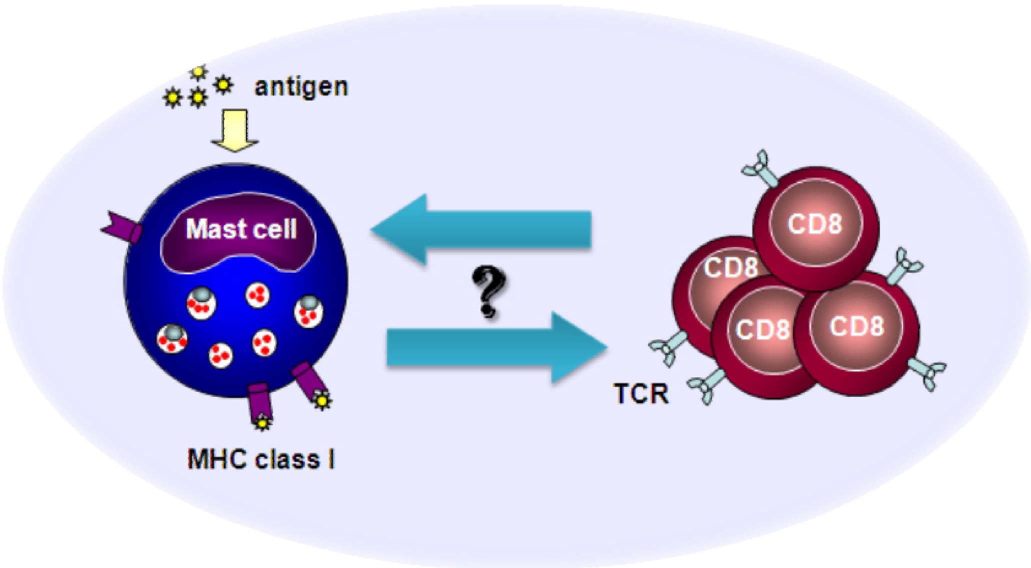
Aus dem Forschungszentrum Borstel
Leibniz - Zentrum für Medizin und Biowissenschaften
Laborgruppe Immunbiologie
Abteilung Immunologie und Zellbiologie
(Direktorin: Prof. Dr. Dr. Silvia Bulfone-Paus)

The role of mast cells in $CD8^+$ T cell-mediated immune responses



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List of abbreviations

%	Per cent
°C	Degree Celsius
AHR	Airway hyperresponsiveness
ANOVA	Analysis of variance
AP-1	Activator protein-1
APC(s)	Antigen-presenting cell(s)
BAL	Bronchoalveolar lavage fluid
BMDC(s)	Bone marrow-derived dendritic cell(s)
BMMC(s)	Bone marrow-derived mast cell(s)
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary DNA
CFDA	Carboxyfluorescein diacetate
CFSE	Carboxyfluorescein succinimidyl ester
CLP	Cecal ligation and puncture
CLIP	Class II-associated Ii peptide
cm	Centimeter
CTL(s)	Cytotoxic T lymphocyte(s)
DC(s)	Dendritic cell(s)
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetracetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell scanning
FcεRI	High affinity I receptor for Fc fragment of IgE

FCS	Fetal calf serum
Fig	Figure
FimH	Tip adhesin of mannose-specific type 1 fimbriae of *Escherichia coli*
FITC	Fluorescein isothiocyanate
FSC	Forward-scatter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIV	Human immunodeficiency virus
hr(s)	Hour(s)
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
Igtp	IFN-γ -induced GTPase
Ii	Invariant chain
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IRF7	IFN regulatory factor 7
LAMP(s)	Lysosomal-associated membrane protein(s)
LPS	Lipopolysaccharide
LTB_4	Leukotriene B4
Lzp	p-lysozyme structural
M	Molar
MACS	Magnetic-associated cell sorting
MAPK	Mitogen-activated protein kinase
MARCO	Macrophage receptor with a collagenous structure
MC(s)	Mast cell(s)
MFI	Mean fluorescence intensity
mg	Milligram
MHC	Major histocompatibility complex
min	Minute
MIP	Macrophage inflammatory protein
mit. C	Mitomycin C
ml	Milliliter

mM	Millimolar
mm	Millimeter
nm	Nanometer
NCS	Newborn calf serum
NFAT	Nuclear factor of activated T cells
NF-kB	Nuclear factor kappa B
ng	Nanogram
NGF	Nerve growth factor
NK cells	Natural killer cells
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PI	Propidium iodide
pIC	Polyinosinic-polycytidylic acid
PLCγ	Phospholipase Cγ
p-value	Probability value
RANTES	Regulated upon activation, normal T cell-expressed and secreted
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
Rsad2	Radical S-adenosyl methionine domain containing 2
RT	Room temperature
Rtp4	Receptor transporter protein 4
SCF	Stem cell factor
SR-A	Scavenger receptor A
SSC	Sideward-scatter
STAT1	Signal transducer and activator of transcription 1
TAP	Transporter associated with antigen processing
TBE	Tris/boric acid/EDTA
TCR	T cell receptor
TGF-β	Transforming growth factor beta
TGM-2	Transglutaminase-2
Th	T helper cell

TLR(s)	Toll-like receptor(s)
Tnfrsf9	Tumour necrosis factor receptor superfamily member 9
TNF-α	Tumor necrosis factor alpha
Tris	Trishydroxymethylaminomethane
WT	Wild-type
x *g*	Gravity force
β2m	Beta-2-microglobulin
μg	Microgram
μl	Microliter

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Preface

"It is generally accepted that the biologic sciences are absolutely splendid. In just the past decade they have uncovered a huge mass of brand-new information, and there is plenty more ahead; the biologic revolution is evidently still in its early stages."

(Thomas Lewis, 1974)

Biological research is indeed splendid (Lewis, 1974); the more information about the cell in general or a particular cell type we discover, the more this discovery reveals new possible functions, which this cell type may exert. This concept applies to mast cells (MCs); the still progressing exploration of these cells and their (patho)physiological role is a longer than a century-lasting investigation which still, and especially during the last decade, reveals novel roles for MCs in health and disease.

When Paul Ehrlich as a 24-year-old student presenting his doctoral thesis in 1878, described a type of ''granular cells of the connective tissue'', which he named ''Mastzellen'' (Ehrlich, 1878), a vivid, multidirectional research to explore the development, phenotype, physiology and pathology of these cells was starting. Since then, the universe of MC biology has been ever extending and the deeper we explore the biology of these cells, the more functions we discover, including some that shatter long-held beliefs on their nature and limitations (Maurer et al., 2003; Maurer and Metz, 2005).

In an attempt to contribute to the unraveling of the (patho)physiological role of these fascinating cells, this study was focused on their involvement in the initiation of an antigen-specific adaptive immune response and their ability to modulate $CD8^+$ T cell responses.

1. Introduction

1.1. General introduction

Life is a dynamic, sophisticated process achieved through various, amazingly organized and interconnected mechanisms, each of which involves several biological pathways. The complexity of this process is attributed to the number of functions the living organism must effectively perform in order to survive. Apart from the apparent need to obtain and successfully process the necessary environmental elements (nutrition, water, air) for its growth and reproduction, a living organism must additionally cope with the challenges of the constantly evolving environment. It is a common phenomenon that the needs of one organism overlap or contradict the needs of another. Thus, a system of protective mechanisms has evolved that secure life from the attack of other organisms. In consistency with the evolution of the organisms, these protective mechanisms have evolved from simple protein secretion and enzymatic digestive pathways, to the development of highly specialized cells and a whole system of complex, still not fully identified interactions between those cells. This defense system that has evolved to protect organisms from the invading pathogens is called the **immune system** (Goldsby et al., 2003). The first ever observation of an existing immune system is considered to be the one mentioned by the Greek historical Thucydides in 430 BC, as he stated that during an epidemic disease in Athens, the patients could be nursed only by those who had recovered from the disease, since they were protected from developing the disease again (Thucydides).

Thus, already during the first observation of its existence, the human immune system was attributed with its two major characteristics: protection and memory. Indeed, the immune system has developed efficient mechanisms not only for inhibiting most kinds of microorganisms, such as viruses, bacteria, protozoa and other parasites to infect the human body (immunological protection), but also for recognizing the same antigens upon a subsequent encounter (immunological memory). Despite the indispensable role of the immune system in protection against pathogens, dysfunction of the immune system may often lead to undesirable pathological situations, such as an undue reaction against non-pathogenic agents (allergy) or even against self-antigens (autoimmunity).

The immune system is sub-divided into two categories: the innate and the adaptive immune system. Each of them includes a series of mechanisms exerted by different cell types, in order to achieve optimal results. Innate and adaptive immunity cooperate with each other and are interconnected by specific cell types and soluble mediators.

1.1.1. Innate immunity

Innate immune responses are identified as unspecific, short-term protective immune responses, present not only in higher evolved organisms, but also in invertebrates and, partially, in plants (Jones and Dangl, 2006; Iriti and Faoro, 2007). They include the secretion of antimicrobial peptides and enzymes, which damage or destroy the membranes of invading microorganisms. In animals, specialized cells, the phagocytes, eradicate foreign microorganisms by internalizing and digesting them with intracellular enzymes. Elimination of invading microorganisms is additionally facilitated by a group of soluble proteins comprising the complement system. Proteins of the complement system recruit phagocytes to the site of infection, damage the membrane of the foreign microorganisms and coat the pathogens, facilitating their uptake by phagocytes. Another important aspect of the innate immune system is the induction of inflammation. Inflammation consists of the release of chemoattractive factors at the site of infection as well as increased blood flow and capillary permeability, which result in increased cell migration to the site of infection and effective clearance of the pathogens. However, dysregulation of the inflammatory response often leads to pathological situations such as chronic inflammation and allergy (Goldsby et al., 2003).

Although the innate and the adaptive immunity are not strictly separated mechanisms, different **cell types** have been characterized as the main executors of the one or the other response. Thus, the mammalian innate immune responses are primarily carried out by phagocytic cells (such as monocytes, macrophages, neutrophils), as well as eosinophils, platelets, NK cells, basophils and MCs (Roitt et al., 2002).

In the center of the detection mechanisms for invading microorganisms in vertebrates lies the family of **Toll-like receptors** (TLRs). Similar to the Toll proteins of Drosophila and highly conserved during evolution, the TLR family has been extensively studied both in human and mice (Gay and Keith, 1991; Medzhitov et al., 1997; Rock et al., 1998). Until now, 10 members (TLRs 1-10) in human and 12 members in mice (TLRs 1-9 and 11-13) have been described. TLRs recognize common molecular structures detected in certain groups of microorganisms, called pathogen associated molecular patterns (PAMPs). They are mainly expressed by antigen-presenting cells (APCs) and upon binding to their ligands, induce activation of the APCs and initiation of immune responses leading to the elimination of the invading microorganisms (Kaisho and Akira, 2006).

In general, the innate immune system can successfully cope with infections at a first stage; however, it cannot guarantee a complete and long-term protection against all infections, since it lacks the equipment for an antigen-specific interaction with pathogens as well as the potential to recognize the pathogenic molecules upon a subsequent encounter.

1.1.2. Adaptive immunity

The encounter of pathogenic microorganisms in our environment is inevitable. The innate immune system provides a prompt defense mechanism, however its lack of specificity towards the pathogens renders it inadequate for efficient elimination of the pathogen upon subsequent encounter. The evolution of the adaptive immune system has provided additional advantages to the vertebrates; specific elimination of the invaders with high efficiency and even, display of efficient equipment for their elimination upon a second encounter.

Adaptive immune responses are carried out by **lymphocytes**, which derive from a common lymphoid progenitor in the bone marrow. There are two major populations of lymphocytes: B lymphocytes (B cells) which mature in the bone marrow and T lymphocytes (T cells) which mature in the thymus. The main function of B cells is to recognize antigens through membrane-bound antibodies (B cell receptor) and subsequently differentiate into effector plasma cells which secrete antigen-specific antibodies. Therefore, B cells are the main participants of the so-called humoral immunity. On the other hand, T cells recognize antigens which are associated with major histocompatibility molecules (MHC) on the membrane of APCs. Upon activation, T cells exert their effector functions and regulate a cellular immune response (Goldsby et al., 2003).

1.1.2.1. T cells and the regulation of adaptive immunity

During their maturation in the thymus, **T cells** express a membrane-bound T cell-receptor (TCR), which has a unique specificity for antigen. T cells are sub-divided into two classes: $CD4^+$ T cells and $CD8^+$ T cells, according to surface expression of CD4 or CD8 antigens respectively. $CD4^+$ T cells recognize MHC class II-associated antigens, while $CD8^+$ T cells recognize MHC class I-associated antigens (Goldsby et al., 2003).

$CD4^+$ T cells mainly modulate the subsequent immune response by the production of cytokines (therefore called T helper cells: Th). Depending on the exposure on cytokines, naïve $CD4^+$ T cells differentiate into IFN-γ-, IL-2- and TNF-α-secreting Th1 cells, IL-4-, IL-5 and IL-13-secreting Th2 cells or IL-17-, IL-6-secreting Th17 cells (Weaver et al., 2006). On the other hand, regulatory $CD4^+$ T cells (Treg) suppress the immune response by secretion of TGF-β as well as by direct cell-cell contact (von Boehmer, 2005).

$CD8^+$ T cells are primarily associated with the elimination of virus- or bacteria-infected cells or tumor cells. Upon interaction with an MHC class I-bound antigen on the surface of APCs, **$CD8^+$ T cells** become activated, proliferate and either mature to effector cytotoxic $CD8^+$ T cells or to long-living memory $CD8^+$ T cells. Cytotoxic $CD8^+$ T cells eliminate virus- or bacteria-infected cells as well as tumor cells, while memory $CD8^+$ T cells preserve the immunological memory for a subsequent encounter with the same antigen (Jabbari and Harty, 2006).

Cytotoxic $CD8^+$ T cells play a central role in the control of microbial and viral infections (Wong and Pamer 2003). The TCR on cytotoxic $CD8^+$ T cell recognize and interact with MHC class I-antigen complexes on the surface of infected cells. Subsequently, cytotoxicity is performed indirectly by the release of cytokines and directly by two different pathways; the Fas-FasL pathway and the granule-exocytosis pathway. The secretion of cytokines such as IFN-γ and TNF-α induces apoptosis of the target cell without requiring direct cell-cell contact (Andersen et al., 2006). On the other hand, the interaction of the surface-expressed Fas-Ligand (FasL) on cytotoxic $CD8^+$ T cells with the surface-expressed Fas on target cells induces aggregation of intracellular death domains and results in apoptosis of the target cell (Hanabuchi et al., 1994). In addition, the granule-exocytosis pathway involves the release of cytotoxic mediators, which are pre-stored in cytolytic granules in the cytoplasm of $CD8^+$ T cells and are released upon degranulation of the $CD8^+$ T cells

(Lieberman, 2003). The best studied cytotoxic mediators of $CD8^+$ T cells are perforin, which forms pores in the cell membrane of the target cell, and granzymes, which induce the caspase-dependent pathway of apoptosis in the target cell. The mechanisms of $CD8^+$ T cell-induced cytotoxicity are schematically depicted in **Fig. 1.1**.

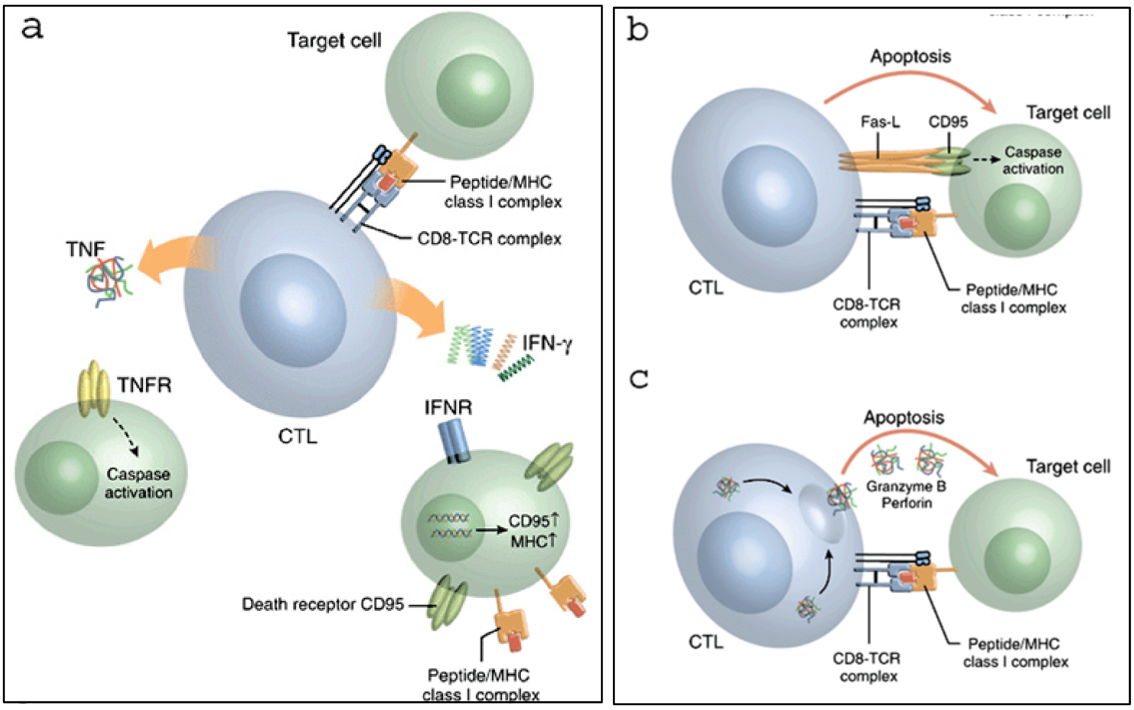

Fig. 1.1. Pathways of $CD8^+$ T cell-induced cytotoxicity. *a) Indirectly induced apoptosis of target cells by cytokines (IFN-γ and TNF-α) released by cytotoxic $CD8^+$ T cell (CTL) b) Cell contact-dependent induced apoptosis of the target cell through the Fas-FasL pathway c) Apoptosis of target cell induced by the release of cytotoxic mediators (granzyme B and perforin) from the CTL (from Andersen et al., 2006, with permission).*

1.1.2.2. Antigen presentation

Naïve B and T lymphocytes can respond to antigens with high specificity and efficiency, initiating an adaptive immune response. The key step for achieving an antigen-specific adaptive immune response is the presentation of antigens by antigen presenting cells (APCs) to B and T lymphocytes.

APCs degrade antigens into peptides (antigen processing) and present them on their surface, associated with MHC molecules and co-stimulatory molecules (**antigen**

presentation) (Brodsky and Guagliardi, 1991). Several factors such as the physical form of the antigen, the site of antigen delivery, the presence of adjuvant, as well as the nature of the APC can influence the processing and presentation of antigens (Trombetta and Mellman, 2005).

Exogenous proteins are internalized by APCs and degraded in endocytic compartments (early endosomes, late endosomes and lysosomes) by hydrolytic enzymes and acidic pH. The assembly of the MHC class II complex occurs in the endoplasmic reticulum (ER), where pairs of MHC class II αβ chains associate with a trimeric protein, the invariant chain (Ii). While the MHC class II complex trafficks from the ER through Golgi to the endocytic compartments, the Ii chain is degraded. However, the degradation of Ii is not complete; a fragment, called CLIP, is left occupying the peptide-binding groove of the MHC class II, thus preventing any immature binding of peptides to the MHC class II. Degradation of CLIP occurs in endosomal compartments by a non-classical MHC molecule called HLA-DM. Thus, the peptide-binding groove becomes available to binding peptides derived from exogenous proteins that have been degraded in endosomes. Finally, the MHC class II-peptide complex is transported to the plasma membrane (Goldsby et al., 2003; Watts, 1997)

In contrast to MHC class II, the loading of the MHC class I complex with the peptide occurs in the ER lumen. Intracellular antigenic proteins (that could be synthesized by virus- or bacteria-infected cells) are degraded into peptides by the proteasome in the cytosol. These peptides are subsequently transported into the ER by the transporter associated with antigen processing (TAP), which is associated with the transmembrane glycoprotein tapasin. Tapasin, on the other hand, associates with the heavy chain of MHC class I molecule, which constitutively forms heterodimers with the beta-2-microglobulin chain (β2m). The association of the MHC class I heterodimers with tapasin enables the loading of the peptides onto the MHC class I, which, in turn, stabilizes the MHC class I complex and initiates its dissociation from tapasin. Subsequently, the loaded MHC class I trafficks to the cell membrane via the Golgi network (Flutter and Gao, 2004; Cresswell et al., 2005; Pamer and Cresswell, 1998). A schematic representation of the MHC class I assembly and loading is shown in **Fig. 1.2**.

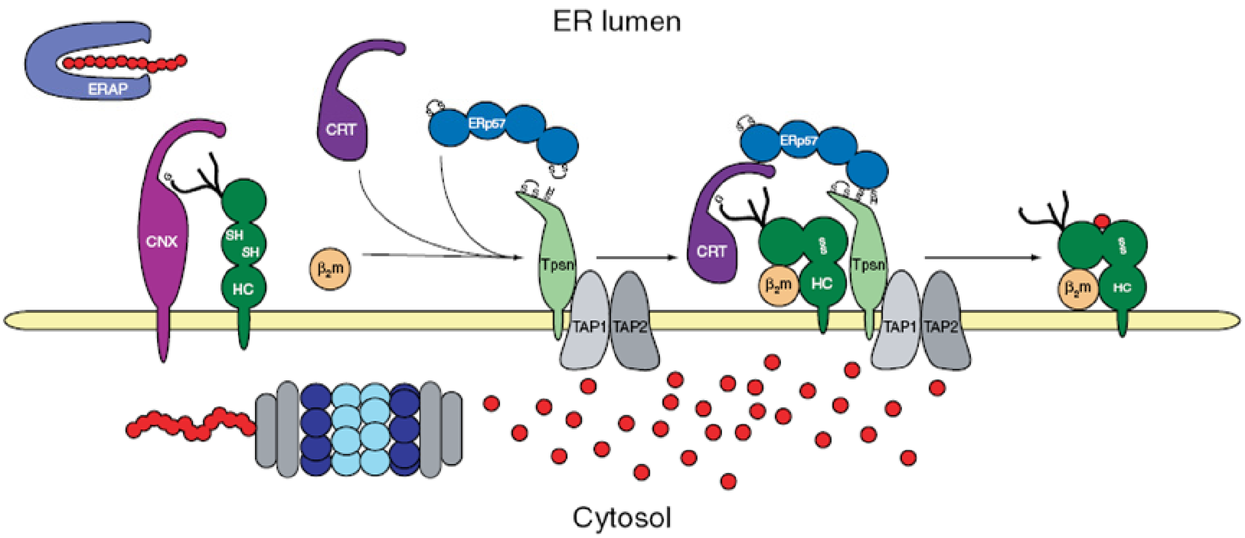

Fig. 1.2. Schematic view of MHC class I assembly and loading pathway. *Peptides derived from the processing of proteins in the cytosol by the proteasome are transported into the ER by TAP. Tapasin provides a link between TAP and the heavy chain of MHC class I molecule, which constitutively forms heterodimers with the β2m chain. The association of the heavy chain-β2m heterodimers with tapasin results in the loading of the peptides onto the MHC class I, which, in turn, stabilizes the MHC class I complex and initiates its dissociation from tapasin (from Cresswell et al., 2005, with permission).*

Despite the well-defined borders between MHC class I and MHC class II route of antigen presentation, both pathways can be crossed in certain cases (Trombetta and Mellman, 2005). Thus, exogenous antigens may escape the dominant route to MHC class II-restricted presentation, access the MHC class I pathway of antigen presentation and induce cytotoxic $CD8^+$ T cell responses. This route of alternative presentation, referred to as **cross-presentation** or cross-priming, can be induced by several, not yet fully understood mechanisms (Groothuis and Neefjes, 2005). Although many different cell types can present antigens through the classical MHC pathways, only DCs and macrophages are so far described as cells cross-presenting exogenous antigens (Trombetta and Mellman, 2005).

DCs are the most highly appreciated **APCs**, due to their high efficiency in internalizing antigens and presenting them *in vitro* and *in vivo* to both $CD4^+$ and $CD8^+$ T cells. However, several other cell types act as APCs with similar or less efficiency. Therefore, the discrimination between “professional” APCs, which generally include DCs, B cells and macrophages, and the “non-professional” or “amateur” APCs has been introduced.

Interestingly, the uptake and presentation of antigens by different cell types might result in distinct, APC-specific epitopes, which induce differential T cell responses (Robadey et al., 1996; Schneider and Sercarz, 1997). Therefore, it is suggested that the presentation of the same antigen by different APCs may result in the induction of distinct T cell responses.

A constructive tool to study antigen presentation is the generation of T cell receptor-**(TCR)-transgenic mice**. The T cell receptor is expressed on the surface of T cells and allows them to recognize antigens associated with MHC molecules. Transgenic mice for the TCR recognize and respond to a specific antigen presented in an MHC class I or class II way, depending on whether the transgenic TCR is expressed on $CD8^+$ or $CD4^+$ T cells, respectively. Since ovalbumin (OVA) comprises a very well characterized model antigenic protein extensively studied in murine models, TCR-transgenic mice have been generated against peptides derived from OVA protein. Thus, OT-I transgenic mice express transgenic TCR on $CD8^+$ T cells, recognizing the $OVA^{257\text{-}264}$ peptide (OT-I peptide) (Hogquist et al., 1994), while OT-II transgenic mice express a transgenic TCR on $CD4^+$ T cells, recognizing the $OVA^{323\text{-}339}$ peptide (OT-II peptide) (Barnden et al., 1998). T cells isolated from those mice are used to investigate antigen-specific immune responses *in vitro* and *in vivo*.

1.1.3. Crosstalk between innate and adaptive immunity

Rather than being two separate systems, the innate and the adaptive immune system are functionally and anatomically linked to each other and synergize for the successful defense against pathogen invasion. Major executors of the innate-adaptive immunity crosstalk are cells that possess functional characteristics of both systems, therefore are able to detect innate signals and forward them to the adaptive immune cells (Getz, 2005).

A key mechanism for the crosslink between the two branches of immunity is the recognition of pathogens by **DCs** through TLRs. First, DCs sense the presence of foreign microorganisms by TLRs. The TLR-PAMP interaction stimulates signal transduction pathways in DCs, which result in maturation of the DCs, up-regulation of co-stimulatory molecules and migration to the lymph nodes. Subsequently, the activated DCs present the pathogen-derived antigens to T cells, therefore initiate an antigen-dependent adaptive immune response. Thus, DCs act as central players at the interface between innate and adaptive immunity by receiving innate immune signals and translating them into antigen-dependent adaptive responses (Clark and Kupper, 2005; Pulendran, 2001).

Apart from the DCs which provide the dominant bridge between innate and adaptive immunity, other innate immune cells have been reported to influence the adaptive immune response. **Macrophages** also express TLRs, as well as scavenger receptors that bind to and mediate phagocytosis of microbial pathogens (Mukhopadhyay and Gordon, 2004). Following phagocytosis of microbes, enhanced surface expression of co-stimulatory molecules is induced, which provides the macrophages with enhanced efficiency to deliver antigen-specific signals to adaptive immune cells (Taylor et al., 2005). Moreover, it is proposed that **NK cells**, apart from their well-accepted role in promoting Th1 responses by IFN-γ production (Stein-Streilein et al., 2000) can also differentiate into Th2 cytokine-secreting NK cells, named NK2 cells (Kimura and Nakayama, 2005). Due to the number of immunologically relevant cytokines secreted by NK cells, it is proposed that NK cells may also serve as a crosslink between innate and adaptive immunity (Kos, 1998).

Accumulating evidence suggest that **MCs** may act as key regulatory cells at the crossroads between innate and adaptive immunity. The functional characteristics that enable MCs to provide a link between innate and adaptive immunity are further discussed in this study.

1.2. Mast cells (MCs)

MCs derive from hematopoietic stem cells (Kitamura et al., 1977; Nabel et al., 1981). MC **progenitors** are either committed to a distinct subtype and selectively recruited to the tissue, or get a tissue-specific character after epigenetic influence by environmental factors. After homing into different tissues, MC progenitors differentiate into mature, long-living, phenotypically diverse MCs (Tsai et al., 2005).

MCs are categorized into two different **subtypes**: connective tissue MCs, mainly found in the skin, lung and peritoneal cavity and mucosal MCs, mainly found in the gastrointestinal mucosa. Mucosal MCs are smaller and contain less granula than the connective tissue MCs (Bienenstock et al., 1982; Miller and Schwartz, 1989). The flexibility of the MC progenitors to give rise to distinct MC subpopulations depending on the environment, may possibly be an explanation for the MC capacity to respond optimally to various stimuli in different environments and be implicated in many biological processes (Galli et al., 2005a).

MC **differentiation** and proliferation is supported by various cytokines and soluble factors, such as stem cell factor (SCF), IL-3, IL-4, IL-9, IL-10 and NGF (Metcalfe et al., 1997), as well as by adhesion to extracellular matrix components, such as laminin (Thompson et al., 1989), vitronectin (Bianchine et al., 1992) and fibronectin (Dastych et al., 1991). Among these factors, IL-3 is the absolutely necessary and sufficient one to induce murine MC differentiation *in vitro*. Therefore, *in vitro* differentiation of murine MCs is commonly achieved by culturing bone marrow cells in the presence of IL-3 for 4-5 weeks (Razin et al., 1981; Razin et al., 1984). Such IL-3-dependent bone marrow-derived MCs (BMMCs) contain histamine and exhibit the characteristic red-purple appearance of their metachromatic granula after staining with toluidine blue (Schueller et al., 1967). A toluidine blue staining of cultured MCs is shown in **Fig. 1.3A**. SCF, the ligand for the c-kit (CD117) receptor, also plays a major role in MC differentiation, and induces MC maturation, activation and proliferation *in vitro* and *in vivo* (Zsebo et al., 1990; Tsai et al., 1991a; Tsai et al., 1991b; Wershil et al., 1992). Therefore, SCF is used in combination with IL-3 for the *in vitro* differentiation of murine BMMCs (Tsai et al., 1991b).

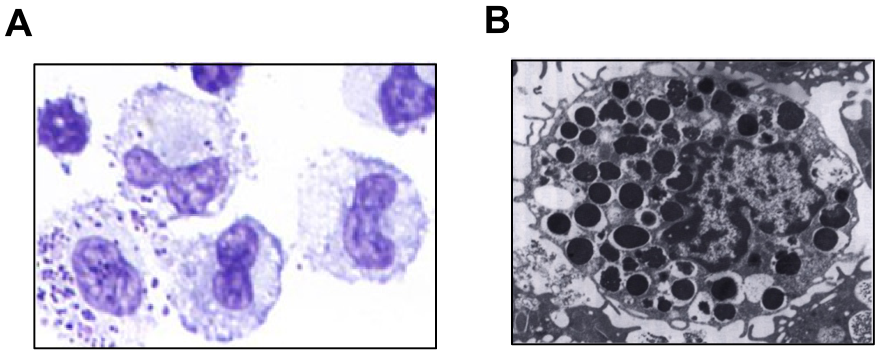

Fig. 1.3. ***Morphological characteristics of MCs. A.*** *Toluidine blue staining of a cultured human MC line showing the characteristic appearance of granula (from Samoszuk et al., 2005).* ***B.*** *Transmission electron micrograph of rat peritoneal MCs depicting the presence of many electron-dense ganula (from Dileepan and Stechschulte, 2006, with kind permission of Springer Science and Business Media).*

A unique characteristic of MCs, possibly a fundamental reason explaining their multi-directional (patho)physiological role, is the large number of their cytoplasmic **granula** (Riley and West, 1952). These electron-dense granules in peritoneal MCs are shown in a transmission electron micrograph in **Fig. 1.3B**. Having been the main issue of MC-related research for decades after MCs were first described, MC-granula contain a large stock of pre-formed soluble mediators; histamine, heparin, proteases, acid hydrolases, chymase and other enzymes (Schwartz and Austen, 1980), occupy the majority of MC-cytoplasmic volume until an appropriate signal will trigger their prompt release in the surrounding microenvironment. Degranulation of MCs, the process of rapid release of pre-stored mediators by fusion of the granula membrane with the plasma membrane, is the quick response of MCs to activating signals. Sometimes beneficial, sometimes detrimental; when pathogens are being attacked, MC degranulation acts as a "first aid" to the threatened organism, when an "innocent" allergen such as grass pollen is the cause, undesired inflammation and anaphylactic shock can be the undue result initiated by MC over-reaction.

In addition to the ability of rapid degranulation and release of pre-stored mediators, MCs have the potential of synthesizing and releasing a large number of **soluble mediators** upon activation. As shown in **Fig. 1.4**, MCs have the capacity to secrete a variety of cytokines, growth factors, chemokines and other classes of newly synthesized soluble factors upon activation.

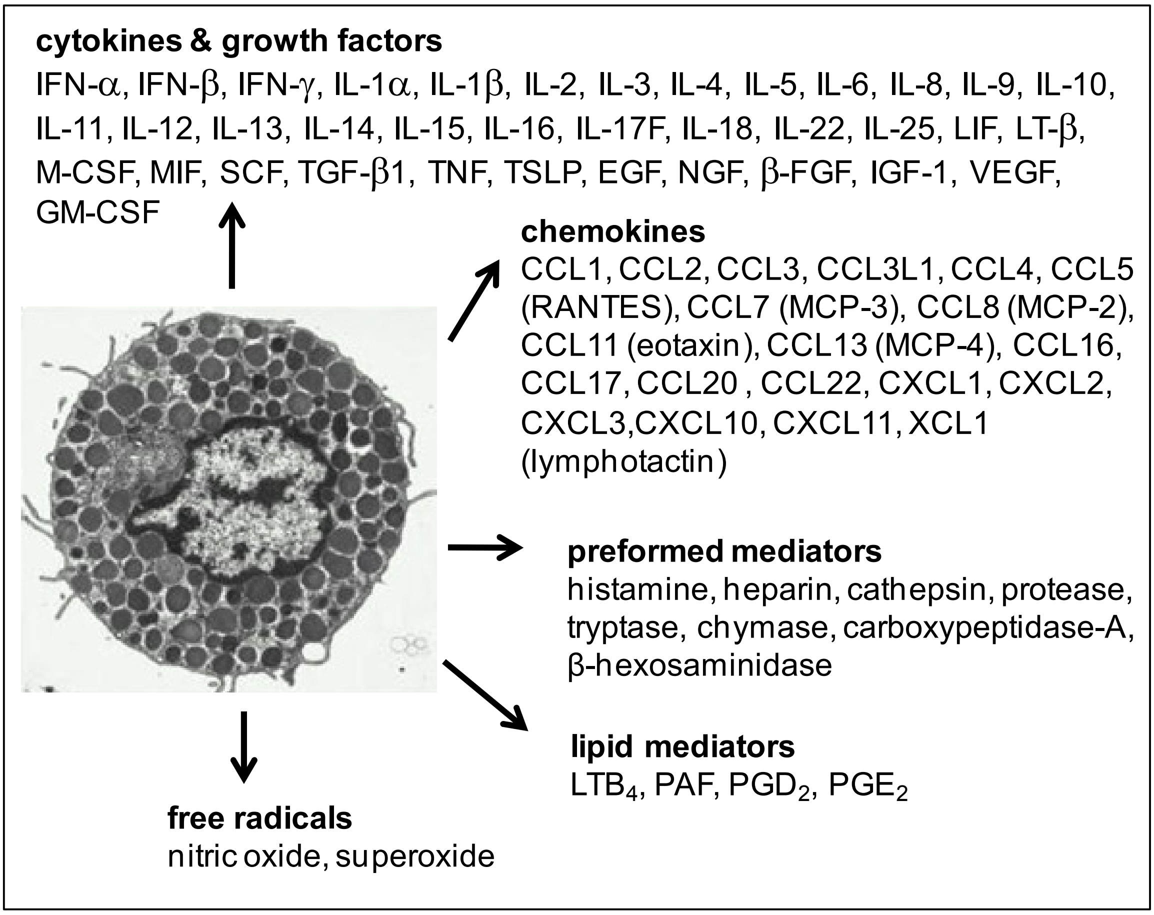

Fig. 1.4. MC-released soluble mediators. *A variety of soluble factors are stored in a preformed state in the MC cytoplasmic granula or are newly generated and secreted upon activation. (modified from Galli et al., 2005b).*

Activation of MCs is induced by various factors, the best studied of which is the crosslinking of the high affinity receptor of IgE (FcεRI) upon specific antigen-binding (Metzger, 1992; Turner and Kinet, 1999). Following FcεRI aggregation, increased mobilization of calcium (Ca^{2+}) mediated by the signaling enzyme phospholipase Cγ (PLCγ), results in MC degranulation. On the other hand, FcεRI signaling also induces phosphorylation of the mitogen-activated protein kinases (MAPK) and extracellular-signal-regulated kinases (ERK), therefore mediates activation of transcription factors (namely AP1, NFAT and NF-kB) which lead to the production of cytokines (Gilfillan and Tkaczyk, 2006). Apart from the antigen-dependent FcεRI-mediated activation of MCs, other stimuli induce MC activation through their interaction with specific receptors. Thus, SCF interacts with the c-kit receptor and induces cytokine and chemokine release and regulates MC growth, differentiation and adhesion to extracellular matrix components (Coleman et al., 1993;

Columbo et al., 1992; Lorentz et al., 2002). Moreover, neuropeptides activate MC degranulation as well as cytokine and chemokine production (Kulka et al., 2007). Interestingly, pathogen-associated molecular patterns (PAMPs) induce MC activation and the release of mediators, via interaction with specific TLRs (Stelekati et al., 2007).

MC functions *in vivo* are studied using **MC-deficient murine models**. MC deficiency in those murine models is based on the concept that the receptor c-kit (CD117) plays a major role in MC differentiation and function. Therefore, mice with c-kit loss of function mutations (WBB6F1-Kit^{W}/Kit^{W-v} mice; Kitamura et al., 1978) or tissue-type specific dysregulation of c-kit expression (Kit^{W-sh}/Kit^{W-sh} mice; Grimbaldeston et al., 2005) lack MCs. An additionally interesting tool using those strains is the possibility to generate “MC-knock-in” mice; after reconstitution with MCs obtained either from bone marrow or from embryonic stem cells or directly isolated, e.g. from peritoneal cavity, these mice can be used to investigate the role of MCs *in vivo* (Nakano et al., 1985).

