT) and 3' (ATGAGGTAGTCTGTCAGGT) primers.

Southern Blot Analysis. Genomic DNA was isolated from the kidneys of C57BL/6 mice according to standard protocols (Sambrook et al., 1989) and 10μg samples were digested for 4 hours with the restriction enzymes indicated. The digests were separated on a 0.8% agarose gel and transferred to nylon membranes (GeneScreen Hybridisation Transfer Membrane, Du Pont (Europe), Brussels, Belgium) using the alkaline transfer protocol described by the manufacturer. Hybridisation was carried out overnight at 42°C using the full length mMAFA cDNA as a probe. Blots were washed for 10 min in 2xSSC at RT, for 20 min in 2xSSC/1%SDS at 42°C and for 20 min in 0.2xSSC/1%SDS at 42°C. Southern blots were exposed overnight to a BIOMAX^{TM}MS film (Amersham International plc, Little Chalfont, United Kingdom).

Western Blot Analysis. 20ml of cell free supernatant of in vitro stimulated CD8^{+} T cells were 100-times concentrated by immunoaffinity chromatography using the IgG fraction of the neutralising anti-βGBP monoclonal antibody clone B2 (Wells and Mallucci, 1991). For electrophoretic analysis of proteins, samples were run on a SDS 12.5% polyacrylamide gel and blotted onto nitrocellulose under standard conditions (Laemmli, 1970). The protein was detected by chemiluminiscence method (ECL-kit,

Amersham International plc, Little Chalfont, United Kingdom) following incubation with monoclonal antibody clone B2 and horseradish peroxidase conjugated anti-mouse IgG.

In Situ Hybridisation. In situ hybridisation was performed on 5-µm-thick frozen sections of the spleen as described (Müller et al., 1989). Labelled sense and antisense RNA probes were derived from the 450-basepair HindIII-BamHI fragment of the LGALS1 gene and the 521-basepair EcoRI-HindIII fragment of the 5′end of the perforin gene. Hybridised tissue sections were dipped into NTB2 nuclear track emulsion (Kodak, Rochester, NY) diluted 1:2 with 600mM ammonium acetate buffer. Sections hybridised with a ^{35}S-labelled RNA antisense or sense probe of LGALS1 were exposed for 28 days at 4°C in a light tight box. Slides were developed with Kodak developer PL-12 for 2.5 min and fixed with Kodak fixer for 5 min at RT. Counter staining was done with nuclear fast red (0.05% (wt/vol) aluminium sulphate).

Retroviral transfection of the T cell line A5 with a chimeric mouse-rat MAFA construct. The chimeric mouse-rat MAFA molecule was generated by combination of the cytoplasmic tail of mMAFA with the extracellular tail of rMAFA. Corresponding cDNA fragments were amplified by PCR from plasmids containing the full length cDNA for mMAFA and rMAFA respectively. Following primers were used for PCR reactions: 5′ primer CTCGAGGTAGAGATGGCTGACAGCTCT introducing a XhoI restriction site and 3′ primer GGAGATCTTCAGTATAGGACCTTC for mMAFA and 5′primer GGAATTCGTGGAGATGGCCGACAAC and 3′primer CGGGATCCGTCA-GGGCAGGACCTTCTC introducing a BamHI restriction site for rMAFA. Both fragments were cloned, sequenced and digested with the according restriction enzymes (AccI and XhoI for mMAFA and AccI and BamHI for rat MAFA). After several cloning steps, the chimeric molecule was inserted into the retroviral vector pLXSN (Clontech) using XhoI and BamHI restriction sites. The moloney murine leukaemia virus derived retroviral vector pLXSN containing a Neomycin resistance gene was used for the delivery and expression of the chimeric mouse-rat MAFA in the A5 T cell line. The T cell line A5 was derived from TCR transgenic mice expressing a TCR (Vα4/Vβ8) specific for the influenza virus peptide HA 110-119 (SFERFEIFPK) in association with I-E^{d} (Kirberg et al., 1994) and was obtained from E. Palmer and K. Karjaleinen (Basel Institute for Immunology, Basel, Switzerland). It expresses a Hygromycin-resistance and the green fluorescence protein (GFP) under the control of the NF-AT-promotor. Activation of these cells by Vβ8-crosslinking leads to GFP expression. pLXSN containing the chimeric mouse-rat MAFA sequence was then transfected into the packaging cell line BOSC (ATCC) by Ca^{2+} precipitation. Briefly,

$2x10^6$ BOSC cells were plated in a T75 flask with 7ml IMDM containing 10% FCS for 8 hours. 10µg plasmid DNA was diluted in up to 500µl H_2O and filtered through a 0.2µm syringe filter into 125µl ice-cold 2M $CaCl_2$. This DNA solution was dropwise added into 1.1ml ice-cold, bubbling 2xHEPES buffered saline. The DNA was added to the flask containing the BOSC cells and incubated over night at 37°C. Medium was exchanged several times and virus-containing supernatant was harvested on day 2. 10^6 A5 T cells were plated in 1ml medium into a well of a 6-well plate. After addition of 2-20µl 4mg/ml DEAE-Dextran (Pharmacia Biotech, Uppsala, Sweden), 1ml of virus-supernatant was added and the cells were incubated for 6-8 hours. After incubation, 5ml G418 containing selection media (IMDM, 5% FCS, 0.5 mg/ml Hygromycin (Boehringer Mannheim), 1mg/ml G418 (Gibco BRL) was added and cells were cultured at 37°C.

For activation, transfected cells and untransfected control cells were coated with anti-Vβ8 mAb (F23.1) (Staerz et al., 1985) followed by incubation for 8 hours at 37°C in 96-well ELISA plates coated with different concentrations of goat anti-mouse IgG (Caltag). For additional crosslinking of rMAFA cells were coated with G63 mAb or an isotype control. The percentage of activated GFP positive cells was determined by flow cytometry.

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8. ABBREVIATIONS

aa	amino acids
APC	antigen presenting cell
βGBP	beta-galactoside-binding protein
bio	biotin
BMMC	bone marrow derived mast cells
cDNA	complementary deoxyribonucleic acid
ConA	Concanavalin A
Cr	chromium
CTL	cytotoxic T lymphocyte
DD	differential display
dNTP	deoxynucleotide triphosphate
FCS	fetal calf serum
FITC	fluoresceinisothiocynat
GFP	green fluorescent protein
GP33	peptide aa 33-41 derived from LCMV-glycoprotein
H-2	major histocompatibility complex of the mouse
IMDM	Iscove's modified Dulbecco Medium
i.p.	intraperitoneal
IR	inhibitory receptor
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
i.v.	intravenous
KIR	killer cell inhibitory receptor (Ig-superfamily)

LAK	lymphokine activated killer (cell)
LCMV	lymphocytic choriomeningitis virus
LGALS1	lectin, galactoside-binding, soluble
LPS	lipopolysaccharide
mAb	monoclonal antibody
mMAFA	mouse mast cell function-associated antigen
rMAFA	rat mast cell function-associated antigen
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NK	natural killer (cell)
NP396-404	peptide aa 396-404 derived from LCMV-nucleoprotein
PBL	peripheral blood lymphocytes
PCR	polymerase chain reaction
PE	phycoerythrin
pfu	plaque forming units
PMA	Phorbolmyristateacatate
RT	room temperature
RT-PCR	reverse transcriptase PCR
SC	spleen cells
SDS	sodium dodecyl phosphate
TCR	T cell receptor
TNF	tumour necrosis factor
VSV	vesicular stomatitis virus
VV	vaccinia virus

9. CURRICULUM VITAE

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Education

1977-1982	Primarschule Schneisingen
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1990	Maturity Typus B
1990-1995	Studies in Biology at the Swiss Federal Institute of Technology (ETH), Zurich, Switzerland
1993-1994	Diplomathesis at the Institute of Experimental Immunology, Zurich under the supervision of Prof. H. Hengartner and Prof. R. M. Zinkernagel. Title: "Aktivierung und Tolerisierung peripherer, zytotoxischer T-Zellen mit Peptiden und Lipopeptiden"
1995	Dipl. Natw. ETH
1995-1999	PhD. student of the ETH Zurich. PhD. thesis at the Institute of Medical Microbiology and Hygiene, Freiburg, Germany under the supervision of Prof. H. Hengartner (Institute of Experimental Immunology, Zurich) and Prof. H. Pircher (Institute of Medical Microbiology and Hygiene, Freiburg). Topic: Identification and characterisation of differentially expressed genes in naive and activated $CD8^+$ T cells.