1.2.1. MCs as participants in allergic responses

Allergy is the result of inappropriate and excessive immune response, often against common, “non-dangerous” antigens. **The allergic responses** either occur within minutes (acute allergic reactions) or within hours (late phase allergic reactions) of allergen exposure. In some cases, symptoms may decrease and revert over time (chronic allergic reactions). When the allergen is encountered systemically, the symptoms occur in several sites simultaneously (anaphylaxis reaction) (Golden, 2007).

For more than a century after their first description by Paul Ehrlich, MCs were only characterized as the main “effector cells” in allergy. The initial role attributed to MCs was their involvement in anaphylactic reactions (Keller, 1962) and in **acute allergic reactions**. Upon antigen/IgE-induced degranulation, MCs release histamine, lipid mediators (prostaglandins, leukotrienes) and proinflammatory cytokines (TNF-α, IL-8) which trigger acute allergic inflammation. The importance of MCs in acute allergic immune responses has been demonstrated *in vivo* by the inability of MC-deficient mice to develop acute IgE-dependent reactions in the skin, respiratory or intestinal tract (Williams and Galli, 2000).

However, the role of MCs in the **late phase allergic reactions** remains controversial and apparently dependent on the immunization procedure and adjuvant used. A commonly used method to study the late phase allergic reactions in mice is based on the intraperitoneal sensitization and airway challenge against ovalbumin (OVA). Consequent symptoms include airway hyperresponsiveness (AHR), increased eosinophilic infiltration in the bronchoalveolar lavage fluid (BAL) and development of OVA-specific serum antibodies (IgE, IgG_1). Takeda et al. (1997) reported that eosinophilic inflammation and AHR in mice sensitized and challenged to OVA is not controlled by MCs. However, using a less potent sensitization protocol, Kobayashi et al. (2000) demonstrated an essential role for MCs in the development of AHR.

1.2.2. MCs and innate immunity

MCs are commonly found at sites exposed to the external environment (Galli et al., 1999; Marshall, 2004). At such places, MCs are capable of encountering exogenous antigens, therefore participating in the innate immune response. So far, MCs are recognized mediators of host defense against parasitic worms, intracellular protozoan parasites and bacteria (Maurer et al., 2003). Less information is available regarding the role of MCs during viral infections.

The crucial contribution of MCs in the defense against **parasites** was defined in the 1980s (Woodbury et al., 1984) and for many years the protective function of MCs was only correlated with parasitic worm infections (Marshall, 2004). MCs also protect against intracellular protozoan parasite infections via degranulation and release of leukotriene B_4 (LTB_4), which induces cytotoxicity against the parasite (Henderson and Chi, 1998; Ben-Rashed et al., 2003).

The recognition of MCs as key players in innate immunity against **bacteria** was promoted by landmark studies of Echtenacher et al. (1996) and Malaviya et al. (1996a). These studies indicated that the presence of MCs is essential for host survival after bacterial infection. MC-deficient mice were shown to be less efficient both in clearing enterobacteria and surviving in the cecal ligation and puncture (CLP)-induced acute septic peritonitis model - the gold standard for sepsis research (Buras et al., 2005).

The role of MCs in **viral infections** is less studied. The synthetic compound polyinosinic-polycytidylic acid (poly[I:C], pIC) is used as a model to study viral infections, since it mimicks dsRNA, which is synthesized by various types of viruses (Der et al., 1997). Upon activation with pIC or virus, MCs release type I IFNs (Kulka, et al., 2004). Moreover, human MCs can be infected by dengue virus, which induces production of chemokines, namely RANTES, MIP-1a and MIP-1b (King et al., 2002). In addition, encephalomyocarditis virus infection results in MC chymase and tryptase production *in vivo* (Kitaura-Inenaga, K., 2003). Interestingly, human MC progenitors, developed from cord blood stem cells of HIV-infected patients, are susceptible to HIV infection (Sundstrom et al., 2004). After maturation in peripheral tissues, HIV-infected MC progenitors mature into latently infected MCs, which comprise a long-living reservoir of the virus in peripheral tissues (Sundstrom et al., 2007). However, the role of MCs in antiviral host defense mechanisms *in vivo* has not been thoroughly investigated.

Lately, functional expression of TLRs has also been detected in human and rodent MCs. **Table 1.1** summarizes the expression of **TLRs on MCs** and the result of TLR-induced MCs activation. The expression of TLRs by MCs suggests that MCs are capable of recognizing innate immune signals and initiating a protective immune response. An interesting question arising from the discovery of TLR-expression on MCs is whether TLR-mediated activation of MCs modulate their ability to direct an adaptive immune response, in a way similar to that of the TLR-mediated activation of DCs.

TLR	Ligand	Result of TLR-induced MC activation		*References*
		in vitro	*in vivo*	
TLR1	lipopeptide	not studied		*Matsushima et al., 2004*
TLR2	peptidoglycan	activation of transcription factors, pro-inflammatory cytokine production	increased vasodilatation, accumulation of neutrophils	*Supajatura et al., 2002; McCurdy et al., 2001; Ushio et al., 2004*
TLR3	dsRNA	transcription of primary response genes, pro-inflammatory cytokine production	up-regulation of co-stimulatory molecules, chemotaxis of $CD8^+$ T cells	*Matsushima et al., 2004; Orinska et al., 2005*
TLR4	LPS, lipid A	activation of transcription factors, MAPK activation, pro-inflammatory cytokine production	neutrophil recruitment, enhanced OVA-induced eosinophilic infiltration	*Ikeda & Funaba, 2003; Masuda et al., 2002; Matsushima et al., 2004; McCurdy et al., 2001; Supajatura et al., 2001; Nigo et al., 2006; Qiao et al., 2006*
TLR6	peptidoglycan, zymosan	not studied		*Ikeda and Funaba, 2003; Matsushima et al., 2004; McCurdy et al., 2001; Supajatura et al., 2001*
TLR7	R-848	pro-inflammatory cytokine production		*Matsushima et al., 2004*
TLR8	R-848	not studied		*Supajatura et al., 2001*
TLR9	CpG	pro-inflammatory cytokine production		*Matsushima et al., 2004*

Table 1.1. TLR expression patterns in MCs and their effect on MC activation. *dsRNA: double-stranded RNA; LPS: lipopolysaccharide; R-848: synthetic imidazoquin-like molecule resiquimod R-848; CpG: unmethylated DNA CpG motifs (modified from Stelekati et al., 2007).*

1.2.3. Emerging role of MCs in adaptive immunity

It is only during the last two decades, that continuously accumulating data suggest a dominant role of MCs in the regulation of adaptive immune responses, by controlling the phenotype and function of the adaptive immunity players (B cells, DCs and T cells), therefore transforming the appreciation of MCs from the pure protagonists of the effector phase of allergy to key regulatory cells at the crossroads between innate and adaptive immunity.

MCs modulate **B cell** responses either via direct cell-cell contact or indirectly via the release of cytokines. Direct cell contact between MCs and B cells occurs via CD40-CD40L interaction and induces IgE production by B cells (Gauchat et al., 1993; Pawankar et al., 1997). Additionally, MCs influence the B cell development and proliferation, as well as the production of immunoglobulins through release of cytokines, namely IL-4, IL-9, IL-13, as well as proteases (Yoshikawa et al., 2001; Stassen et al., 2001; Tkaczyk et al., 1996; Tkaczyk et al., 2000).

Furthermore, MCs influence the **DC** biology. Several MCs products, such as IL-1β, IL-18, RANTES, TNF-α and prostaglandin E_2 have been reported to promote DC migration (Yamazaki et al., 1998; Cumberbatch et al., 2001; Kabashima et al., 2003; Suto et al., 2006), while IgE-mediated activation of MCs directly induces Langerhans cell migration *in vivo* (Jawdat et al., 2004). In addition, MCs regulate the functional maturation of DCs by up-regulating integrin and co-stimulatory molecule expression (Caron et al., 2001b; Skokos et al., 2003). A key function of MCs in regulating DC-mediated immune responses is their ability to down-regulate IL-12 production by DCs via histamine and prostaglandins release and therefore promote a Th-2 T cell response (Caron et al., 2001a; Mazzoni et al., 2006).

A highly effective, most recently studied mechanism, by which MCs exhibit their regulatory effect on B cells and DCs, is dependent on the release of **exosomes**. Heterogeneous in size and shape and stored inside the cytoplasmic granules, MC-derived exosomes contain a pool of MHC class II-, costimulatory- and adhesion-related molecules and are released during the process of exocytosis (Raposo et al., 1997; Skokos et al., 2001a). Exosomes can be transferred between cells of the immune system (Denzer et al., 2000), thus interact with B cells or DCs in an adhesion molecule-dependent way (Skokos et al., 2001b; Skokos et al., 2001c), induce maturation of DCs and initiate adaptive immune responses (Skokos et al., 2003).

An optimal **T cell response** is the center of an antigen-specific cell-mediated immune response. MCs have been shown to modulate $CD4^+$ T cell responses either directly **(Fig. 1.5i)** or indirectly **(Fig. 1.5ii)**. MCs release Th-2-related cytokines such as IL-4, which induce Th-2 polarization of naïve T cells (Mekori and Metcalfe, 1999). In contrast, exosome release by MCs has been correlated with Th-1 differentiation of naïve T cells *in vitro* (Skokos et al., 2001a). On the other hand, the MC-B cell interaction promotes a Th-1 differentiation of naïve T cells (Tkaczyk et al., 2000; Skokos et al., 2001b; Skokos et al., 2001c), while MC-DC interaction, resulting in down-regulation of IL-12 production by DCs promotes a Th-2 polarization of naïve T cells (Caron et a., 2001b; Mazzoni et al., 2006). These interactions suggest that there is a dual role for MCs in regulating the Th1/Th2 equilibrium, depending on the encounter of different cells in every microenvironment (Stelekati et al., 2007).

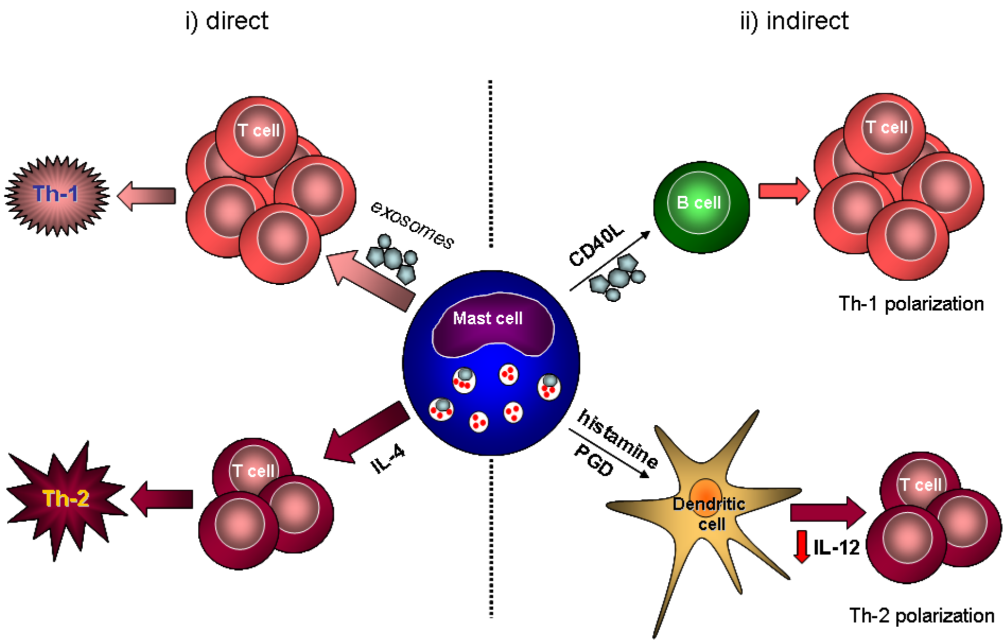

Fig. 1.5. Antigen-independent, MC-mediated modulation of T cell responses. *MCs modulate T cell responses either (i) directly or (ii) indirectly. (i) The release of IL-4 by MCs favours Th-2 polarization of naïve T cells, while exosome release induces Th-1 differentiation. (ii) MCs interact with B cells either directly (e.g. via CD40-CD40L interaction) or via exosome release and this interaction promotes Th-1 differentiation of naïve T cells. On the other hand, MCs interact with DCs via histamine and prostaglandins release, resulting in down-regulation of IL-12 production by DCs and subsequent Th-2 polarization of naïve T cell (from Stelekati et al., 2007).*

A critical question arising after the exciting discovery that MCs influence T cell responses is whether MCs have the potential to **encounter with T cells *in vivo***. Indeed, MCs have been detected in close proximity to T cells at sites of allergic inflammation (Mekori, 2004; Nakae et al., 2005). On one hand, a variety of MC-derived chemokines induce T cell migration towards the site of inflammation (Mekori and Metcalfe, 1999). Furthermore, MC-derived TNF-α induces recruitment of circulating T cells into the draining lymph nodes during bacterial infection (McLachlan et al., 2003). In addition, FcεRI-mediated degranulation of MCs results in chemotaxis of effector but not central memory $CD8^+$ T cells via leukotriene B_4 (LTB_4) secretion (Ott et al., 2003). $CD8^+$ T cell recruitment is also induced by TLR-3-dependent activation of MCs *in vivo* (Orinska et al., 2005). On the other hand, MCs have been also reported to migrate from the site of allergen encounter to the draining lymph nodes, where they recruit T lymphocytes (Wang et al., 1998). This evidence suggests that MCs actively regulate T cell migration, therefore a potential MC-T cell interaction *in vivo* is speculated.

1.2.4. MCs and antigen presentation

Presentation of antigens is of major importance for the initiation of an appropriate adaptive immune response, which will lead to the successful elimination of pathogens as well as to the induction of immunological memory. Antigen presentation in every microenvironment is dependent on the presence and function of the corresponding **APCs** (Unanue, 2007). DCs, B cells and macrophages are regarded as “professional” APCs, however constantly more cell types are suggested to execute similar function. Several hints of MCs exerting such a physiological role have emerged during the last decade; however the admission of MCs as “antigen presenting cells” has not yet been entirely approved by the scientific community.

The first suggestion that **MCs may act as APCs** arouse after the observation that cultured MC progenitors (Wong et al., 1982) as well as primary rat MCs (Banovac et al., 1989) express MHC class II. Thereafter, MHC class II and co-stimulatory molecule expression has been detected on mouse (Frandji, et al., 1993), rat (Fox et al., 1994; Warbrick et al., 1995) and human (Love et al., 1996; Poncet et al., 1999) MCs. Further studies investigated the ability of MCs to present MHC class II-restricted antigens to T cells *in vitro*: mouse BMMCs (Frandji, et al., 1993) as well as rat (Fox et al., 1994) and human (Poncet et al., 1999) primary MCs can efficiently induce antigen-specific proliferation of $CD4^+$ T cell lines *in vitro*. Despite the reports demonstrating the ability of MCs to induce antigen-specific $CD4^+$ T cell responses by presentation of MHC class II-restricted antigens, less information has been obtained so far regarding the ability of MCs to modulate $CD8^+$ T cell responses. A valuable report in this direction by Malaviya *et al.,* demonstrates that BMMCs present bacterial antigens and induce proliferation of a $CD8^+$ T cell line after phagocytosis of living bacteria (Malaviya et al., 1996b). Nevertheless, neither the mechanism of the antigen-specific interaction between MCs and $CD8^+$ T cells, nor its relevance *in vivo* has been investigated so far.

1.3. The goal of this study

Increasing evidence of MCs playing an important regulatory role in adaptive immunity suggests a re-evaluation of their designation as pure innate immune participants and a detailed investigation of their diverse physiological and pathological role. It has been reported that MCs induce CD8$^+$ T cell chemotaxis, however little is known about the potential of MCs to induce further CD8$^+$ T cell responses. The crucial contribution of CD8$^+$ T cells in the defense against intracellular pathogen infections as well as their dominant role in allergic reaction renders essential the detailed examination of the mechanisms that induce their effector functions. A possible crosstalk between MCs and CD8$^+$ T cells could be a key regulatory mechanism to modulate the activities of both MCs and CD8$^+$ T cells in various pathophysiological situations. For this reason, the purpose of this study was to investigate the ability of MCs to interact with CD8$^+$ T cells, present an MHC class I-restricted antigen and initiate antigen-specific primary CD8$^+$ T cell responses *in vitro* and *in vivo*. Furthermore, since MCs are resident cells in the periphery, therefore have the potential to contact activated T cells, the effect of MC interactions with activated CD8$^+$ T cells was studied. Finally, the investigation of the regulatory effects that a MC-CD8$^+$ T cell interaction has on the MCs was pursued.

In summary, the questions addressed during this study were the following:

1. Are MCs able to modulate primary CD8$^+$ T cell responses in an antigen-independent manner?
2. Can MCs initiate antigen-specific CD8$^+$ T cell responses?
    - What are the outcomes (e.g. activation, proliferation, cytokine production, cytotoxicity) of a MC-CD8$^+$ T cell antigen-specific interaction?
    - What is the mechanism of a MC-CD8$^+$ T cell antigen-specific interaction?
3. Do MCs regulate CD8$^+$ T cell responses in antigen-driven manner *in vivo*?
    - Do MCs contribute to the modulation of CD8$^+$ T cell activities in an allergic murine model?
4. Do MCs tune the activities of effector CD8$^+$ T cells?
5. Do CD8$^+$ T cells after the MC encounter affect the activities of their MC partners?

The questions aroused during this study are schematically depicted in **Fig. 1.6**.

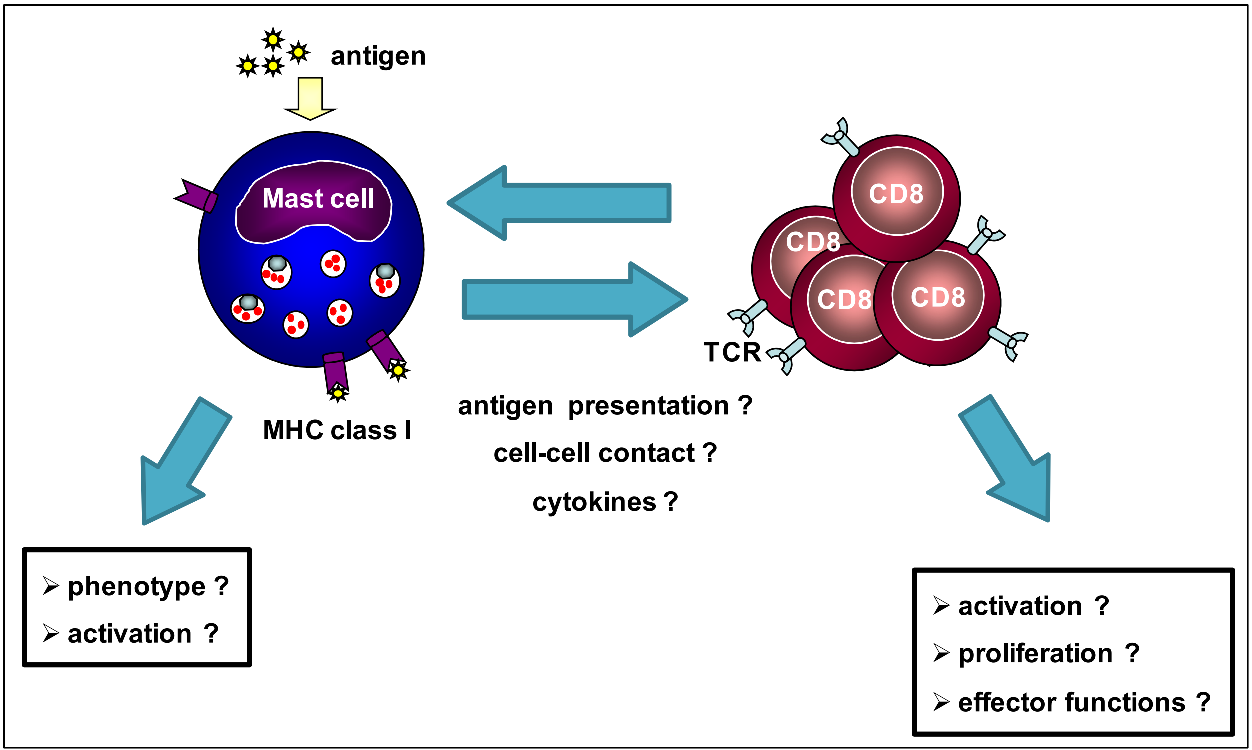

Fig. 1.6. Questions aroused during the present study. *MCs have been shown to induce chemotaxis of CD8*$^{+}$ *T cells, however little is known about their further interaction with CD8*$^{+}$ *T cells. Therefore, this study investigated the ability of MCs to induce CD8*$^{+}$ *T cell responses in an antigen-dependent and antigen-independent manner as well as the ability of CD8*$^{+}$ *T cells to modulate MC activities.*

2. Materials and Methods

2.1. Materials

2.1.1. Animals

C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). TCR-transgenic **OT-I** mice (Hogquist et al., 1994) were bred in the Animal Care Facility of the Research Center Borstel, Germany. **Beta-2-microglobulin-deficient** mice ($\beta 2m^{-/-}$) (Koller et al., 1989) were kindly provided by F. Winau (MPI for Infection Biology, Berlin, Germany). Mast cell (MC)-deficient **WBB6F1-Kit^{W}/Kit^{W-v}** mice (Kitamura et al., 1978) were kindly provided by Prof. M. Maurer (Charite, Berlin, Germany) and bred in FEM Berlin.

2.1.2. Chemicals and other reagents

Reagent:	**Purchased from:**
Accutase	PAA Laboratories, Pasching, Austria
Agarose	Invitrogen, Paisley, U.K.
Alum Imject ($Al(OH)_3$)	Pierce Biotechnology, Rockford, U.S.A.
Ammonium chloride (NH_4Cl)	Merck, Darmstadt, Germany
Aqua B. Braun H_2O	Melsungen, Germany
Bio-Plex Cytokine Assay (10-plex, mouse Th1/Th2)	Bio-Rad Laboratories, Munich, Germany
Boric acid (H_3BO_3)	Merck, Darmstadt, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, Steinheim, Germany
Brefeldin A	Sigma-Aldrich, Steinheim, Germany
CFDA SE Cell Tracer Kit (CFSE)	Molecular Probes, Leiden, The Netherlands
Chloroform ($CHCl_3$)	Merck, Darmstadt, Germany
Ciprofloxacinhydrochlorid	Wald-Apotheke, Wahlstedt, Germany
Collagenase Type IV (from Clostridium histolyticum)	Sigma-Aldrich, Steinheim, Germany

Deoxyribonuclease I	Sigma-Aldrich, Steinheim, Germany
Diethylpyrocarbonate (DEPC)-H_2O	Sigma-Aldrich, Steinheim, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Steinheim, Germany
Disodium hydrogen phosphate (Na_2HPO_4)	Merck, Darmstadt, Germany
dNTP (10 mM)	Roche, Mannheim, Germany
DuoSet ELISA Development Kit (IFN-γ)	R&D Systems, Wiesbaden, Germany
DuoSet ELISA Development Kit (IL-2)	R&D Systems, Wiesbaden, Germany
Ethanol (C_2H_5OH)	Merck, Darmstadt, Germany
Ethidium bromide	Merck, Darmstadt, Germany
Ethylene diamine tetraacetic acid (EDTA)	Merck, Darmstadt, Germany
Fetal calf serum (FCS)	Biochrom, Berlin, Germany
Fluorescein Isothiocyanate Isomer I (FITC)	Sigma-Aldrich, Steinheim, Germany
Hydrochloric acid (HCl)	Merck, Darmstadt, Germany
Hydrogen peroxide (H_2O_2)	Merck, Darmstadt, Germany
Hematoxylin	Merck, Darmstadt, Germany
Hydrogen citrate (citric acid) ($C_6H_8O_7$)	Merck, Darmstadt, Germany
IMDM	PAA Laboratories, Pasching, Austria
Isopropanol (C_3H_8O)	Gibco, Rockville, USA
L-glutamine with penicillin/streptomycin	PAA Laboratories, Pasching, Austria
Loading dye (6-fold)	MBI Fermentas, St.Leon-Rot, Germany
LPS (from *Salmonella enterica*)	provided by H. Brade, Borstel
Lysine	Merck, Darmstadt, Germany
Magnesium chloride ($MgCl_2$)	Merck, Darmstadt, Germany
Microarray (Maus MG 430 2.0)	Affymetrix, Santa Clara, U.S.A.
Microbeads (anti-FITC)	Miltenyi Biotec, Bergisch Gladbach, Germany
Microbeads (streptavidin)	Miltenyi Biotec, Bergisch Gladbach, Germany
Mitomycin C	Sigma-Aldrich, Steinheim, Germany
Molecular weight marker	MBI Fermentas, St.Leon-Rot, Germany
Newborn calf serum (NCS)	Gibco, Rockville, USA
Non-essential amino acids	Gibco, Rockville, USA
Ovalbumin (OVA) grade V	Sigma-Aldrich, Steinheim, Germany
Ovalbumin (OVA) grade VI	Sigma-Aldrich, Steinheim, Germany
$OVA^{257-264}$ (SIINFEKL)	NeoMPS, Strasbourg, France

Paraformaldehyde	Fluka, Steinheim, Germany
PBS for cell culture	
(without Magnesium, without Calcium)	PAN Biotec, Aidenbach, Germany
Polyinosinic-polycytidylic acid (pIC)	Sigma-Aldrich, Steinheim, Germany
Potassium chloride (KCl)	Merck, Darmstadt, Germany
Potassium phosphate (KH_2PO_4)	Merck, Darmstadt, Germany
Propidium iodide (PI)	Sigma-Aldrich, Steinheim, Germany
Random Primer	New England Biolabs, Frankfurt, Germany
Ribonuclease Inhibitor	MBI Fermentas, St.Leon-Rot, Germany
RPMI-1640	Biochrom, Berlin, Germany
Sephadex G50	GE Healthcare, Freiburg, Germany
Sodium azide (NaN_3)	Merck, Darmstadt, Germany
Sodium carbonate (Na_2CO_3)	Merck, Darmstadt, Germany
Sodium chloride (NaCl)	Merck, Darmstadt, Germany
Sodium pyruvat ($C_3H_3NaO_3$)	Gibco, Rockville, U.S.A.
Streptavidin-HRP	R&D Systems, Wiesbaden, Germany
Succrose	Sigma-Aldrich, Steinheim, Germany
Sulfuric acid (H_2SO_4)	Merck, Darmstadt, Germany
Superscript II Reverse Transcriptase Kit	Invitrogen, Paisley, U.K.
Taq-DNA-Polymerase (SAWADY)	Peqlab Biotechnologie, Erlangen, Germany
Tetramethylbenzidin	Fluka, Steinheim, Germany
Toluidine blue	Sigma-Aldrich, Steinheim, Germany
Tris	Serva GmbH, Heidelberg, Germany
Trizol-Reagent	Invitrogen, Paisley, U.K.
Trypan blue	
(0.5% w/v in physiological saline)	Biochrom, Berlin, Germany
Tween 20	Sigma-Aldrich, Steinheim, Germany
Vitamin	Gibco, Rockville, U.S.A.
β-mercaptoethanol	Gibco, Rockville, U.S.A.

2.1.3. Cytokines

Cytokine:	**Purchased from:**
Recombinant mouse IL-3	R&D Systems, Wiesbaden, Germany
Recombinant mouse SCF	R&D Systems, Wiesbaden, Germany
Recombinant mouse GM-CSF	Peprotec, London, U.K.

2.1.4. Monoclonal antibodies and detection reagents for FACS

Antibody (specific for mouse):	**Purchased from:**
CD117 (c-Kit, 2B8)	BD PharMingen, Heidelberg, Germany
CD11b (Integrin α_M chain, M1/70)	BD PharMingen, Heidelberg, Germany
CD11c (Integrin α_x chain, HL3)	BD PharMingen, Heidelberg, Germany
CD16/32 (Fcγ III/II, 2.4G2)	BD PharMingen, Heidelberg, Germany
CD25 (IL-2 receptor α chain, p55)	BD PharMingen, Heidelberg, Germany
CD3ε (CD3 ε chain, 145–2C11)	BD PharMingen, Heidelberg, Germany
CD4 (L3T4, RM4-5)	BD PharMingen, Heidelberg, Germany
CD44 (Pgp-1, Ly-24, IM7)	BD PharMingen, Heidelberg, Germany
CD45R/B220 (RA3-6B2)	BD PharMingen, Heidelberg, Germany
CD49b/Pan-NK Cells (DX5)	BD PharMingen, Heidelberg, Germany
CD62L (L-selectin, LECAM-1, Ly-22, MEL-14)	BD PharMingen, Heidelberg, Germany
CD69 (very early activation antigen, H1.2F3)	BD PharMingen, Heidelberg, Germany
CD80 (B7.1, 16-10A1)	BD PharMingen, Heidelberg, Germany
CD86 (B7.2, GL1)	Southern Biotechnology, Alabama, U.S.A.
CD8α (Ly-2, 53-6.7)	BD PharMingen, Heidelberg, Germany
F(ab´)2 anti-rat IgG	AbD Serotec, Dusseldorf, Germany
F4/80 antigen (MCA497FB)	AbD Serotec, Dusseldorf, Germany
FcεRI alpha (MAR-1)	eBioscience, Frankfurt, Germany
Gr-1 (Ly-6C and G, 53-6.7)	BD PharMingen, Heidelberg, Germany
Granzyme B (16G6)	eBioscience, Frankfurt, Germany
H2-kb (MHC class I) (AF6-88.5)	BD PharMingen, Heidelberg, Germany

I-A/I-E (MHC class II) (M5/114.15.2)	BD PharMingen, Heidelberg, Germany
IFN-γ	BD PharMingen, Heidelberg, Germany
IL-2	BD PharMingen, Heidelberg, Germany
Isotype controls	BD PharMingen, Heidelberg, Germany
LAMP-1	DSHB, Iowa, U.S.A.
Streptavidin-APC	BD PharMingen, Heidelberg, Germany
Streptavidin-FITC	Genzyme, Cambridge
Streptavidin-PE	Dianova, Hamburg, Germany
T1/ST2 (DJ8)	Morwell Diagnostics, Switzerland

2.1.5. Primers

Primers for PCR were purchased from Metabion (Martinsried, Germany) at stock concentration of 100 µM. Following primer pairs were used:

mouse Lzp-s-F: 5´-AGA ATG CCT GTG GGA TCA AT- 3´
mouse Lzp-s-R: 5´-CTG GGA CAG ATC TCG GTT TT- 3´

mouse 4-1BB-F: 5´-AGT GTC CTG TGC ATG TGA- 3´
mouse 4-1BB-R: 5´-AGT TAT CAC AGG AGT TCT GC- 3´

mouse 4-1BBL-F: 5´-CGC TTT GGT TTT GCT GCT TCT G- 3´
mouse 4-1BBL-R: 5´-CAT CTA CCT GAG GCT TTG CTT GC- 3´

mouse Tgm-2-F: 5´-TCA GCC AGC AGC CTC TAG AC- 3´
mouse Tgm-2-R: 5´-CCT ACT GCC TGC TTG GAA CC- 3´

mouse IFN-γ-F: 5´-AGC GGC TGA CTG AAC TCA GAT TGT AG- 3´
mouse IFN-γ-R: 5´-GTC ACA GTT TTC AGC TGT ATA GGG- 3´

mouse β-actin-F: 5´-CTC CTT AAT GTC ACG CAC GAT TTC- 3´
mouse β-actin-R: 5´-GTG GGG CGC CCC AGG CAC CA- 3´

2.1.6. Buffers

2.1.6.1. Buffers for cell culture

Phosphate buffered saline (PBS) (PAN Biotec, Aidenbach, Germany)

IMDM complete medium = Iscove´s modified DMEM (IMDM) + 10% heat-inactivated FCS + 50 µM β-mercaptoethanol + 2 mM L-glutamine + 100 U/mL penicillin + 100 µg/mL streptomycin + 1-fold non-essential amino acids + 1 mM sodium pyruvat

RPMI complete medium = RPMI-1640 + 10% heat-inactivated FCS + 50 µM β-mercaptoethanol + 2 mM L-glutamine + 100 U/mL penicillin + 100 µg/mL streptomycin

Erythrocyte lysis buffer = H_2O + 0.83% NH_4Cl + 0.168% Na_2CO_3 + 1 mM EDTA, pH=7.3, sterile filtrated

2.1.6.2. Buffers for cell isolation

MACS buffer = PBS + 5% heat-inactivated FCS

2.1.6.3. Buffers for flow cytometry

FACS buffer = PBS + 2% NCS + 0.1% NaN_3 + 2 mM EDTA, pH=7.4 - 7.5

Phosphate buffered saline (PBS) = H_2O + 3.2 mM Na_2HPO_4 + 0.5 mM KH_2PO_4+ 1.3 mM KCl + 135 mM NaCl, pH = 7.4

<u>For intracellular FACS staining:</u>

IC Fixation buffer (eBioscience, Frankfurt, Germany), containing 4% paraformaldehyde

10-fold Permeabilization buffer (eBioscience, Frankfurt, Germany), diluted 1:10 with Aqua B. Braun H_20 (working solution), containing 0.1% saponin and 0.09% sodium azide at the 1-fold final working solution

Flow cytometry staining buffer (eBioscience, Frankfurt, Germany), containing fetal bovine serum and sodium azide

2.1.6.4. Buffers for ELISA

Blocking buffer = PBS + 1% BSA + 5% sucrose + 0.05% sodium azide

Washing buffer = PBS + 0.05% Tween 20, pH=7.2-7.4

Reagent diluent buffer = Tris-buffered saline (20 mM Tris, 150 mM NaCl) + 0.1% BSA+ 0.05% Tween 20, pH=7.2-7.4

2.1.6.5. Buffers for molecular biological techniques

Reaction buffer for cDNA synthesis = 5.0 µl 5-fold Strand-Buffer + 2.5 µl DTT-Buffer (0.1 M) + 5.0 µl dNTP´s (10 mM) + 0.75 µl Ribonuclease Inhibitor + 4.5 µl DEPC-H_2O

5-fold strand-buffer (Invitrogen, Paisley, U.K.), containing 250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, pH=8.3 at room temperature (RT)

DTT-Buffer (Invitrogen, Paisley, U.K.)

10-fold PCR-buffer for SAWADY Taq-DNA-polymerase (Peqlab Biotechnologie, Erlangen, Germany), containing 100 mM Tris-HCl, 500 mM KCl, 0.1% Tween 20, 15 mM $MgCl_2$, pH = 8.8

Reaction mix for PCR = 2 µl 10-fold PCR-Buffer + 1 µl forward primer + 1 µl reverse primer + 0.3 µl dNTP´s (10 mM) + 0.2 µl Taq Polymerase + 14.5 µl Aqua B. Braun H_2O

Electrophoresis buffer (Tris/boric acid/EDTA; TBE) = 89 mM Tris + 89 mM boric acid + 2 mM EDTA, pH = 8.0

2.1.7. Laboratory supplies

Supply:	**Purchased from:**
Anopore membrane (0.02 µm, 10 µm)	Nunc, Roskilde, Dennmark
Anopore membrane (0.2 µm, 10 µm)	Nunc, Roskilde, Dennmark
Gel casting tray	Bio-Rad Laboratories, Munich, Germany
Microcentrifuge tubes (1.5 ml, 2 ml)	Sarstedt, Nümbrecht, Germany
Microscope cover slips (24 x 40 mm)	Gerhard Menzel Glasbearbeitungswerk, Braunschweig, Germany
Microscope slides (76 x 26 mm)	Waldemar Knittel Glasbearbeitung, Braunschweig, Germany
Multidish with 24 wells	Nunc, Roskilde, Dennmark
Multidish with 48 wells	Nunc, Roskilde, Dennmark
Multidish with 96 wells (flat-bottom)	Nunc, Roskilde, Dennmark
Needles (23 G, 27 G)	Becton Dickinson S.A., Madrid, Spain
Neubauer counting chamber	Paul Marienfeld, Königshofen, Germany
Petri dish (100 x 15 mm, sterile)	Becton Dickinson Labware, Franklin Lakes, U.S.A.
Plastic pipette tips	Sarstedt, Nümbrecht, Germany
Plastic pipettes (5 ml, 10 ml, 25 ml)	Greiner bio-one, Frickenhausen, Germany
Plastic tubes (15 ml, 50 ml)	Sarstedt, Nümbrecht, Germany
Syringe (1 ml, 5 ml, 10 ml)	Becton Dickinson S.A., Madrid, Spain
Tissue culture flask (non-pyrogenic, 250 ml, 75 cm^2, sterile)	Greiner bio-one, Frickenhausen, Germany
Tubes for FACS (5 ml, 75 x 12 mm)	Sarstedt, Nümbrecht, Germany

2.1.8. Laboratory equipment

Equipment:	**Purchased from:**
Automacs	Miltenyi Biotec, Bergisch Gladbach, Germany
Bench	Kojair, Vilppula, Finland
Bio-Plex array reader	Bio-Rad Laboratories, Munich, Germany

Centrifuge Mikro 22	Hettich Zentrifugen, Tuttlingen, Germany
Centrifuge Rotina 46 R	Hettich Zentrifugen, Tuttlingen, Germany
Cytocentrifuge (Cytospin3)	Shandon, Frankfurt, Germany
ELISA reader	Tecan, Salzburg, Austria
ELISA washer	Tecan, Salzburg, Austria
FACS Calibur flow cytometer	BD Biosciences, Heidelberg, Germany
Fluorescence microscope (Diaphot 300)	Nikon, Japan
Glassware	Duran Group, Mainz, Germany
Incubator	Kendro Laboratory Products, Langenselbold, Germany
Microplate shaker	Laborbedarf Hassa, Lübeck, Germany
Microwave oven (1026L)	Privileg, Korea
Microscope	Olympus Optical, Japan
pH Meter	Baack Laborbedarf, Schwerin, Germany
Photometer (automatic)	Eppendorf, Hamburg, Germany
Pipettes	Eppendorf, Hamburg, Germany
Power supply (Power PAC300)	Bio-Rad Laboratories, Munich, Germany
Precision Balance	A&D Instruments, Japan
Thermoblock	Biometra, Goettingen, Germany
Thermocycler	Eppendorf, Hamburg, Germany
Transluminator with camera	Biometra, Goettingen, Germany
Vortex (Minishaker MS2)	IKA Works, Wilmington, USA
Waterbath	Medingen, Germany

2.1.9. Software

Bio-Plex Manager Software	Bio-Rad Laboratories, Munich, Germany
Cell Quest	Becton Dickinson, Immunocytometry Systems, California, U.S.A.
Magellan	Tecan, Salzburg, Austria
Microsoft Office 2007	Microsoft Cooperation, CA, U.S.A.

2.2. Methods

2.2.1. Cell culture techniques

2.2.1.1. Cell counting

Counting of cells was performed with exclusion of dead cells by trypan blue. Trypan blue permeates the membrane of only dead cells, therefore dead cells appear blue. Working solution of trypan blue was prepared by diluting trypan blue 1:10 in 0.9% NaCl. The cell suspension to be counted was diluted 1:10 in the working solution of trypan blue and cells were counted using a Neubauer counting chamber under optical microscope.

2.2.1.2. Generation and cultivation of BMMCs

As a model of studying mast cell (MC) phenotype and function, bone marrow-derived mast cells (BMMCs) were used. BMMCs were obtained as described previously (Razin et al., 1984; Dvorak et al., 1994) with some modifications. Bone marrow was isolated from femurs and tibia of C57BL/6 wild-type mice or of $\beta 2m^{-/-}$ mice. The bone marrow was flushed with phosphate buffered saline (PBS) and washed once with PBS. All cell washing steps were carried out in centrifuge at 270 x g for 10 min at 4° C. Erythrocytes were lysed by incubation in erythrocyte lysis buffer for 15 min at room temperature (RT). Next, cells were washed with IMDM complete medium and cultured in a 75 cm^2 tissue culture flask. Cells were maintained in IMDM complete medium supplemented with 5 ng/ml murine recombinant IL-3 and 10 ng/ml murine recombinant SCF. Once per week, cells were collected from the flask, centrifugated, resuspended in fresh IMDM complete medium supplemented with 5 ng/ml IL-3 and 10 ng/ml SCF and plated in a new flask. During the first 2 weeks of culture, ciprofloxacinhydrochlorid was added at a concentration of 10 µg/ml. The cells were maintained in incubator with 7.5% CO_2 at 37° C. Five weeks after the initiation of the culture, the maturation and purity of BMMCs were tested with toluidine blue/hematoxylin staining **(2.2.1.3)** and FACS analysis **(2.2.4.1)** for cell surface expression of CD117 (c-Kit), FcεRI, CD11c, CD11b, CD45R/B220 and F4/80 .

2.2.1.3. Toluidine blue/hematoxylin staining of BMMCs

Toluidine blue staining of MCs was performed in order to test MC maturation. Toluidine blue is a metachromatic dye, which binds to biologically active mediators in MC granula. Cytospins of MCs were prepared using a cytocentrifuge on 76 x 26 mm microscope slides. Cells were fixed in 50% ethanol for 15 min at RT and then transferred into toluidine blue working solution, consisting of 0.2% toluidine blue and 1.92% hydrogen citrate in 50% ethanol. Cells were stained with toluidine blue for 30 min at RT and subsequently washed with distilled water. Afterwards, cells were stained with hematoxylin for 3 min at RT and then washed with water. Slides were left to dry on air and covered with microscope cover slips before being examined using a microscope.

2.2.1.4. Generation of BMDCs

As a positive control for antigen presentation experiments, bone marrow-derived dendritic cells (BMDCs) were used. BMDCs were derived as previously described (Scheicher et al., 1992; Inaba et al., 1992). Bone marrow isolated from femurs and tibia of C57BL/6 wild-type mice was flushed with phosphate buffered saline (PBS) and washed once with PBS. All cell washing steps were performed at 270 x g for 10 min at 4° C. Erythrocytes were lysed by incubation in erythrocyte lysis buffer for 15 min at RT. Next, cells were washed with RPMI complete medium and viable cells were counted after staining with trypan blue. A number of 3 x 10^6 bone marrow cells were seeded in a 100 x 15 mm petri dish in 10 ml complete RPMI medium supplemented with 20 ng/ml murine recombinant GM-CSF. The cells were maintained in incubator with 5% CO_2 at 37° C. On day 3, 10 ml RPMI complete medium supplemented with 20 ng/ml GM-CSF were added to the cells. On day 6, 10 ml of the cells were removed in a plastic sterile tube, centrifugated, resuspended in fresh 10 ml RPMI complete medium supplemented with 10 ng/ml GM-CSF and added to the rest of the cells in the petri dish. On day 8, the maturation of BMDCs was tested by FACS analysis **(2.2.4.1)** for surface expression of CD11c, CD80 and MHC class II expression.

2.2.1.5. Detachement of BMDCs

BMDCs are adherent cells at the bottom of the petri dish. On day 8 of BMDC differentiation, cells were detached from the bottom of the petri dish with the use of accutase. The medium of the cell culture was removed into a plastic sterile 50 ml tube. 5 ml PBS were used to wash the petri dish and subsequently added to the tube with the rest of the cells. 3 ml of pre-warmed accutase were added into the petri dish and incubated for 20 min at 5% CO_2 in 37° C in an incubator. Next, the petri dish was washed with PBS until the cells were detached. The supernatant was given to the rest of the cells in the plastic tube and the petri dish was washed twice with 5 ml RPMI complete medium. Finally, the collected cells were added to the rest of the cells in the plastic tube and centrifugated at 270 x g for 10 min, at 4° C.

2.2.2. Cell purification

2.2.2.1. CD8$^+$ T cell separation

CD8$^+$ T cells were obtained from TCR-transgenic (OT-I) mice using magnetic-associated cell sorting (MACS). Mice were euthanized with CO_2 inhalation and inguinal, cervical, axillary, brachial, mesenteric and lumbar lymph nodes were excised and homogenized in PBS by forcing them through a metallic mesh. All cell washing steps were carried out at 270 x g for 10 min, at 4° C. Erythrocytes were lysed by incubation in erythrocyte lysis buffer for 15 min at RT. Afterwards, cells were washed with RPMI complete medium, counted and resuspended in MACS buffer at a concentration of 10^8 cells/ml.

Negative selection of CD8$^+$ T cells from the lymph node cell suspension was performed according to a standard protocol provided by Miltenyi Biotec (www.miltenyibiotec.com). The cells were incubated with 1 μg/ml FITC-labeled monoclonal antibodies against CD4, CD45R/B220, CD49b/Pan-NK, CD11c and F4/80 for 20 min on ice, in the dark. After a washing step with MACS buffer, cells were incubated with anti-FITC microbeads for 15 min at 4° C in the dark, according to the manufacturer´s instructions. Finally, magnetic associated cell sorting using the program "DEPLETES" (for increased purity) of automacs was performed, resulting in two separate fractions: a negative fraction containing the unlabeled $CD8^+$ T cells and a positive fraction containing the rest of the lymph node cells (FITC-labeled). The purity of the $CD8^+$ T cell fraction was tested by FACS analysis **(2.2.4.1)**.

2.2.2.2. Purification of MCs

In some experiments, MCs were purified after co-culture with $CD8^+$ T cells *in vitro*. For purification of MCs, positive selection with MACS was used. Cells were washed with MACS buffer, counted and resuspended at a concentration of 10^8 cells/ml. Cells were incubated with 2.5 µg/ml biotin-labeled monoclonal antibody against CD117 for 20 min on ice, in the dark. After a washing step with MACS buffer, cells were incubated with streptavidin-micobeads for 15 min at 4° C in the dark, according to the manufacturer´s instructions. Magnetic associated cell sorting using the program "POSSEL" (for increased purity) of automacs resulted in two separate fractions: a negative fraction containing the unlabeled $CD8^+$ T cells and a positive fraction containing the labeled MCs. To increase the purity of MCs, the positive fraction was re-labeled with streptavidin-microbeads and sorted with automacs for a second time. The purity of the MC fraction was tested by FACS analysis **(2.2.4.1)**.

2.2.3. *In vitro* activation of $CD8^+$ T cells by MCs

2.2.3.1. Pulsing of BMMCs with $OVA^{257\text{-}264}$peptide

In order to test the ability of MCs to induce antigen-specific $CD8^+$ T cell responses, BMMCs were pulsed with the OT-I specific $OVA^{257\text{-}264}$ peptide. The pulsing of BMMCs with the $OVA^{257\text{-}264}$ peptide was performed according to standard protocols used for DCs (Kukutsch et al., 2000; Rückert et al., 2003). BMMCs were resuspended in IMDM complete medium at a concentration of 10^7 cells/ml. The $OVA^{257\text{-}264}$ peptide was added to the cell suspension at a final concentration of 4 µg/ml and cells were incubated for 3 hrs in a waterbath at 37° C. Cells were gently shaken every 30 min. Afterwards, cells were washed 4 times with 5 ml IMDM complete medium, resuspended in IMDM complete medium and counted after staining with trypan blue.

In some experiments, the cytokine release from BMMCs was inhibited by mitomycin C or paraformaldehyde treatment of the BMMCs. For this reason, following the pulsing with $OVA^{257-264}$ peptide, BMMCs were treated with 10 µg/ml mitomycin C for 30 min in a waterbath at 37° C or with 1% paraformaldehyde for 3 hrs at RT, in the dark. After mitomycin C or paraformaldehyde treatment, the BMMCs, as well as their untreated controls, were washed 3 times with 5 ml IMDM complete medium. Finally, cells were counted and resuspended in IMDM complete medium.

2.2.3.2. In vitro MC-CD8$^+$ T cell co-culture

Purified $CD8^+$ T cells were plated in the wells of a 48-well multidish plate at a concentration of 1 x 10^6 cells/ml. In parallel, 0.01 x 10^6 - 0.5 x 10^6 of $OVA^{257-264}$ -pulsed or control unpulsed BMMCs were added to the $CD8^+$ T cells, as indicated in each experiment. 1 µg/ml murine recombinant IL-3 was added to the wells and the final volume was brought to 500 µl by addition of IMDM complete medium.

In some experiments, the direct cell-cell contact between $CD8^+$ T cells and BMMCs was inhibited by placing a 0.2 µm or a 0.02 µm anopore membrane in a 24-well multidish plate. In this case, $CD8^+$ T cells were plated at the bottom part of the wells, while BMMCs were placed inside the upper chamber of the transwell.

Unless otherwise stated, the MC-$CD8^+$ T cell co-culture lasted for 48 hrs. However, for measuring the $CD8^+$ T cell proliferation by CFSE **(2.2.5.1)**, the MC-$CD8^+$ T cell co-culture lasted for 72 hrs. In either case, supernatants were collected for measurement of secreted cytokines **(2.2.6)** and cells were analyzed by flow cytometry **(2.2.4.)**. A schematic representation of the *in vitro* MC-$CD8^+$ T cell co-culture experimental setup is depicted in **Fig. 2.1**.

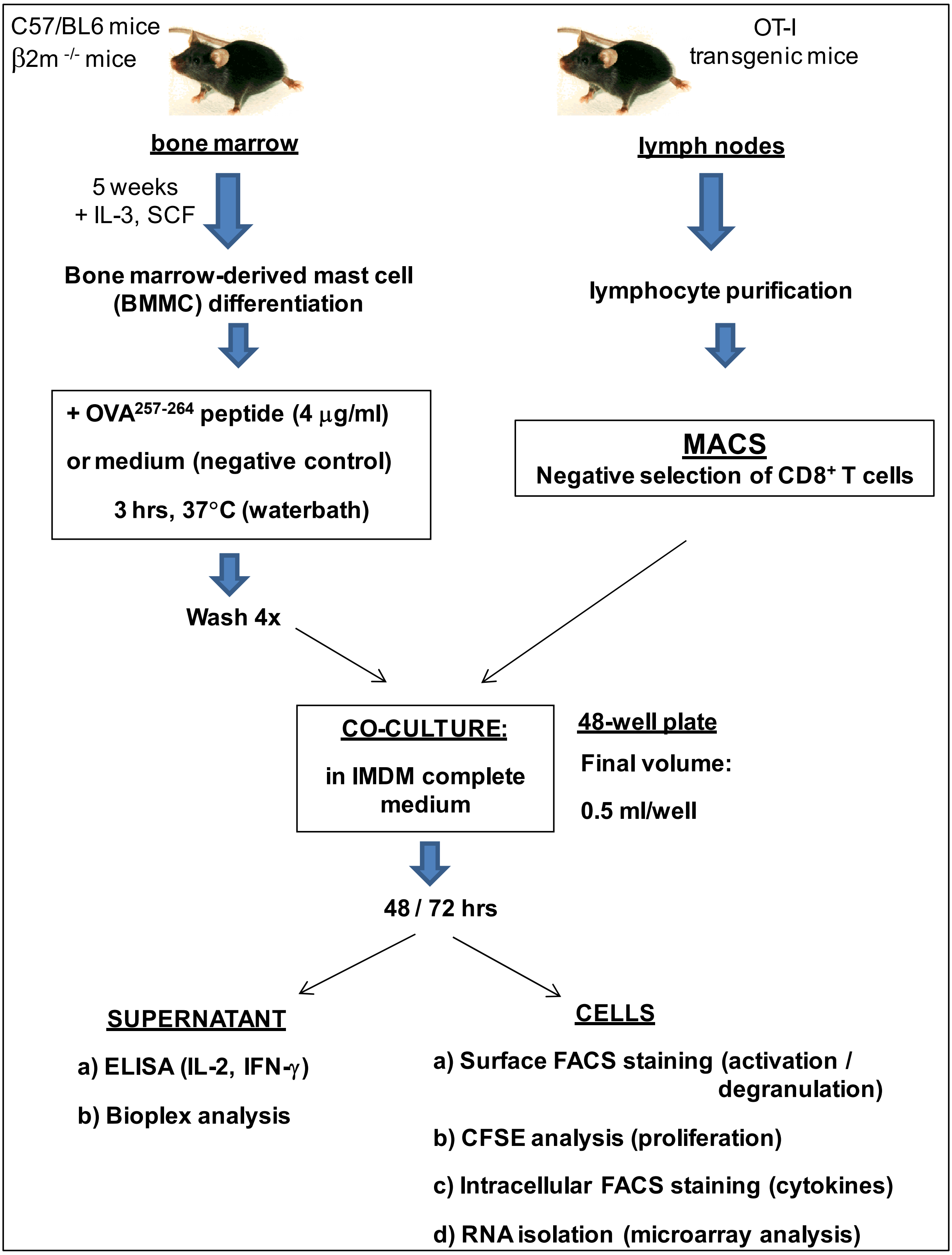

Fig. 2.1. Experimental setup for studying the interactions between MCs and CD8^{+} T cells in vitro.

2.2.4. Flow cytometry

2.2.4.1. Surface staining

For surface FACS staining, 2 x 10^5 – 5 x 10^5 cells were washed once, resuspended in 100 µl FACS buffer and stained with phycoerythrin-(PE), allophycocyanin-(APC), or fluorescein isothyocyanate-(FITC) conjugated monoclonal antibodies. The antibodies used for surface FACS staining are shown in **2.1.4**. Cells were distributed into 5 ml FACS tubes and each sample was stained with a maximum of three antibodies: FITC-, PE- or APC-conjugated. Unstained cells or isotype-control stained cells served as a negative control, as indicated in each experiment. Unless otherwise specified, the antibodies for FACS staining were added to the cells at a concentration of 0.2 µg/ml. Cells were incubated for 20 min at 4° C in the dark and subsequently washed with 1µg/ml propidium iodide in FACS buffer, in order to stain for dead cells.

In some experiments, biotin-labeled antibodies were used for surface FACS staining. These samples were stained with 0.2 µg/ml biotin-labeled antibodies for 20 min, at 4° C in the dark and subsequently washed with FACS buffer. Cells were then stained with a secondary antibody, namely FITC-, PE- or APC- conjugated streptavidin for 20 min, at 4° C in the dark and finally washed with 1µg/ml propidium iodide in FACS buffer.

For analysis of MC phenotype, the unspecific binding of antibodies to the Fc receptor of MCs was blocked by the use of purified anti-mouse CD16/32 antibody. MCs were incubated with 10 µg/ml purified anti-CD16/32, for 15 min at 4° C. The antibodies for surface staining of MCs were added without washing the anti-CD16/32 antibodies away. Samples were incubated for 20 min at 4° C in the dark and subsequently washed with 1µg/ml propidium iodide in FACS buffer.

All samples were analyzed using a FACS Calibur flow cytometer. Gate on viable cells was set according to exclusion of propidium iodide staining. Further gates were set on forward- sideward- scatter, or a particular stained population, as indicated in each experiment. Data were analyzed with CellQuest software.

2.2.4.2. Intracellular staining

In order to examine the presence of cytokines in a particular cell population, intracellular FACS staining was performed according to a standard protocol provided by eBioscience (www.eBioscience.com). To inhibit the release of cytokines from the cells, brefeldin A was added in the cell suspension at a concentration of 10 µg/ml during the last 7-9 hrs of cell culture. Cells were then washed once in IMDM complete medium and surface FACS staining was performed for phenotypical discrimination of the different cell types in the culture. Surface staining was performed in IMDM complete medium by addition of 0.4 µg/ml monoclonal antibodies **(2.1.4)** for 20 min, at 4° C in the dark. Next, cells were washed with IMDM complete medium and fixed with 100 µl IC fixation buffer for 30 min at RT, in the dark. Cells were washed with 500 µl of 1-fold permeabilization buffer and resuspended in 100 µl of permeabilization buffer. Intracellular staining was performed by incubating the cells with 1 µg/ml monoclonal antibodies against the cytokines of interest (FITC-conjugated IL-2, IFN-γ or granzyme B) for 30 min at RT, in the dark. After a washing step with permeabilization buffer, cells were resuspended in 200 µl of flow cytometry staining buffer. Samples were analyzed in a FACS Calibur flow cytometer using the software CellQuest.

2.2.4.3. Assessment of $CD8^+$ T cell degranulation

Degranulation of $CD8^+$ T cells after activation with MCs was assessed by FACS analysis for surface expression of lysosomal-associated membrane protein 1, LAMP-1 (CD107a) (Alter et al. 2004). Cells were washed and resuspended in FACS buffer and stained with 0.4 µg/ml APC-conjugated anti-CD3ε monoclonal antibody for discrimination between $CD8^+$ T cells and MCs. Simultaneously, cells were stained with rat anti-mouse LAMP-1 antibody, according to the manufacturer´s instructions. Staining was performed for 20 min, at 4° C in the dark. Subsequently, the cells were washed with FACS buffer and stained with F(ab´)2 anti-rat IgG for 20 min, at 4° C in the dark. Finally, cells were washed with 1µg/ml propidium iodide in FACS buffer, resuspended in FACS buffer and analyzed in a FACS Calibur flow cytometer. For evaluation of $CD8^+$ T cell degranulation, gates were set on viable cells according to exclusion of propidium iodide staining, on lymphocytes according to forward- sideward- scatterplot analysis and on $CD3^+$ cells.

2.2.5. Quantification of cell proliferation

2.2.5.1. CFSE labeling

For assessment of $CD8^+$ T cell proliferation, the method of CFSE labeling of $CD8^+$ T cells was used. CFSE labeling was performed before the co-culture of $CD8^+$ T cells with MCs. Purified $CD8^+$ T cells were resuspended in PBS at concentration of 8 x 10^7 cells/ml and an equal volume of 6 µM CFDA SE in PBS was added. Cells were continuously vortexed for 6 min at RT and an equal volume of ice-cold FCS was added to stop the reaction of CFSE labeling. This was followed by a centrifugation step at 270 x g for 10 min at 4° C and two washing steps in PBS. The efficiency of the CFSE labeling was determined by FACS. CFSE-labeled $CD8^+$ T cells were co-cultured with MCs **(2.2.3.2)** and proliferation of $CD8^+$ T cells was measured by FACS after 72 hrs.

2.2.6. Measurement of cytokines in culture supernatants

2.2.6.1. Enzyme-Linked ImmunoSorbent Assay (ELISA)

Supernatants of MC-$CD8^+$ T cell co-cultures were kept frozen at -20° C. ELISA was performed using a DuoSet ELISA Development Kit according to the manufacturer´s instructions. 96-well, flat bottom plates were coated with 100 µl of 4 µg/ml capture antibody diluted in PBS and incubated overnight at 4° C. Next, the plates were washed and incubated overnight with 200 µl/well ELISA blocking buffer. All washing steps were performed by washing the plates three times with ELISA washing buffer in ELISA washer. Samples (100 µl) and standard dilutions of the cytokines were diluted in reagent diluent buffer, were added in the wells and incubated for 2 hrs at RT. After a washing step, the detection antibody was diluted in reagent diluent buffer and added in the wells at a concentration of 400 ng/ml (100 µl/well) for 2 hrs at RT. Plates were then washed and 100 µl/well streptavidin-HRP were added for 20 min. Following a washing step, the reaction was visualized by addition of 100 µl/well substrate solution, consisting of 1:1 H_2O_2:Tetramethylbenzidin. Finally, the reaction was stopped by addition of 50 µl/well of 1 N H_2SO_4 and the optical density of each well was determined using ELISA reader set to 450 nm.

2.2.6.2. Bioplex

For screening of the cytokine content in the MC-$CD8^+$ T cell co-culture supernatants, the Bio-Plex Cytokine Assay (10-plex, mouse Th1/Th2) was used according to the manufacturer´s instructions. Briefly, the wells of a 96-well flat-bottom microplate were prewet with 100 µl of Bio-Plex assay buffer and the buffer was removed by vacuum filtration. Next, 50 µl of the multiplex bead working solution were added into the wells and subsequently removed by vacuum filtration. 100 µl of Bio-Plex wash buffer were dispensed in each well and subsequently removed by vacuum filtration. Samples and pre-diluted standard dilutions of the cytokines were added at a final volume of 50 µl and incubated for 30 min during continuous shaking of the plate on a microplate shaker. Next, the plate was washed three times with 100 µl of Bio-Plex wash buffer. The Bio-Plex detection antibody was added at a final volume of 25 µl and incubated for 30 min during continuous shaking of the plate on a microplate shaker. Following three washings with 100 µl of Bio-Plex wash buffer, 50 µl of streptavidin-PE were added for 10 min. Finally, after additional three washings with 100 µl of Bio-Plex wash buffer, the beads in each well were resuspended with 100 µl Bio-Plex assay buffer. The absorbance of the wells was measured in the Bio-Plex array reader and the concentration of the cytokines was automatically calculated by Bio-Plex Manager Software.

2.2.7. Generation of OVA-FITC conjugates

2.2.7.1. Labeling of OVA protein to FITC

In order to visualize the uptake of ovalbumin (OVA) protein by MCs, OVA was coupled with fluorescein isothiocyanate (FITC). OVA grade V was diluted in 0.1 M sodium carbonate at a concentration of 2 mg/ml. FITC was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml. For each 1 ml of protein solution, 50 µl of FITC solution were added. The FITC solution was added very slowly and the protein solution was continuously stirred during the addition. The reaction was left in the dark for 8 hrs at 4° C. The reaction was stopped by addition of 1.5 M lysine for 2 hrs at RT during continuous shaking.

2.2.7.2. Column purification of OVA-FITC

In order to purify the OVA-FITC protein from the unbound FITC, gel filtration was performed in Sephadex G50 Column (1.5 cm diameter, 50 cm length). The sample was carefully layered on top of the column and was allowed to flow into the column until it entered the column bed. PBS was used as elution buffer. The speed of the elution was set to 1.5 ml / 10 min. The OVA-conjugated FITC was eluted first and could be easily distinguished under room light from the unbound FITC, which was eluted afterwards.

The concentration of protein after the gel filtration was calculated by measuring the absorbance at 280 nm. The ratio of FITC to protein was calculated by measuring the absorbance at 495 (A_{495}) nm and at 280 nm (A_{280}). Fractions with a ratio of A_{495} / A_{280} between 0.3 and 1 were considered optimal (Harlow and Lane, 1988) and were pooled together for further use. The degree of labeling in the final fraction was 0.92 according to the following formula:

dye per protein molecule = (A_{495} x dilution factor) / (68000 x protein concentration (M))

2.2.8. Molecular biological techniques

2.2.8.1. RNA isolation

For preparation of RNA, cells were thoroughly resuspended in 1 ml Trizol-Reagent and frozen at -70° C. Shortly before the RNA isolation, samples were thawed slowly on ice. A volume of 200 µl chloroform was added and the cells were vortexed and centrifugated at 16000 x g for 15 min, at 4° C. As a result of this centrifugation, three phases were obtained:

an upper, colourless phase containing the RNA, a middle phase containing proteins and DNA and a lower light red coloured phase, containing trizol and chloroform. The upper phase containing the RNA was transferred to a new microcentrifuge tube containing 500 µl isopropanol to induce RNA precipitation. Samples were vortexed, left at RT for 10 min and centrifugated at 16000 x g (4° C) for 15 min before the supernatant was removed. To wash the RNA pellets, 500 µl of 75% ethanol were carefully added without resuspending the pellets. Samples were centrifugated again at 16000 x g for 10 min and the supernatants were carefully decanted. Pellets were left to air-dry and then resuspended in 30-50 µl DEPC-H_2O.

Quantification of RNA was carried out using an automated photometer. Purity was determined by calculating the ratio of absorbance at 260 nm (A_{260}) to absorbance at 280 nm (A_{280}). Pure RNA should have an A_{260}/A_{280} ratio of 2.

2.2.8.2. cDNA synthesis

Complementary DNA (cDNA) was synthesized from purified RNA using random oligonucleotides and Superscript II TM Kit. A volume of 1.5 µl (120 ng) random primer was added to 2.5 µg RNA and the total volume was brought to 11.5 µl with DEPC-H_2O. The reaction was incubated for 10 min in a 70° C pre-heated thermoblock. Samples were briefly centrifugated and cDNA synthesis was performed by addition of 100 U (0.5 µl) Superscript II reverse transcriptase in reaction buffer. The reaction was incubated for 1 hr at 37° C and subsequently inactivated by 5 min incubation at 100° C in a pre-heated thermoblock. Finally, the samples were transferred into ice and PCR was performed for amplification of cDNA.

2.2.8.3. Polymerase chain reaction (PCR)

The sequences of the primers used for PCR are shown in **2.1.5**. PCR was performed using 1 U (0.2 µl) of Taq DNA polymerase in a PCR-reaction mixture of 20 µl. Samples were amplified in a DNA Thermocycler for 30 cycles. Each cycle consisted of denaturation at 95° C for 30 sec, annealing for 30 sec, and elongation at 72° C for 30 sec, preceded by initial denaturation at 95° C for 3 min and followed by a final extension step at 72° C for 10 min. Annealing temperature for each primer was experimentally produced by running the same reaction at different annealing temperatures using a gradient thermocycler.

Amplification of β-actin message was used to normalize the amount of cDNA. For β-actin amplification, PCR was carried out for 25 cycles, each consisting of denaturation at 95° C for 25 sec, annealing at 60° C for 25 sec, and elongation at 72° C for 25 sec, preceded by initial denaturation at 95° C for 3 min and followed by a final extension step at 72° C for 10 min. A mock PCR (without cDNA) was included to exclude contamination in all experiments. Aliquots of PCR products were electrophoresed on 1.5% agarose gel and visualized under UV light after ethidium bromide staining.

2.2.8.4. Electrophoresis

Agarose gel (1.5%) was prepared by heat-dissolving agarose in Tris/boric acid/EDTA (TBE) using a microwave oven. Melted agarose was allowed to cool to 55° C before a volume of 40 ml was poured into a beaker. To this volume, 10 µl of 1 mg/ml ethidium bromide were added, gently swirled and poured into a small gel casting tray fitted with a 12 well comb. Samples were loaded at volumes of 6 µl which contained 5 µl PCR product plus 1 µl 6-fold loading dye along with 5 µl (0.1 µg) molecular weight marker. Gel electrophoresis was carried out in TBE buffer at 10 V/cm gel width (approximately 70 V) for 1 hr. Visualization and photography of the gel were done using a transluminator equipped with a camera.

2.2.8.5. Microarray

Microarray analysis was performed by Dr. Reinhardt Hoffmann and Dr. Jörg Mages in the facilities of the Institute for Medical Microbiology, Immunology and Hygiene, at the Technical University of Munich. The purified RNA samples were labeled and hybridized on a mouse (MG 430 2.0) DNA-microarray (Affymetrix, Santa Clara, U.S.A.), according to the manufacturer´s instructions.

2.2.9. Animal experiments

All *in vivo* animal experiments were approved by the “Ministerium für Landwirtschaft, Umwelt und ländliche Räume des Landes Schleswig-Holstein, Germany“.

2.2.9.1. Adoptive transfer of BMMCs, BMDCs and CD8$^+$ T cells

BMMCs and BMDCs were pulsed with 4 µg/ml OVA$^{257-264}$, as described in **2.2.3.1**. 5 x 10^6 of OVA$^{257-264}$ -pulsed BMMCs or BMDCs or equal amount of unpulsed BMMCs (control) in 200 µl of PBS were injected into the peritoneum of C57BL/6 or β2m$^{-/-}$ mice. One day later, CD8$^+$ T cells were purified from the lymph nodes of OT-I transgenic mice as described in **2.2.2.1** and labeled with CFSE **(2.2.5.1.)**. A number of 8 x 10^6 CFSE-labeled CD8$^+$ T cells in 200 µl PBS were injected into the peritoneum of the recipient mice. Four days after transfer of the CD8$^+$ T cells, mice were euthanized with CO_2 inhalation. Peritoneal lavage was obtained after injection of 10 ml ice-cold 0.9% NaCl in the peritoneal cavity. Inguinal and mesenteric lymph nodes, spleen and blood were isolated and analyzed for CFSE proliferation of transgenic CD8$^+$ T cells by FACS.

2.2.9.2. Allergic airway sensitization

WBB6F1-Kit^W/Kit^{W-v} (MC-deficient) female mice, as well as their congeneic wild-type mice (WBB6F1-Kit^W/Kit^W) were immunized against ovalbumin (OVA), according to an established protocol of sensitization against OVA (Rückert et al., 2005; Beisswenger et al., 2006). Sensitization of mice was performed by three intraperitoneal injections of alum-absorbed OVA. 20 µg OVA (grade VI) were diluted in 200 µl PBS containing 1.5 mg $Al(OH)_3$ and the solution was injected in the peritoneum of the mice with a 27 G needle on day 0, day 14 and day 21. Mice were challenged by exposure to aerosolized OVA (1% OVA grade V in PBS) for 40 min and were left in the aerosol-chamber for additional 10 min. One day after the last OVA-aerosol challenge, mice were sacrificed by cervical dislocation.

The lung and the draining lymph nodes of the lung were isolated after injection of PBS in the right ventricle of the heart, in order to remove the blood. Next, the lung and the draining lymph nodes of the lung were digested by incubation in collagenase /

deoxyribonuclease buffer (1 mg/ml collagenase and 0.2 mg/ml deoxyribonuclease in RPMI) for 30-60 min in incubator with 5% CO_2, at 37° C. Subsequently, cells were washed with FACS buffer at 270 x g, for 10 min, at 4° C, counted and analyzed for the content and phenotype of $CD8^+$ T cells by FACS **(2.2.4.1)**.

2.2.10. Statistical analysis

Results are represented as mean values ± standard deviation from pooled data of two to six independent experiments, as indicated. Statistical analysis of the results, unless otherwise stated, was performed by Student's t test. A p value of <0.05 was considered statistically significant (*).

3. Results

Mast cells (MCs) have been well characterized as main “effector” cells in the acute phase of allergic reactions and as participants of the innate immune response. Recent results, however, suggest that MCs also have a key role in the regulation of adaptive immunity, since they recruit and interact with B cells, dendritic cells (DCs) and T cells (Galli et al., 2005b). Moreover, bone marrow-derived MCs (BMMCs) have been shown to process bacterial antigens and induce proliferation of $CD8^+$ T cell line *in vitro* (Malaviya et al., 1996b). A potential MC-$CD8^+$ T cell interaction is also supported by the fact that MCs induce chemotaxis of $CD8^+$ T cells *in vivo* (McLachlan et al., 2003; Ott et al., 2003; Orinska et al., 2005). However, the ability of MCs to modulate primary $CD8^+$ T cell activation and effector functions, as well as the identification of the factors that regulate this interaction remain unknown. $CD8^+$ T cells play a central role in the induction of a protective immune response against intracellular pathogens. Moreover, $CD8^+$ T cells are important for the induction of allergic sensitization and atopic diseases (Haczku et al., 1995a; Haczku et al., 1995b; Hamelmann et al., 1996). Therefore, a potential ability of MCs to regulate $CD8^+$ T cell responses could be crucial for the modulation of host defense mechanisms as well as for the development of allergic diseases. For this reason, this study investigated the potential of MCs to interact with $CD8^+$ T cells *in vitro* and *in vivo*, as well as the effects of this interaction on MCs.

3.1. Obtaining a pure MC population from the bone marrow

As a model to study the role of MCs, bone marrow-derived MCs (BMMCs) were used. BMMCs were obtained after cultivating the bone marrow of C57/BL6 mice in the presence of IL-3 and SCF for 5 weeks (Dvorak et al., 1994). The maturation of the cells was tested with toluidine blue/hematoxylin staining, which results in appearance of the MC-specific red-purple metachromatic granula **(Fig. 3.1A)**. The morphology of the cells reproduced the typical MC-phenotype, as shown by the high granularity (sideward-scatter) and size (forward-scatter) of the cells, analyzed by FACS **(Fig. 3.1B)**. The presence of dead cells in the cultures was measured by FACS after propidium iodide (PI) staining **(Fig. 3.1C)**, which verified that the cultures contained only 1-5% dead cells (PI positive cells). The cells displayed the MC-specific surface expression of c-kit (CD117), FcεRI and T1/ST2, as shown by FACS analysis **(Fig. 3.1D)**. In order to exclude the presence of other cell types in the cultures, that could possibly interact with T cells and affect the results of the following experiments, the cultures were tested for the presence of DCs, myeloid cells, B cells and macrophages by analyzing surface expression of CD11c, CD11b, B220 and F4/80 respectively by FACS **(Fig. 3.2)**. IL-3- and SCF- differentiated BMMCs displayed no significant surface expression of those markers, indicating that no other antigen presenting cells (APCs) were present in the culture. Therefore, the cells obtained after 5 weeks of *in vitro* culture were considered as pure BMMCs and used as a model to investigate the role of MCs in modulating $CD8^+$ T cell responses.

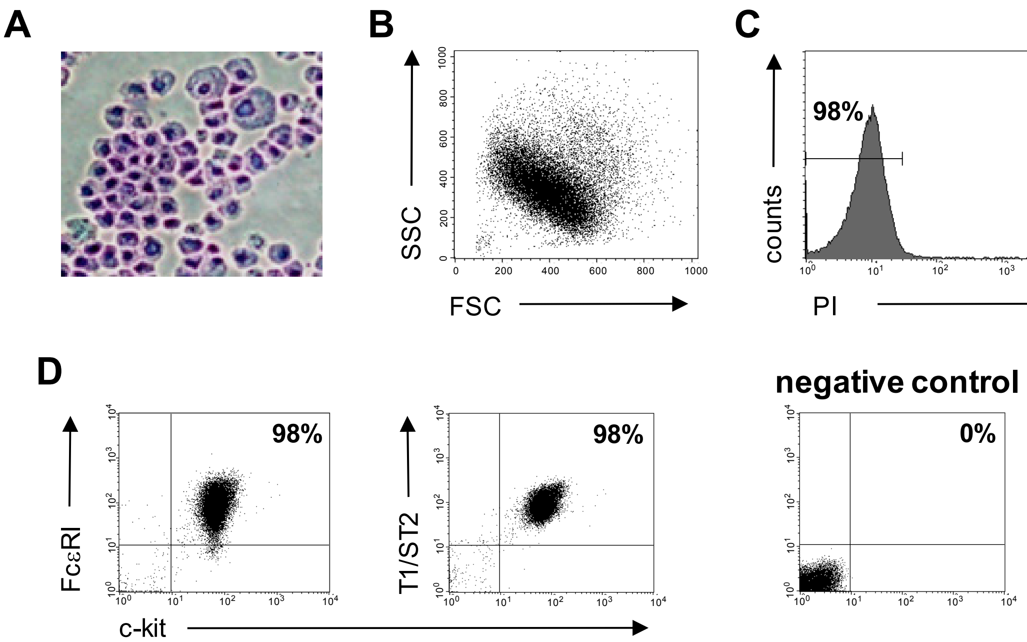

Fig. 3.1. The in vitro culture of BMMCs results in a pure population dsiplaying the MC-phenotype. *Bone marrow cells of C57/BL6 mice were cultured in the presence of IL-3 (10 ng/ml) and SCF (5 ng/ml) for 5 weeks.* ***A.*** *Maturation of BMMCs was tested with toluidine blue/hematoxylin staining.* ***B.*** *Morphology of BMMCs was analyzed by FACS. (FSC: forward-scatter, SSC: sideward-scatter)* ***C.*** *Viability of the cells was tested with propidium iodide (PI) staining* ***D.*** *Purity of BMMCs was analyzed by FACS staining for MC-related surface markers (c-kit, FcεRI and T1/ST2) (gated on viable cells). Cells stained with isotype control antibodies of irrelevant specificity served as negative control. Representative results of more than six independent experiments are shown.*

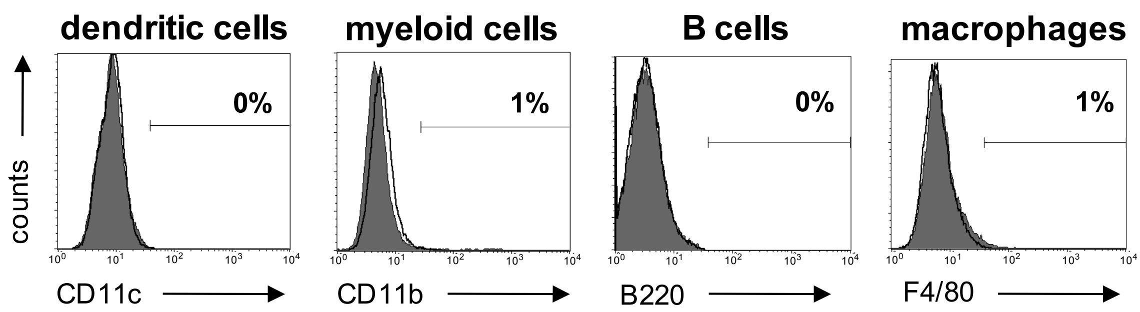

Fig. 3.2. The in vitro cultures of BMMCs were not contaminated with "professional" antigen-presenting cells (APCs). *Bone marrow cells of C57/BL6 mice were cultured in the presence of IL-3 (10 ng/ml) and SCF (5 ng/ml). Following 5 weeks of culture, the presence of potential APCs was tested with FACS after staining for CD11c, CD11b, B220 and F4/80 (gated on viable cells). Staining of the cells with control isotype matched antibodies of irrelevant specificity is shown in bold black lines. Representative results of more than six independent experiments are shown.*

3.2. Obtaining a pure DC population from the bone marrow

DCs are highly efficient APCs, capable of inducing $CD4^+$ as well as $CD8^+$ T cell responses. For this reason, DCs were used as a comparison to MCs in following experiments investigating the antigen-specific MC-$CD8^+$ T cell interactions *in vivo*. Bone marrow-derived DCs (BMDCs) were obtained after cultivating the bone marrow of C57/BL6 mice in the presence of GM-CSF for 7-9 days, as previously described (Scheicher et al., 1992; Inaba et al., 1992). On day 7, the phenotype of DCs was analyzed by FACS for maturation and viability of the cells. The morphology of the cells reproduced the typical DC-phenotype, as shown by the low granularity (sideward-scatter) and moderate size (forward-scatter) of the cells **(Fig. 3.3A)**. The presence of dead cells in the culture was measured by propidium iodide (PI) staining **(Fig. 3.3B)**, which verified that the culture contained only 5-10% dead cells (PI positive cells). The cells displayed the DC-specific surface expression of CD11c, **(Fig. 3.3C)**. The DCs obtained by this method were mature, since they expressed surface MHC class II (I-A/I-E) and the co-stimulatory molecule CD80 **(Fig. 3.3C)**. Therefore, the BMDCs obtained according to this protocol were used as potent APCs in comparison to MCs in following experiments.