10. LIST OF PUBLICATIONS

C. Zimmermann, K. Brduscha-Riem, **C. Blaser**, R. M. Zinkernagel and H. Pircher (1996). Visualisation, characterisation and turnover of $CD8^+$ memory T cells in virus-infected hosts. J. Exp. Med. 183:1367-1375

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M. Kaufmann, **C. Blaser**, S.Takashima, R. Schwartz-Albiez, S. Tsuji and H. Pircher (1999). Identification of an α2,6 sialyltransferase induced early after lymphocyte activation. Int. Immunol. In press.

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12. REPRINTS

β-galactoside-binding protein (β-GBP) secreted by activated T cells inhibits antigen-induced proliferation of T cells. Eur. J. Immunol. 1998, 8:2311-2319

Virus-activated CD8 T cells and lymphokine-activated NK cells express the mast cell function-associated antigen MAFA, an inhibitory C-type lectin. J. Immunol. 1998, 161:6451-6454.

β-Galactoside-binding protein secreted by activated T cells inhibits antigen-induced proliferation of T cells

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We have used mRNA differential display PCR to search for genes induced in activated T cells and have found the LGALS1 (lectin, galactoside-binding, soluble) gene to be strongly up-regulated in effector T cells. The protein coded by the LGALS1 gene is a β-galactoside-binding protein (βGBP), which is released by cells as a monomeric negative growth factor but which can also associate into homodimers (galectin-1) with lectin properties. Northern blot analysis revealed that *ex vivo* isolated $CD8^+$ effector T cells induced by a viral infection expressed high amounts of LGALS1 mRNA, whereas LGALS1 expression was almost absent in resting $CD8^+$ T cells. LGALS1 expression could be induced in $CD4^+$ and $CD8^+$ T cells upon activation with the cognate peptide antigen and high levels of LGALS1 expression were found in concanavalin A-activated T cells but not in lipopolysaccharide-activated B cells. Gel filtration and Western blot analysis revealed that only monomeric βGBP was released by activated $CD8^+$ T cells and *in vitro* experiments further showed that recombinant βGBP was able to inhibit antigen-induced proliferation of naive and antigen-experienced $CD8^+$ T cells. Thus, these data indicate a role of βGBP as an autocrine negative growth factor for $CD8^+$ T cells.

Key words: β-Galactoside-binding protein / T cell / Growth inhibition / LGALS1 / Differential display

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1 Introduction

The identification of genes induced in activated T cells may lead to new molecular markers useful for the better characterization and the understanding of functional events in effector or memory T cells. In this report we have analyzed gene expression patterns in naive, effector and memory $CD8^+$ T cells in the murine model of LCMV infection using a modified form of the recently developed technique of mRNA differential display [1]. Using this technique we have identified a gene which is strongly expressed in activated but not in resting T cells. Sequence analysis of the cDNA isolates revealed 100 % homology with the coding sequence of the LGALS1 gene [2, 3], which encodes the 14.735-kDa β-galactoside-binding protein (βGBP). Cells release this protein in its native state as a monomer [4, 5] which can also associate as a lectin homodimer (galectin-1) [3, 5, 6].

Galectin-1 has been assigned to the family of soluble (S-type) lectins which have been isolated from many tissues. Presence of galectin-1 in the extracellular matrix [7] suggests a possible role in cell-cell interaction and cell adhesion [8] via glycoconjugate binding on opposing surfaces. This includes also tumor cell adhesion to the endothelium, an interaction with possible relevance in metastasis [9]. Galectin-1 is unstable in the absence of reducing agents [10, 11] and it dissociates into monomers with a K_d of 7 μM [5]. Recently it has been shown that activated peripheral T cells and thymocytes undergo apoptosis when treated with high concentrations (20 μM) of recombinant galectin-1 [12, 13]. Apoptosis is thought to occur via cross-linking of glycosylated surface pro-

[I 17935]

Abbreviations: βGBP: β-galactoside-binding protein **LGALS1:** Lectin, galactoside-binding, soluble

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teins, an effect well in line with the lectin nature of the dimeric form of the protein.

In its monomeric form the protein acts as a negative regulator of the cell cycle. Secreted by the cell, monomeric βGBP binds with high affinity to about 5×10^4 receptor sites per cell with a K_d of 10^{-10} M through molecular domains other than those that link saccharide determinants to control G_0/G_1 and S/G_2 transition [4]. The data presented here provide the first evidence for the expression of the LGALS1 gene and the release of monomeric βGBP by activated T cells and show that T cells respond to the growth inhibitory effect of βGBP.

2 Results

2.1 LGALS1 mRNA is expressed in CD8+ effector and memory T cells isolated *ex vivo*

We performed mRNA differential display PCR to identify genes differentially expressed in naive, effector and memory $CD8^+$ T cells. We identified a band that appeared only in the cDNA from effector and memory T cells but not in the cDNA from naive T cells. DNA eluted from this band was amplified by PCR with the original primers and sequenced. The sequence revealed 100 % homology with the coding sequence of the LGALS1 gene. To confirm the results of the differential display analysis, Northern blot analysis of the RNA from naive, effector and memory cells was performed using a full-length LGALS1 cDNA probe. As shown in Fig. 1, naive $CD8^+$ T cells expressed low levels of LGALS1 mRNA (Fig. 1, lane 1), while *ex vivo* isolated $CD8^+$ effector T cells expressed very high levels (Fig. 1, lane 2). LGALS1 mRNA was still expressed in $CD8^+$ memory T cells (Fig. 1, lane 3) but to a lower extent than in effector T cells. To test whether LGALS1 expression was also induced in $CD8^+$ T cells in normal mice during viral infection, Northern blot analysis of mRNA from spleen cells of acutely LCMV-infected C57BL/6 mice was performed. As shown in Fig. 1, lane 4, spleen cells from day 8 LCMV immune mice expressed LGALS1 at levels comparable to those of purified TCR-transgenic effector T cells (lane 2). To demonstrate that increased LGALS1 expression was due to $CD8^+$ effector T cells, RNA was isolated from day 8 LCMV immune spleen cells that were depleted of $CD8^+$ T cells by negative selection *in vitro*. As shown in Fig. 1, lane 5, immune spleen cells depleted of $CD8^+$ T cells expressed only a small amount of LGALS1 mRNA close to that of the naive $CD8^+$ T cells (lane 1). This result confirms that the high level of LGALS1 expression can be attributed to $CD8^+$ effector T cells and is not due to contamination of the cell preparations.

2.2 Kinetics of LGALS1 expression induced by peptide antigen *in vivo*

Injection of the LCMV glycoprotein peptide 33–41 (GP33) peptide into P14 TCR-transgenic mice leads to transient activation of TCR-transgenic T cells [14]. To test

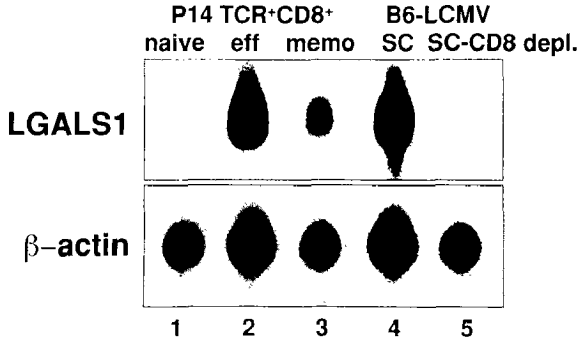

Figure 1. Northern blot analysis of LGALS1 expression in different T cell populations isolated *ex vivo*. Lane 1: naive $CD8^+$ T cells isolated from uninfected P14 TCR-transgenic mice. Lane 2: $CD8^+$ P14 TCR-transgenic effector T cells isolated 8 days after LCMV infection. Lane 3: $CD8^+$ P14 TCR-transgenic memory T cells isolated 4 weeks after LCMV infection. Lane 4: spleen cells (SC) from day 8 LCMV immune C57BL/6 mice. Lane 5: $CD8^+$ T cell-depleted spleen cells (SC) from day 8 LCMV immune C57BL/6 mice. For each lane 5 μg of total RNA were loaded. Hybridization with a β-actin probe shows equivalent sample loading.