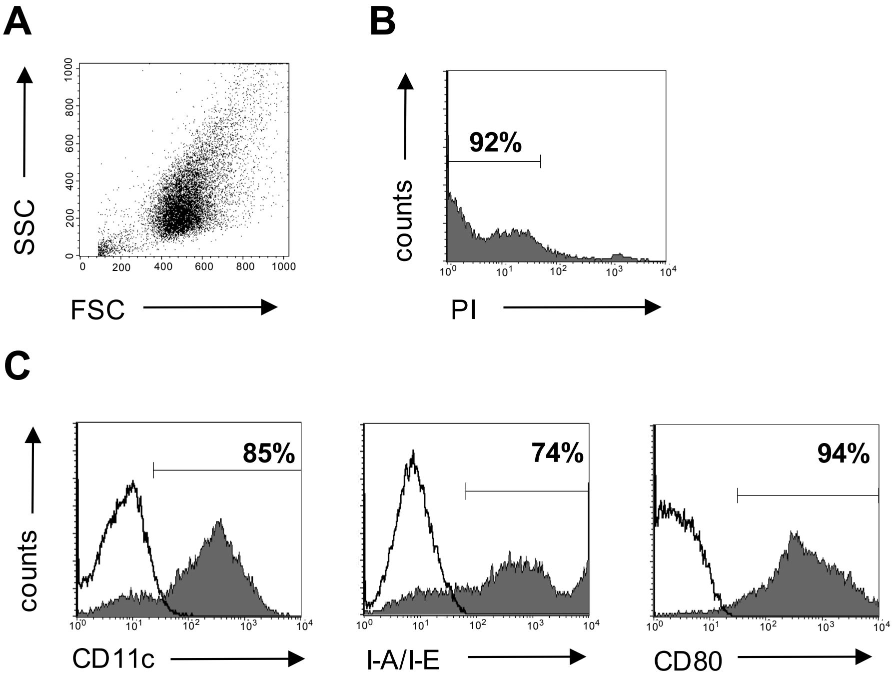

Fig. 3.3. The in vitro culture of BMDCs results in a pure population displaying the DC-phenotype. *Bone marrow cells of C57/BL6 mice were cultured in the presence of GM-CSF for 7-9 days.* ***A.*** *Morphology of BMDCs was analyzed by FACS. (FSC: forward-scatter, SSC: sideward-scatter)* ***B.*** *Viability of the cells was measured by propidium iodide (PI) staining.* ***C.*** *Maturation of BMDCs was analyzed by FACS staining for surface markers (CD11c, I-A/I-E and CD80) (gated on viable cells). Staining of the cells with control isotype matched antibodies of irrelevant specificity is shown in bold black lines. Representative results of more than six independent experiments are shown.*

3.3. Obtaining a pure population of primary CD8$^+$ T cells

CD8$^+$ T cells were purified from the lymph nodes of OT-I transgenic mice. These mice express a transgenic TCR on CD8$^+$ T cells, which recognizes the OVA$^{257\text{-}264}$ peptide in association with MHC class I molecules. The viability, purity and activation status of the isolated CD8$^+$ T cells were tested by FACS analysis **(Fig. 3.4)**. The isolated cells displayed the typical low size and low granularity of naïve lymphocytes according to forward-/ sideward-scatterplot analysis **(Fig. 3.4A)**. The isolated CD8$^+$ T cells were viable (97 ± 2% PI negative) **(Fig. 3.4B)**, highly pure (96 ± 2% CD8$^+$) **(Fig. 3.4C)** and not activated, as shown by the minimal surface expression of the activation markers CD69, CD25 and CD44 **(Fig. 3.4D)** at the time of the isolation.

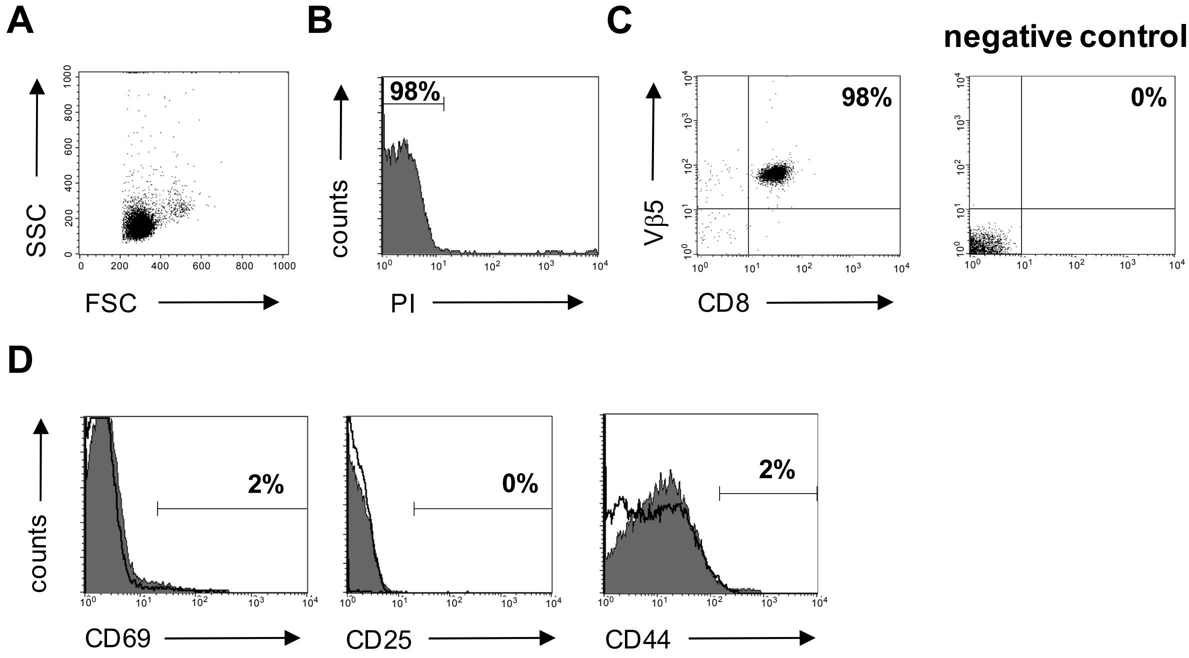

Fig. 3.4. The isolated CD8$^+$ T cells comprise a pure population of naïve primary CD8$^+$ T cells. *Lymph nodes were excised from OT-I transgenic mice and CD8$^+$ T cells were purified by negative selection with MACS. The phenotype of the CD8$^+$ T cells was analyzed by FACS.* ***A.*** *Forward-/ sideward- scatterplot analysis of the purified CD8$^+$ T cells shows that the isolated cells displayed the typical morphology of naïve lymphocytes.* ***B.*** *Propidium iodide (PI) staining was performed in order to test the viability of the cells.* ***C.*** *Purity of the cells was analyzed by surface expression of CD8 and the transgenic TCR β chain (Vβ5).* ***D.*** *Activation of the isolated CD8$^+$ T cells was analyzed by surface expression of activation markers (CD69, CD25 and CD44) (gated on viable cells). Staining of the cells with control isotype matched antibodies of irrelevant specificity is shown in bold black lines. Representative results of more than six independent experiments are shown.*

In order to exclude the presence of any other possible APCs in the purified CD8$^+$ T cell fraction, the cells were tested for the presence of B cells, DCs, macrophages and NK cells by analyzing the surface expression of B220, CD11c, F4/80 and NK1.1 respectively by FACS **(Fig. 3.5)**. None of those markers displayed a significant expression, showing that the MACS depletion of contaminating cells was up to 96 ± 2% (n=6) efficient. Therefore, the CD8$^+$ T cells isolated from the OT-I transgenic mice were considered a pure population of naïve primary CD8$^+$ T cells and were used in the following experiments to study the interaction between MCs and CD8$^+$ T cells.

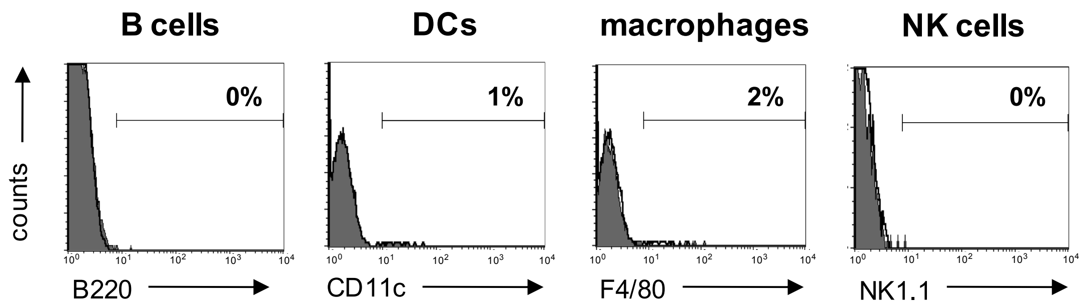

Fig. 3.5. The isolated CD8$^+$ T cell population was not contaminated with potential APCs. *CD8$^+$ T cells were isolated from lymph nodes of OT-I mice and purified by negative selection with MACS. The presence of APCs in the culture was tested with FACS staining for surface expression of B220, CD11c, F4/80 and NK1.1 (gated on viable cells). Staining of the cells with control isotype matched antibodies of irrelevant specificity is shown in bold black lines. Representative results of more than six independent experiments are shown.*

3.4. Antigen-independent effects of MCs on CD8$^+$ T cells *in vitro*

The interaction between APCs and T cells may occur both in an antigen-dependent and antigen-independent way. In the absence of a specific antigen, DCs have been shown to form functional synapses with T cells and induce a small Ca^{2+} response and weak proliferation, mainly on CD4$^+$ and, to a minimal extent, on CD8$^+$ T cells (Revy et al., 2001). Interestingly, DCs, but neither B cells nor monocytes, increase the survival of naïve T cells (both CD4$^+$ and CD8$^+$) in an antigen-independent manner (Revy et al., 2001; Kondo et al., 2001). Since an antigen-independent interaction between APCs and T cells is potentially relevant for the homeostasis of the naïve T cell pool, the cells that participate in this interaction should be characterized in detail. Several investigators suggest that MCs modulate CD4$^+$ T cell activities in an antigen-independent manner, by influencing the Th1/Th2 balance via release of soluble mediators (Stelekati et al., 2007). Therefore, it was suggested that the CD8$^+$ T cell responses might also be influenced by MCs in an antigen-independent manner. For this reason, the survival as well as the activation of CD8$^+$ T cells was tested in the presence of MCs and in the absence of a specific stimulus (namely the OVA$^{257\text{-}264}$ peptide).

3.4.1. MCs support the survival of naïve $CD8^+$ T cells

MCs are able to release a variety of cytokines (Introduction, Fig.1.3). Some of the MC-released cytokines, namely IL-2, IL-7 and IL-15, support and regulate the survival of $CD8^+$ T cells (Marrack and Kappler, 2004). Therefore, it was examined whether MCs are able to directly regulate the survival of $CD8^+$ T cells. For this reason, the survival of naïve $CD8^+$ T cells in the presence or absence of MCs was evaluated by propidium iodide (PI) staining of the $CD8^+$ T cells. Indeed, the addition of MCs at a ratio 1:2 to $CD8^+$ T cells increased the percentage of viable (PI-negative) $CD8^+$ T cells by 32 ± 9% (n=7), while addition of MCs at a ratio 1:10 increased the survival of $CD8^+$ T cells by 16 ± 3% (n=3) **(Fig. 3.6A)**.

It has been previously reported that DCs promote the survival of naïve T cells in an antigen-independent and cell contact-dependent manner (Revy et al., 2001; Kondo et al., 2001), suggesting that not only cytokine production but also physical cell-cell interactions are essential for the increased T cell survival. For this reason, the importance of direct cell contact between MCs and $CD8^+$ T cells for the increased survival of $CD8^+$ T cells was examined. Thus, $CD8^+$ T cells were cultured with MCs in the presence of a membrane, which inhibited direct cell contact between MCs and $CD8^+$ T cells. As shown in **Fig. 3.6B**, the presence of the membrane reduced the survival of $CD8^+$ T cells by 25 ± 18% (n=4), suggesting that direct cell contact between MCs and $CD8^+$ T cells is essential for the enhanced survival of $CD8^+$ T cells.

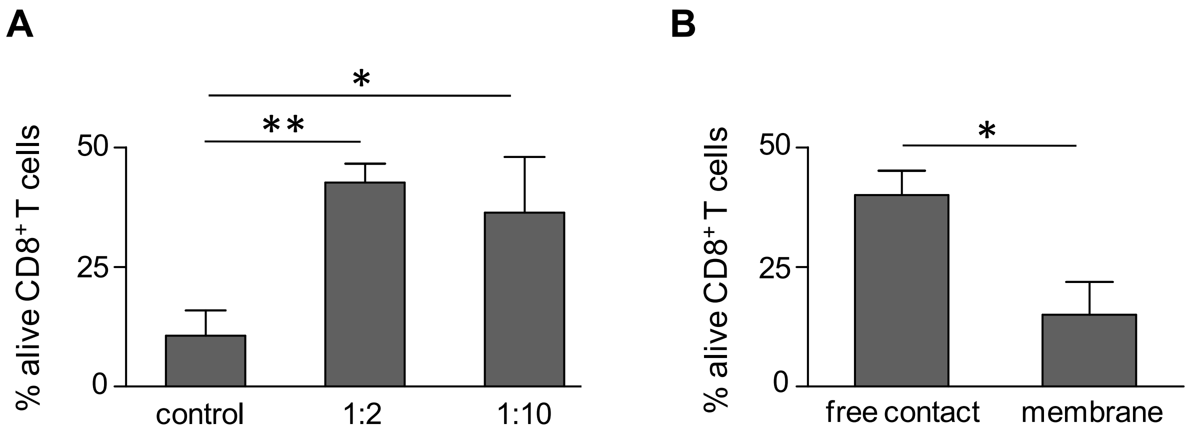

Fig. 3.6. MCs enhance the survival of primary naïve CD8$^+$ T cells. *CD8$^+$ T cells were purified from lymph nodes by negative MACS separation.* ***A.*** *1 x 10^6 CD8$^+$ T cells were cultured in the absence* ***("control")*** *or in the presence of 0.5 x 10^6 MCs* ***("1:2")*** *or 0.1 x 10^6 MCs* ***("1:10")*** *in IMDM complete medium containing IL-3 (5 ng/ml) for 48 hours.* ***B.*** *1 x 10^6 CD8$^+$ T cells were cultured with 0.5 x 10^6 MCs and the direct cell contact between MCs and CD8$^+$ T cells was either allowed* ***("free contact")*** *or inhibited by the presence of a 0.2 μm anopore membrane* ***("membrane")****. Cells were stained with propidium iodide (PI) and analyzed by FACS. Graphs show the percentage of PI-negative cells (% viable CD8$^+$ T cells), gated on CD8$^+$ T cells. Mean values with standard deviation of two to five independent experiments are shown (n=4-7). * $p<0.05$, ** $p<0.01$*

These data suggest that MCs promote the survival of CD8$^+$ T cells in an antigen-independent and cell-cell contact-dependent manner and propose that MCs contribute to the homeostasis of naïve CD8$^+$ T cells.

3.4.2. MCs do not induce CD8$^+$ T cell activation in the absence of antigen

The antigen-independent activation of T cells may be important for the establishment of protective immunity or play a role in autoimmune diseases (Kondo et al., 2001). Therefore, the question whether MCs regulate CD8$^+$ T cell activation in an antigen-independent manner was addressed. For this reason, CD8$^+$ T cells were isolated from lymph nodes by MACS and cultured in the presence of MCs for 48 hours. Subsequently, the cells were analyzed by FACS for surface expression of activation markers on CD8$^+$ T cells. Surface expression of CD69 (very early activating antigen) has been shown to become rapidly up-regulated after activation of T cells (Ziegler et al., 1994). CD25 (IL-2 receptor α) is also reported to be expressed on T cells upon activation (Santos et al., 1991), while CD44 (cell adhesion receptor) surface expression has been correlated not only with activation but also with memory differentiation of T cells (Budd et al., 1987). Therefore, the combination of these markers was chosen in order to provide a clear estimation of the activation status of the $CD8^+$ T cells. In order to focus on the $CD8^+$ T cell population during further analysis, gates were set on the lymphocyte population according to forward-/ sideward-scatterplot analysis **(Fig. 3.7A, left plot)**, alive cells, as demonstrated by exclusion of PI staining **(Fig. 3.7A, middle histogram)** and $CD8^+$ cells, as shown by staining with specific CD8 antibody **(Fig. 3.7A, left histogram).** Further analysis on $CD8^+$ T cells included the set of all these three gates. As shown in **Fig. 3.7B**, MCs did not induce any activation on naïve $CD8^+$ T cells in the absence of a specific antigen, as depicted by the absence of surface expression of activation markers CD69, CD25 and CD44 on $CD8^+$ T cells.

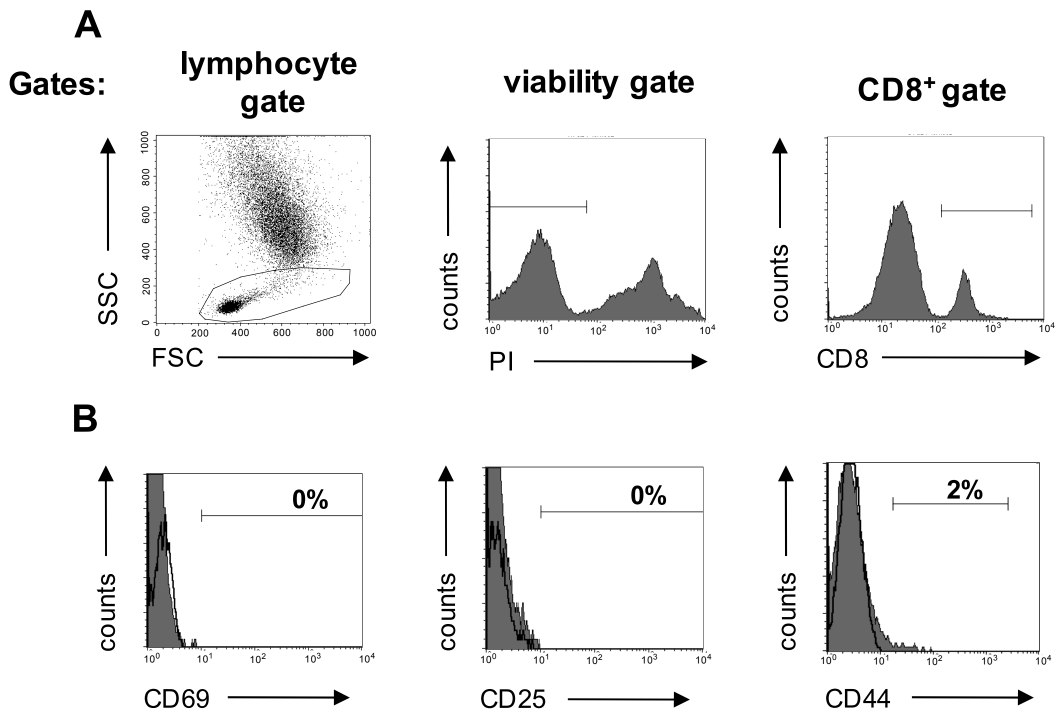

Fig. 3.7. MCs do not induce activation of naïve T cells in the absence of a specific antigen. *$CD8^+$ T cells were isolated from lymph nodes by MACS and cultured with MCs in the presence of IL-3 (5 ng/ml) for 48 hours.* ***A.*** *Gates were set in order to select the $CD8^+$ T cell population for further analysis.* ***Lymphocyte gate*** *was set according to FSC/SSC localization of lymphocytes.* ***Viability gate*** *was set on the propidium iodide (PI) negative cells in order to exclude dead cells from further analysis.* ***$CD8^+$ gate*** *was set according to surface expression of CD8. Further analysis was focused on the population selected according to these three gates.* ***B.*** *Histograms depict FACS staining for surface expression of CD69, CD25 and CD44, gated on viable $CD8^+$ T cells. Staining of the cells with control isotype matched antibodies of irrelevant specificity is shown in bold black lines. Representative results of five independent experiments are shown.*

These results suggest that an antigen-independent interaction between MCs and $CD8^+$ T cells occurs only in terms of enhanced $CD8^+$ T cell survival, and not $CD8^+$ T cell activation. Therefore it is proposed that MCs might contribute only to the homeostasis of naïve $CD8^+$ T cells, but do not induce activation of naïve $CD8^+$ T cells in the absence of a specific antigen.

3.5. Antigen-dependent effects of MCs on $CD8^+$ T cells *in vitro*

The presentation of antigens to T cells is the initiative step for an adaptive cellular immune response. APCs internalize proteins, degrade them into peptides and present them on their surface associated with MHC class I or MHC class II molecules for priming $CD8^+$ or $CD4^+$ T cells, respectively. Endogenous proteins are generally presented on an MHC class I-restricted manner, while exogenous proteins are presented on an MHC class II-restricted manner. However, through the process of cross-presentation, exogenous antigens may also be presented on an MHC class I-dependent manner to induce $CD8^+$ T cell responses (Trombetta and Mellman, 2005). MCs have been shown to present MHC class II-related antigens to $CD4^+$ T cells (Frandji, et al., 1993). However, less is known about the antigen-specific interaction between MCs and $CD8^+$ T cells; MCs induce *in vitro* proliferation of $CD8^+$ T cell lines (Malaviya et al., 1996b), but neither the mechanism of MC-$CD8^+$ T cell interaction nor its relevance *in vivo* has been investigated so far.

3.5.1. MCs internalize the OVA protein

The first step of presentation or cross-presentation of exogenous antigens consists of the internalization of antigenic proteins. In order to test the potential of MCs to interact with $CD8^+$ T cells in an antigen-dependent manner, MCs were initially tested for their ability to internalize the ovalbumin (OVA) protein. OVA is a model antigenic protein (Herz et al., 1996) and was chosen on the concept that the OT-I $CD8^+$ T cells used in this study express the transgenic TCR for the peptide $OVA^{257-264}$, derived from the OVA protein. In order to visualize the possible uptake of the OVA protein by MCs, OVA protein was coupled to FITC and the OVA-unbound FITC was removed by column purification. First, MCs were either incubated with 20 µg/ml OVA-FITC for 4 hours or were left untreated, washed and FITC-internalization was observed in a fluorescence microscope. As shown in **Fig. 3.8A**, MCs were able to internalize the protein. Next, the effect of the protein concentration on the internalization result was studied in the same experimental settings. MCs were incubated with different concentrations of OVA-FITC, subsequently washed and analyzed by FACS. As depicted in **Fig. 3.8B**, the internalization of the protein by MCs occured at protein concentrations of at least 20 µg/ml. These results suggest that MCs are capable of internalizing antigenic proteins in a dose-dependent manner, therefore have the potential to act as APCs.

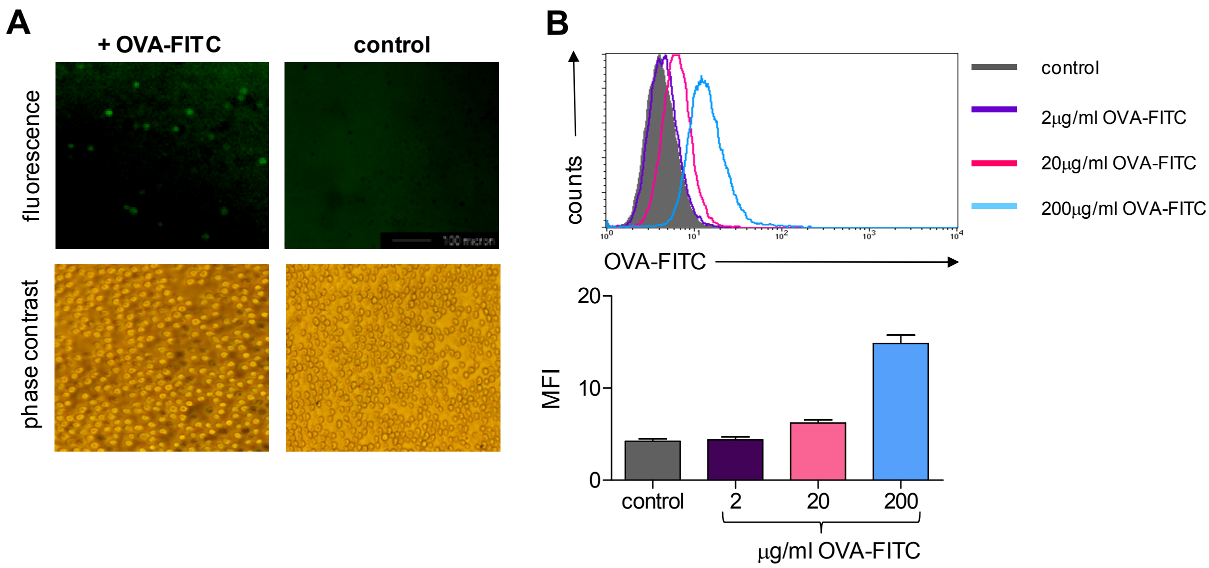

Fig. 3.8. MCs internalize the OVA protein in vitro. *OVA protein was coupled with FITC and added to the MC culture.* ***A.*** *MCs were incubated with OVA-FITC (20 µg/ml) for 4 hours* ***("+OVA-FITC")*** *or left untreated* ***("control")****, subsequently washed and visualized under fluorescence microscope.* ***B.*** *MCs were incubated with different concentrations of OVA-FITC, as indicated or left untreated* ***("control")*** *for 4 hours and analyzed by FACS. Histogram (upper row) shows OVA-FITC staining of MCs (gated on viable, c-kit positive cells). Graph in lower row shows mean fluorescence intensity (MFI) of MCs after OVA-FITC treatment. Bars represent mean values of MFI with standard deviation of two independent experiments.*

3.5.2. MCs induce antigen-specific $CD8^+$ T cell responses

The fact that MCs internalize the OVA-protein supports the initial hypothesis that MCs may interact with $CD8^+$ T cells in an antigen-dependent manner. In order to study the ability of MCs to induce antigen-dependent $CD8^+$ T cell responses, BMMCs were pulsed with the $OVA^{257-264}$ peptide and cultured with TCR-transgenic (OT-I) $CD8^+$ T cells recognizing the peptide $OVA^{257-264}$. Subsequently, the $CD8^+$ T cell responses were measured in terms of activation, proliferation and cytokine production.

3.5.2.1. MCs induce antigen-specific $CD8^+$ T cell activation

In order to test the ability of MCs to induce antigen-dependent activation of $CD8^+$ T cells, MCs were pulsed with $OVA^{257-264}$ peptide and cultured with OT-I transgenic $CD8^+$ T cells. After 48 hours of co-culture, $CD8^+$ T cell activation was measured by surface expression of activation markers with FACS. Analysis was focused on the $CD8^+$ T cells by setting of gates on the lymphocyte population, PI-negative (viable) cells and CD8-expressing cells **(Fig. 3.9A)**. A first hint that $CD8^+$ T cells become activated after antigen-dependent contact with MCs was given by the fact that $CD8^+$ T cells exhibited increased size (FSC) and granularity (SSC) **(Fig. 3.9A, left plot)** compared to the $CD8^+$ T cells which experienced antigen-independent contact with MCs **(Fig. 3.7A, left plot)**. Indeed, already after 18 hours (data not shown) and, to a greater extent, after 48 hours **(Fig. 3.9B)**, antigen-pulsed MCs induced activation of $CD8^+$ T cells, as shown by the marked up-regulation of the activation markers CD69 (very early activating antigen), CD25 (IL-2 receptor α) and CD44 (cell adhesion receptor) on $CD8^+$ T cells, measured by FACS analysis. Since CD69 is a very sensitive marker of activation, rapidly induced upon T cell activation, it was used in combination with CD25 to estimate the activation status of the $CD8^+$ T cells. The activation of $CD8^+$ T cells was dependent on the numbers of $OVA^{257-264}$-pulsed MCs. All three activation markers showed the maximal up-regulation at a ratio of MC:CD8 = 1:2. However, even at a much lower ratio of MC:CD8 = 1:10, $CD8^+$ T cells displayed up to 90 ± 4% activation (n=6). As depicted in **Fig. 3.9C**, CD69 appeared to be a more sensitive activation marker, as shown by its higher up-regulation compared to CD25 and CD44, mainly at the lower MC:CD8 ratios. Thus, at the lowest ratio of MC:CD8 = 1:100, CD69 still exhibited an up-regulation of 45 ± 14% (n=6), while the corresponding up-regulation for CD25 and CD44 were 29 ± 9% and 35 ± 5%, respectively.

It is therefore concluded that MCs are indeed able to initiate an antigen-dependent activation of $CD8^+$ T cells and induce up-regulation of CD69 and subsequently CD25 and CD44 surface expression.

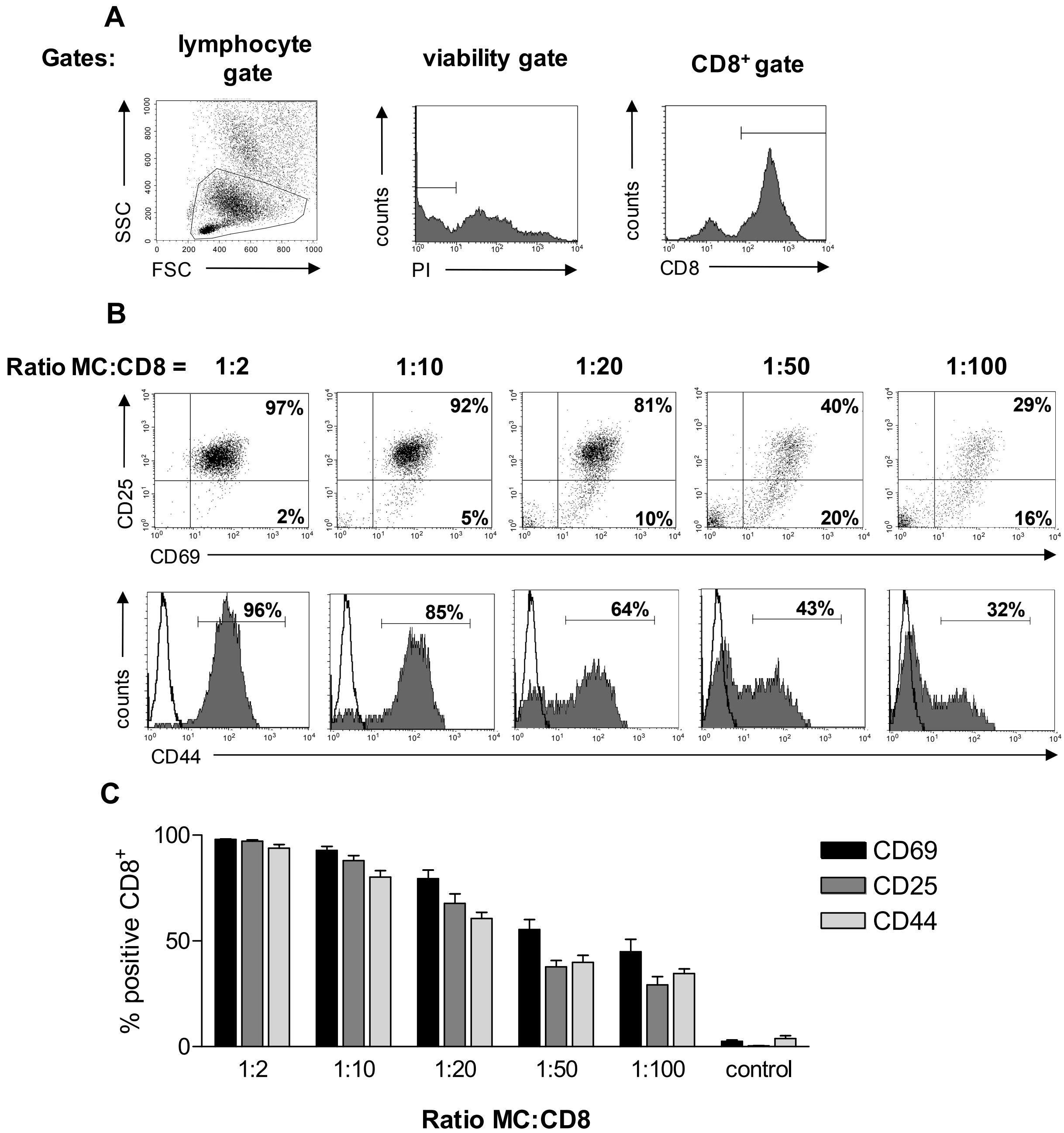

Fig. 3.9. MCs induce antigen-specific CD8⁺ T cell activation in vitro. *MCs were pulsed with OVA$^{257\text{-}264}$ peptide (4 µg/ml), washed and cultured with naïve, purified OT-I transgenic CD8$^+$ T cells. 1 x 10^6 CD8$^+$ T cells were incubated with different numbers of antigen-pulsed MCs.* ***A.*** *Analysis of CD8$^+$ T cell population was performed after setting appropriate gates on lymphocyte population (left plot), alive cells (middle histogram) and CD8-expressing cells (right histogram). Further analysis focused on the population selected according to these three gates.* ***B.*** *Activation of CD8$^+$ T cells was determined by analysis of CD69 and CD25 (upper row) and CD44 (second row) expression by FACS (gated on viable CD8$^+$ T cells). Control isotype staining is shown in bold black lines. One representative of six independent*

experiments is shown. ***C.*** *Mean values of activated $CD8^+$ T cells (according to CD69, CD25 or CD44 surface expression) with standard deviation of six independent experiments are shown.*

To exclude a TCR-independent, unspecific activation of $CD8^+$ T cells, naïve $CD8^+$ T cells were purified from lymph nodes of C57BL/6 wild-type mice and cultured with $OVA^{257-264}$-pulsed MCs. After 48 hours of co-culture, no surface expression of CD69 and CD25 and minimal surface expression of CD44 were detected on the wild-type $CD8^+$ T cells **(Fig. 3.10)**. These results demonstrate that the antigen-specific activation of $CD8^+$ T cells by MCs is TCR-dependent.

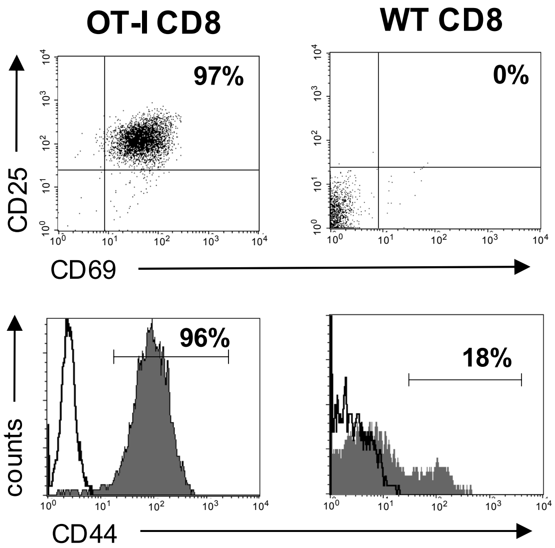

Fig. 3.10. The antigen-specific activation of $CD8^+$ T cells by MCs is TCR-dependent. *MCs were pulsed with $OVA^{257-264}$ peptide and cultured with naïve $CD8^+$ T cells purified from lymph nodes of OT-I transgenic mice* ***("OT-I CD8")*** *or C57BL/6 wild-type mice* ***("WT CD8")*** *at a ratio of 1:2. Activation of $CD8^+$ T cells was determined by analysis of CD69 and CD25 (upper row) and CD44 (lower row) expression by FACS (gated on viable $CD8^+$ T lymphocytes). Control isotype staining is shown in bold black line. Representative results of three independent experiments are shown.*

To verify that the antigen-specific activation of $CD8^+$ T cells was dependent on the functional expression of MHC class I on MCs, BMMCs derived from beta-2-microglobulin deficient mice ($\beta2m^{-/-}$), were used for co-culture with $CD8^+$ T cells. Absence of β2m expression in these mice leads to the lack of a functional expression of MHC class I and therefore, to the inability to present in an MHC class I-dependent manner. $\beta2m^{-/-}$ MCs were pulsed with OVA$^{257\text{-}264}$ and co-cultured with OT-I transgenic CD8$^+$ T cells. As shown in **Fig. 3.11**, after 48 hours of co-culture, the β2m$^{-/-}$ MCs induced only a minimal activation of the CD8$^+$ T cells, measured by surface expression of CD69, CD25 and CD44, as compared with wild-type MCs. Therefore, these data confirm that the bulk of CD8$^+$ T cell activation induced by MCs is due to MHC class I-dependent antigen presentation.

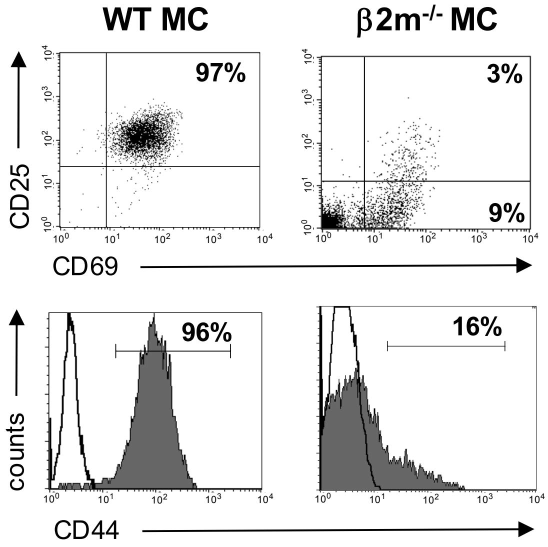

Fig. 3.11. The antigen-specific activation of CD8$^+$ T cells by MCs is MHC class I-dependent. *BMMCs were derived from the bone marrow of C57BL/6 wild-type mice* ***("WT MC")*** *or β2m-deficient mice* ***("β2m$^{-/-}$ MC")****. MCs were pulsed with OVA$^{257\text{-}264}$ peptide and cultured with naïve, purified, OT-I transgenic CD8$^+$ T cells at a ratio of 1:2. Activation of CD8$^+$ T cells was determined by analysis of CD69 and CD25 (upper row) and CD44 (lower row) expression by FACS (gated on viable CD8$^+$ T cells). Control isotype staining is shown in bold black line. Representative results of three independent experiments are shown.*

3.5.2.2. MCs induce antigen-specific CD8$^+$ T cell proliferation

Antigen-specific activation of CD8$^+$ T cells by "professional" APCs leads on the one hand to proliferation and clonal expansion, and on the other hand to their differentiation into effector cells or long-living memory cells (Roitt et al., 2002). In order to study the result of the MC-induced MHC class I-dependent antigen presentation, the proliferation of CD8$^+$ T cells after antigen-specific contact with MCs was examined using the method of CFSE labeling of CD8$^+$ T cells. CFSE is a cell tracking reagent, which passively diffuses into the cells. Upon reaction with intracellular amines it forms fluorescent conjugates that are detectable by flow cytometry. The dye is distributed equally with the cytoplasmic proteins to the daughter cells after cell division. Therefore, cell division can be tracked by the sequential halving of CFSE fluorescence resulting in a fluorescence histogram in which the peaks represent successive generations of daughter cells.

Thus, the MC-induced, antigen-specific proliferation of $CD8^+$ T cells was examined by incubating $OVA^{257-264}$-pulsed MCs with OT-I transgenic, CFSE-labeled $CD8^+$ T cells. Peptide-pulsed MCs stimulated $CD8^+$ T cells to undergo up to five cell divisions, as shown by the five successive peaks in the histogram plot of CFSE dilution **(Fig. 3.12A)**, demonstrating that MCs are capable of inducing proliferation of naïve, primary $CD8^+$ T cells. Maximal proliferation was detected at a ratio MC:CD8 = 1:20, with 92 ± 2% (n=3) of the $CD8^+$ T cells proliferating. At the lowest tested ratio of MC:CD8 = 1:100, clearly less cells appeared in each cell division, however a total of 57 ± 16% (n=4) of the $CD8^+$ T cell population proliferated. No proliferation could be detected in the presence of unpulsed MCs **(Fig. 3.12B, "control")** or with $\beta2m^{-/-}$, peptide-pulsed MCs **(Fig. 3.12B, "$\beta2m^{-/-}$")**. Therefore, it is concluded that MCs are able to induce antigen-specific proliferation of $CD8^+$ T cells in a MHC class I-dependent manner.

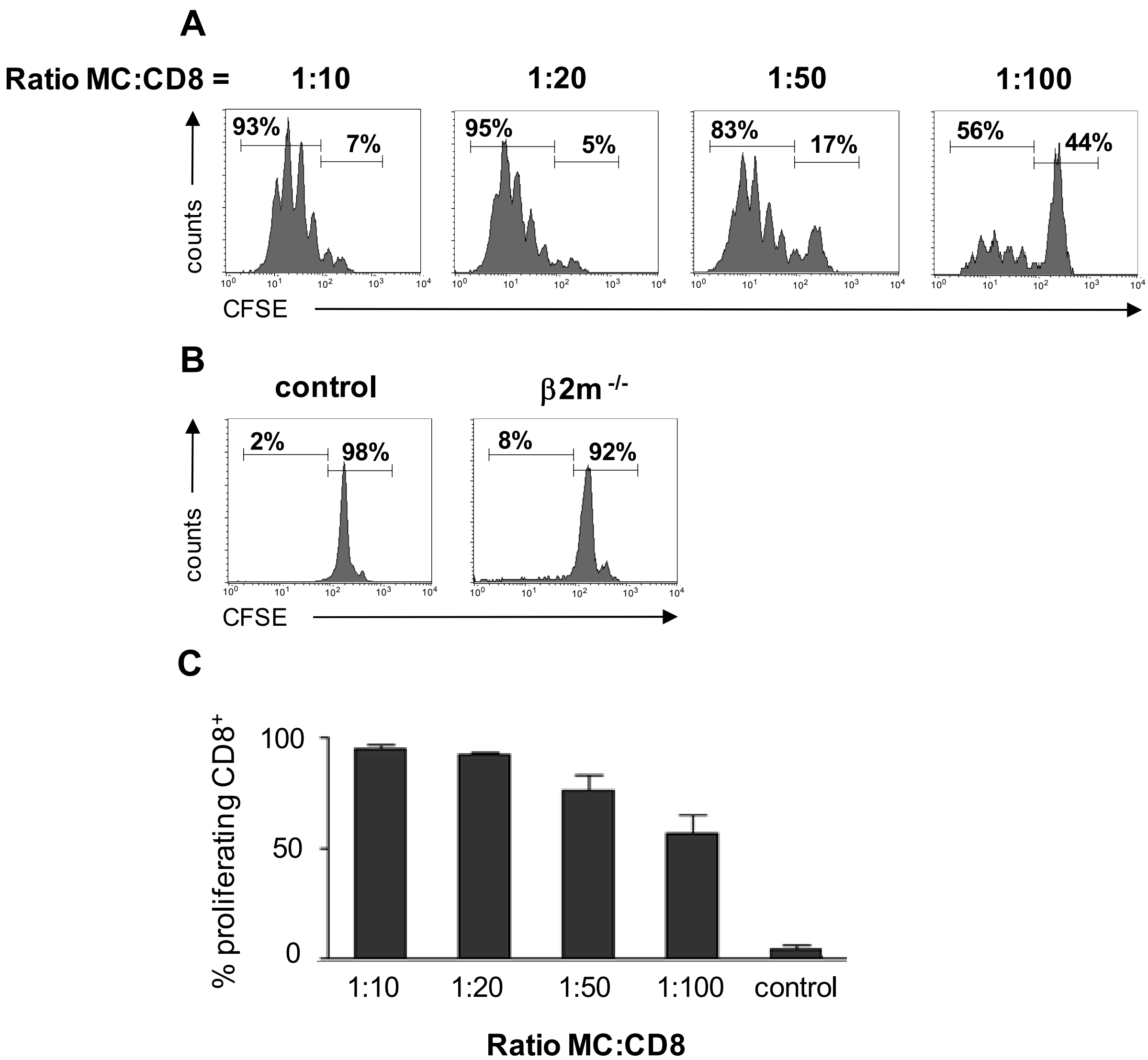

Fig. 3.12. MCs induce antigen-specific CD8$^+$ T cell proliferation in vitro. *MCs were pulsed with OVA$^{257\text{-}264}$ peptide and cultured with CFSE-labeled OT-I transgenic CD8$^+$ T cells.* ***A.*** *1 x 10^6 CD8$^+$ T cells were incubated with different numbers of antigen-pulsed MCs.* ***B.*** *1 x 10^6 CD8$^+$ T cells were cultured with 0.5 x 10^6 unpulsed MCs* ***("control")*** *or with 0.5 x 10^6 β2m$^{-/-}$, peptide-pulsed MCs* ***("β2m$^{-/-}$")****. Histograms depict CFSE dilution as measured by FACS (gated on viable CD8$^+$ T cells). Representative results of five independent experiments are shown.* ***C.*** *Mean values of the percentages of proliferating CD8$^+$ T cells with standard deviation of three to four independent experiments are shown.*

3.5.2.3. MCs induce antigen-specific cytokine production by $CD8^+$ T cells

Effector functions of activated $CD8^+$ T cells are exerted by secretion of cytokines, mainly IL-2 and IFN-γ (Roitt et al., 2002). Therefore, it was questioned whether the MC-induced antigen-dependent activation of $CD8^+$ T cells resulted also in differentiation of $CD8^+$ T cells into effector, cytokine-producing cells. For this reason, the supernatants of the MC-$CD8^+$ T cell co-cultures were screened for their cytokine content with Bio-Plex assay. Cytokines known to be produced at high amounts after antigen-specific T cell activation (IL-2, IFN-γ), as well as cytokines that regulate T cell differentiation (IL-4, IL-10, IL-12) and cytokines that contribute to the inflammatory response (TNF-α, GM-CSF) were measured. High amounts of the T cell-related cytokines IL-2 [5.6 ± 0.9 ng/ml] (n=3) and IFN-γ [6.0 ± 0.2 ng/ml] (n=2) and lower amounts of TNF-α [312 ± 180 pg/ml] (n=3) and GM-CSF [400 ± 116 pg/ml] (n=3) were detected after antigen-specific MC-$CD8^+$ T cell contact (n=3) **(Fig. 3.13)**. In contrast, none, or hardly any, production of IL-4, IL-5, IL-10 and IL-12 was detectable.

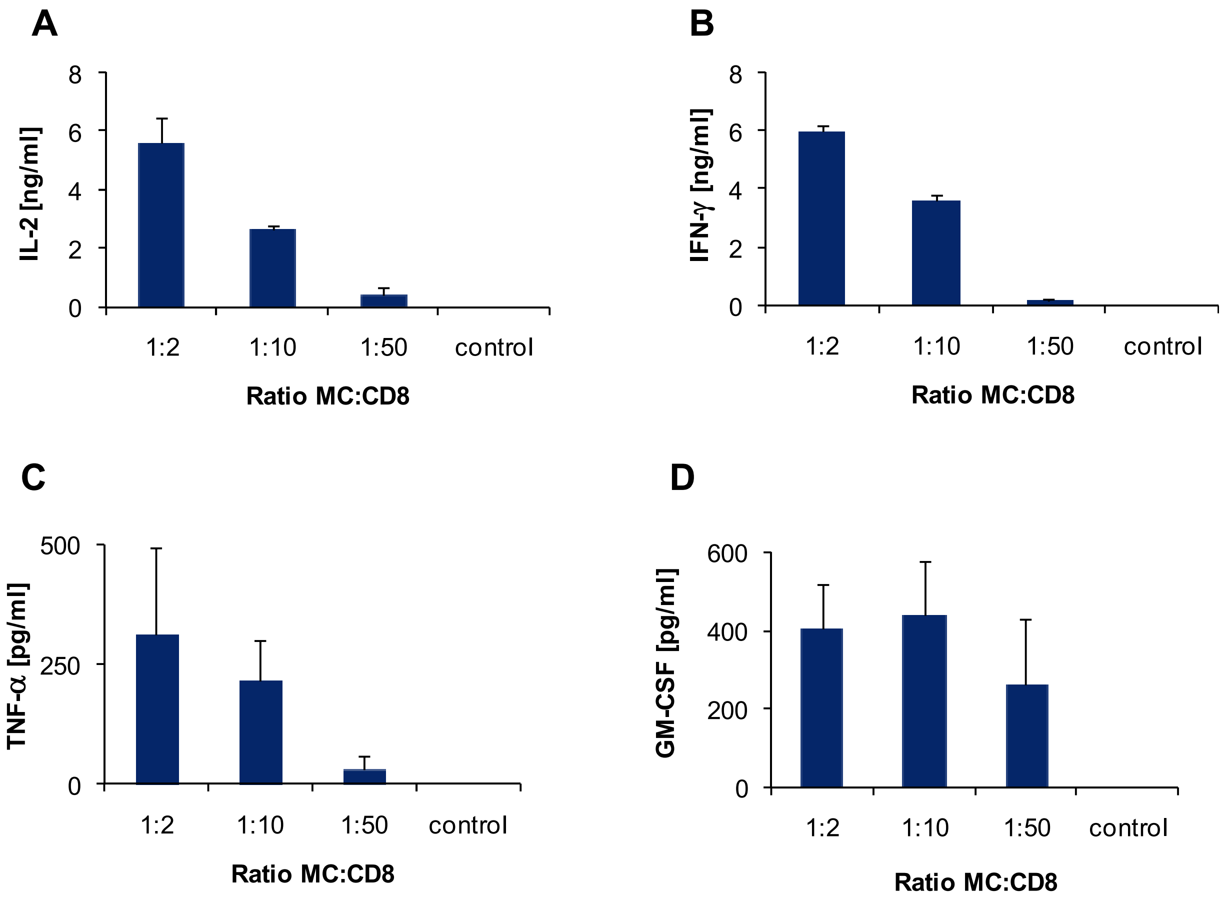

Fig. 3.13. Cytokines are released during antigen-specific MC-CD8$^+$ T cell contact. *MCs were pulsed with OVA$^{257-264}$ peptide or left untreated* ***("control")*** *and cultured with 1 x 10^6 OT-I transgenic CD8$^+$ T cells at different cell ratios for 48 hours. Supernatants of the co-culture were analyzed for cytokine content with Bio-Plex assay. Mean values with standard deviation of two to three independent experiments are shown.*

In order to investigate whether the detected cytokines were produced by the activated CD8$^+$ T cells, intracellular cytokine staining for the T cell-related cytokines IL-2 and IFN-γ was performed. Indeed, both IL-2 and IFN-γ were detected intracellularly in CD8$^+$ T cells after the MC-mediated antigen-dependent activation **(Fig. 3.14)**, at a percentage of 5.5 ± 2.9% and 3.1 ± 1.5% (n=10) respectively. Thus, it was demonstrated that IL-2 and IFN-γ were produced by the activated CD8$^+$ T cells, although their production by MCs as well was not excluded.

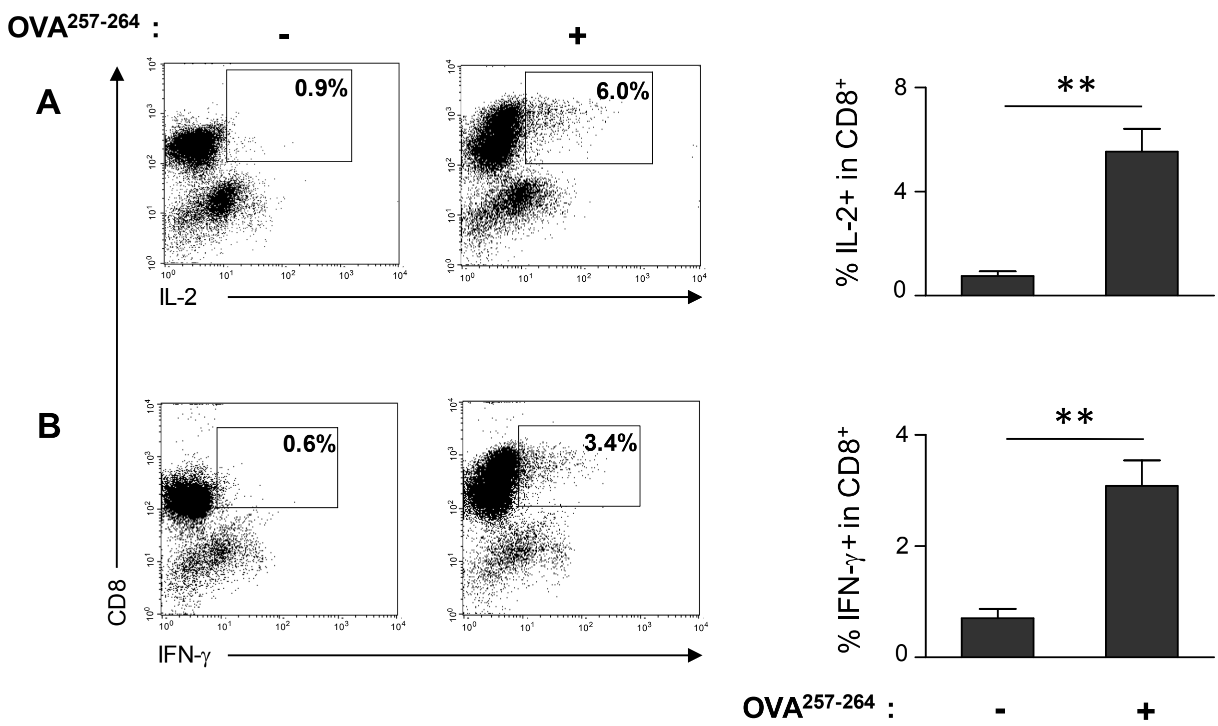

Fig. 3.14. MCs induce antigen-specific cytokine production by $CD8^+$ T cells. *1 x 10^6 OT-I transgenic $CD8^+$ T cells were incubated with 0.1 x 10^6 unpulsed MCs* ***(" - $OVA^{257-264}$")*** *or with an equal number of antigen-pulsed MCs* ***(" + $OVA^{257-264}$")*** *for 48 hours. Cells were stained for surface expression of CD8, subsequently stained for intracellular expression of IL-2* ***(A)*** *or IFN-γ* ***(B)*** *and analyzed by FACS. Percentages show cytokine-positive $CD8^+$ T cells. Dot plots show one representative of five independent experiments. Right panel shows mean values of cytokine-positive $CD8^+$ T cells with standard deviation of five independent experiments (n=10).*

** *$p<0.01$*

Conclusively, these results demonstrate that MCs are capable of inducing significant antigen-specific $CD8^+$ T cell responses, as shown by the activation, proliferation and cytokine production by primary $CD8^+$ T cells. Thus, MCs act as efficient antigen-presenting cells *in vitro*, by presenting MHC class I-related antigens and inducing effector $CD8^+$ T cell responses.

3.5.3. MCs increase the cytotoxic potential of $CD8^+$ T cells

A key effector function of $CD8^+$ T cells is their cytotoxic activity (e.g. against infected host cells) (Wong and Pamer, 2003). A crucial step in $CD8^+$-mediated cytotoxicity is the exocytosis of specialized granules containing cytotoxic proteins, namely granzymes and perforin (Lieberman, 2003). Therefore, it was examined whether MCs were able to enhance the cytotoxic potential of the $CD8^+$ T cells by increasing their intracellular content of granzyme B upon antigen-specific interaction. Indeed, as shown in **Fig. 3.15**, intracellular granzyme B expression was increased by 27 ± 9% (n=9) in $CD8^+$ T cells after antigen-mediated activation by MCs.

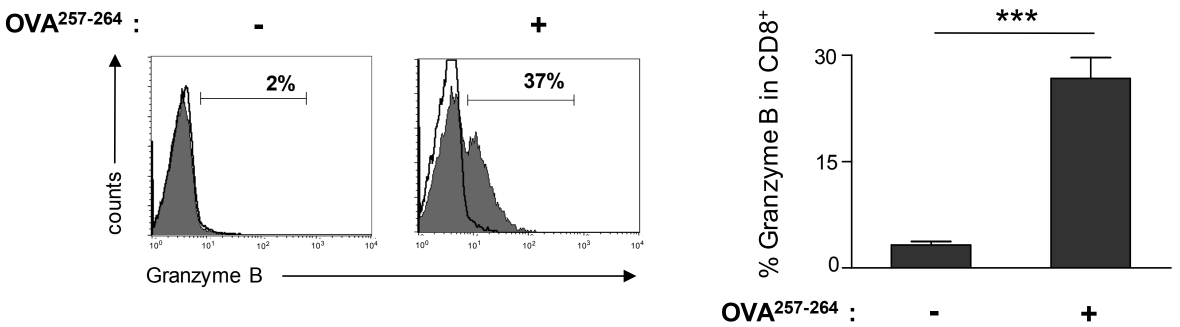

Fig. 3.15. MCs increase the cytotoxic potential of $CD8^+$ T cells. *1 x 10^6 $CD8^+$ T cells were incubated with 0.1 x 10^6 unpulsed MCs **(" - $OVA^{257-264}$")** or with an equal number of antigen-pulsed MCs **(" + $OVA^{257-264}$")** for 48 hours. Cells were stained for surface expression of CD8, subsequently stained for intracellular expression of granzyme B and analyzed by FACS. Histograms show one representative of five independent experiments. Control isotype staining is shown in bold black lines. Graph in the right panel shows mean values of granzyme B-positive $CD8^+$ T cells with standard deviation of five independent experiments (n=9). *** $p<0.001$*

Since an essential step for exhibiting cytolytic activity is the exocytosis of the preformed cytotoxic granules, it was further examined whether the $CD8^+$ T cells degranulated after MC-dependent antigen-specific activation. A novel method for detecting cytotoxic $CD8^+$ T cell degranulation has been developed based on the detection of cell surface expression of lysosomal associated membrane glycoproteins (LAMPs) on $CD8^+$ T cells (Betts and Koup, 2004; Burkett et al., 2005). LAMPs are embedded in the membrane of the cytotoxic granules; upon activation and subsequent degranulation of cytotoxic $CD8^+$ T cells, the granule membrane fuses with the plasma membrane, resulting in exposure of LAMPs on the cell surface. Therefore, CD8$^+$ T cell exocytosis was evaluated by measuring the expression of LAMP-1 (CD107a) on the surface of CD8$^+$ T cells by FACS after MC-mediated activation. Upon activation with antigen-pulsed MCs, the CD8$^+$ T cells displayed increased surface LAMP-1 expression, which was dependent on the numbers of antigen-pulsed MCs **(Fig. 3.16)**.

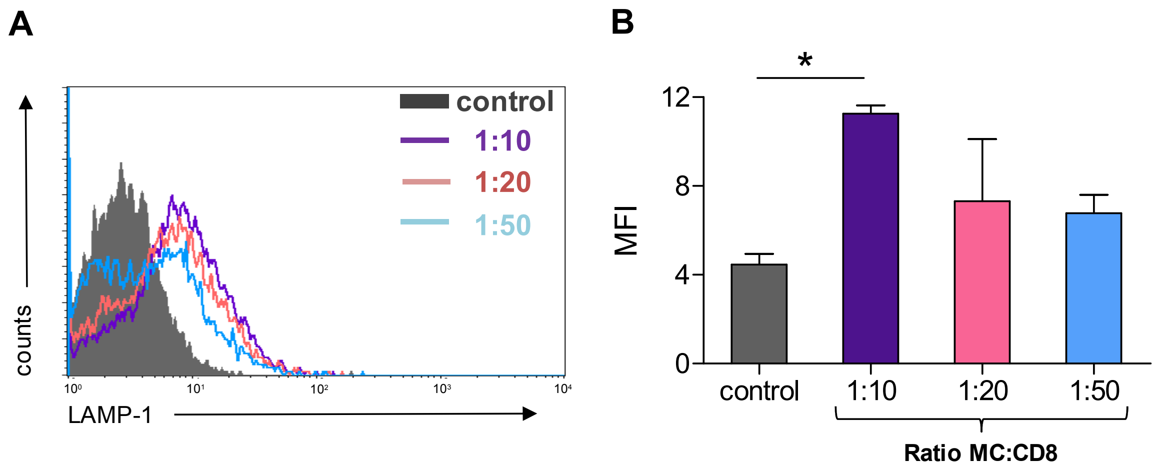

Fig. 3.16. MCs induce degranulation of CD8$^+$ T cells. *MCs were pulsed with OVA$^{257\text{-}264}$ peptide or left untreated and cultured with OT-I transgenic CD8$^+$ T cells. 1 x 10^6 CD8$^+$ T cells were incubated with unpulsed MCs* ***("control")*** *or with antigen-pulsed MCs at different cell ratios* ***(1:10, 1:20 or 1:50)****. Cells were stained for surface expression of CD8 and LAMP-1 and analyzed by FACS. Histogram shows one representative of three independent experiments for surface expression of LAMP-1 (gated on viable CD3$^+$ T cells). Right graph shows mean values of mean fluorescence intensity (MFI) for LAMP-1 with standard deviation of two to three independent experiments. * $p<0.05$*

Thus, it is suggested that MCs modulate the effector functions of CD8$^+$ T cells in an antigen-dependent manner by increasing their cytotoxic content, as well as by promoting the release of cytotoxic granula. For this reason, the MC-CD8$^+$ T cell interaction should be taken into consideration when processes involving cytotoxicity, such as viral infections, are studied.

3.5.4. The antigen-specific interaction between MCs and CD8$^+$ T cells is cell-cell contact-dependent

The finding that MCs induce antigen-specific CD8$^+$ T cell activities raises questions about the requirements involved in this interaction. The first step of interaction between "professional" APCs (DCs) and T cells consists of physical cell-cell contacts leading to long-lasting stable conjugates between the two cell types, which allow the formation of immunological synapse (Gunzer et al., 2000; Mempel et al., 2004). It was therefore hypothesized that direct cell-cell contact is essential for the induction of CD8$^+$ T cell activation by MCs.

To examine this hypothesis, antigen-pulsed MCs were cultured with CD8$^+$ T cells either allowing free cell-cell contact or in the presence of a semi-permeable membrane. The diverse size of mediators produced by MCs instructed the inhibition of the direct MC-CD8$^+$ T cell contact with two different kinds of membrane: either one which allows the passive diffusion of any soluble factor produced by MCs (0.2 μm anopore membrane), or one which allows soluble factors of only less than 0.02 μm diameter to pass through (0.02 μm anopore membrane) and therefore excludes the diffusion of, e.g., exosomes. The inhibition of cell contact between MCs and CD8$^+$ T cells by both membranes inhibited the subsequent activation, as shown by FACS analysis for activation markers **(Fig. 3.17A)** and proliferation, as shown by the CFSE dilution of the CD8$^+$ T cells, measured with FACS **(Fig. 3.17B)**. Thus, it was demonstrated that direct cell-cell contact between antigen-pulsed MCs and CD8$^+$ T cells is essential for the induction of antigen-specific CD8$^+$ T cell responses.

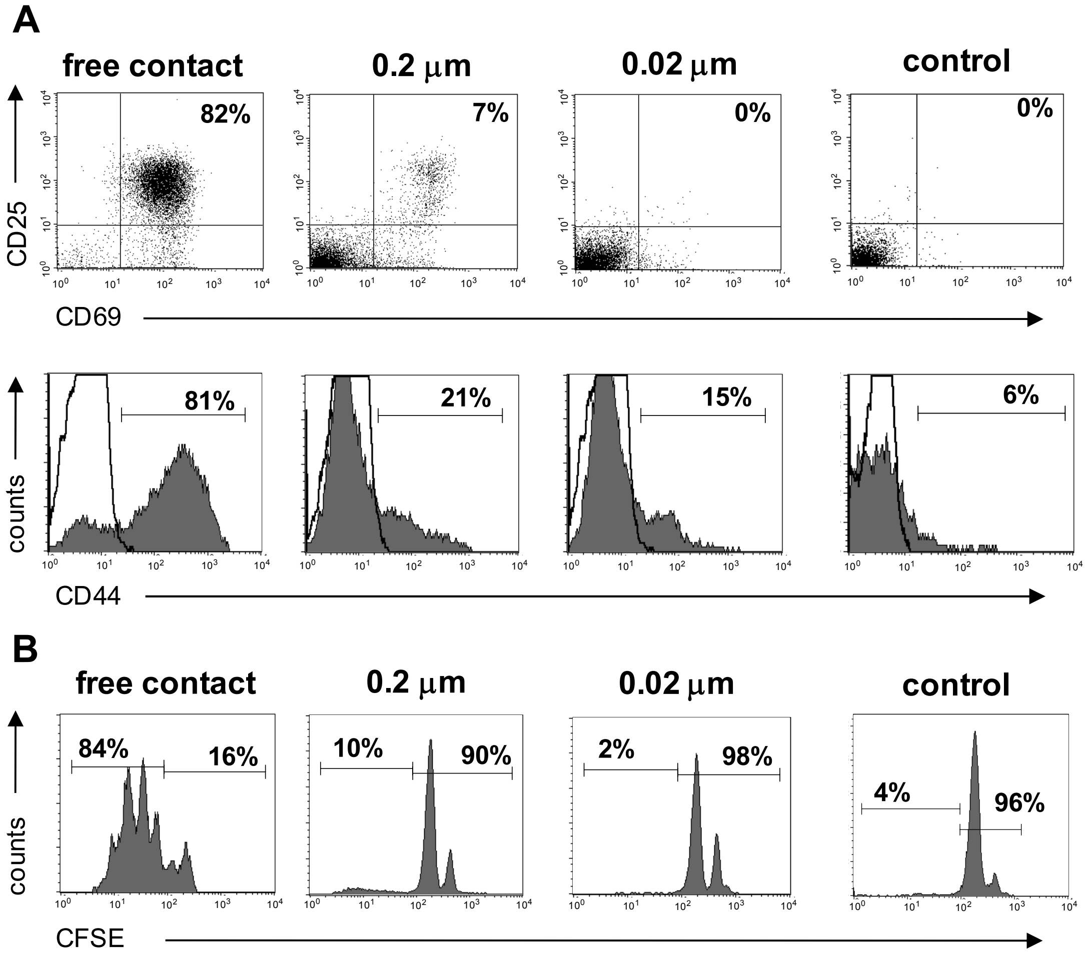

Fig. 3.17. The antigen-dependent activation and proliferation of CD8$^+$ T cells induced by MCs are cell-cell contact-dependent. *MCs were pulsed with OVA$^{257\text{-}264}$ peptide or left untreated* ***("control")*** *and cultured with CD8$^+$ T cells for 48 hours. Direct cell contact between MCs and CD8$^+$ T cells was either allowed* ***("free contact")*** *or inhibited by the presence of a 0.2 μm or a 0.02 μm-anopore membrane, as indicated.* ***A.*** *Activation of CD8$^+$ T cells was measured by analysis of CD69, CD25 (upper row) and CD44 (lower row) expression by FACS. Staining of the cells with control isotype matched antibodies of irrelevant specificity is shown in bold black lines.* ***B.*** *Proliferation of CD8$^+$ T cells was visualized by CFSE dilution. All graphs are gated on viable CD8$^+$ T cells. Representative results of three independent experiments are shown.*

3.5.5. The antigen-specific interaction between MCs and $CD8^+$ T cells is dependent on cytokines released by MCs

Since it was demonstrated that direct cell-cell contact is essential for the antigen-specific interaction between MCs and $CD8^+$ T cells, it was further enquired whether direct cell-cell contact is the only requirement for MCs to induce antigen-specific $CD8^+$ T cell activation, or soluble factors produced by MCs contribute additionally to this activation. For this reason, the release of soluble factors by MCs was inhibited by treatment with mitomycin C.

Initially, in order to exclude the possibility that the mitomycin C treatment of MCs induces activation of the $CD8^+$ T cells in the absence of the $OVA^{257-264}$ peptide, unpulsed MCs were left untreated or treated with mitomycin C and were subsequently co-cultured with $CD8^+$ T cells. As shown in **Fig. 3.18A**, in the absence of the peptide, the $CD8^+$ T cells did not exhibit any activation, whether MCs were pretreated with mitomycin C or not.

Thus, it was further examined whether mitomycin C treatment of MCs would influence the ability of MCs to activate $CD8^+$ T cells in an antigen-dependent manner. For this reason, MCs were treated with mitomycin C immediately after their pulsing with the $OVA^{257-264}$ peptide. As compared with untreated antigen-pulsed MCs, the treatment of MCs with mitomycin C resulted in 54 ± 18% (n=3) decrease of their capacity to activate $CD8^+$ T cells, as measured by the surface expression of activation markers (CD69+CD25+) on $CD8^+$ T cells **(Fig. 3.18B)**.

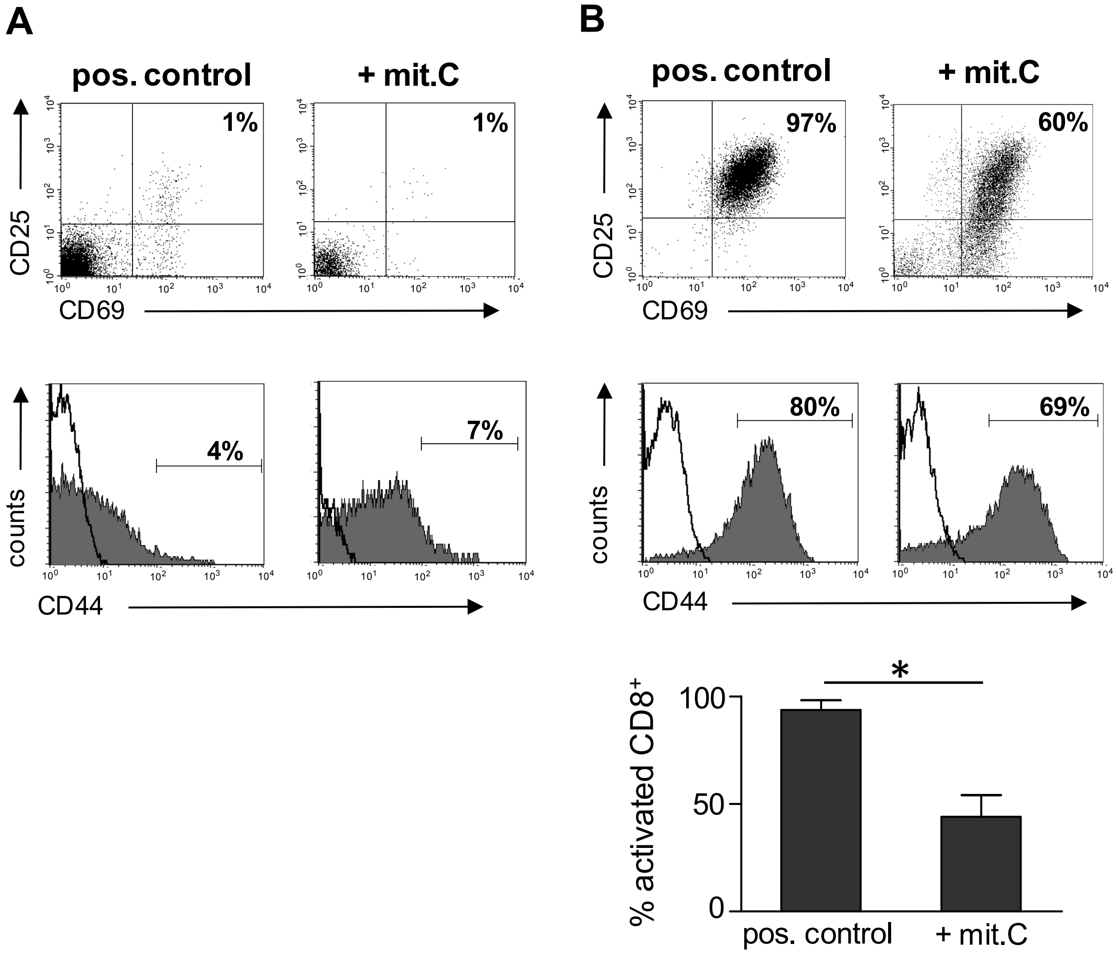

Fig. 3.18. Mitomycin C treatment of MCs reduces their ability to activate CD8⁺ T cells. *Unpulsed MCs **(A)** or OVA257-264-pulsed MCs **(B)** were left untreated **("pos. control")** or were treated with mitomycin C (10 µg/ml) **("mit. C")** prior to their addition to OT-I transgenic CD8+ T cells. Activation of CD8+ T cells was measured 48 hours later by analysis of CD69 and CD25 expression (upper row) and CD44 expression (middle row) by FACS (gated on viable CD8+ T cells). Control isotype staining is shown in bold black lines. Representative results of one out of three independent experiments are shown. Graph in the lowest row (in B) shows mean values of activated (CD69+CD25+) CD8+ T cells with standard deviation of three independent experiments. * p<0.05*

Mitomycin C inhibits nucleic acid synthesis, therefore blocks the synthesis of cytokines. However, MCs contain granula with many preformed cytokines and soluble factors, which are released upon MC degranulation. For this reason, the effect of blocking both the synthesis as well as the release of preformed soluble factors was examined by paraformaldehyde treatment of MCs.

First, in order to exclude the possibility that the paraformaldehyde treatment of MCs would induce activation of the $CD8^+$ T cells in the absence of the peptide, peptide-unpulsed MCs were left untreated or treated with paraformaldehyde and subsequently co-cultured with $CD8^+$ T cells. In the absence of the peptide, the $CD8^+$ T cells did not exhibit any activation, whether MCs were pre-treated with paraformaldehyde or not **(Fig. 3.19A)**.

Subsequently, MCs were treated with paraformaldehyde immediately after their pulsing with the antigen and were used to activate $CD8^+$ T cells in an antigen-dependent manner. As shown in **Fig. 3.19B**, the paraformaldehyde treatment of MCs resulted in 69% ± 16% (n=4) decrease of their capacity to activate $CD8^+$ T cells, as measured by the surface expression of activation markers (CD69, CD25 and CD44) on $CD8^+$ T cells.