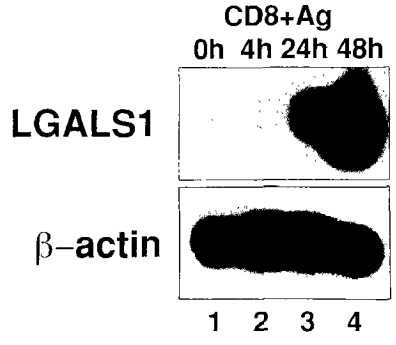

Figure 2. Northern blot analysis of LGALS1 expression in $CD8^+$ T cells after peptide antigen treatment *in vivo*. P14 TCR-transgenic mice were injected once with 500 μg LCMV GP33 peptide i.p. The mice were killed at the indicated time points after peptide injection. The blot shows LGALS1 expression in $CD8^+$ T cells isolated from untreated animals (lane 1) and from peptide antigen-treated mice 4 h, 24 h and 48 h after peptide injection (lane 2, 3 and 4, respectively). For each lane 5 μg of total RNA were loaded. Equivalent sample loading was assured by hybridization with a β-actin probe.

whether stimulation with a soluble peptide antigen also induces LGALS1 mRNA expression, $CD8^+$ T cells were isolated from P14 TCR-transgenic mice that had been treated once with 500 μg of the LCMV GP33 peptide. Fig. 2 shows Northern blot analysis of $CD8^+$ T cells isolated at various time points after peptide application. $CD8^+$ T cells from untreated animals (Fig. 2, lane 1) expressed low levels of LGALS1 mRNA. Twenty-four hours after peptide treatment, LGALS1 expression was induced (Fig. 2, lane 3) and further increased at 48 h after peptide treatment (Fig. 2, lane 4) reaching levels comparable to those seen in effector T cells (Fig. 1, lanes 2 and 4). This result indicates that in $CD8^+$ T cells LGALS1 expression is induced by 24 h and further enhanced until 48 h after interaction of their TCR with the cognate peptide antigen in the absence of a viral infection.

2.3 $CD4^+$ and $CD8^+$ T cells, but not B cells, express high levels of LGALS1 mRNA upon stimulation *in vitro*

To assess LGALS1 expression after stimulation *in vitro* $CD8^+$ T cells from P14 TCR-transgenic mice were stimulated *in vitro* with LCMV GP33 peptide. At 3 and 8 days after peptide stimulation the cultures were harvested. RNA was isolated and Northern blot analysis was performed. As shown in Fig. 3, lanes 2 and 3, *in vitro* activation of $CD8^+$ T cells induced high levels of LGALS1 mRNA. Naive spleen cells (Fig. 3, lane 1) showed only marginal expression of LGALS1.

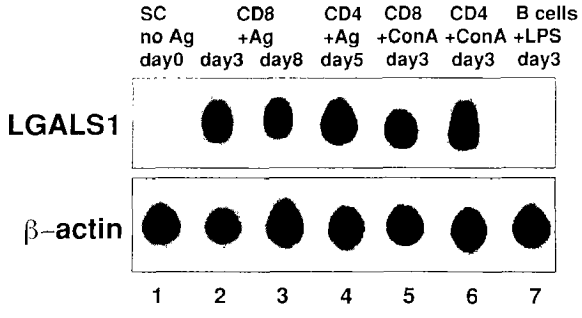

Figure 3. Northern blot analysis of LGALS1 expression in $CD8^+$ and $CD4^+$ T cells and B cells activated *in vitro*. Lane 1: Spleen cells (SC) from naive C57BL/6 mice. Lanes 2 and 3: $CD8^+$ T cells from P14 TCR-transgenic mice 3 and 8 days after peptide stimulation. Lane 4: $CD4^+$ T cells from 2B4 TCR-transgenic mice 5 days after peptide stimulation. Lanes 5 and 6: Con A-activated $CD8^+$ and $CD4^+$ T cells from C57BL/6 mice 3 days after mitogen stimulation. Lane 7: LPS-activated B cells from C57BL/6 mice 3 days after mitogen stimulation. Of total RNA, 5 μg was loaded per lane. Hybridization with a β-actin probe shows equivalent sample loading.

To examine whether activated $CD4^+$ T cells also express LGALS1 mRNA, spleen cells from 2B4 TCR-transgenic mice were stimulated *in vitro* with the cognate MCC peptide. Five days after peptide stimulation $CD4^+$ T cells were purified and RNA was isolated. Lane 4 in Fig. 3 shows that peptide antigen-activated $CD4^+$ T cells also expressed LGALS1 mRNA. Similarly, Con A-activated $CD8^+$ as well as $CD4^+$ T cells both expressed high LGALS1 mRNA levels (Fig. 3, lanes 5 and 6). Interestingly, however, LPS-activated B cells only expressed LGALS1 mRNA at low levels close to those of the naive spleen cells (Fig. 3, lanes 7 and 1). Thus, in contrast to antigen- or mitogen-activated $CD4^+$ and $CD8^+$ T cells (Fig. 3, lanes 2, 3, 4, 5 and 6), LPS-activated B cells showed only weak LGALS1 expression.

2.4 LGALS1 mRNA expression in cell lines of lymphoid origin

LGALS1 expression was analyzed in a number of T and B cell lines by Northern blotting. As shown in Fig. 4, LGALS1 mRNA was expressed in all cell lines of T cell origin tested (Fig. 4, lanes 1–7) including EL4, RMA-S and BW 5147 lymphoma cells as well as cloned CTL lines (CTLL-2, Hy-Ad9) and T cell hybridomas (BWLy2-3, IT H6/A11). In contrast, J558 and P3-X63Ag8 myeloma cells (Fig. 4, lanes 8 and 9) and three B cell hybridomas tested (Fig. 4, lanes 10–12) did not express LGALS1 mRNA at detectable level. These results agree well with the findings above, namely that high LGALS1 mRNA expression is a characteristic of activated T cells but not of B cells.

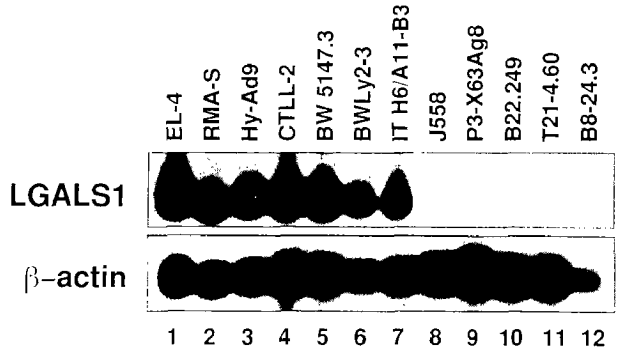

Figure 4. Northern blot analysis of LGALS1 expression in cell lines. Five micrograms of total RNA isolated from lymphoma (EL4, RMA-S, BW 5147), cloned CTL (CTLL-2, Hy-Ad9), T cell hybridoma (BWLy2-3, IT H6/A11), myeloma (J558, P3-X63Ag8) and B cell hybridoma (B22.249, T21-4.60, B8-24-3) cell lines were loaded per lane. Equivalent sample loading was assured by hybridization with a β-actin probe.

2.5 LGALS1 mRNA expression analyzed by *in situ* hybridization

LGALS1 expression in the spleens of uninfected and LCMV-infected C57BL/6 mice at day 8 was analyzed by *in situ* hybridization using a radiolabeled LGALS1 antisense RNA probe. A strong signal was observed in LCMV-infected mice (Fig. 5B) but not in the uninfected controls (Fig. 5A). The LGALS1-positive cells were mainly localized in the red pulp. A similar pattern was found when the splenic sections were analyzed by *in situ* hybridization using a radiolabeled perforin antisense RNA probe which specifically detects $CD8^+$ effector CTL (Fig. 5C, D); however, the signal obtained for LGALS1 was much stronger than the one for perforin.