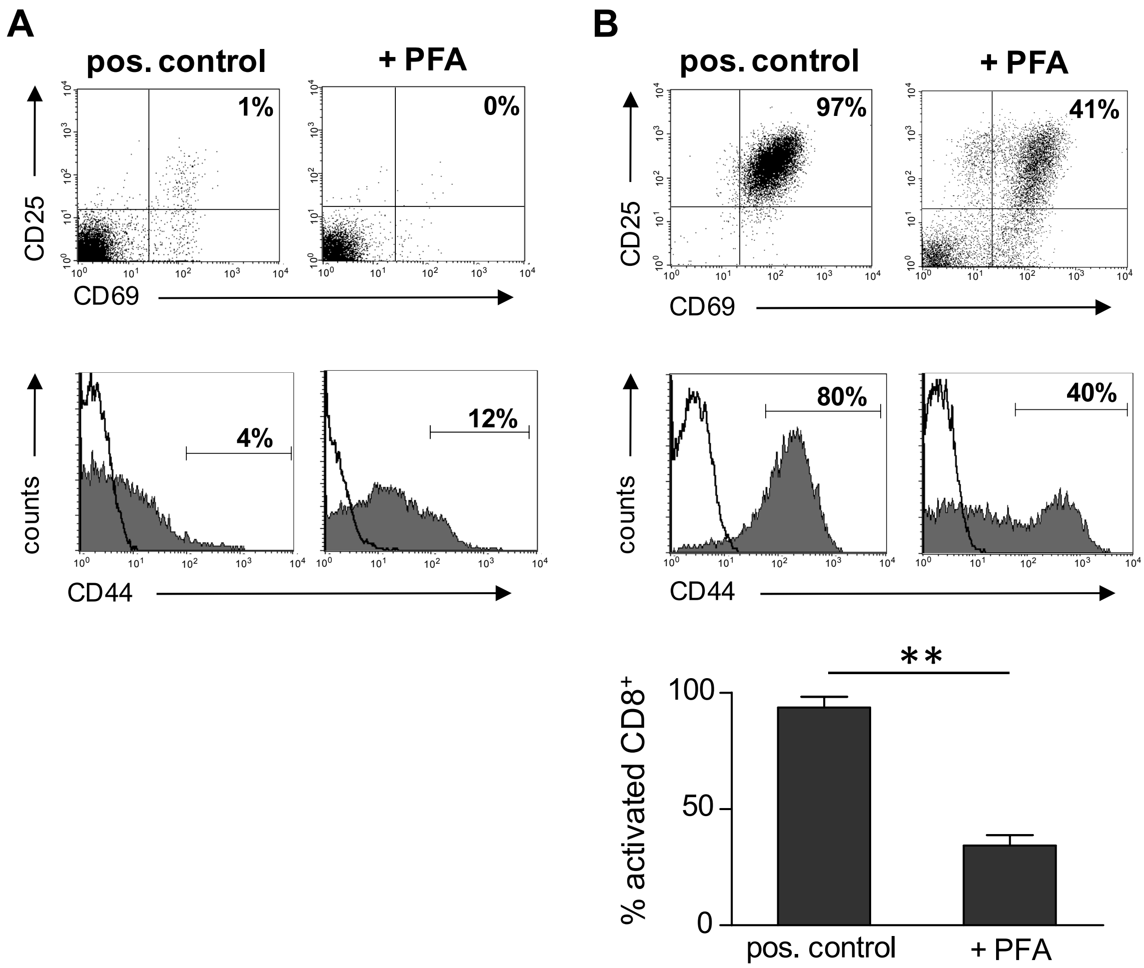

Fig. 3.19. Paraformaldehyde treatment of MCs reduces their ability to activate $CD8^+$ T cells. *Unpulsed MCs* ***(A)*** *or peptide-pulsed MCs* ***(B)*** *were left untreated* ***("pos. control")*** *or were treated with paraformaldehyde* ***("PFA")****, prior to their addition to the $CD8^+$ T cells. Activation of $CD8^+$ T cells was measured by analysis of CD69 and CD25 expression (upper row) and CD44 expression (middle row) by FACS (gated on viable $CD8^+$ T cells). Control isotype staining is shown in bold black lines. Representative results of four independent experiments are shown. Graph in the lowest row (in B) shows mean values of activated (CD69+CD25+) $CD8^+$ T cells with standard deviation of four independent experiments. ** $p<0.01$*

Conclusively, the MC-mediated, antigen-specific $CD8^+$ T cell activation is dependent both on direct cell-cell contact as well as on soluble factors released by MCs. It is therefore proposed that direct cell-cell contact between MC and $CD8^+$ T cells is essential for the formation of cell conjugates and the presentation of MHC class I-bound antigen. Once this first step of interaction is achieved, pre-stored and newly generated soluble factors released by MCs contribute additionally to the antigen-dependent activation of $CD8^+$ T cells. However, the formation of cell conjugates between MCs and $CD8^+$ T cells during MHC class I-dependent antigen presentation remains to be elucidated.

3.5.6. TLR-ligand exposure of MCs enhances their potential to activate $CD8^+$ T cells

MCs are resident cells in peripheral tissues, especially at sites exposed to the external environment (skin, airways, gastrointestinal tract) (Marshall, 2004). At such places, MCs encounter invading pathogens, which they can recognize by TLRs. It has been shown that TLR3-induced stimulation of MCs mediates $CD8^+$ T cell recruitment *in vivo* (Orinska et al., 2005). Therefore, it was suggested that a potential TLR-ligand exposure of MCs might have a modulatory effect on the capacity of MCs to induce $CD8^+$ T cell responses.

To investigate this hypothesis, first it was examined whether TLR-activation of MCs enhances the surface expression of MHC class I molecules, since this is the case for MHC class II surface expression on MCs (Frandji et al., 1993). Therefore, the level of MC surface-expressed MHC class I after stimulation with LPS (100 ng/ml) or pIC (10 μg/ml) was examined by FACS. As shown in **Fig. 3.20**, compared to untreated MCs, LPS-exposed MCs, exhibited significantly higher expression of surface MHC class I molecules. The surface expression of MHC class I on pIC-exposed MCs appeared to be slightly, but not significantly increased.

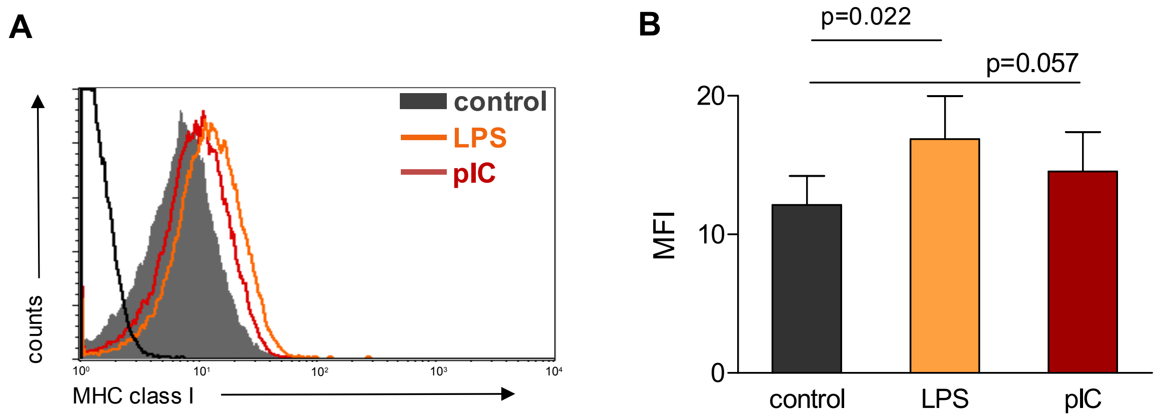

Fig. 3.20. TLR-activation of MCs enhances surface expression of MHC class I. *MCs were stimulated with LPS (100 ng/ml) or pIC (10 µg/ml) or left untreated* ***("control")*** *for 48 hours. Surface expression of MHC class I on MCs was analyzed by FACS (gated on viable, c-kit positive MCs).* ***A.*** *Histogram shows one representative of four independent experiments (n=5). Control isotype staining is shown in bold black line.* ***B.*** *Mean values of mean fluorescence intensity (MFI) for MHC class I on MCs with standard deviation of four independent experiments are shown (n=5).*

The suggestion that MCs may regulate $CD8^+$ T cell responses upon TLR-ligand exposure was supported by the up-regulation of MHC class I expression by MCs. Therefore, it was further examined whether the activation of MCs with TLR-ligands influenced their ability to induce $CD8^+$ T cell responses. To investigate this hypothesis, MCs were cultured in the presence of LPS (100 ng/ml) or pIC (10 µg/ml) for 48 hours prior to their loading with $OVA^{257-264}$ peptide. Subsequently, the antigen-pulsed MCs were incubated with OT-I transgenic $CD8^+$ T cells. The activation of $CD8^+$ T cells (surface CD69 and CD25 expression), as well as the cytokines in the supernatants were measured. MCs that have been exposed to LPS or pIC exhibited a greater capacity to induce antigen-specific $CD8^+$ T cell activation **(Fig. 3.21A)**. At a ratio of MC:CD8 = 1:10, MCs exhibited the maximum capacity to activate $CD8^+$ T cells, therefore the exposure to TLR-ligands did not significantly enhance the $CD8^+$ T cell activation. However, at a lower ratio of 1:20, LPS- or pIC- activated MCs displayed an increased capacity to activate $CD8^+$ T cells by 20 ± 8% or 20 ± 11% respectively. Moreover, secretion of IL-2 **(Fig. 3.21B)** was significantly enhanced upon TLR-activation of MCs. Regarding IL-2 production, the effect of LPS-stimulation was more prominent at the highest ratio of MC:CD8 (1:2), while the effect of pIC-stimulation was more prominent at the ratio of 1:10. In contrast to secretion of IL-2, IFN-γ secretion was not significantly enhanced upon TLR-ligand exposure of MCs **(Fig. 3.21B)**.

These results suggest that TLR-ligand exposure of MCs controls the antigen-dependent interaction between MCs and $CD8^+$ T cells. Therefore, it is proposed that the encounter of PAMPs by MCs in peripheral tissues is an important factor modulating the contribution of MCs in the regulation of adaptive immune responses.

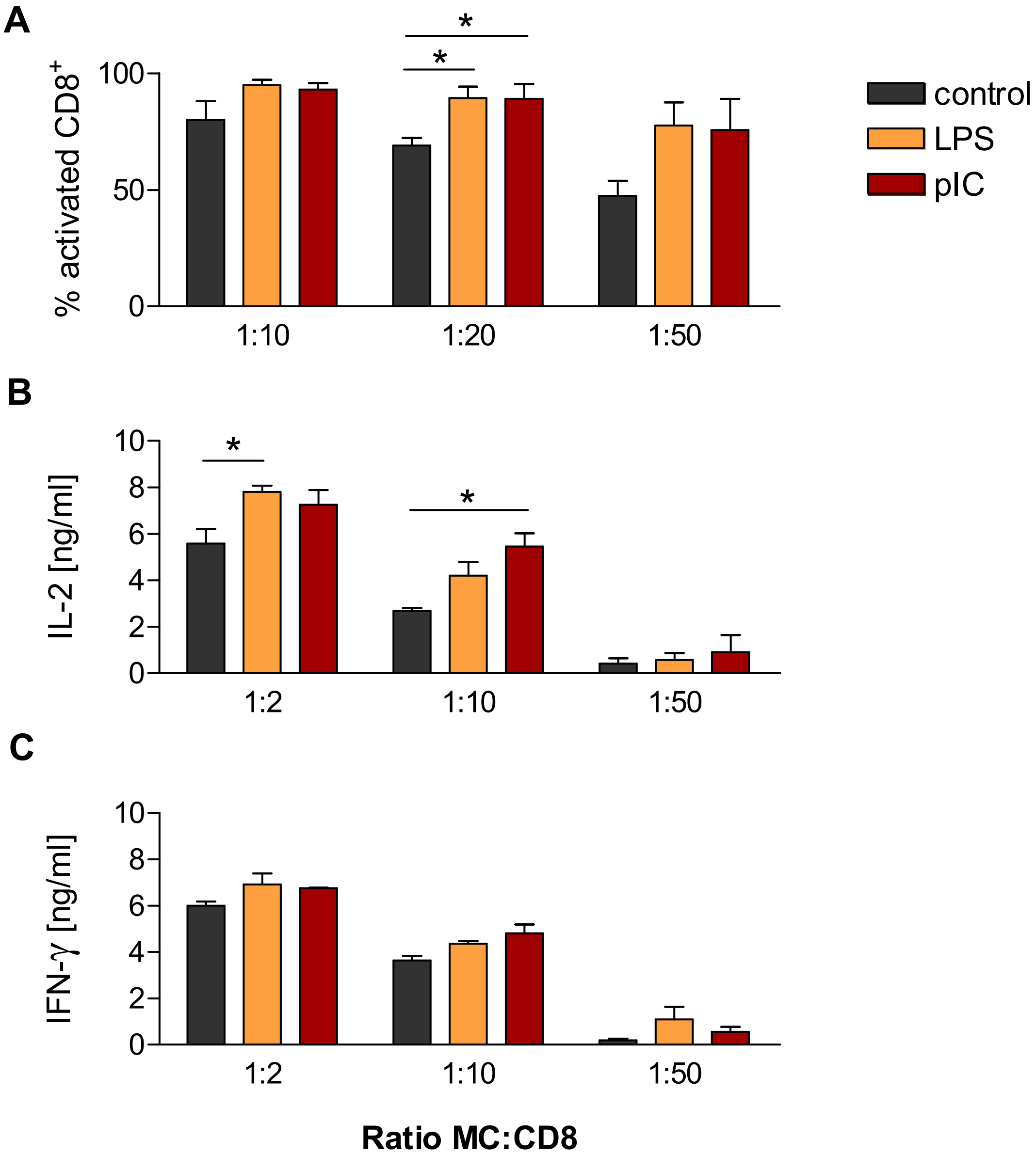

Fig. 3.21. TLR-ligand exposure of MCs enhances their capacity to activate CD8+ T cells. *MCs were stimulated with LPS (100 ng/ml) or pIC (10 µg/ml) or left untreated* ***("control")*** *prior to their loading with OVA257-264 peptide and were cultured with OT-I transgenic CD8+ T cells.* ***A.*** *Activation of CD8+ T cells was measured by analysis of CD69 and CD25 expression by FACS. Percentage of activated (double-positive CD69+CD25+) CD8+ T cells is shown.* ***B.*** *IL-2 production was measured in the supernatants of the co-cultures by ELISA.* ***C.*** *IFN-γ production was measured in the supernatants of the co-cultures by ELISA. Bars represent mean values with standard deviation of two to three independent experiments. * $p < 0.05$*

3.6. Antigen-dependent effects of MCs on $CD8^+$ T cells *in vivo*

Since MCs were proven to be efficient activators of $CD8^+$ T cell responses, the question whether this is only an *in vitro* observed effect or also an *in vivo* occurring phenomenon was raised. Therefore, the potential of MCs to interact with $CD8^+$ T cells in an antigen-dependent manner *in vivo* was investigated. First, MCs were examined for their ability to induce antigen-dependent proliferation of primary $CD8^+$ T cells *in vivo*. Furthermore, since MCs are well characterized cells during allergic reactions, the potential of MCs to modulate antigen-specific $CD8^+$ T cell responses in an *in vivo* murine model of allergy was tested.

3.6.1. MCs induce antigen-specific proliferation of $CD8^+$ T cells *in vivo*

In order to investigate whether MCs induce antigen-specific $CD8^+$ T cell responses *in vivo*, adoptive transfer experiments were performed. First, 5 x 10^6 antigen-pulsed MCs were administered intraperitoneally in C57/BL6 wild-type mice. As positive control, an equal number of antigen-pulsed bone marrow-derived DCs were administered, while an equal number of unpulsed MCs served as negative control. One day later, recipient mice were injected intraperitoneally with 5 x 10^6 CFSE-labeled, primary $CD8^+$ T cells purified from lymph nodes of OT-I transgenic mice. Proliferation of the transferred $CD8^+$ T cells was examined by FACS analysis four days later. As shown in **Fig. 3.22** MCs did not induce any antigen-independent proliferation of $CD8^+$ T cells **("control")**, while they were indeed able to induce the proliferation of $CD8^+$ T cells in the presence of the $OVA^{257\text{-}264}$ peptide **(Fig. 3.22, "MC")**. The relative amount of $CD8^+$ T cells in each of the successive peaks of CFSE dilution corresponds to the relative amount of $CD8^+$ T cells in each cell division. Thus, it is shown that DCs pulsed with the $OVA^{257\text{-}264}$ peptide induced higher proliferation of the $CD8^+$ T cells, as compared with MCs, since the percentages of the $CD8^+$ T cells in the later cell divisions are higher **(Fig. 21, "DC")**.

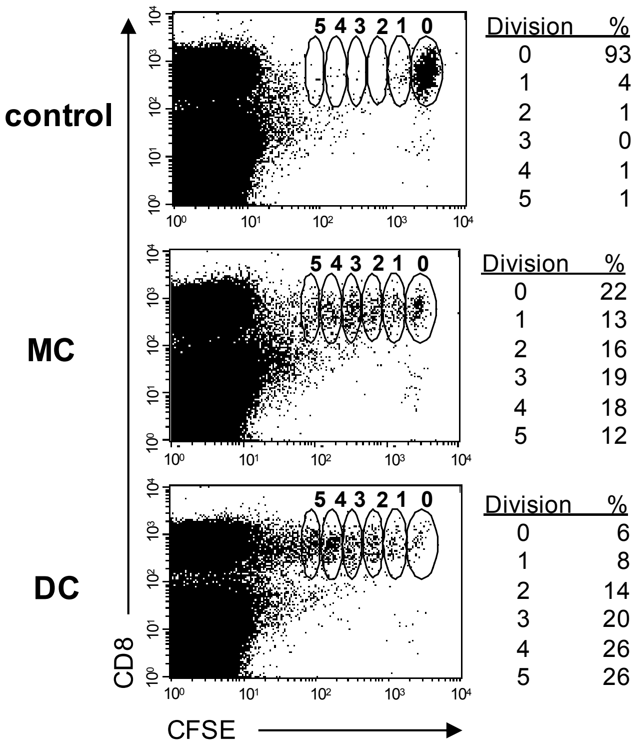

Fig. 3.22. MCs induce antigen-specific proliferation of $CD8^+$ T cells in vivo. *5 x 10^6 MCs were left untreated* ***("control")*** *or were pulsed with the $OVA^{257\text{-}264}$ peptide* ***("MC")*** *and injected intraperitoneally in C57BL/6 recipients. As positive control, 5 x 10^6 DCs were pulsed with the $OVA^{257\text{-}264}$ peptide* ***("DC")*** *and injected intraperitoneally in C57BL/6 recipients. 24 hours later, 8 x 10^6 purified CFSE-labeled $CD8^+$ OT-I transgenic T cells were transferred intraperitoneally. 4 days later, lymph node cells were isolated and analyzed for proliferation of the CFSE-labeled $CD8^+$ T cells by the dilution of CFSE (gated on viable lymphocytes). Representative results of three independent experiments with at least seven mice per group are shown (n=7-9).*

In order to prove that the antigen presentation was performed exclusively by the antigen-pulsed, transferred MCs, and not by other resident APCs, the same adoptive transfer experiment was performed in $\beta2m^{-/-}$ mice. These mice do not possess the β2m chain of MHC class I, therefore lack the ability to efficiently present MHC class I-related antigens. A comparable stimulation of $CD8^+$ T cell proliferation by the transfer of antigen-pulsed MCs was observed in wild-type **(Fig. 3.23, "WT")** and in $\beta2m^{-/-}$ mice **(Fig. 3.23, "$\beta2m^{-/-}$")**, excluding the possibility that other resident cells which present MHC class I-dependent antigens significantly influenced the $CD8^+$ T cell proliferative response.

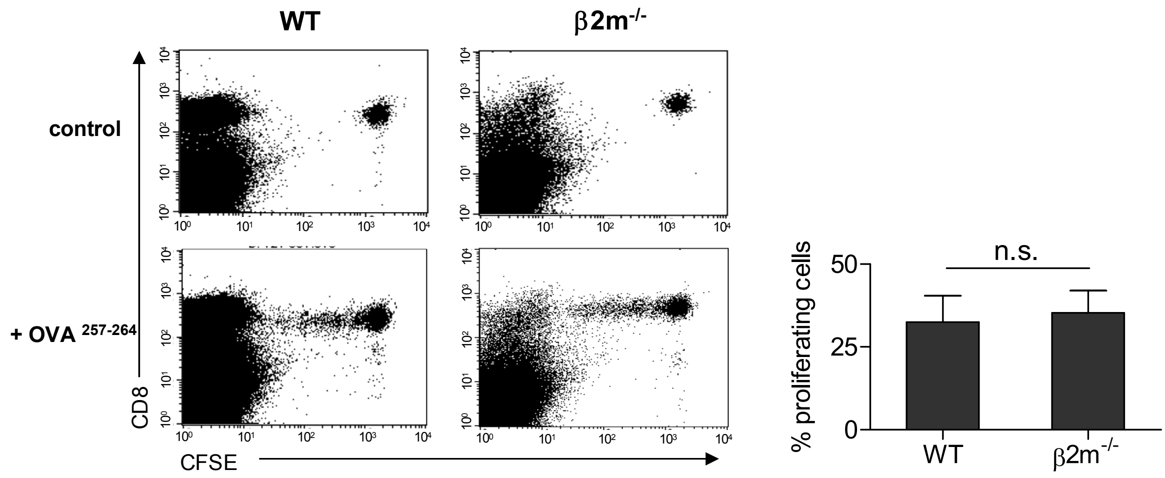

Fig. 3.23. MCs induce antigen-specific CD8$^+$ T cell proliferation in β2m$^{-/-}$ mice. *5 x 10^6 MCs were left untreated **("control")** or pulsed with the OVA$^{257\text{-}264}$ peptide **("+ OVA$^{257\text{-}264}$")** and injected intraperitoneally in C57BL/6 **("WT")** or in β2m$^{-/-}$ **("β2m$^{-/-}$")** recipients. 24 hours later, 8 x 10^6 purified, CFSE-labeled, OT-I transgenic CD8$^+$ T cells were transferred intraperitoneally. 4 days later, lymph node cells were isolated and analyzed for proliferation of the CD8$^+$ T cells by the dilution of CFSE (gated on viable lymphocytes). Representative results of three independent experiments with at least seven mice per group are shown. Bars in the right graph represent mean values for the percentages of proliferating CFSE-labeled CD8$^+$ T cells with standard deviation of three independent experiments with at least seven mice per group (n=7-9). n.s.:not significant*

With those experiments, showing that MCs induce proliferation of CD8$^+$ T cells in wild-type as well as in β2m$^{-/-}$ mice, it was determined that MCs are efficient APCs, capable of interacting with CD8$^+$ T cells upon presentation of MHC class I-related antigens *in vivo.*

3.6.2. MCs do not significantly influence CD8$^+$ T cell responses in a murine model of allergic airway sensitization

Since MCs were proven to induce antigen-specific CD8$^+$ T cell responses *in vitro* and *in vivo*, an *in vivo* pathological situation was investigated, in which the MC-CD8$^+$ T cell interaction could be relevant. MCs are well characterized as main participants of an allergic immune response. In addition, evidence in different models suggests that $CD8^+$ T cells are also important for the induction of allergic sensitization (Haczku et al., 1995a; Haczku et al., 1995b). Several parameters of allergic sensitization have been shown to be directly dependent on the presence of $CD8^+$ T cells (Hamelmann et al., 1996). For this reason, the effect of MCs in inducing antigen-specific $CD8^+$ T cell responses during a well established murine model of allergic sensitization against OVA was examined.

To investigate this question, MC-deficient mice (Kit-W/Wv) as well as their congeneic wild-type mice (Kit-W/W) were immunized against ovalbumin (OVA), according to a well established protocol of sensitization against OVA, schematically represented in **Fig. 3.24**. Mice were sensitized with three intraperitoneal injections of OVA (20 µg/mouse) and subsequently challenged with OVA aerosol (1% OVA in PBS). One day after the last OVA-aerosol challenge, the lung and the draining lymph nodes of the lung were analyzed for the content and phenotype of $CD8^+$ T cells.

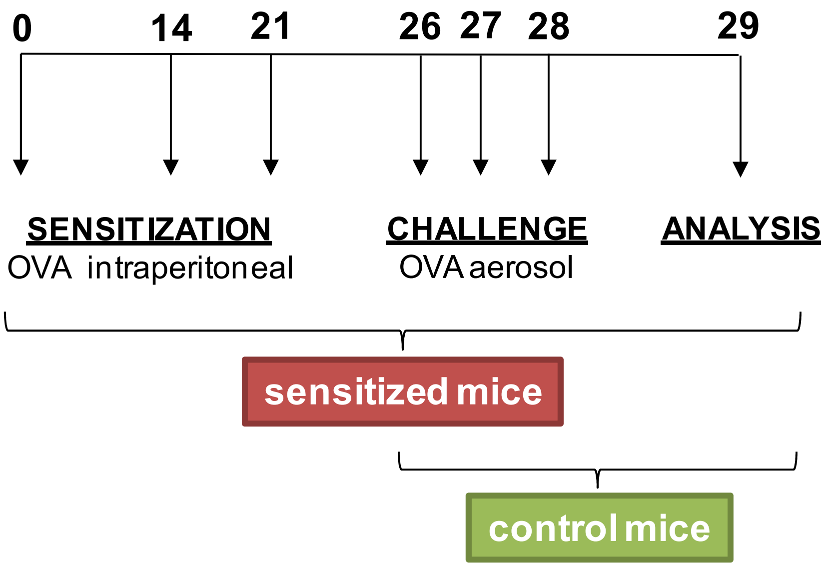

Fig. 3.24. Protocol for inducing allergic immune response against ovalbumin (OVA). *MC-deficient mice (Kit-W/Wv) as well as their congeneic wild-type mice (Kit-W/W) received intraperitoneal injection of OVA (20 µg/mouse) on days 0, 14 and 21. Subsequently, mice were challenged with inhalation (aerosol) of 1% OVA in PBS for 40 min on days 26, 27 and 28. Control mice were only treated with OVA aerosol. Mice were analyzed for the* $CD8^+$ *immune response in the lung and in the draining lymph nodes on day 29.*

Using the above protocol of OVA immunizations in wild-type and MC-deficient mice, four groups of mice were formed: "control" (= unimmunized) wild-type and MC-deficient mice as well as "sensitized" (= sensitized and challenged with OVA) wild-type and MC-deficient mice **(Fig. 3.25)**. Thus, initially the CD8$^+$ T cell response was compared between "control" wild-type and MC-deficient mice, in order to examine whether the absence of MCs results in an altered CD8$^+$ T cell phenotype in naïve mice. Subsequently, the CD8$^+$ T cell response was compared between "sensitized" wild-type and MC-deficient, in order to further investigate whether the CD8$^+$ T cell response during an allergic sensitization is dependent on the presence of MCs.

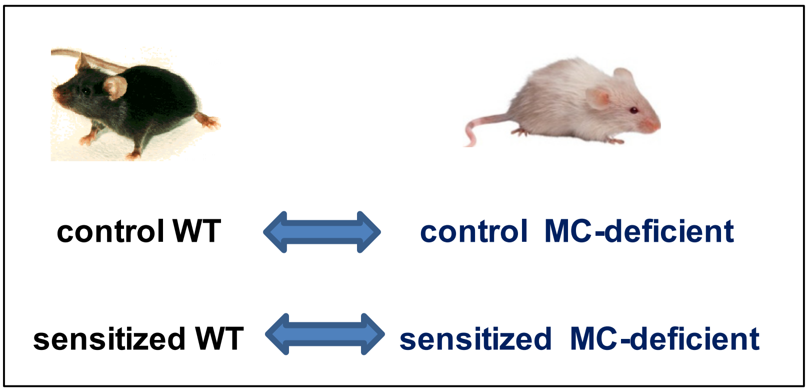

Fig. 3.25. The groups of mice that were formed and compared after OVA immunizations. *Unimmunized* ***("control")*** *wildtype (WT) mice were compared to their congeneic MC-deficient mice (W/Wv) to examine if the CD8$^+$ T cell phenotype in naïve mice is dependent on the presence of MCs. OVA-sensitized and -challenged mice* ***("sensitized")*** *WT mice were compared to MC-deficient mice to examine whether the CD8$^+$ T cell response during a murine model of allergic sensitization is dependent on the presence of MCs.*

First, the percentage as well as the total number of CD8$^+$ T cells in the lung and in the draining lymph nodes of the lung was determined by FACS analysis. MC-deficient "control" mice exhibited a reduced percentage and reduced number of CD8$^+$ T cells in the lung, but not in the draining lymph nodes of the lung **(Fig. 3.26, left columns)**. However, these differences were not visible after sensitization and challenge against OVA **(Fig. 3.26, right columns)**. This evidence suggests that MCs may regulate the amount of CD8$^+$ T cells in the lung of naïve mice. However, it should be taken into consideration that the lack of c-kit function in Kit-W/Wv mice causes additional abnormalities in these mice, such as macrocytic anemia, lack of melanocytes, intestinal TCRγδ intraepithelial lymphocytes and interstitial cells of Cajal. Therefore, results in terms of MC-mediated effects obtained from comparisons between Kit-W/Wv and their congeneic wild-type mice should be interpreted carefully. For this reason, the amount of $CD8^+$ T cells in Kit-W/Wv mice reconstituted with BMMCs should be examined, in order to distinguish whether the above demonstrated decreased number of $CD8^+$ T cells in the MC-deficient mice is exclusively a MC-dependent phenomenon, or results from a dysregulated T cell development in the Kit-W/Wv mice.

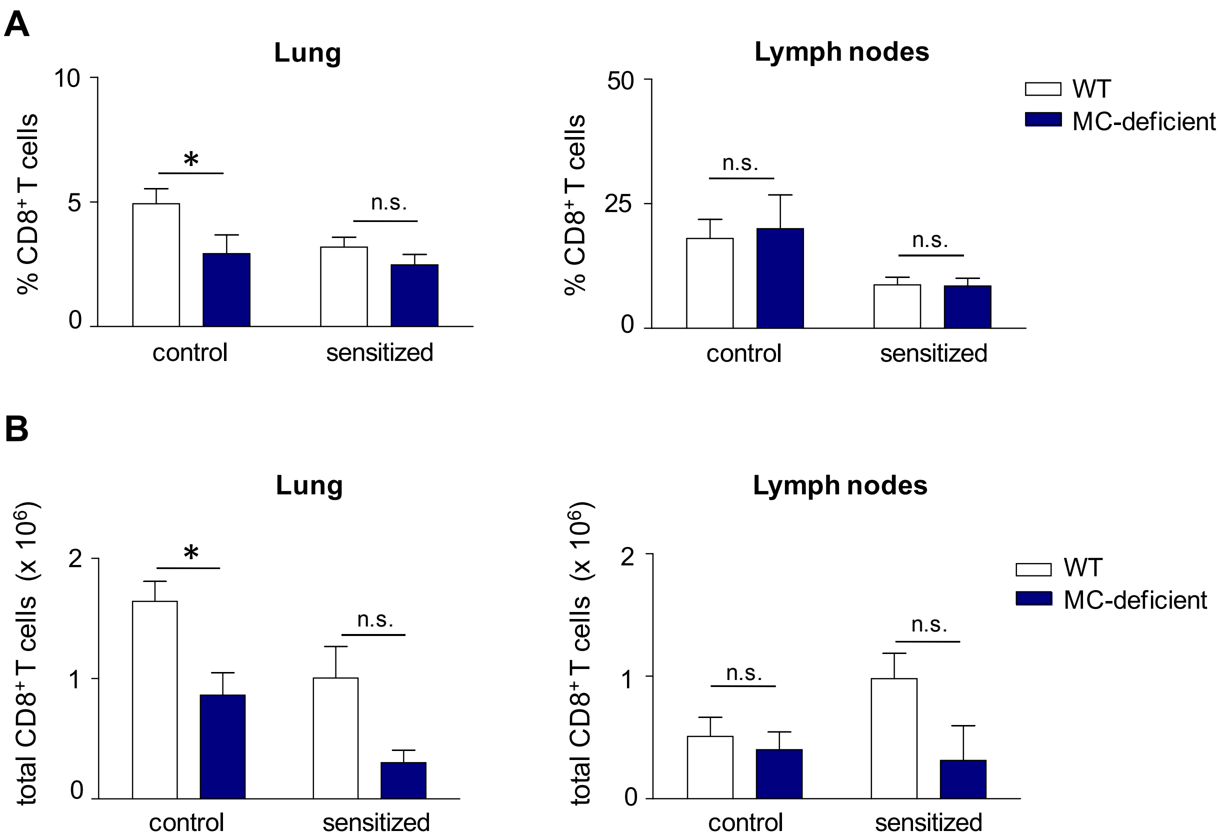

Fig. 3.26. MCs do not regulate the number of $CD8^+$ T cells in an in vivo model of allergic airway sensitization against OVA. *MC-deficient mice (Kit-W/Wv,* ***blue bars****), and their congeneic wild-type mice (Kit-W/W,* ***white bars****), were sensitized and challenged* ***("sensitized")*** *or only challenged* ***("control")*** *against OVA according to the protocol shown in Fig. 3.24. Lung and the draining lymph nodes of the lung were analyzed by FACS.* ***A.*** *Percentage of $CD8^+$ T cells is shown (gated on viable cells) (n=7-9).* ***B.*** *Total number of viable $CD8^+$ T cells is shown (n=3-5). * $p<0.05$, n.s.:not significant*

Furthermore, the potential of MCs to modulate the activation or memory differentiation of $CD8^+$ T cells in this allergic model was analyzed. Activation of $CD8^+$ T cells was measured by surface expression of CD69 and CD25, while effector memory differentiated $CD8^+$ T cells were characterized as $CD44^+CD62L^-$ by FACS. As shown in **Fig.**

3.27, MC-deficient mice did not exhibit a significant difference in the activation of $CD8^+$ T cells **(Fig. 3.27A)** or in the development of effector memory $CD8^+$ T cells **(Fig. 3.27B)** after sensitization against OVA.

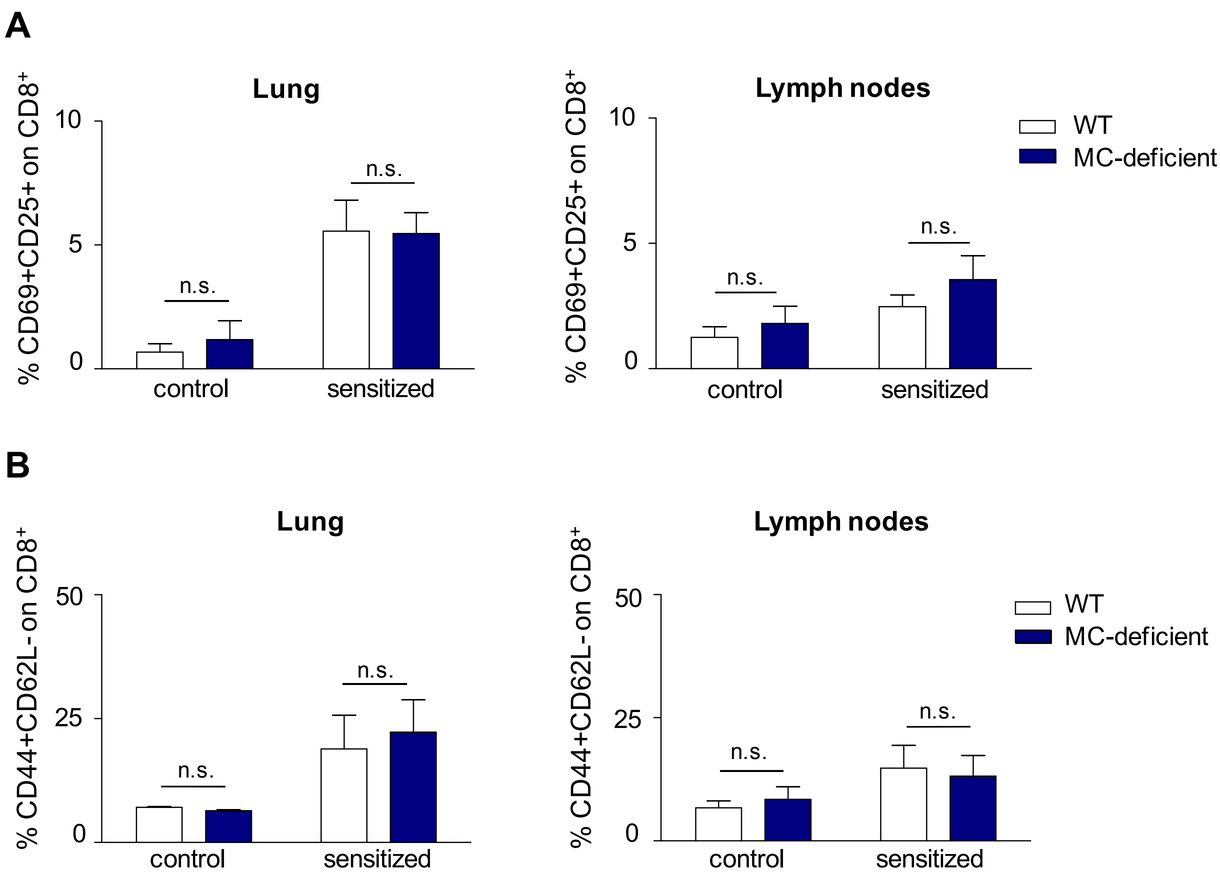

Fig. 3.27. MCs do not modulate the $CD8^+$ T cell response in an in vivo model of allergic airway sensitization against OVA. *MC-deficient mice (Kit-W/Wv,* ***blue bars****), as well as their congeneic wild-type mice (Kit-W/W,* ***white bars****), were sensitized and challenged* ***("sensitized")*** *or only challenged* ***("control")*** *against OVA according to the protocol shown in Fig. 3.24. Lung and the draining lymph nodes of the lung were analyzed by FACS.* ***A.*** *Percentage of $CD69^+CD25^+$ on viable $CD8^+$ T cells is shown.* ***B.*** *Percentage of $CD44^+CD62L^-$ on viable $CD8^+$ T cells is shown. Bars represent mean values with standard deviation of three independent experiments with at least seven mice per group (n=7-9). n.s.:not significant*

This data indicate that, although MCs are able to induce antigen-specific proliferation of primary $CD8^+$ T cells *in vitro* and *in vivo*, this antigen-specific interaction between MCs and $CD8^+$ T cells does not have a significant impact on the outcome of the murine allergic model studied here. However, MC-$CD8^+$ T cell interactions could play an important role in other *in vivo* situations, for example viral infections. The role of MC-$CD8^+$ T cell crosstalk in such pathological situations remains to be investigated.

3.7. The effect of MCs on pre-activated $CD8^+$ T cells

MCs are long-lived, resident cells in the peripheral tissues (Marshall, 2004). Therefore, the next hypothesis formulated here was that MCs can influence $CD8^+$ T cells that have been primed and pre-activated by the migrating DCs in the lymph nodes. Thus, MCs may play a role also in a secondary immune response, by regulating the activities of DC-mediated pre-activated $CD8^+$ T cells. To investigate the effect of MCs on pre-activated $CD8^+$ T cells two different approaches were used. First, the effect of the presence of MCs during the DC-mediated activation of $CD8^+$ T cells and second, the role of MCs in regulating the proliferation of pre-activated $CD8^+$ T cells were examined.