2.6 βGBP protein release by $CD8^+$ T cells after peptide stimulation *in vitro*

Supernatant of $CD8^+$ spleen cells from P14 TCR-transgenic mice, which had been stimulated *in vitro* with the LCMV GP33 peptide, was analyzed by Western blotting. We found a marked expression of monomeric βGBP in its 15000 M_r form and a faint expression of monomeric βGBP in its glycan-linked 18000 M_r form [15]. No evidence of release of the protein in its dimeric galectin-1 form was found as examined by gel filtration (data not shown). βGBP released in the cell-free supernatant by activated $CD8^+$ T cells was detected on day 1 (Fig. 6, lane 2) with a further strong increase on day 2 (lane 3) and 3 (lane 4) after antigen stimulation. $CD8^+$ T cells which had been stimulated for only 4 h did not show marked release of βGBP (lane 1). These results are in full accordance with the expression of LGALS1 mRNA shown in Fig. 2. Densitometric analysis using recombinant βGBP as a standard revealed that the amount of βGBP released corresponded to 31.2 ng/ml (2 nM), a 7–8 fold increase over the basal level of the naive cells. It is noteworthy that this amount of βGBP secreted by the activated T cells is about 100- to 1000-fold higher than that constitutively released by mouse embryonic fibroblasts (data not shown).

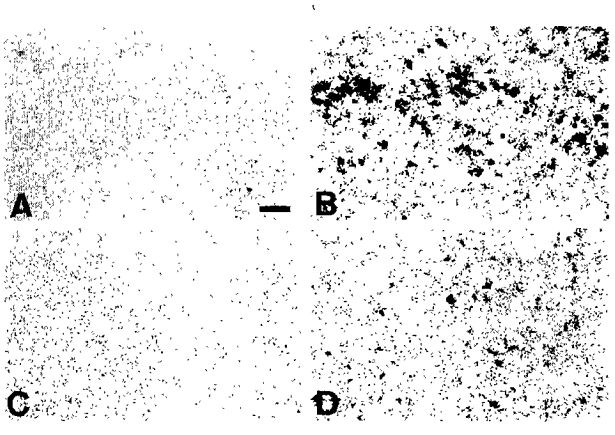

Figure 5. In situ hybridization of LGALS1 and perforin mRNA. Spleen sections from uninfected C57BL/6 mice (A, C) and from day 8 LCMV immune mice (B, D) were hybridized with a ^{35}S-labeled RNA antisense probe for the LGALS1 gene (A, B) or the perforin gene (C, D). Hybridization was specific since labeling with the corresponding LGALS1/perforin sense probe did not yield significant staining (not shown). Bar in A = 64 μm.

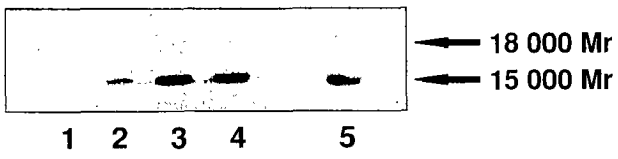

Figure 6. Western blot analysis of βGBP release. Spleen cells of P14 TCR-transgenic mice were incubated with 10^{-6} M GP33 peptide and cell-free supernatant was collected at 4 h (lane 1), 1 day (lane 2), 2 days (lane 3) and 3 days (lane 4) after peptide stimulation. Supernatants were concentrated 100 times by affinity chromatography. A 15000 M_r and an 18000 M_r band comigrate with the corresponding bands of recombinant murine βGBP (200 ng, lane 5).

2.7 Recombinant βGBP inhibits antigen-induced proliferation of naive and primed $CD8^+$ T cells

To address the functional role of βGBP secretion by activated T cells, we analyzed the growth inhibitory activity of βGBP on T cells. P14 TCR-transgenic T cells were stimulated *in vitro* with the LCMV GP33 peptide in the presence of varying concentrations of recombinant βGBP (r-mβGBP) [4]. As depicted in Fig. 7a, TCR-transgenic T cells proliferated vigorously during *in vitro* culture with the cognate peptide antigen. The proliferative response of the TCR-transgenic T cells was inhibited in a dose-dependent manner after addition of r-mβGBP: 350 ng/ml r-mβGBP (25 nM) reduced the antigen-specific proliferation to 50 %, and 1400 ng/ml r-mβGBP (100 nM) effectively abolished proliferation. Addition of r-mβGBP to the cultures did not affect cell viability and early steps in T cell activation since CD69 and CD25 up-regulation and TCR down-regulation of TCR-transgenic T cells were identical in the presence or absence of r-mβGBP (data not shown). This finding also rules out any generally toxic effect of r-mβGBP.

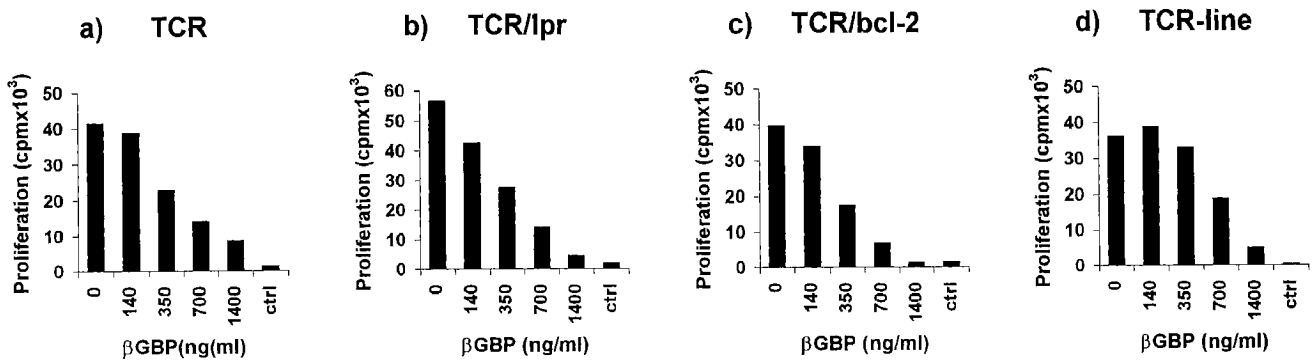

Figure 7. Inhibition of LCMV GP33 peptide-induced T cell proliferation by βGBP. Spleen cells from P14 TCR (a), P14 TCR/lpr (b), P14 TCR/bcl-2 mice (c) and an antigen-experienced $CD8^+$ T cell line derived from P14 TCR mice (d) were stimulated with LCMV GP33 peptide (a–c) or GP33 peptide-loaded B6 spleen cells (d) in the presence of the indicated concentrations of r-mβGBP. Proliferation was measured after 2 days by [^{3}H]thymidine incorporation. As a control (ctrl), proliferation of cells without GP33 peptide and without βGBP is shown. Shown is one representative experiment of two.

To examine the role of apoptosis-regulating genes, such as Fas/CD95 and bcl-2, in the context of the growth inhibitory activity of r-mβGBP, spleen cells of Fas-deficient P14 TCR/lpr mice and from P14 TCR/bcl-2 doubly transgenic mice overexpressing the anti-apoptotic protein Bcl-2 were stimulated with LCMV GP33 peptide *in vitro* in the presence of r-mβGBP. As shown in Fig. 7 b and c, spleen cells from TCR/lpr and TCR/bcl-2 mice were as susceptible to the growth inhibitory activity of r-mβGBP as the TCR-transgenic spleen cells (Fig. 7 a). This result argues against a crucial role of these two genes in the βGBP-induced inhibitory signaling pathway.

Finally, we tested whether βGBP also affected antigen-induced proliferation of antigen-experienced T cells. As shown in Fig. 7 d, r-mβGBP inhibited LCMV GP33 peptide-induced proliferation of a $CD8^+$ T cell line derived from P14 TCR-transgenic mice in a dose-dependent manner. Taken together, these data show that nanomolar amounts of r-mβGBP were able to block antigen-induced proliferation of naive and primed $CD8^+$ T cells and that the growth inhibitory activity of r-mβGBP was independent of Fas/CD95 and Bcl-2 expression.

3 Discussion

The product of the LGALS1 gene can be expressed as a homodimer (galectin-1) with lectin properties and as a monomer (βGBP) with negative cell growth regulatory properties. In the present report we demonstrate (1) the induction of LGALS1 expression upon T cell activation, (2) the release of monomeric βGBP by activated T cells and (3) the growth inhibitory properties of recombinant βGBP on antigen-activated T cells.