3.7.1. MCs reduce the ability of DCs to activate $CD8^+$ T cells *in vitro*

DCs are "professional" APCs capable of inducing optimal $CD8^+$ T cell responses in an antigen-dependent manner. MCs have been shown to interact with DCs and induce their functional maturation by induction of integrins and co-stimulatory molecule expression (Galli et al., 2005b). In order to understand whether MCs also modulate the capacity of DCs to prime $CD8^+$ T cells, the DC-induced activation of $CD8^+$ T cells was studied in the presence or absence of MCs. For this reason, $OVA^{257\text{-}264}$-pulsed DCs were used to stimulate naïve, OT-I transgenic $CD8^+$ T cells in the absence of MCs or in the presence of different amounts of MCs in the culture. As read-out, the surface expression of CD69 and CD25 was measured by FACS analysis. The result indicated that the presence of MCs in the culture inhibited the antigen-dependent, DC-induced $CD8^+$ T cell activation. This effect occurred in a MC dose-dependent way. Low numbers ($1 \times 10^4 – 5 \times 10^4$) of MCs did not significantly influence the DC-mediated $CD8^+$ T cell activation. However, 1×10^5 MCs reduced the activation of $CD8^+$ T cells, as shown by the 12.5% ± 3.5% (n=2) reduced surface expression of activation markers CD69 and CD25, measured by FACS **(Fig. 3.28)**.

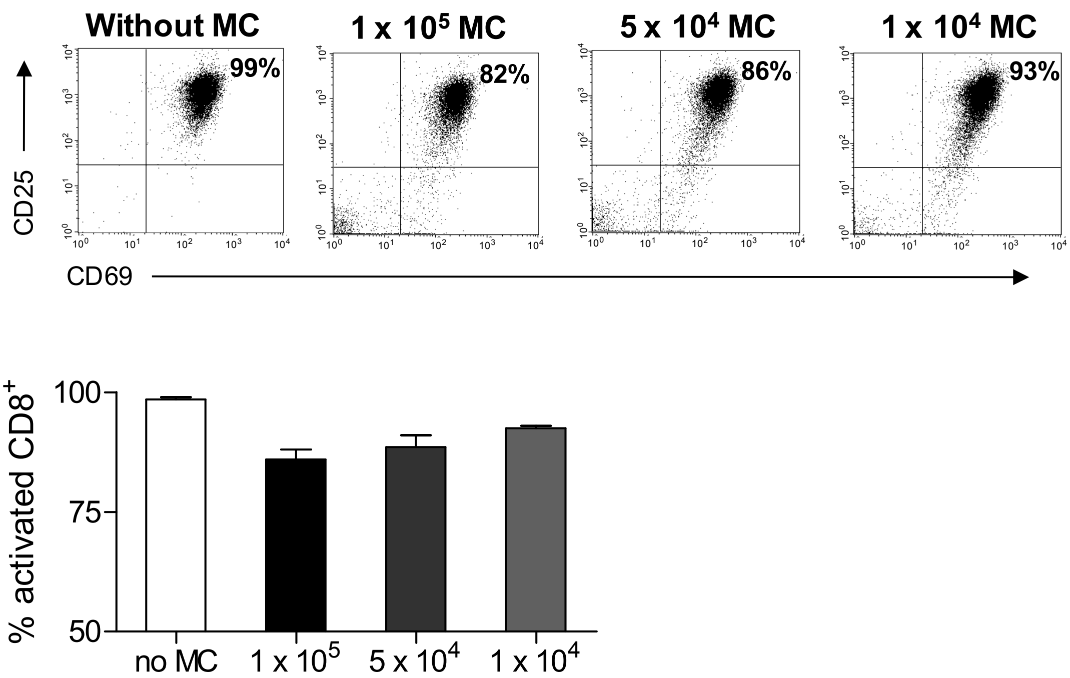

Fig. 3.28. The presence of MCs reduces the antigen-dependent, DC-mediated CD8$^+$ T cell activation. *DCs were pulsed with OVA$^{257\text{-}264}$ peptide and co-cultured with naïve, OT-I transgenic CD8$^+$ T cells without MCs or in addition of 1 x 10^5, 5 x 10^4 or 1 x 10^4 MCs, as indicated. After 48 hours, cells were analyzed by FACS for their activation status. Dot plots show surface expression of activation markers (CD69 and CD25) on CD8$^+$ T cells (gated on viable CD8$^+$ T cells) (n=2). Graph in lower row shows mean values of activated (CD69+CD25+) CD8$^+$ T cells with standard deviation of two independent experiments.*

3.7.2. MCs inhibit the proliferation of pre-activated CD8$^+$ T cells *in vitro*

Furthermore, it was investigated whether MCs modulate the proliferation of DC-mediated, pre-activated CD8$^+$ T cells. Therefore, CD8$^+$ T cells were antigen-dependently activated by OVA$^{257\text{-}264}$-pulsed DCs. Subsequently, the CD8$^+$ T cells were isolated from the co-culture by MACS, and co-cultured with MCs at different cell ratios. The presence of MCs in the culture inhibited the proliferation of CD8$^+$ T cells, as shown by the reduced CFSE dilution of the CD8$^+$ T cells **(Fig. 3.29)**.

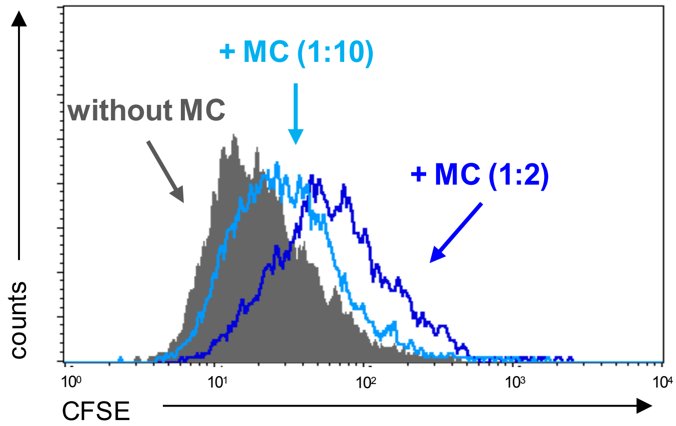

Fig. 3.29. MCs reduce the proliferation of pre-activated CD8$^+$ T cells. *OVA$^{257\text{-}264\text{-}}$-pulsed DCs were cultured with OT-I transgenic lymph node cells for 24 hours. Following, CD8$^+$ T cells were isolated by negative selection with MACS and labeled with CFSE. Purified activated CD8$^+$ T cells were cultured in the presence of MCs at a MC:CD8 ratio of 1: 2* ***(dark blue line)*** *or 1:10* ***(light blue line)*** *or in the absence of MCs* ***(grey filled histogram)****. Proliferation was analyzed by CFSE dilution of CD8$^+$ T cells, 48 hours after the addition of MCs, as measured by FACS (gated on viable CD8$^+$ T cells). Representative results of two independent experiments are shown.*

These results suggest that MCs might regulate the CD8$^+$ T cell responses during a secondary immune response, by down-regulating the DC-mediated CD8$^+$ T cell activation as well as by reducing the proliferation of pre-activated CD8$^+$ T cells. Thus, MCs may act as regulatory cells during adaptive immune responses: when encounter with naïve CD8$^+$ T cells occurs, MHC class I-dependent presentation of antigens results in antigen-specific CD8$^+$ T cell responses; however when MCs encounter pre-activated CD8$^+$ T cells, reduced CD8$^+$ T cell activation and proliferation occurs.

3.8. The effect of $CD8^+$ T cells on MCs

With the above demonstrated experiments, it was proven that MCs modulate $CD8^+$ T cell responses in an antigen-dependent manner *in vitro* and *in vivo*. The contact with activated $CD8^+$ T cells could, in turn, modulate the phenotype of MCs, as it is the case for DCs (Bernhard et al., 2000). In particular, activated T cells have been shown to induce maturation of DCs by enhancing surface expression of MHC and co-stimulatory molecules (Bernhard et al., 2000). Moreover, the $CD8^+$ T cell response detected in our settings comprised the secretion of cytokines, such as IFN-γ and GM-CSF, which are reported to affect MC differentiation and phenotype (Frandji et al., 1995). Therefore, it was suggested that the MC phenotype was also regulated by the contact with $CD8^+$ T cells. The modulation of MC activities upon contact with activated $CD8^+$ T cells could have a feedback in the MC-mediated regulation of adaptive immune responses. For this reason, it was further investigated whether $CD8^+$ T cells are able to modulate MC activities in an antigen-independent or antigen-dependent manner.

3.8.1. $CD8^+$ T cells regulate MHC class I protein expression on MCs

Activated T cells induce up-regulation of surface expressed MHC molecules on DCs (Bernhard et al., 2000). Since a surface expression of MHC class I molecules is important for the presentation of antigens to $CD8^+$ T cells, the ability to $CD8^+$ T cells to induce surface MHC class I on MCs was examined. For this reason, unpulsed or $OVA^{257\text{-}264}$ -pulsed MCs were cultured with $CD8^+$ T cells. As negative control, $OVA^{257\text{-}264}$ -pulsed MCs that did not contact $CD8^+$ T cells were used. After 48 hours of co-culture, MCs were analyzed for surface MHC class I expression by FACS. As shown in **Fig. 3.30**, antigen-dependent contact with $CD8^+$ T cells induced surface expression of MHC class I on MCs. Thus, it is proposed that $CD8^+$ T cells upon antigen-dependent, MC-mediated activation, increase the expression of MHC class I molecules on MCs, therefore enhance their potential for further interaction with $CD8^+$ T cells.

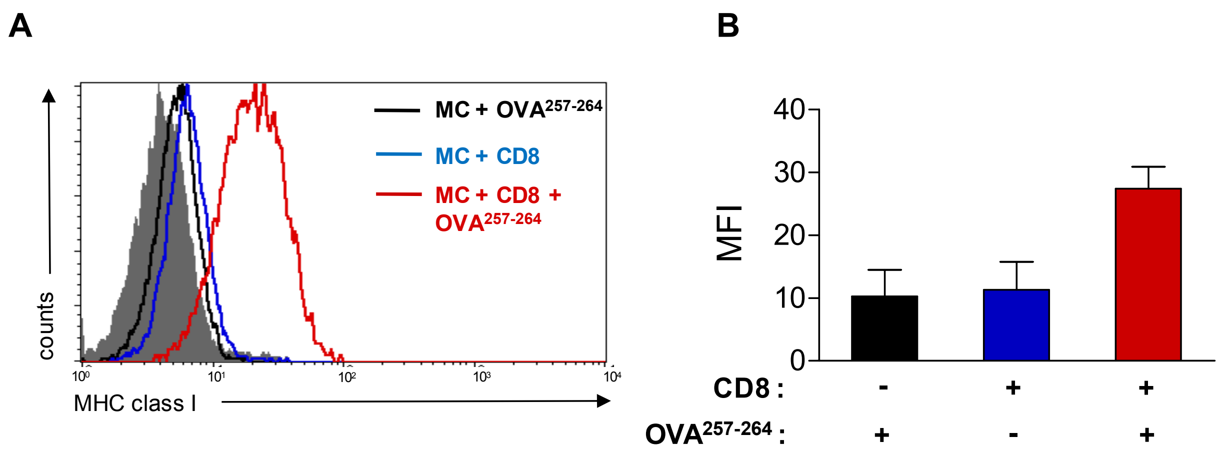

Fig. 3.30. CD8$^+$ T cells induce MHC class I surface protein expression on MCs. *Unpulsed MCs* ***(blue line)*** *or OVA$^{257\text{-}264}$ -pulsed MCs* ***(red line)*** *were cultured with CD8$^+$ T cells. As negative control, OVA$^{257\text{-}264}$ -pulsed MCs that did not contact CD8$^+$ T cells were used* ***(black line)****. After 48 hours of co-culture, MCs were analyzed for surface expression of MHC class I by FACS. Control isotype staining is shown by the grey filled histogram.* ***A.*** *Expression of MHC class I in one representative of three independent experiments is shown.* ***B.*** *Mean values of MFI for MHC class I with standard deviation of two independent experiments are shown.*

3.8.2. CD8$^+$ T cells modulate the gene expression profile of MCs

The enhanced MHC class I expression by MCs after antigen-dependent contact with CD8$^+$ T cells suggests that activated CD8$^+$ T cells are able to modulate MC activities. To further investigate the impact of CD8$^+$ T cells on MCs, first the gene expression profile of MCs after antigen-independent or antigen-dependent contact with CD8$^+$ T cells was compared to the one of MCs not having contacted CD8$^+$ T cells. Therefore, untreated or OVA$^{257\text{-}264}$-pulsed MCs were co-cultured with CD8$^+$ T cells for 48 hours and subsequently purified by MACS separation. As negative control, OVA$^{257\text{-}264}$-pulsed MCs not having encountered CD8$^+$ T cells were used. The purity of MCs after double MACS separation was tested with FACS staining for surface expression of c-kit (CD117) and FcεRI. As shown in **Fig. 3.31**, MCs after MACS purification were 96.8 ± 1.8% (n=3) pure for the case of OVA$^{257\text{-}264}$-pulsed MCs co-cultured with CD8$^+$ T cells, 97.9 ± 0.5% (n=3) for the case of unpulsed MCs co-cultured with CD8$^+$ T cells and 95.7 ± 0.1% (n=2) for the case of OVA$^{257\text{-}264}$-pulsed MCs not co-cultured with CD8$^+$ T cells.

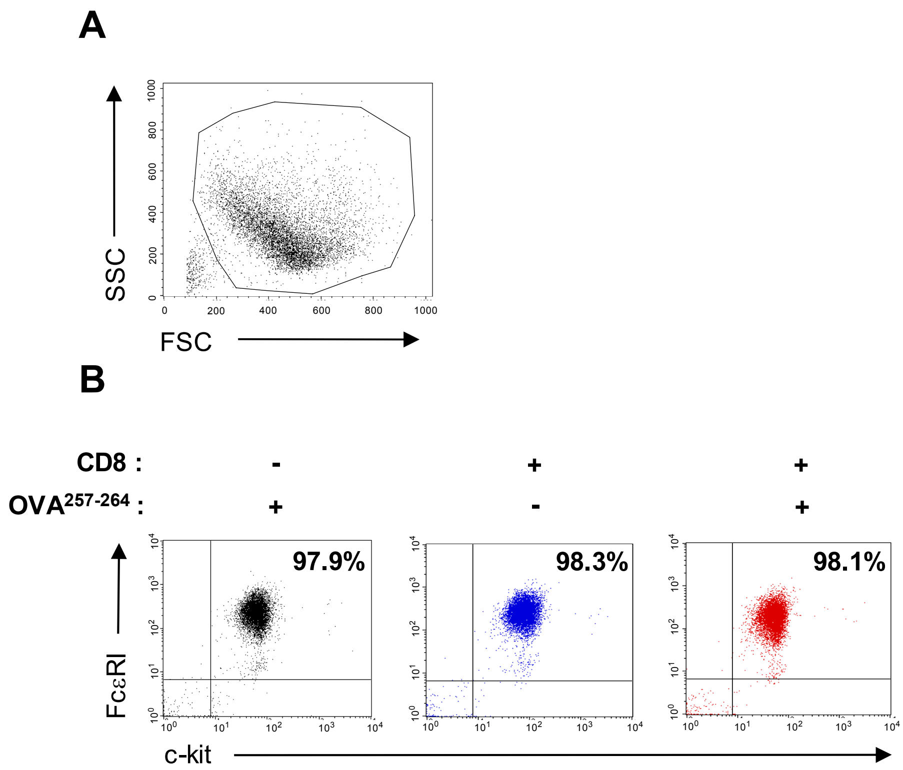

Fig. 3.31. Purity of MCs after MACS separation following co-culture with CD8$^+$ T cells. *OVA$^{257\text{-}264}$-pulsed MCs or unpulsed MCs were co-cultured with CD8$^+$ T cells for 48 hours and subsequently purified by two successive MACS separations. As negative control, OVA$^{257\text{-}264}$-pulsed MCs not having encountered CD8$^+$ T cells were used.* ***A.*** *Gate was set on all viable cells excluding cell debris and fractions of dead cells by FSC/SSC analysis.* ***B.*** *The purity of MCs after MACS separation was tested with FACS staining for surface expression of c-kit and FcεRI. Dot plots show representative results of two to three independent experiments.*

The experiment was performed three times and RNA was isolated from the purified MCs. Microarray analysis was performed in order to detect genes in MCs which were differentially expressed upon contact with CD8$^+$ T cells. The experimental design for this part of the study is schematically represented in **Fig. 3.32.**

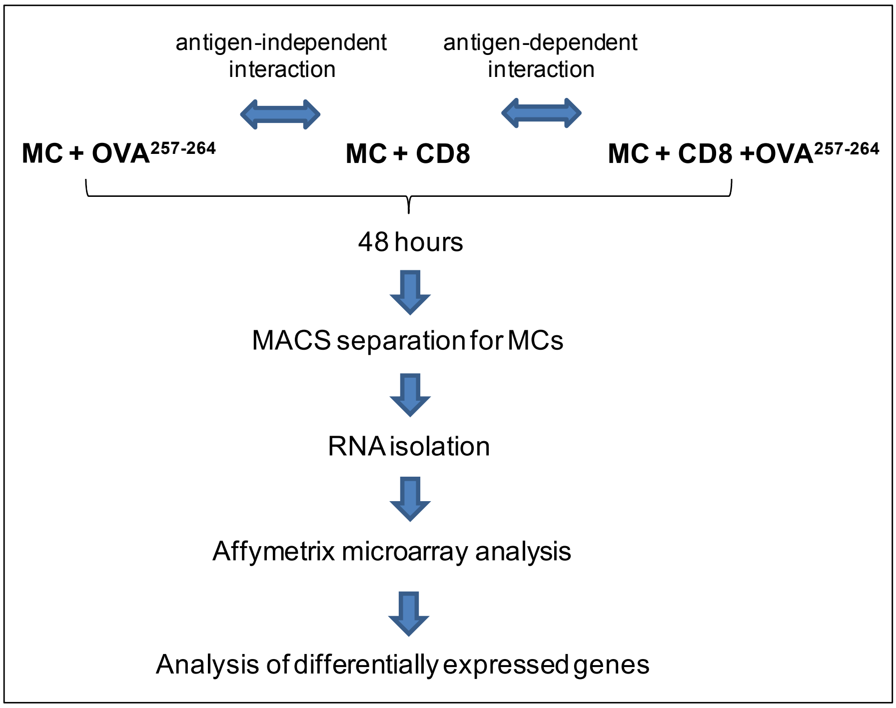

Fig. 3.32. Schematic representation of the experimental design for analyzing the differential gene expression in MCs after contact with CD8⁺ T cells.

The labeling and hybridization of the MC RNA in Affymetrix GeneChips, as well as the evaluation of the primary data obtained from the microarray experiment, were performed by Dr. Reinhardt Hoffmann and Dr. Jörg Mages in the Technische Universität München. Statistical analysis was performed by limma analysis (Smyth, 2005), in which each condition was compared with each other. The F.p.-value derived from a summarization of the all these tests, similarly to a one-way ANOVA analysis. p-values derived from the comparison of pairs of MC sets. All p-values derived from the limma analysis were subsequently corrected for multiple testing using Benjamini Hochberg correction (Benjamini and Hochberg, 1995). Further evaluation of the results was focused only on the genes that revealed a significant differential expression between the different sets of MCs (F.p.-value < 0.05). The experiment was performed three times and the mean values of each set of MCs were used for further analysis.

The genes that displayed differential expression (more than 5 times) in the different sets of MCs are summarized in **Table 3.1**. Briefly, 29 genes appeared to be more than 5 times differentially expressed in MCs after contact with $CD8^+$ T cells. Of these, only 3 **(Table 3.1, green colour)** were down-regulated after contact with $CD8^+$ T cells. These results demonstrate that $CD8^+$ T cells direct a potent MCs response.

Gene symbol	Gene title	MC+ OVA$^{257-264}$ (Mean)	MC+ CD8 (Mean)	MC+OVA$^{257-264}$ +CD8 (Mean)	max/m in means	F.p.- value	Entrez. Gene
Rtp4	receptor transporter protein 4	12,96	560,55	546,74	43,25	0,03	67775
LOC630509 /// LOC674192	similar to H-2 class I histocompatibility antigen, Q7 alpha chain precursor (QA-2	34,17	213,82	1387,46	40,60	0,00	
Ifng	interferon gamma	5,93	12,25	143,20	24,15	0,00	15978
Mpa2l	macrophage activation 2 like	11,18	132,18	240,09	21,48	0,01	
Ak3l1	adenylate kinase 3 alpha-like 1	8,87	18,70	174,42	19,67	0,01	11639
AI451557	expressed sequence AI451557	10,76	182,40	210,13	19,54	0,01	102084
Tnfrsf9	tumor necrosis factor receptor superfamily, member 9	70,37	68,91	1238,48	17,97	0,00	21942
Lzp-s	P lysozyme structural	356,11	247,35	34,18	10,42	0,00	17110
H2-Q1 /// 0610037M1	histocompatibility 2, Q region locus 1 /// RIKEN cDNA	3,94	6,98	39,73	10,08	0,00	
Rsad2	radical S-adenosyl methionine domain containing 2	14,21	132,36	136,58	9,61	0,05	58185
Tgm2	transglutaminase 2, C	22,95	39,95	202,67	8,83	0,00	21817
Lpl	lipoprotein lipase	79,47	27,20	9,17	8,67	0,00	16956
Zbp1	Z-DNA binding protein 1	10,77	59,06	92,90	8,63	0,02	58203
Stat1	signal transducer and activator of transcription 1	74,80	509,32	643,01	8,60	0,01	20846
Irf7	interferon regulatory factor 7	32,56	277,03	200,01	8,51	0,03	54123
Iigp1	interferon inducible GTPase 1	7,67	33,85	60,67	7,91	0,04	60440
Ms4a6b	membrane-spanning 4-domains, subfamily A, member 6B	12,25	16,36	90,87	7,42	0,05	69774
AW112010	expressed sequence AW112010	22,26	80,97	162,29	7,29	0,01	107350
Mcf2l	mcf.2 transforming sequence-	56,96	46,85	305,35	6,52	0,00	17207
Plod2	procollagen lysine, 2-oxoglutarate 5-dioxygenase 2	40,28	41,96	259,84	6,45	0,00	26432
Irgm	immunity-related GTPase family,	122,08	589,89	761,01	6,23	0,04	15944
Rgs11	regulator of G-protein signaling	17,16	35,83	105,81	6,17	0,04	50782
Socs3	suppressor of cytokine	401,19	243,35	1470,84	6,04	0,00	12702
H2-Ab1	histocompatibility 2, class II antigen A, beta 1	45,36	66,46	261,66	5,77	0,00	14961
Tgfbi	transforming growth factor, beta induced	23,12	7,28	4,24	5,45	0,01	21810
Igtp	interferon gamma induced	226,87	1041,43	1195,15	5,27	0,03	16145
H2-Q8	histocompatibility 2, Q region	17,22	24,29	90,11	5,23	0,00	15019
Pdk1	pyruvate dehydrogenase kinase, isoenzyme 1	47,65	69,48	246,26	5,17	0,02	228026
Irf1	interferon regulatory factor 1	217,34	756,57	1087,75	5,00	0,01	16362

Table 3.1. Differential gene expression in MCs after contact with CD8^{+} T cells in an antigen-dependent or antigen-independent manner. *OVA$^{257-264}$-pulsed MCs were left untreated **(MC + OVA$^{257-264}$)** or were co-cultured with OT-I transgenic CD8^{+} T cells **(MC + CD8 + OVA$^{257-264}$)**. In parallel, unpulsed MCs were co-cultured with OT-I transgenic CD8^{+} T cells **(MC + CD8)**. After 48 hours MCs were isolated by positive selection with MACS and RNA was isolated. Microarray analysis (Affymetrix, Mouse MG 430 2.0) was performed. Statistical analysis was performed by limma analysis. Table summarizes the genes that were*

more than 5 times down-regulated ***(green colour)*** *or up-regulated* ***(red colour)*** *with F.p.-value <0.05. Mean values of gene expression from three independent experiments are shown.*

3.8.2.1. CD8$^+$ T cells modulate the gene expression profile of MCs in an antigen-independent manner

In an attempt to understand the relevance of the CD8$^+$ T cell-induced gene regulation in MCs, the differentially expressed genes were analyzed according to whether their differential expression was induced in an antigen-independent or antigen-dependent manner. First, CD8$^+$ T cells were tested for their ability to modulate the expression of genes in MCs in the absence of a specific antigen. As shown in **Fig. 3.33**, MCs isolated after antigen-independent contact with CD8$^+$ T cells **(MC + CD8)** displayed an up-regulated expression of several genes, each of them represented by a separate dot in the upper left triangle of the plot. Therefore, it is suggested that CD8$^+$ T cells are able to induce a MC response in an antigen-independent manner.

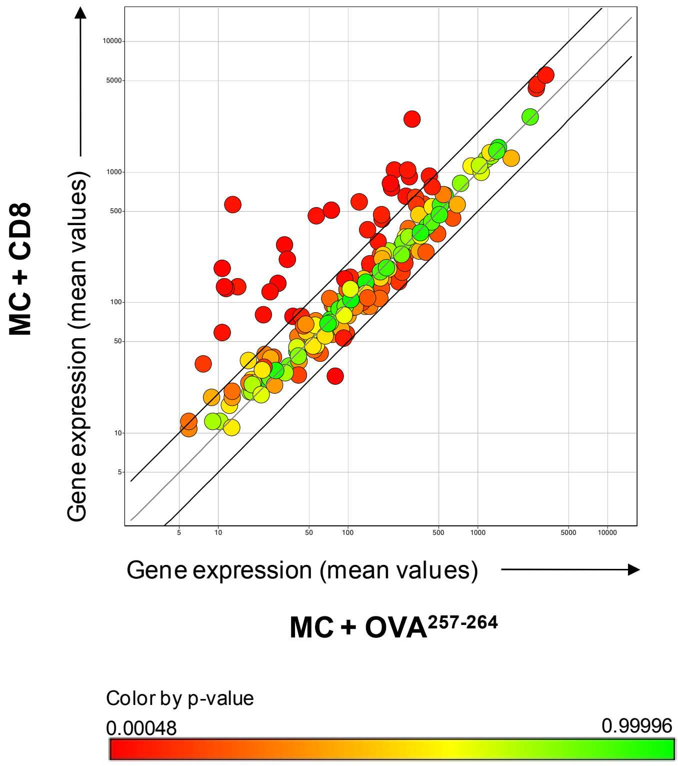

Fig. 3.33. CD8⁺ T cells induce differential gene expression in MCs in an antigen-independent manner. *MCs were co-cultured with OT-I transgenic CD8⁺ T cells **(MC + CD8)**. In parallel, OVA257-264-pulsed MCs were cultured alone **(MC + OVA257-264)**. After 48 hours, MCs were isolated by positive selection with MACS and RNA was isolated. Microarray analysis (Affymetrix, Mouse MG 430 2.0) was performed. Statistical analysis was performed by limma analysis. Scatterplot shows the differential expression of genes with F.p.-value of <0.05. Mean values of gene expression from three independent experiments are shown. Genes appearing in the upper left triangle of the plot displayed an up-regulation in the set of MCs having experienced contact with CD8⁺ T cells (MC + CD8), while genes appearing in the low right triangle of the plot displayed an up-regulation in the set of MCs not having contacted CD8⁺ T cells.*

Examination of the genes that were up-regulated after $CD8^+$ T cell contact revealed that several IFN-related genes, such as the receptor transporter protein 4 (Rtp4), IFN regulatory factor 7 (IRF7), signal transducer and activator of transcription 1 (STAT1) and IFN-γ induced GTPase (Igtp) were induced in MCs in an antigen-independent manner. In addition, radical S-adenosyl methionine domain containing 2 (Rsad2), an IFN-inducible gene encoding for the antiviral protein viperin (Chin and Cresswell, 2001), was in average 9 times up-regulated in MCs upon contact with $CD8^+$ T cells. Therefore, the suggestion that $CD8^+$ T cells induce an IFN signal transduction pathway in MCs, leading to a possible protective antiviral response is proposed. A summary of the genes that were induced in MCs upon antigen-independent contact with CD8$^+$ T cells is shown in **Table 3.2**.

Gene symbol	Gene title	MC+ OVA$^{257\text{-}264}$ (Mean)	MC+ CD8 (Mean)	max/min means	p-value	Entrez Gene
Rtp4	receptor transporter protein 4	12.96	560.55	43.25	0.04	67775
AI451557	expressed sequence AI451557	10.76	182.40	16.96	0.01	102084
Mpa2l	macrophage activation 2 like	11.18	132.18	11.83	0.02	
Rsad2	radical S-adenosyl methionine domain containing 2	14.21	132.36	9.32	0.05	58185
Irf7	interferon regulatory factor 7	32.56	277.03	8.51	0.02	54123
Stat1	signal transducer and activator of transcription 1	74.80	509.32	6.81	0.01	20846
LOC630509 /// LOC674192	similar to H-2 class I histocompatibility antigen, Q7 alpha chain precursor (QA-2 antigen)	34.17	213.82	6.26	0.02	
Zbp1	Z-DNA binding protein 1	10.77	59.06	5.48	0.04	58203
Igtp	interferon gamma induced GTPase	226.87	1041.43	4.59	0.04	16145

Table 3.2. CD8$^+$ T cells induce differential gene expression in MCs in an antigen-independent manner. *MCs were co-cultured with OT-I transgenic CD8$^+$ T cells* ***(MC + CD8)****. In parallel, OVA$^{257\text{-}264}$-pulsed MCs were left untreated* ***(MC + OVA$^{257\text{-}264}$)****. After 48 hours, MCs were isolated by positive selection with MACS and RNA was isolated. Microarray analysis (Affymetrix, Mouse MG 430 2.0) was performed. Statistical analysis was performed by limma analysis. Table summarizes the differential expression of genes with p-value of <0.05 and differential expression (max/min averages) of >4.5. Mean values of gene expression from three independent experiments are shown.*

3.8.2.2. CD8$^+$ T cells modulate the gene expression profile of MCs in an antigen-dependent manner

Since the antigen-independent effect of CD8$^+$ T cells on MCs was analyzed, it was further examined whether the presence of a specific MHC class I-related antigen (OVA$^{257\text{-}264}$) inducing CD8$^+$ T cell responses upon presentation by MCs, would have a different effect on the gene expression profile of MCs. For this reason, the gene expression profile of OVA$^{257\text{-}264}$-pulsed MCs after contact with CD8$^+$ T cells **(Fig. 3.34A, MC + CD8 + OVA$^{257\text{-}264}$)** was compared to the gene expression profile of unpulsed MCs after contact with CD8$^+$ T cells **(Fig. 3.34A, MC + CD8)**. As shown in **Fig. 3.34**, MCs isolated after antigen-dependent contact with CD8$^+$ T cells **(MC + CD8 + OVA$^{257\text{-}264}$)** displayed an up-regulation of gene expression of several genes, each of them represented by a separate dot in the low right triangle of the plot, as compared to MCs isolated after antigen-independent contact with CD8$^+$ T cells **(MC + CD8)**.

In order to exclude the possibility that the presence of the peptide induces gene up-regulation in MCs independently of CD8$^+$ T cells, the gene expression profile of OVA$^{257\text{-}264}$-pulsed MCs **(Fig. 3.34B, MC + OVA$^{257\text{-}264}$)** was compared to the gene expression profile of OVA$^{257\text{-}264}$-pulsed MCs after co-culture with CD8$^+$ T cells **(Fig. 3.34B, MC + CD8 + OVA$^{257\text{-}264}$)**. It was shown that the MC response was indeed dependent on the presence of CD8$^+$ T cells and not induced by the pulsing with the OVA$^{257\text{-}264}$ peptide. Therefore, it is suggested that CD8$^+$ T cells induce a MC response in an antigen-dependent manner.

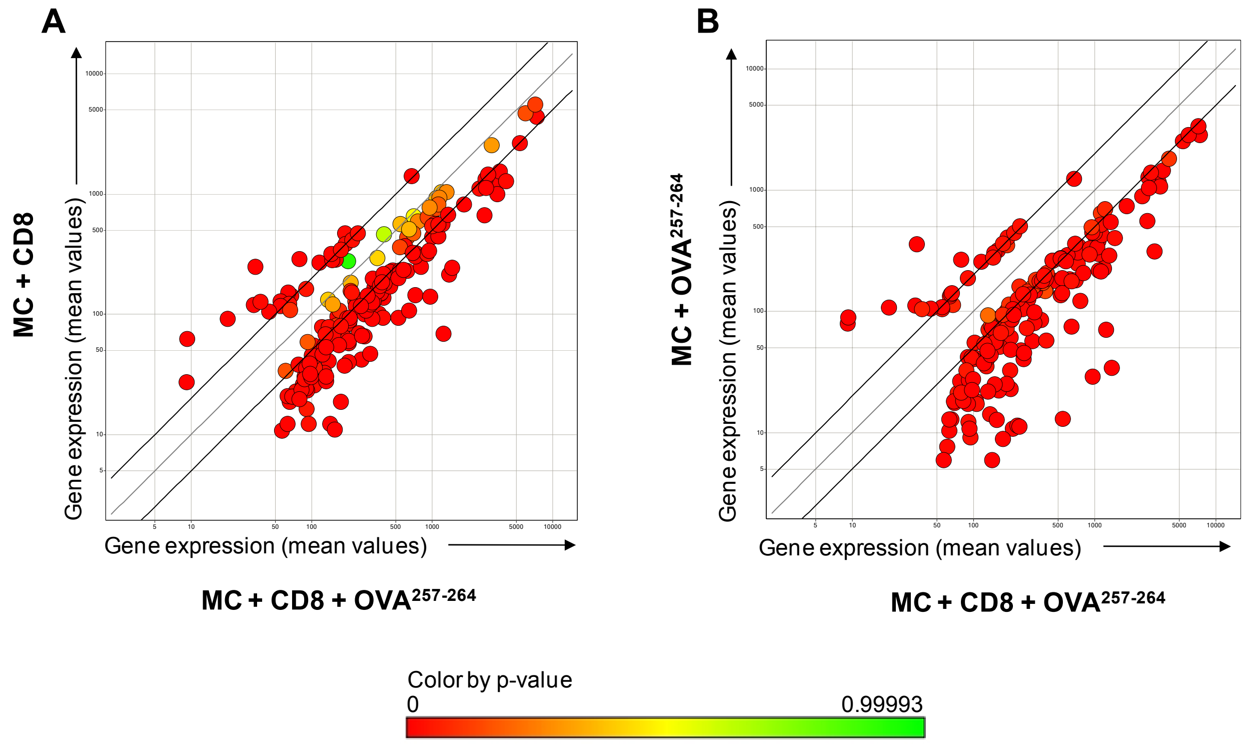

Fig. 3.34. CD8$^+$ T cells induce differential gene expression in MCs in an antigen-dependent manner. *OT-I transgenic CD8$^+$ T cells were cultured with OVA$^{257\text{-}264}$-pulsed MCs* ***(MC + CD8 + OVA$^{257\text{-}264}$)*** *or with untreated MCs* ***(MC + CD8)****. As control, OVA$^{257\text{-}264}$-pulsed MCs were left untreated* ***(MC + OVA$^{257\text{-}264}$)****. After 48 hours, MCs were isolated by positive selection with MACS and RNA was isolated. Microarray analysis (Affymetrix, Mouse MG 430 2.0) was performed. Statistical analysis was performed by limma analysis. Scatterplot shows the differential expression of genes with F.p.-value of <0.05. Mean values of gene expression from three independent experiments are shown.* ***A.*** *Comparison of the gene expression profile of MCs after antigen-dependent or antigen-independent contact with CD8$^+$ T cells is shown.* ***B.*** *Comparison of the gene expression profile of OVA$^{257\text{-}264}$ -pulsed MCs after contact or not with CD8$^+$ T cells is shown.*

A summary of the genes that were induced in MCs upon antigen-dependent contact with CD8$^+$ T cells is shown in **Table 3.3**. Noteworthy is the fact that several genes related with the induction of adaptive immune response were up-regulated. Thus, the co-stimulatory molecule tumour necrosis factor receptor superfamily member 9 (Tnfrsf9) and antigens related to major histocompatibility complex (MHC) class I and II (Qa-2 antigen, H2-Q1, H2-Ab1 and H2-Q8) displayed enhanced expression upon antigen-dependent contact with CD8$^+$ T cells. MHC class molecules act in collaboration with co-stimulatory molecules on the surface of APCs in order to induce antigen-specific immune responses. It is therefore proposed that the role of MCs in adaptive immunity is instructed by antigen-dependent contact with CD8$^+$ T cells.

Gene symbol	Gene title	MC+ OVA$^{257-264}$ (Mean)	MC+ CD8 (Mean)	MC+OVA$^{257-264}$ +CD8 (Mean)	max/min means	F.p.-value	Entrez Gene
LOC630509 /// LOC674192	similar to H-2 class I histocompatibility antigen, Q7 alpha chain precursor (QA-2 antigen)	34.17	213.82	1387.46	40.60	0.00	
Ifng	interferon gamma	5.93	12.25	143.20	24.15	0.00	15978
Mpa2l	macrophage activation 2 like	11.18	132.18	240.09	21.48	0.01	
Ak3l1	adenylate kinase 3 alpha-like 1	8.87	18.70	174.42	19.67	0.01	11639
Tnfrsf9	tumor necrosis factor receptor superfamily, member 9	70.37	68.91	1238.48	17.97	0.00	21942
H2-Q1 /// 0610037M15Rik	histocompatibility 2, Q region locus 1 /// RIKEN cDNA 0610037M15 gene	3.94	6.98	39.73	10.08	0.00	
Tgm2	transglutaminase 2, C polypeptide	22.95	39.95	202.67	8.83	0.00	21817
Zbp1	Z-DNA binding protein 1	10.77	59.06	92.90	8.63	0.02	58203
Iigp1	interferon inducible GTPase 1	7.67	33.85	60.67	7.91	0.04	60440
Ms4a6b	membrane-spanning 4-domains, subfamily A, member 6B	12.25	16.36	90.87	7.42	0.05	69774
AW112010	expressed sequence AW112010	22.26	80.97	162.29	7.29	0.01	107350
Mcf2l	mcf.2 transforming sequence-like	56.96	46.85	305.35	6.52	0.00	17207
Plod2	procollagen lysine, 2-oxoglutarate 5-dioxygenase 2	40.28	41.96	259.84	6.45	0.00	26432
Rgs11	regulator of G-protein signaling 11	17.16	35.83	105.81	6.17	0.04	50782
Socs3	suppressor of cytokine signaling 3	401.19	243.35	1470.84	6.04	0.00	12702
H2-Ab1	histocompatibility 2, class II antigen A, beta 1	45.36	66.46	261.66	5.77	0.00	14961
H2-Q8	histocompatibility 2, Q region locus 8	17.22	24.29	90.11	5.23	0.00	15019
Pdk1	pyruvate dehydrogenase kinase, isoenzyme 1	47.65	69.48	246.26	5.17	0.02	228026

Table 3.3. CD8$^+$ T cells induce differential gene expression in MCs in an antigen-dependent manner. *OT-I transgenic CD8$^+$ T cells were cultured with OVA$^{257-264}$-pulsed MCs **(MC + CD8 + OVA$^{257-264}$)** or with untreated MCs **(MC + CD8)**. As control, OVA$^{257-264}$-pulsed*

MCs were left untreated ***(MC + OVA$^{257\text{-}264}$)****. After 48 hours, MCs were isolated by positive selection with MACS and RNA was isolated. Microarray analysis (Affymetrix, Mouse MG 430 2.0) was performed. Statistical analysis was performed by limma analysis. Table summarizes the differential expression of genes with F.p.-value <0.05 and differential expression (max/min averages) of >5. Mean values of gene expression from three independent experiments are shown.*

These results demonstrated that $CD8^+$ T cells direct a strong MC response both in an antigen-dependent as well as in an antigen-independent manner. Therefore, the crosstalk between MCs and $CD8^+$ T cells is bidirectional, consisting of both the MC-induced presentation of MHC class I-related antigens to $CD8^+$ T cells, and of the $CD8^+$ T cell-induced up-regulation of gene expression in MCs. Taken into consideration that many of these genes are IFN-related, the previously formulated hypothesis that the MC-$CD8^+$ T cell contact plays a significant role in the defense against virus is highly strengthened.