Different scenarios relating to the dimeric and monomeric form of the LGALS1 gene product in immunomodulation need to be discussed. Perillo et al. [12, 13] have recently demonstrated that at a concentration of 20 μM the dimer galectin-1 can rapidly induce apoptosis of immature thymocytes and activated, but not naive, peripheral T cells. The apoptotic effect could be inhibited by the addition of lactose and, therefore, appeared to be mediated by cross-linking of cell surface glycoproteins on T cells. Based on these results galectin-1 was proposed to be involved in the modulation of immune responses. In this context our study might suggest that effector T cells could kill each other by secreting galectin-1, which would ensure that the immune response mounted would decline appropriately after antigen is cleared.

In the murine system monomeric βGBP acts as an autocrine negative growth factor in embryonic fibroblasts and inhibits cell proliferation by blocking the cells both in G_0 and at S/G_2 traverse [4]. In contrast to the apoptotic effect of recombinant galectin-1 on activated T cells which requires micromolar concentrations, the negative growth factor activity on somatic cells is evident at nanomolar concentrations. This growth inhibitory effect is independent of the carbohydrate binding site, since (1) addition of lactose does not block the inhibitory activity and (2) the 18 000 M_r form of βGBP, in which the carbohydrate binding site is blocked by a glycan complex, has full growth inhibitory activity [15].

Activation of T cells leads to a sequential induction of several genes which can be divided into immediate (< 12 h), early (1–2 days) and late (> 3 days) genes [16]. LGALS1 expression was found to be high in $CD8^+$ T cells 8 days after virus infection and expression was still vis-

ible in memory T cells 4 weeks after infection. After antigen stimulation *in vitro* LGALS1 expression and βGBP secretion by $CD8^+$ T cells reached its peak 2–3 days after stimulation. Thus, compared to other T cell activation markers, LGALS1 expression and βGBP secretion appears rather late in the process of activation. Our data further showed that βGBP inhibited antigen-induced proliferation of $CD8^+$ T cells at nanomolar concentrations. The growth inhibitory effect of βGBP on T cells was not Fas/CD95 mediated and could not be blocked by overexpression of Bcl-2 in T cells. These data indicate a role of βGBP as a down-regulatory cytokine during the silencing phase of a T cell immune response: βGBP secreted by effector T cells at the peak of the response could inhibit their own proliferation and/or prevent further induction of naive T cells. Such an autocrine mechanism involving βGBP as a negative growth factor could complement other down-regulating pathways such as CD95- and/or growth factor deprivation-mediated apoptosis. Since we also found LGALS1 expression in activated $CD4^+$ T cells, it will be important to test whether βGBP belongs to the family of Th0, Th1 or Th2 cytokines.

4 Materials and methods

4.1 Animals

C57BL/6 (B6.Thy-1.2) mice were obtained from Harlan Winkelmann (Borchen, Germany) and B6.PL-$Thy1^a$ (B6.Thy-1.1) mice were a generous gift of Dr. H. Mossmann (Max-Planck-Institute for Immunobiology, Freiburg, Germany). The (B6.Thy-1.1 × B6.Thy-1.2) F1 mice, the P14 TCR-transgenic mice [17], the 2B4 TCR-transgenic mice [18], the TCR/bcl-2 doubly transgenic mice [19] and the TCR/lpr mice [20] were bred locally and have been described previously.

4.2 Virus and peptides

LCMV-WE was originally obtained from F. Lehmann-Grube (Heinrich-Pette-Institute for Experimental Virology and Immunology, University of Hamburg, Hamburg, Germany) and was propagated on L929 fibroblast cells and the virus titer was quantified in a virus plaque assay as described [21]. Mice were infected i.v. with 200 PFU of LCMV-WE. The LCMV GP33 peptide (KAVYNFATM) was purchased from Neosystem Laboratoire (Strasbourg, France) and the moth cytochrome c peptide 88–103 (ANERADLIAYLKQATK) was kindly provided by Klaus Eichmann (Max-Planck-Institute for Immunobiology, Freiburg, Germany).

4.3 *In vitro* stimulation of T and B cells

For stimulation of $CD8^+$ T cells, spleen cells of P14 TCR-transgenic mice were incubated in Iscove's modified Dulbecco's medium (IMDM, Gibco BRL, Paisley, GB) supplemented with 10 % FCS and 10^{-6} M of the LCMV GP33 peptide. $CD4^+$ T cells from 2B4-transgenic mice were stimulated with 10 μg/ml moth cytochrome c peptide. To obtain mitogen-activated T cells, C57BL/6 spleen cells were incubated for 3 days with 5 μg/ml Con A. To obtain activated B cells, spleen cells from C56BL/6 mice were incubated for 3 days with 10 μg/ml LPS.

4.4 Proliferation assay *in vitro*

Spleen cells from P14 TCR, P14 TCR/lpr [20] or P14 TCR/bcl-2 [19] mice containing 5×10^4 TCR^+ transgenic T cells were preincubated for 4 h in 96-well plates with indicated concentrations of r-mβGBP. Afterwards, LCMV GP33 peptide (10^{-9} M) was added to the culture and after 2 days the cultures were pulsed with 1 mCi [^{3}H]thymidine per well for 8 h before harvesting onto filter paper.

4.5 Cell lines

Cells were cultured in IMDM supplemented with 10 % FCS, penicillin/streptomycin and glutamine. The following cell lines were used: EL4, RMA-S, CTLL-2, BW 5147.3, P3-X63Ag8, J558 (all obtained from ATCC, Manassas, VA), Hy-Ad9 [22], BWLy2-3 [23], IT H6/A11 [24], T21-4.60 [25], B8-24.3 [26] and B22.249 [25]. The P14 TCR^+ $CD8^+$ T cell line was generated by cultivation of spleen cells from P14 TCR-transgenic mice with LCMV GP33 peptide. Afterwards the line was kept in culture by weekly restimulation with GP33-loaded C57BL/6 spleen cells in the presence of 20 U/ml IL-2.

4.6 Generation of effector and memory T cells

Effector and memory $CD8^+$ T cells were generated *in vivo* using an adoptive transfer system as described [27]. The transgenic cells were transferred into (B6.Thy-1.1 × B6.Thy-1.2) F1 to allow separation of the transferred transgenic cells from the endogenous host cells. Mice were killed 8 days after infection to isolate $CD8^+$ effector T cells. $CD8^+$ memory T cells were isolated from mice killed 4 weeks after infection. Uninfected P14 TCR-transgenic mice were used as a source of naive $CD8^+$ T cells.

$CD8^+$ T cells were purified from spleen cells by a two-step negative selection with Dynabeads. RBC were lysed by hypotonic shock and B cells were removed using sheep anti-mouse IgG Dynabeads. To isolate naive $CD8^+$ T cells, $CD4^+$ cells were removed using anti-CD4-coupled Dynabeads. Afterwards, cells were coated with hybridoma supernatant (100 μl per 10^7 spleen cells) of the following mAb: anti-Mac-1 mAb (M1-70) [28] and anti-FcγRII/III mAb (2.4G2, obtained from ATCC, Manassas, VA). To isolate $Thy1.2^+$ TCR^+ transgenic effector cells from the (B6.Thy-1.1 ×

B6.Thy-1.2) F1 recipient mice, the following mAb were used: anti-FcγRII/III mAb (2.4G 2) and anti-Thy1.1 mAb (19E-12) [29]. For the isolation of Thy1.2$^+$ TCR$^+$ transgenic memory cells, the following mAb were used: anti-Mac-1 mAb (M1-70), anti-FcγRII/III mAb (2.4G 2) and anti-Thy1.1 mAb (19E-12). Antibody-coated cells were removed using sheep anti-rat and sheep anti-mouse IgG Dynabeads. After this two-step negative selection procedure 90–95 % of the remaining cells were CD8$^+$ T cells. CD8 depletion of spleen cells was performed with anti-CD8-coupled Dynabeads.

4.7 Differential display

Total RNA was isolated with an RNA Isolation Kit (Fluka Chemie AG, Buchs, Switzerland) according to the manufacturer's protocol. For cDNA synthesis, total RNA was first treated with DNase to remove genomic DNA. Of total RNA from naive, effector and memory T cells, 2 μg was used for first-strand cDNA synthesis (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The differential display PCR reactions were performed using the DELTA RNA Fingerprinting Kit (Clontech Laboratories, Inc. Palo Alto, CA), Advantage Klen Taq Polymerase Mix (Clontech) and [α-^{33}P] dATP (Amersham International plc, Little Chalfont, GB) according to the manufacturer's protocol. For re-amplification, bands were excised and standard PCR was performed using the appropriate differential display primers and the PCR products were directly sequenced. The LGALS1 cDNA was amplified using LGALS1-specific primers [TTGAGGGATCCAGGCTGGCTGGCTTCACTC (5' primer) and CTTCGCTTAAGCTTCAATCATGGCCTGTGG (3' primer)] and cloned into the pGEM-T vector (Promega, Madison, WI). The 450-bp HindIII-BamH1 fragment was used for Northern blot analysis and *in situ* hybridization.

4.8 Northern blot analysis

Of total RNA 5–10 μg were separated on a 1.2 % agarose/1 × MOPS/3.7 % formaldehyde gel and transferred overnight to nylon membranes (GeneScreen Hybridisation Transfer Membrane, Du Pont Europe, Brussels, Belgium). DNA probes were synthesized using the DECAprimeTM II Kit (Ambion, Inc., Austin, TX) and [α-^{32}P] dCTP (Amersham) according to the manufacturer's protocol. Northern blots were hybridized at 42 °C overnight and exposed for 6–8 h at −70 °C to a BIOMAX ™MS film (Amersham).

4.9 Western blot analysis

Cell-free supernatant (20 ml) of *in vitro* stimulated CD8$^+$ T cells was concentrated 100 times by immunoaffinity chromatography using the IgG fraction of the neutralizing anti-βGBP mAb clone B2 [4]. For electrophoretic analysis of proteins, samples were run on an SDS-12.5 % polyacrylamide gel and blotted onto nitrocellulose under standard conditions [30]. The protein was detected by the chemiluminiscence method (ECL-kit, Amersham) following incubation with mAb clone B2 and horseradish peroxidase-conjugated anti-mouse IgG.

4.10 *In situ* hybridization

In situ hybridization was performed on 5-μm-thick frozen sections of the spleen as described [31]. Labeled sense and antisense RNA probes were derived from the 450-bp HindIII-BamH1 fragment of the LGALS1 gene and the 521-bp EcoRI-HindIII fragment of the 5' end of the perforin gene.

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CUTTING EDGE

Cutting Edge: Virus-Activated CD8 T Cells and Lymphokine-Activated NK Cells Express the Mast Cell Function-Associated Antigen, An Inhibitory C-Type Lectin[1]

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The mast cell function-associated Ag (MAFA) is an inhibitory C-type lectin that was originally identified on the cell surface of a rat mucosal mast cell line, RBL-2H3. We have cloned the mouse homologue of the rat MAFA gene, and Northern blot analysis revealed that mouse MAFA (mMAFA) gene expression was strongly induced in effector CD8 T cells and lymphokine-activated NK cells but not in effector CD4 T cells and in mouse mast cells. Moreover, mMAFA gene expression was only found in effector CD8 T cells that had been primed in vivo with live virus because in vitro activated CD8 T cells did not express mMAFA. Primary sequence comparison revealed a high degree of conservation (89% similarity) between rat MAFA and mMAFA. Thus, the MAFA molecule in the mouse is a putative inhibitory receptor on anti-viral CD8 T cells induced in vivo and on NK cells. ***The Journal of Immunology*****, 1998, 161: 6451–6454.**

The mast cell secretory response is triggered by clustering of Fcε receptors (FcεRI) by IgE immune complexes. A cell surface protein has been identified on the rat mucosal mast cell line RBL-2H3 that inhibits FcεRI-mediated degranulation and cytokine release after aggregation by a specific mAb (1). This molecule has been named mast cell function-associated antigen (MAFA),[3] and molecular analysis revealed that MAFA is a type II membrane protein that belongs to the C-type lectin superfamily (2). MAFA exists as both a monomer and a disulfide-linked homodimer, and the intracellular domain contains a putative tyrosine-based inhibition motif that was found to be constitutively phosphorylated. These characteristics reveal a close relationship of MAFA with other members of the C-type lectin superfamily that function as inhibitory receptors (i.e., Ly49 and CD94/NKG2) in NK cells and also in T cells. However, MAFA expression in the rat analyzed with the sensitive RT-PCR technique has been reported to be mast cell specific (3).

We have used the "differential display" technique to identify genes in activated T cells, and in this context we have now cloned the mouse homologue of the rat MAFA gene. Surprisingly, we found that the MAFA gene in the mouse was strongly expressed both by effector CD8 T cells after priming in vivo by a viral infection and by lymphokine-activated NK cells. The present study represents the first characterization of this gene in the mouse; its product exhibits the molecular characteristics of a regulatory molecule for CD8-effector T cells.

Materials and Methods

Animals

C57BL/6 (B6) mice were purchased from Harlan Winkelmann (Borchen, Germany), and B6.PL-Thy1[a] (B6.Thy-1.1) mice were obtained from Dr. H. Mossmann (Max-Planck Institute for Immunobiology, Freiburg, Germany). CD8 lymphocytic choriomeningitis virus (LCMV) TCR transgenic mice (4) and CD4 LCMV TCR transgenic mice (5) were bred locally.

Viral strains

LCMV-WE was propagated on L929 cells, vesicular stomatitis virus (VSV) Indiana (Mudd-Summers isolate) was grown on baby hamster kidney cells and vaccinia virus (VV) strain WR was produced by infecting BSC 40 cells. Mice were infected i.v. with 200 plaque-forming units (PFU) of LCMV-WE, 10^6 PFU LCMV-docile, 2×10^6 PFU VSV, and 2×10^6 PFU VV WR.

Cell cultures and cell lines

T cells from CD8 LCMV TCR transgenic mice were stimulated weekly with LCMV-infected, irradiated (2500 rad) B6 peritoneal macrophages in Iscove's modified Dulbecco's medium (IMDM, Life Technologies, Paisley, U.K.) supplemented with 10% FCS and 20 U/ml IL-2 (PharMingen, San Diego, CA). CD8 memory T cells isolated from LCMV-immune B6 mice (4–6 wk after infection) were cultured and stimulated in the same manner. Bone marrow-derived mast cells were isolated from B6 mice as described (6) and stimulated for 8 h with 0.5 μM ionomycine (Sigma-Aldrich, Deisenhofen, Germany). To generate lymphokine-activated killer cells, B6 spleen cells (SC) were depleted after lysis of RBC from T and B

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[3] Abbreviations used in this paper: MAFA, mast cell function-associated Ag; mMAFA, mouse MAFA; LCMV, lymphocytic choriomeningitis virus; VSV, vesicular stomatitis virus; SC, spleen cells; PFU, plaque-forming unit; VV, vaccinia virus; GP, glycoprotein.

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      1                                30
Mouse MADSSIYSTL ELPEAPQVQD ESRWKLKAVL
Rat   ...N...... ...A..R... D....V....

      31                               60
Mouse HRPHLSRFAM VALGLLTVIL MSLLMYQRIL
Rat   ...CV.YLV. .......... ....L...T.

      61                               90
Mouse CCGSKDSTCS HCPSCPILWT RNGSHCYYFS
Rat   .....GFM.. Q.SR..N..M ..........

      91                               120
Mouse MEKKDWNSSL KFCADKGSHL LTFPDNQGVK
Rat   ...R...... .......... .........N

      121                              150
Mouse LFGEYLGQDF YWIGLRNIDG WRWEGGPALS
Rat   ..Q..V.E.. ......D... ....D.....

      151                              180
Mouse LRILTNSLIQ RCGAIHRNGL QASSCEVALQ
Rat   .S..S..VV. K..T...C.. H.........

      181    188
Mouse WICKKVLY
Rat   ...E...P
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FIGURE 1. Amino acid sequence alignment of mouse and rat MAFA. Sequence numbering starts with the N terminus, which is located in the cytoplasma. The putative immunoreceptor tyrosine-based inhibition motif is shown in bold and the putative transmembrane region is underlined. The complete nucleotide sequence of mMAFA is available from the EMBL database under accession number AJ010751.

cells by negative selection with Dynabeads (Dynal, Hamburg, Germany). The remaining cells were cultured for 6 days in IMDM supplemented with 10% FCS and 1000 U/ml IL-2. After 6 days, the cells (90–95% NK1.1$^+$) were harvested.

Generation of effector and memory T cells

CD4 and CD8 effector and memory T cells were generated in vivo using an adoptive transfer system as described (7). Uninfected LCMVTCR transgenic mice were used as a source of naive CD4 or CD8 T cells. CD8 T cells were purified from SC by a two step negative selection procedure with Dynabeads as described (8). CD4-effector T cells were purified by depletion of CD8 T cells and B cells by negative selection with Dynabeads.

Differential display

The differential display analysis was performed as described (8) using the DELTA RNA Fingerprinting Kit (Clontech Laboratories, Palo Alto, CA). Full-length cDNA was obtained using long-distance RT-PCR and the SMART-PCR cDNA Synthesis Kit (Clontech) according to the manufacturer's instructions.

Northern blot analysis

Total RNA was isolated from SC or purified cell populations with an RNA Isolation Kit (Fluka Chemie, Buchs, Switzerland). Northern blot analysis was performed as described (8) using 5–10 μg of total RNA per lane. The 600-bp fragment of the mouse MAFA (mMAFA) cDNA representing the entire coding region was used as mMAFA probe. The mouse cytoskeletal β-actin probe (560 bp) was amplified from cDNA by PCR using 5′ (ATGGATGACGATATCGCT) and 3′ (ATGAGGTAGTCTGTCAGGT) primers.

Results and Discussion

Identification of mMAFA

We performed mRNA differential display PCR to identify genes induced in effector CD8 T cells and isolated a PCR product that revealed significant sequence homology to the rat MAFA gene (2). Full-length cDNA was obtained by RT-PCR with mRNA from CD8 effector T cells and the full primary sequence was determined. The primary sequence was aligned with that of the rat MAFA, and sequence comparison revealed 80% identity and 89% similarity of the mouse to the rat sequence (Fig. 1). This result

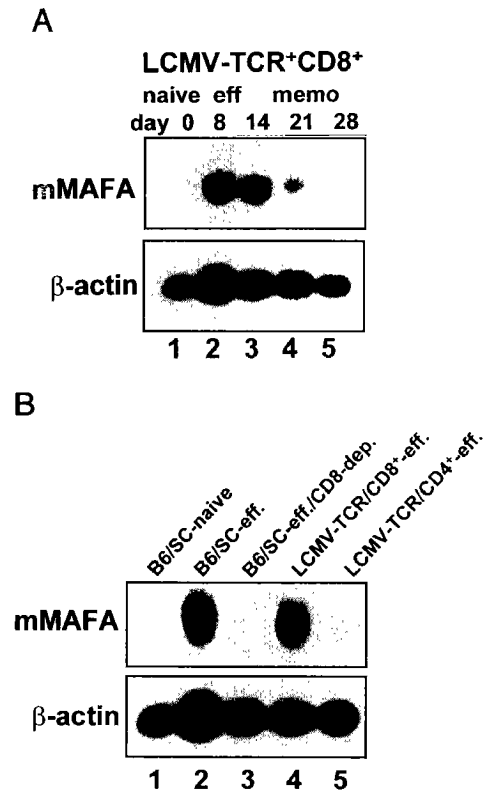

FIGURE 2. *A*, Northern blot analysis of mMAFA expression in CD8 T cells from CD8 LCMV TCR transgenic mice isolated at the indicated time points after LCMV infection. *Lane 1*, Naive CD8 T cells. *Lanes 2–5*, Effector/memory CD8 T cells isolated on day 8 (*lane 2*), day 14 (*lane 3*), day 21 (*lane 4*), and day 28 (*lane 5*) after infection. *B*, Northern blot analysis of mMAFA expression in B6 SC and in CD4 and CD8 LCMV TCR transgenic cells after LCMV infection. *Lane 1*, SC from naive B6 mice. *Lane 2*, SC from day 8 LCMV-immune B6 mice. *Lane 3*, CD8 T cell-depleted SC from day 8 LCMV-immune B6 mice. *Lane 4*, CD8 LCMV TCR transgenic effector cells isolated 8 days after LCMV infection. *Lane 5*, CD4 LCMV TCR transgenic effector cells isolated 8 days after LCMV infection. For each lane, 10 μg of total RNA were loaded. Equivalent sample loading was assured by hybridization with a β-actin probe.

suggested that the mouse homologue of the rat MAFA gene had been isolated. Southern blot analysis of genomic DNA using a full-length mMAFA cDNA as a probe revealed a hybridization pattern suggesting that MAFA is a single gene in the mouse (not shown). The same conclusion has been drawn for the MAFA gene in the rat (3).

mMAFA is induced in CD8 but not in CD4 T cells after LCMV infection

Northern blot analysis using the mMAFA cDNA as a probe demonstrated that CD8 T cells from LCMV TCR transgenic mice isolated on both day 8 and day 14 after LCMV infection expressed high levels of mMAFA mRNA (Fig. 2*A*, *lanes 2* and *3*). In contrast, naive CD8 T cells from these mice did not express mMAFA at detectable levels (Fig. 2*A*, *lane 1*). In CD8 memory T cells, mMAFA gene expression was still detectable 3–4 wk after infection, but to a lower extent (Fig. 2*A*, *lanes 4* and *5*). Importantly, mMAFA gene expression was also induced in CD8 effector T cells from normal C57BL/6 mice isolated 8 days after LCMV infection (Fig. 2*B*, *lane 2*). To test whether SC other than CD8 effector T cells expressed mMAFA, SC from B6 mice taken on day 8 after LCMV infection were depleted of CD8 T cells by negative selection in vitro. As shown in Fig. 2*B*, *lane 3*, these CD8-depleted

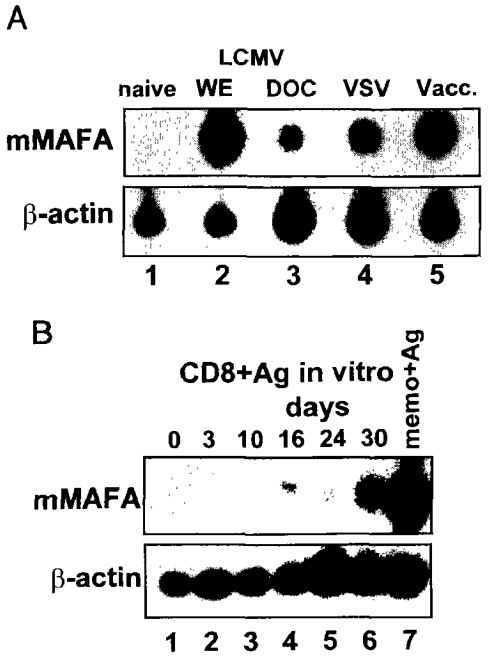

FIGURE 3. *A*, Northern blot analysis of mMAFA expression in the spleen of B6 mice after the viral infections indicated. *Lane 1*, SC from naive B6 mice. *Lane 2*, SC from day 8 LCMV-WE-immune mice. *Lane 3*, SC from day 8 LCMV-docile-immune mice. *Lane 4*, SC from day 6 VSV_{IND}-immune mice. *Lane 5*, SC from day 6 VV-immune mice. The docile strain of LCMV exhibited a decreased capacity to induce CD8 effector T cells (9). *B*, Northern blot analysis of mMAFA expression in in vitro stimulated CD8 T cells from CD8 LCMV TCR transgenic mice. Naive CD8 T cells from LCMV TCR transgenic mice (*lane 1*) were stimulated once a week with LCMV-infected macrophages, and CD8 T cells were harvested from the culture on the days indicated (*lanes 2–6*). *Lane 7*, CD8 T cells from LCMV memory (memo) mice were restimulated for 3 days in vitro with LCMV-infected macrophages. For each lane, 10 μg of total RNA were loaded; hybridization with a β-actin probe shows constant sample loading.

immune SC did not express mMAFA at significant levels, indicating that mMAFA was virtually exclusively expressed in activated CD8 T cells. To directly compare mMAFA expression in activated CD4 and CD8 T cells, effector T cells were generated using CD4 and CD8 TCR transgenic mice, which both express an Ag receptor specific for LCMV glycoprotein (GP). T cells from CD8 LCMV TCR transgenic mice are specific for LCMV GP amino acid 33–41 in the context of H-$2D^b$ (4), and CD4 LCMV TCR transgenic mice express an Ag receptor specific for LCMV GP amino acid 61–80 together with I-A^b (5). CD4 and CD8 T cells from both TCR transgenic lines were activated in vivo using the same adoptive transfer system, which allows the same vigorous expansion of the transferred transgenic T cells (5, 7). As shown in Fig. 2*B*, *lane 5*, effector cells from CD4 LCMV TCR transgenic mice expressed mMAFA at low levels when compared with effector cells from CD8 LCMV TCR transgenic mice (*lane 4*). Thus, these results indicated that mMAFA expression on effector T cells was CD8 specific.

Induction of mMAFA expression in CD8 T cells is not restricted to LCMV infection but requires in vivo priming with virus

To examine whether viral infections other than LCMV were also capable of inducing mMAFA expression, SC from B6 mice acutely infected with LCMV, VV, or VSV were examined. Fig. 3*A* shows that mMAFA expression levels were comparable in splenocytes from mice infected with either LCMV, VSV, or VV. It is noteworthy that mMAFA expression was lower in splenocytes from mice infected with a high dose (10^6 PFU) of the docile strain of LCMV, a virus strain known to induce clonal exhaustion of CD8 effector T cells (9). To test whether Ag stimulation in vitro also induced mMAFA expression, CD8 T cells from LCMV TCR transgenic mice were stimulated with LCMV-infected APC. Ag stimulation of CD8 T cells from LCMV TCR transgenic mice in vitro is known to lead to vigorous proliferation and induction of CTL activity (10). However, these in vitro conditions were not sufficient to induce significant mMAFA expression (Fig. 3*B*, *lanes 2–4*). Only after repeated restimulation and prolonged in vitro culture (3–4 wk), low level mMAFA expression was apparent in these cultures (Fig. 3*B*, *lanes 5* and *6*). In striking contrast, high levels of mMAFA expression were induced in memory CD8 T cells within 3 days of in vitro restimulation (Fig. 3*B*, *lane 7*); these memory CD8 T cells had been primed in vivo with LCMV.

mMAFA expression occurs in NK but not in mast cells

Expression of mMAFA mRNA was not detected by Northern blotting in brain, heart, kidney, liver, lymph node, lung, skeletal muscle, spleen, thymus, and testis (not shown). As depicted in Fig. 4*A*, mMAFA was also not expressed in EL-4, RMA-S, BW5147 lymphoma cells, or in CD4 and CD8 T-cell hybridomas (BWLy2–3, IT H6/A11). Similarly, J558 and P3-X63Ag8 myeloma cells and B cell hybridomas did not express mMAFA. However, the CTL clone HY-Ad9 (Fig. 4*A*, *lane 3*), which exhibits NK-like activity (11), expressed mMAFA levels comparable to CD8-effector T cells. This result led us to examine mMAFA expression in lymphokine-activated $NK1.1^+$ killer cells. As shown in Fig. 4*B*, *lane 3*, lymphokine-activated $NK1.1^+$ cells (95% purity) generated by culturing T and B cell-depleted SC in the presence of IL-2 expressed high levels of mMAFA mRNA. The expression pattern of mMAFA is reminiscent of KIR and CD94/NKG2 molecules, which function as inhibitory receptors for both human NK cells (12, 13) and T cells (14–16). Similar to our data with mMAFA, KIRs are expressed on NK cells and on effector/memory phenotype CD8 T cells (17), and attempts to induce KIR expression on T cells by in vitro stimulation were unsuccessful (17, 18).

The MAFA gene was originally identified in the rat mucosal mast cell line RBL-2H3, and, in the rat, MAFA gene expression appears to be mast cell specific because signals were obtained from lung tissue but not from other organs including spleen and lymph nodes when the sensitive RT-PCR technique was used (3). Surprisingly, we did not observe mMAFA gene expression in IL-3/IL-4 induced bone marrow-derived mast cell cultures, which are thought to represent an in vitro analogue of mucosal mast cells (6) (Fig. 4*C*, *lane 2*). In addition, stimulation of these cells with ionomycine did not lead to mMAFA gene expression (Fig. 4*C*, *lane 3*). Our results indicate that MAFA gene expression in the mouse is not mast cell specific and may not even be expressed on mucosal mast cells in this species.

In analogy to the inhibitory effect on mast cell degranulation by rat MAFA, we postulate that the mouse homologue has a similar function and serves as an inhibitory molecule on CD8-effector T cells and on NK cells. Due to the lack of the appropriate serological reagents, we could not yet test this hypothesis directly. However, it is important to stress that the inhibitory function of the rat MAFA molecule has been clearly established (1). In contrast to rat MAFA, which is constitutively expressed in the rat mast cell line RBL-2H3, mMAFA expression in CD8 T cells was induced by Ag stimulation. Compared with other T cell-activation markers,

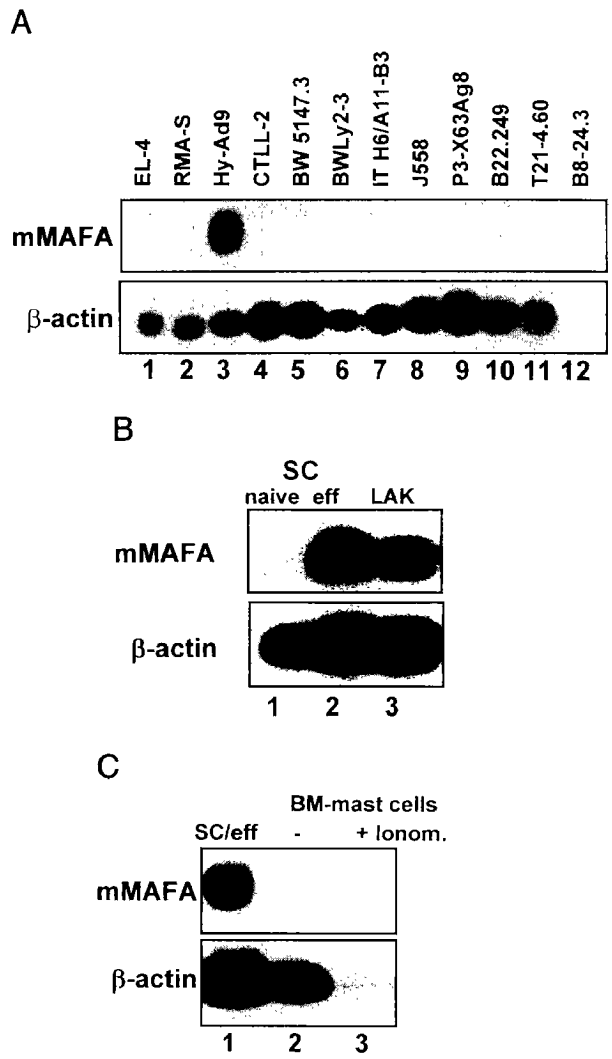

FIGURE 4. Northern blot analysis of mMAFA expression. *A*, Expression in lymphomas (EL-4, RMA-S, BW 5147), cloned CTLs (CTLL-2, HY-Ad9), T cell hybridomas (BWLy2–3, IT H6/A11), myelomas (J558, P3-X63Ag8), and B cell hybridomas (B22.249, T21-4.60, B8-24-3). *B*, mMAFA expression in lymphokine-activated NK1.1$^+$ cells. *Lane 1*, SC from naive B6 mice. *Lane 2*, SC from day 8 LCMV immune B6 mice. *Lane 3*, NK 1.1$^+$ lymphokine-activated killer cells from B6 mice. *C*, mMAFA expression in bone marrow-derived mast cells (BM-mast cells). *Lane 1*, SC from day 8 LCMV-immune B6 mice. *Lane 2*, BM-mast cell culture isolated from B6 mice. *Lane 3*, Ionomycine-treated BM-mast cell culture. For each lane, 10 μg of total RNA were loaded; constant sample loading was controlled by hybridization with a β-actin probe.

mMAFA appears rather late in the course of a CTL response. This late kinetic of mMAFA expression fits in well with the postulated inhibitory role of this molecule for CD8 effector T cells.

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