3.8.2.3. Confirmation of the microarray results

The microarray analysis has revealed a significantly differential (>5 times) expression of 29 genes in MCs after contact with $CD8^+$ T cells. In order to verify some of these results, PCR analysis for the cDNA of the MACS-purified MCs was performed. The results of the PCR analysis confirmed the corresponding data of the microarray experiment. Thus, transglutaminase 2 (TGM-2) was up-regulated in MCs after contact with $CD8^+$ T cells, as shown both by the microarray experiment as well as by the PCR verification **(Fig. 3.35)**. In addition, p-lysozyme structural was one of the three genes that were down-regulated in the microarray experiment after antigen-independent contact with $CD8^+$ T cells and the same result was obtained with PCR analysis **(Fig. 3.35)**. IFN-γ was significantly up-regulated in MCs after contact with $CD8^+$ T cells, as shown by the microarray analysis. As shown in **Fig. 3.35**, the PCR analysis showed the same tendency of increased IFN-γ in MCs upon contact with $CD8^+$ T cells, however this result was not statistically significant.

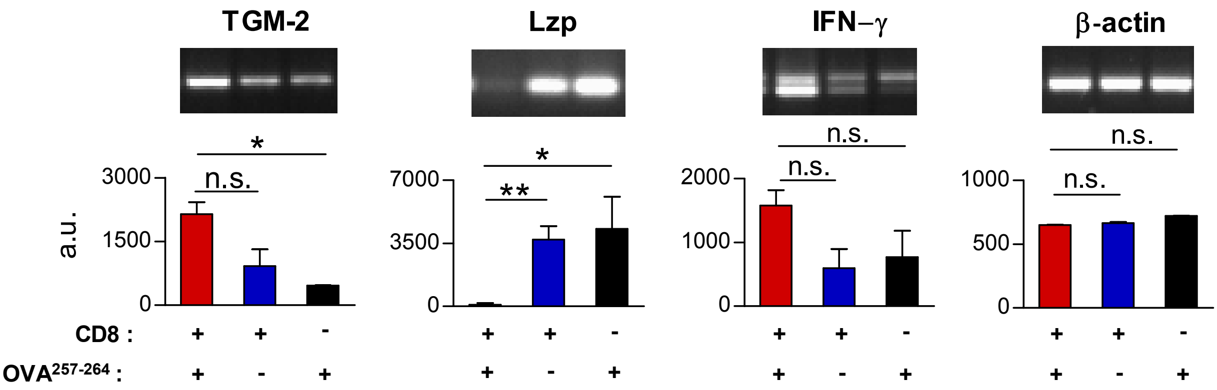

Fig. 3.35. ***PCR analysis confirmed part of the data obtained by microarray analysis.*** *OVA$^{257\text{-}264}$ -pulsed MCs* ***(red bars)*** *or unpulsed MCs* ***(blue bars)*** *were cultured with CD8^{+} T cells. As negative control, OVA$^{257\text{-}264}$ -pulsed MCs that did not contact CD8^{+} T cells were used* ***(black line)****. After 48 hours, MCs were isolated by positive selection with MACS, RNA was isolated and cDNA was synthesized. PCR analysis revealed the expression of genes of interest in MCs, in comparison to β-actin control expression. Graphs show mean values of arbitrary units (a.u.) as measured with Optimas Software, with standard deviation of three independent experiments.* ***Lzp****: p-lysozyme structural,* ***TGM-2****: transglutaminase-2. * $p<0.05$, ** $p<0.01$, n.s.:not significant*

A prominent up-regulation of the co-stimulatory molecule tnfrsf9 (4-1BB) was revealed from the microarray analysis. Next to its role as co-stimulatory molecule during APC-T cell interaction, 4-1BB/4-1BBL interaction also mediates DC as well as MC activities. Interestingly enough, DCs (Futagawa et al., 2002) as well as MCs (Nishimoto et al., 2005) express both 4-1BB and its ligand, 4-1BBL, and 4-1BB/4-1BBL interactions mediate activation and cytokine production by both DCs and MCs. These data inspired a further investigation of 4-1BBL expression on MCs after co-culture with CD8^{+} T cells. For this reason, PCR analysis was performed on the cDNA of the purified MCs after co-culture with CD8^{+} T cells. As shown in **Fig. 3.36**, 4-1BB displayed a significant up-regulation in MCs upon antigen-dependent contact with CD8^{+} T cells; a result which verified the data obtained by the microarray analysis. In addition, 4-1BBL expression was slightly but not significantly up-regulated in MCs after antigen-dependent contact with CD8^{+} T cells.

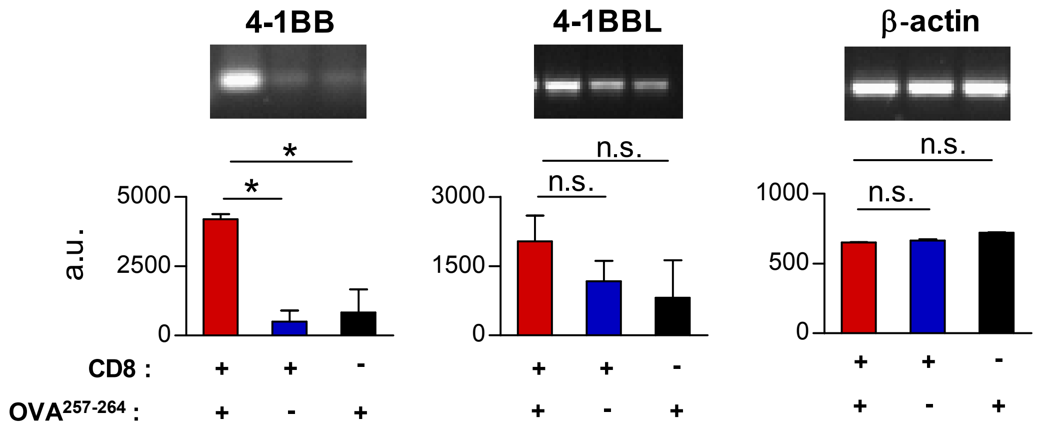

Fig. 3.36. Expression of 4-1BB but not 4-1BBL in MCs is up-regulated after antigen-dependent contact with CD8^{+} T cells. *OVA$^{257\text{-}264}$ -pulsed MCs* ***(red bars)*** *or unpulsed MCs* ***(blue bars)*** *were cultured with CD8^{+} T cells. As negative control, OVA$^{257\text{-}264}$ -pulsed MCs that did not contact CD8^{+} T cells were used* ***(black line)****. After 48 hours MCs were isolated by positive selection with MACS, RNA was isolated and cDNA was synthesized. Expression of 4-1BB and 4-1BBL was tested by PCR. Graphs show mean values of arbitrary units (a.u.) as measured by Optimas Software, with standard deviation of three independent experiments.* ***4-1BB****: tumour necrosis factor receptor superfamily member 9 (tnfrsf9, 4-1BB),* ***4-1BBL****: 4-1BB-ligand. * $p<0.05$, n.s.: not significant*

In conclusion, this part of the study demonstrated that the interaction between MCs and CD8^{+} T cells is bidirectional. Thus, CD8^{+} T cells instruct a potent MC response in an antigen-dependent as well as in an antigen-independent manner. The contact with CD8^{+} T cells stimulates MCs to activate the IFN-related pathway and presumably contribute to an antiviral response. Moreover, upon antigen-dependent activation, CD8^{+} T cells induce MHC and co-stimulatory molecule expression on MCs, therefore potentially facilitate the MC contribution in adaptive immune responses. Thus, MCs may act as key regulatory cells at the crossroads between innate and adaptive immunity.

4. Discussion

An optimal $CD8^+$ T cell response is essential for the defense against pathogens, such as viral, intracellular bacterial or protozoal infections (Wong and Pamer, 2003). Moreover, several pathological situations, including allergic responses (Haczku et al., 1995a; Haczku et al., 1995b; Hamelmann et al., 1996) and autoimmune reactions (Huseby et al., 2001; Sun et al., 2001) implicate antigen-specific $CD8^+$ T cell activities. An antigen-specific $CD8^+$ T cell response is clearly initiated by "professional" antigen presenting cells (APCs), namely dendritic cells (DCs), B cells and macrophages. However, recent results suggest that mast cells (MCs) communicate with $CD8^+$ T cells, by inducing $CD8^+$ T cell chemotaxis upon activation *in vivo* (McLachlan et al., 2003; Ott et al., 2003; Orinska et al., 2005). Moreover, it has been demonstrated that MCs phagocytose living bacteria, process bacterial antigens and induce antigen-specific proliferation of $CD8^+$ T cell lines upon presentation of bacterial-expressed proteins *in vitro* (Malaviya et al., 1996a; Malaviya et al., 1996b). However, the consequences of this crosstalk between MCs and $CD8^+$ T cells, as well as the requirements for this interaction, have not been further investigated. Therefore, the main purpose of this study was to provide a better understanding of the antigen-dependent, as well as the antigen-independent MCs-$CD8^+$ T cell interactions.

4.1. The use of BMMCs for studying MC functions *in vitro*

In order to investigate MC activities and their potential interaction with $CD8^+$ T cells, the model of *in vitro*-differentiated murine bone marrow-derived MCs (BMMCs) was used. An essential requirement for the further experiments of this study was the high purity of the MC cultures and the absence of potential APCs in the MC cultures. Adequate numbers of highly pure MC populations were technically difficult to obtain by isolating primary MCs from the mouse skin, lung or peritoneal cavity. Therefore, highly pure, homogeneous BMMC cultures were chosen as the suitable model to study MC functions in this study.

The differentiation of BMMCs *in vitro* was performed in the presence of IL-3 and SCF for 5 weeks. Although IL-3 is dispensable for the generation of murine MCs *in vivo* under physiological conditions (Lantz et al., 1998) as well as for the *in vitro*-generation of human MCs from bone marrow cells, peripheral blood mononuclear cells or cord blood cells

(Valent et al., 1992; Mitsui et al., 1992), it is the absolutely essential factor to support murine MC survival *in vitro* (Mekori et al., 1993). Moreover, IL-3 promotes, at least partially, the maturation of mouse MCs, in contrast to human MCs (Valent et al., 1989; Valent et al., 1990). The addition of SCF in BMMCs *in vitro* results in a synergistic effect in the maturation of MCs, since it enhances the expression of MC proteases and proteoglycans (Tsai et al., 1991a; Tsai et al., 1991b; Gurish et al., 1992) and promotes the formation of cytoplasmic granules (Dvorak et al., 1994). For this reason, mouse BMMCs in this study were differentiated in the presence of both IL-3 and SCF.

MC maturation is routinely tested by the specific staining of their metachromatic granula. The BMMC cultures used in this study displayed the typical appearance of the MC metachromatic granula upon toluidine blue staining; however, this staining was weaker compared to the staining of *ex vivo* isolated peritoneal MCs (data not shown). Thus, it is suggested that, even when the maturation of BMMCs is significantly enhanced by the addition of SCF in the culture, it remains weaker compared to MCs *in vivo*. The reduced maturation of *in vitro*-generated BMMCs is also proven by the fact that BMMCs, when transferred intracutaneously, intraperitoneally or intravenously into mice *in vivo*, require 5-10 weeks for optimal maturation to be achieved (Nakano et al., 1985). However, although the maturation of BMMCs compared to MCs *in vivo* appears to be reduced, BMMCs display specific phenotypical and functional MC characteristics, such as intracellular storage of proteases, histamine, proteoglycans and heparin (Tsai et al., 1991a; Tsai et al., 1991b; Gurish et al., 1992), surface expression of c-kit (CD117) and FcεRI (Dvorak et al., 1994), and degranulation upon antigen/IgE-specific stimulation (Levi-Schaffer et al., 1993). For this reason, BMMCs generated according to the method used in this study, comprise a well-accepted and widely-used model to study MC functions *in vitro* (Kawakami et al., 2006).

4.2. Antigen-independent control of $CD8^+$ T cell activities by MCs *in vitro*

A potential interaction between MCs and $CD8^+$ T cells in the absence of a specific antigen might be relevant for the homeostasis of the naïve $CD8^+$ T cell pool. Therefore, the crosstalk between MCs and $CD8^+$ T cell was initially studied in the absence of a specific antigen. MCs were found to promote the survival of $CD8^+$ T cells in an antigen-independent and cell-cell contact-dependent manner. Similar observations regarding DCs, demonstrate that in the absence of a specific antigen, DCs form functional synapses with T cells, induce tyrosine phosphorylation and a small Ca^{2+} response and promote the survival of naïve $CD4^+$ and $CD8^+$ T cells (Revy et al., 2001; Kondo et al., 2001). Consistent with the present findings, the increased survival of T cells was promoted by DCs only in a cell-cell contact-dependent manner (Kondo et al., 2001). It is noteworthy that the tyrosine kinase Lck was suggested to be a central mediator of antigen-independent DC-T cell interactions, since inhibition of Lck activity reduces the formation of stable conjugates between DCs and T cells (Revy et al., 2001; Kondo et al., 2001). Although the signal transduction pathways that promote $CD8^+$ T cell survival upon antigen-independent contact with MCs were not the focus of the present study, it would be interesting to examine whether this phenomenon is similarly dependent on Lck tyrosine kinase activation.

However, MCs did not induce any antigen-independent activation of $CD8^+$ T cells, as measured by surface expression of activation markers. Evidence that MCs interact with T cells in an antigen-independent manner was provided by Nakae *et al.* (2005). However, in their settings MCs facilitated the proliferation and cytokine production by T cells, only when T cells were simultaneously stimulated by sub-optimal doses of anti-CD3. In light of these findings, the present study suggests that MCs do not induce antigen-independent $CD8^+$ T cell response *de novo*.

In conclusion, MCs promote the survival of naïve $CD8^+$ T cells, suggesting that in a physiological situation MCs contribute to the homeostasis of naïve $CD8^+$ T cells without inducing their activation. Thereby, MCs are possible key regulators of naïve $CD8^+$ T cell homeostasis at peripheral tissues and may potentially modulate the dimension of $CD8^+$ T cell-dependent processes.

4.3. Antigen-dependent control of $CD8^+$ T cell activities by MCs *in vitro*

Several studies demonstrate the ability of MCs to induce antigen-specific $CD4^+$ T cell responses in an MHC class II-dependent manner (Frandji, et al., 1993; Fox et al., 1994; Poncet et al., 1999). Despite the central role of antigen-specific $CD8^+$ T cells in host defense mechanisms, the potential of MCs to also induce antigen-specific $CD8^+$ T cell responses remains inadequately studied. Therefore, this study attempted to investigate the antigen-specific interaction between MCs and $CD8^+$ T cells.

4.3.1. MCs internalize the OVA protein

An essential requirement for a cell type to be considered as APC is the ability to uptake, process and present antigenic proteins. Thus, it was initially examined whether MCs are able to uptake the OVA protein. Indeed, MCs internalized the OVA protein coupled with FITC, as shown by fluorescence microscopy and FACS, in a dose-dependent manner. The finding that MCs internalize an antigenic protein is in line with previous investigations reporting that MCs phagocytose bacteria after binding to FimH, a mannose-binding subunit expressed on *Escherichia coli* and other enterobacteria (Malaviya et al., 1994). In addition, MCs were recently reported to express the scavenger receptors SR-A and MARCO and internalize silica particles of size 1.5-2 μm (Brown et al., 2007). The fact that MCs internalize antigenic proteins and particles or phagocytose bacteria, supports the hypothesis that MCs may act efficiently as APCs for presentation of exogenous or bacterial proteins to T cells.

Following protein uptake, intracellular processing should occur in order to present antigenic peptides to T cells. The ability of MCs to process the OVA protein for presentation of antigenic peptides to T cells was not the direct focus of the present study. However, in a pilot experiment where OVA-pulsed MCs were used to stimulate $CD8^+$ T cells, no significant $CD8^+$ T cell activation was detected (data not shown). This was attributed to the fact that either a) the $CD8^+$ T cells used in this system (OT-I transgenic) were skewed for the recognition of only a specific peptide ($OVA^{257\text{-}264}$), therefore the magnitude of the $CD8^+$ T cell response against the whole protein was restricted, or b) the reduced maturation of BMMCs *in vitro* did not allow efficient processing of the OVA protein. However, previous investigations have suggested the ability of MCs to process antigenic proteins. Thus, the uptake of live bacteria resulted in the processing of bacterial-produced proteins and the presentation of antigenic peptides to peptide-specific T cell clones (Malaviya et al., 1996b). In addition, MCs apparently possess the required machinery to degrade foreign proteins, since they have non-secretory lysosomes, containing acid-hydrolases (Schwartz and Austen, 1980), where the processing of the ingested proteins might occur. However, whether the processing of proteins for presentation is a feature of mature MCs *in vivo* should be further elucidated.

4.3.2. MCs induce antigen-specific $CD8^+$ T cell responses

The ability of MCs to induce antigen-specific $CD8^+$ T cell responses *in vitro* was examined by the co-culture of antigen ($OVA^{257\text{-}264}$) -pulsed MCs with TCR-transgenic (OT-I) $CD8^+$ T cells. $OVA^{257\text{-}264}$-pulsed MCs induced activation and proliferation of $CD8^+$ T cells in a dose-dependent manner. The use of the $OVA^{257\text{-}264}$ peptide for studying antigen presenting functions of different cell types *in vitro* has been widely used by various investigators (Met et al., 2003; Rückert et al., 2003; Winau et al., 2007). A widely used APC:T cell ratio for *in vitro* antigen presentation experiments is 1:2 - 1:10 (Chefalo and Harding, 2001; Brandt et al., 2003; Rückert et al., 2003; Setterblad et al., 2004; Castiglioni et al., 2005; Winau et al., 2007). However, it was acknowledged that MCs are generally found at sites where low numbers of T cells are encountered (skin, lung, peritoneal cavity), although during a primary immune response MCs may migrate to the lymph nodes, where the encounter of high numbers of T cells occurs (Wang et al., 1998; Dabak et al., 2004). Thus, in order to reproduce a more physiological situation regarding the MC:$CD8^+$ T cell ratio, successively reduced MC numbers as stimulators of $CD8^+$ T cells were used. MCs were proven to be efficient activators of $CD8^+$ T cells, even at the lowest ratio of MC:CD8 = 1:100, which was sufficient to induce activation and proliferation of approximately 50% of the $CD8^+$ T cell population. This ratio is comparable to the one required for LPS-matured DCs to induce proliferation of heterologous T cells *in vitro* (Brandes et al., 2005) as well as for *E.coli*-infected DCs to induce proliferation of up to 15% of the $CD8^+$ T cell population (Billard et al., 2007). Thus, MCs are suggested to act as highly potent inducers of antigen-specific $CD8^+$ T cell responses *in vitro*.

The antigen-specific $CD8^+$ T cell response after contact with antigen-pulsed MCs was dependent on MHC class I-TCR-signalling, since neither MHC class I-deficient MCs ($\beta 2m^{-/-}$ MCs) induced significant $CD8^+$ T cell activation, nor did non-TCR-transgenic $CD8^+$ T cells respond to the TCR-specific peptide (OT-I) presented. Thus, it is excluded that the here detected MC-mediated antigen-specific $CD8^+$ T cell response was induced by contamination of the peptide (e.g. from LPS) or by any other artificial factor except for the active presentation of the peptide from functional MHC class I on the MCs to functional TCR on the $CD8^+$ T cells. $\beta 2m^{-/-}$ mice represent a valuable model for studying MHC class I-dependent antigen presentation (Koller et al., 1989; Zijlstra et al., 1990). These mice do not express the β2m chain, therefore they lack functional MHC class I expression, and subsequently the ability to present MHC class I-restricted antigens. The minimal $CD8^+$ T cell response observed after co-culture of OT-I $CD8^+$ T cells with $OVA^{257\text{-}264}$-pulsed $\beta 2m^{-/-}$ MCs is attributed to the fact that minimal amounts of the peptide could remain in the culture even after extensive washings of the MCs and may be presented from $CD8^+$ T cells to themselves. In support of this hypothesis, $CD8^+$ T cells have been previously reported to present soluble peptides to other $CD8^+$ T cells (Walden and Eisen, 1990; Su et al., 1993; Schott et al., 2002), thus suggesting that traces of free $OVA^{257\text{-}264}$ peptide could be responsible for a minimal *in vitro* activation of OT-I specific $CD8^+$ T cells. However, since this MHC class I-independent activation is considered minimal in comparison to the MHC class I-dependent (10% in comparison to 95%), MCs are suggested to act as efficient APCs of MHC class I-restricted antigens.

In addition to the antigen-specific activation and proliferation of the $CD8^+$ T cells, MCs induced also effector $CD8^+$ T cell functions, such as cytokine production. IFN-γ, a cytokine known to be produced at high levels upon antigen-specific activation of $CD8^+$ T cells (Harty et al., 2000), as well as IL-2, a major regulator of T cell-survival (Marrack and Kappler, 2004), were detected at high levels in the supernatants after antigen-specific, MC-mediated $CD8^+$ T cell stimulation. In order to prove that IFN-γ and IL-2 were secreted by the $CD8^+$ T cells, intracellular cytokine staining was performed. Indeed, $CD8^+$ T cells were the source of IL-2 and IFN-γ, without excluding the possibility that also MCs contribute additionally to the accumulation of these cytokines in the co-culture supernatant. The high levels of IFN-γ and IL-2 produced during antigen-dependent $CD8^+$ T cell activation suggest that $CD8^+$ T cells may actively regulate many immunological processes upon antigen-dependent contact with MCs. First, IFN-γ is identified as a main cytokine with cytotoxic activities, since it enhances the anti-microbial activity and killing of intracellular pathogens by macrophages and neutrophils (Young and Hardy, 1995) and also mediates the cytotoxic activity of CTL by promoting the Fas-mediated target cell-lysis (Andersen et al., 2006). On the other hand, IL-2 is a growth factor for all subpopulations of T cells and stimulates proliferation of B and T lymphocytes at high efficiency (Murray, 1996). Both IFN-γ and IL-2 are characterized as Th1-associated cytokines, therefore promote inflammatory reactions (Abbas et al., 1996). Thus, it is suggested that upon MC-mediated antigen-presentation $CD8^+$ T cells contribute to cytotoxic reactions and to the elimination of intracellular pathogens on one hand, and on the other hand contribute to the magnitude of an inflammatory response and support the survival of T and B lymphocytes.

Moreover, the inflammatory cytokines TNF-α and GM-CSF were detected in the supernatants upon antigen-dependent MC-$CD8^+$ T cell co-culture. The release of TNF-α and GM-CSF suggests that during the antigen-specific MC-$CD8^+$ T cell contact, inflammatory cytokines are secreted, which promote neutrophil recruitment and inflammation (Thomas, 2001; Broide et al., 2001; Shi et al., 2006) and modulate the adaptive immune response by regulating the maturation of DCs (Kimber and Cumberbatch, 1992; Jonuleit et al. 1996; Thomas and Lipsky, 1996). However, it would be interesting to determine by intracellular staining the source of TNF-α and GM-CSF, since both cytokines can be produced at high amounts by MCs (Galli et al., 2005b). Moreover, it is interesting to examine whether $CD8^+$ T cells induced degranulation of MCs, since TNF-α can be released by MCs by secretion and also upon degranulation (Okayama, 2005).

It is noteworthy that none of the Th-2-associated cytokines examined here (IL-4, IL-5, IL-10) (Abbas et al., 1996) were detected after the antigen-specific MC-mediated $CD8^+$ T cell activation. Collectively, the cytokine detection after MC-mediated antigen-specific $CD8^+$ T cell activation suggests that MCs mediate an antigen-specific activation of $CD8^+$ T cells, which results in the production of cytokines favouring the Th1 responses, thus, promoting inflammatory reactions and the development of cytotoxic T cells. These findings support the proposal that MCs have a diverse, complex role in regulating the Th1/Th2 cytokine milieu (Gregory and Brown, 2006; Stelekati et al., 2007). Therefore, next to their traditional, well-characterized role in inducing Th2 responses through the release of IL-4 (Huels et al., 1995; Mekori and Metcalfe, 1999), MCs can also favour the Th1 response via interacting with $CD8^+$ T cells and inducing considerable production of IFN-γ and IL-2. Whether a degranulation of MCs is required for this function remains to be investigated.

4.3.3. MCs increase the cytotoxic potential of $CD8^+$ T cells

Given the central role of IFN-γ in the facilitation of cytolytic activity performed by $CD8^+$ T cells (Andersen et al., 2006), the hypothesis that MCs modulated the cytolytic activities of the $CD8^+$ T cells was examined. The intracellular content of granzyme B, a key component of the cytotoxic machinery of cytolytic $CD8^+$ T cells, which induces the caspase-dependent pathway of apoptosis in target cells (Talanian et al., 1997), was increased in $CD8^+$ T cells after antigen-specific activation by MCs. Moreover, $CD8^+$ T cell degranulation was induced by MCs upon MHC class I-dependent antigen presentation. These findings strongly suggest that MCs enhance the cytotoxic potential of $CD8^+$ T cells upon antigen-specific activation.

There has been little information obtained regarding the possible implication of MCs in directly inducing cytotoxicity. Human MCs (Strik et al., 2007) as well as mouse MCs (Kataoka et al., 2004; Pardo et al., 2007) express granzyme B, the release of which upon MC degranulation induces cell death in target cells by a perforin-independent way (Pardo et al., 2007). The present study suggests that MCs participate in cytotoxic reactions by an additional mechanism; that is, by directly inducing cytotoxic $CD8^+$ T cells upon antigen-specific activation. This suggestion is supported by the most recent findings of Heib et al. (2007), who reported that MC-deficient mice display a deficiency in initiating peptide-specific CTL responses upon TLR-7 activation *in vivo*. It would be interesting to examine whether the MCs are subsequently targets of the $CD8^+$ T cell cytotoxicity, or they have mechanisms to prevent apoptosis upon contact with CTLs. It is possible that, in response to contact with CTLs, MC degranulation is induced in order to increase the release of cytotoxic mediators (such as granzyme B) and enhance the efficiency of cytotoxicity. However, the effect of such a contact with CTLs on MCs remains to be investigated.

4.3.4. The antigen-dependent activation of $CD8^+$ T cells by MCs requires direct cell-cell contact and the release of soluble mediators

The mechanism of the MC-induced $CD8^+$ T cell activation was further investigated in this study. Thus, it was first demonstrated that inhibition of cell contact between MCs and $CD8^+$ T cells blocks the antigen-specific MC-$CD8^+$ T cell interaction. It is therefore suggested that the presentation of MHC class I-antigen complexes on the surface of the MCs is the initiative and essential step for the antigen-specific interaction with $CD8^+$ T cells. This is explained by the fact that physical APC-T cell contact is essential for the formation of the immunological synapse, for the MHC-TCR interaction and for the subsequent stimulation of the TCR signaling pathway, which results in the activation of the T cells. Thus, consistent with the present study, a similar requirement of direct cell-cell contact has been reported for DCs, in order to induce antigen-specific activation of $CD8^+$ T cells (Gunzer et al., 2000; Mempel et al., 2004).

MC-released cytokines were also found to contribute to the antigen-dependent interaction with $CD8^+$ T cells, since blocking of cytokine synthesis or secretion by MCs significantly reduced their ability to induce antigen-specific $CD8^+$ T cell activation. Thus, it is suggested that once direct presentation of the antigen via MHC class I-TCR interaction occurs, additional soluble mediators produced by MCs enhance the efficiency of antigen presentation. Consistent with this observation, the secretion of cytokines, such as IL-1, IL-6, TNF-α, IL-12 and IL-15, by APCs was found to be important for priming naïve T cells and promoting their differentiation (Roitt et al. 2002). MC-released cytokines among their diverse functions also contribute in the regulation of $CD8^+$ T cell activities. For example, important mediators of naïve T cell homeostasis, such as IL-4 and IL-6 (Vella et al., 1997; Teague et al., 2000) are produced by MCs (Brown et al., 1987; Burd et al., 1989; Plaut et al., 1989). Interestingly, IL-15, which is characterized as the main survival factor for memory $CD8^+$ T cells (Zhang et al., 1998), has been detected in MC intracellular granula (Orinska et al., 2007), although stimuli inducing its release have not been characterized so far. Moreover, MC-derived TNF-α has been reported to mediate T cell activation in an antigen-independent way (Nakae et al., 2005). However, the identification of the MC-released soluble mediators that mediate the antigen-dependent $CD8^+$ T cell activation, as observed in the present study, remains to be investigated.

4.3.5. TLR-ligand exposure of MCs enhances their potential to activate $CD8^+$ T cells

In order to understand how an antigen-dependent interaction between MCs and $CD8^+$ T cells can be further modulated, the effect of TLR-stimulation of MCs in this interaction was investigated. It was shown that LPS- and, less potently, pIC-stimulated MCs exhibited an enhanced capacity to activate $CD8^+$ T cells, due, at least partially, to their increased surface expression of MHC class I. Thus, it was demonstrated that the MC ability to induce antigen-specific $CD8^+$ T cell responses is modulated by TLR-stimulation.

The effect of TLR-stimulation of MCs has been extensively studied so far in the context of enhanced cytokine production (Introduction, Table 1.1). Thus, TLR-4 mediated activation of MCs induces TNF-α and other pro-inflammatory cytokine production, without stimulating MC degranulation. Therefore, it is suggested that the enhanced activation of $CD8^+$ T cells by MCs upon TLR-stimulation of MCs, described in this study, is mediated partially by the enhanced MHC class I expression on MCs and partially by cytokines, such as TNF-α, released by MCs upon TLR-activation.

TLR-4-mediated stimulation of MCs by LPS has been identified as a key causal agent for the LPS-induced exacerbation of allergic inflammation *in vivo,* since it results in increased cellular infiltration in the lung and enhanced Th2 cytokine production (Nigo et al., 2006; Murakami et al., 2007). The finding of the present study that LPS enhances also the ability of MCs to activate $CD8^+$ T cells, proposes an additional effect of the LPS-exposure on MCs; upon TLR4-engagement, MCs additionally initiate an adaptive, $CD8^+$ T cell-dependent immune response. Taking into consideration that TLR-3-mediated activation of MCs induces $CD8^+$ T cell recruitment *in vivo* (Orinska et al., 2005), it is here proposed that MCs serve as key mediators between innate and adaptive immunity in peripheral tissues; upon encounter of pathogens and TLR-induced stimulation, they lead to an enhanced adaptive immune response, mediated by increased $CD8^+$ T cell recruitment and subsequent activation.

4.4. Antigen-dependent control of $CD8^+$ T cells by MCs *in vivo*

As soon as it was demonstrated that MCs efficiently induce primary $CD8^+$ T cell responses *in vitro*, the necessity to investigate whether this phenomenon occurs also *in vivo* arose. For this reason, first the ability of MCs to induce antigen-dependent responses of primary $CD8^+$ T cells *in vivo* was investigated. Following, the role of MCs in regulating the $CD8^+$ T cell response during an allergic airway sensitization model was examined.

4.4.1. MCs induce antigen-dependent proliferation of $CD8^+$ T cells *in vivo*

In order to prove that this interesting MC-$CD8^+$ T cell interaction occurs *in vivo*, adoptive transfer experiments were performed in wild-type and in $\beta 2m^{-/-}$ mice. $\beta 2m^{-/-}$ mice lack the expression of the β2m chain of MHC class I, therefore, exhibit a deficiency in generating $CD8^+$ T cells and they are unable to efficiently present antigens in an MHC class I-dependent manner. For this reason, transfer of antigen-loaded, wild-type MCs and OT-I transgenic $CD8^+$ T cells into $\beta 2m^{-/-}$ mice provided a model that lacks endogenous MHC-I-restricted antigen presentation. Interestingly, MCs induced a similar antigen-dependent $CD8^+$ T cell proliferation in wild-type as well as in $\beta 2m^{-/-}$ mice. This finding completely excludes the possibility that other resident APCs contributed to the antigen-dependent $CD8^+$ T cell responses demonstrated in this study, thus underestimating the role of MCs in this phenomenon. The proof that MCs are able to induce antigen-specific proliferation of primary $CD8^+$ T cells upon MHC class I-dependent antigen presentation *in vivo* is of central significance for the understanding of the role of MCs in the modulation of adaptive immune responses. Thus, this is the first report to demonstrate that MCs are able to induce proliferation of primary $CD8^+$ T cells in an antigen-dependent manner *in vivo*, in mice. The investigation whether primary, freshly isolated MCs exhibit the same function, as well as whether a similar role for MCs in the human system exists, is considered to be of great interest.

An interesting question arising upon the demonstration that MCs induce antigen-specific $CD8^+$ T cell responses *in vivo* is the physiological conditions under which the organism benefits by this function of MCs. MCs are mostly resident cells at peripheral sites. Therefore, it is possible that upon pathogen or allergen encounter they internalize antigens and preserve them in the periphery, thus comprising a reservoir of the antigen in the periphery. In support of this hypothesis, it was recently reported that latently HIV-infected MCs comprise a long-living, inducible reservoir of the virus at peripheral tissues in humans. The release of infectious virus from those latently infected MCs could be induced upon IgE crosslinking *in vitro* (Sundstrom et al., 2007). Thus, it is here hypothesized that MCs may have a central contribution in the establishment of memory $CD8^+$ T cell compartment and the regulation of immune responses at the periphery. Although subpopulations of MCs have also been reported to migrate to the lymph nodes upon infection or inflammatory stimuli (Wang et al., 1998; Dabak et al., 2004), the differences between MCs and DCs in the kinetics and degree of migration may highlight the individuality of these two different cell types as APCs. Taken together, all these attribute to the MCs the unique role of controlling the $CD8^+$ T cell responses in the periphery, in contrast to DCs, which normally execute their antigen presenting function in the lymph nodes.

Interesting evidence accumulate demonstrating that in addition to the "professional" APCs (DCs, B cells and macrophages), several other cell types may act as APCs in different organs and at different sites of the organism. The fact that endothelial cells (Marelli-Berg and Jarmin, 2004), fibroblasts (Kundig et al., 1995), MCs (Frandji et al., 1993), hepatic stellate cells (Winau et al., 2007) may act as APCs indicates that antigen presentation might be regulated in different ways at the various sites of the body. A possible meaning of the existence of several APCs at different sites was interestingly proposed in a recent report by Unanue, E. (2007); the antigen presentation in every microenvironment is dependent on anatomical and physiological considerations such as patterns of blood flow, barriers to vascular permeability, organization of connective tissue and expression of adhesion molecules and chemokines. Therefore, the existence of several APCs, next to the "professional" ones, is not redundancy; the "non-professional" APCs, such as MCs, are needed to mount optimal responses to antigens, meeting the requirements of the exact surrounding conditions and to perform the fine-tuning of the antigen presentation in the particular environment.

4.4.2. MCs do not significantly influence $CD8^+$ T cell responses in a murine model of allergic airway sensitization *in vivo*

As soon as the ability of MCs to modulate antigen-specific $CD8^+$ T cell responses *in vivo* was determined, the question of how this phenomenon is relevant for the function of the organism arose. Therefore, a pathological situation in which the MC-mediated antigen-specific $CD8^+$ T cell responses are potentially important was investigated. Since MCs are key mediators of the allergic immune reactions, it was hypothesized that their capacity to present antigens to $CD8^+$ T cells may regulate the outcome of allergic responses. Increasing evidence in different models suggest that $CD8^+$ T cells are important for the induction of allergic sensitization and atopic diseases (Haczku et al., 1995a, Haczku et al., 1995b). Interestingly, in a murine model of allergic sensitization to OVA, the development of airway hyper-reactivity, eosinophil influx, and IL-5 production was absolutely dependent on the presence of $CD8^+$ T cells (Hamelmann et al., 1996). For this reason, the role of MCs in the induction of antigen-specific $CD8^+$ T cell responses was studied using a well-established murine model of allergic sensitization against OVA, in which antigen-specific $CD8^+$ T cell proliferation was observed (Rückert et al., 2005).

An interesting observation arising from the analysis of naïve (control unimmunized) mice was that MC-deficient mice exhibit a reduced percentage and reduced total number of $CD8^+$ T cells in the lung compared to their congeneic wild-type mice. An important role for MCs in regulating naïve $CD8^+$ T cell homeostasis in the lung could be a possible explanation for this $CD8^+$ T cell deficiency in MC-deficient mice. Such a role for MCs in promoting $CD8^+$ T cell survival has been investigated earlier in this study and discussed in the paragraph **4.2**. However, it should be taken into consideration that Kit-W/Wv mice exhibit several developmental abnormalities such as macrocytic anemia, lack of melanocytes, intestinal TCRγδ intraepithelial lymphocytes and interstitial cells of Cajal (Galli and Kitamura, 1985; Grimbaldeston et al., 2005). Therefore, it is not excluded that the $CD8^+$ T cell deficiency observed here is an additional defect caused by a dysregulated T cell development in the Kit-W/Wv mice. In order to evaluate if MCs were the cause of this deficiency, the $CD8^+$ T cell development in the lung of Kit-W/Wv mice reconstituted with MCs should be investigated.

In contrast to the initial expectations, the $CD8^+$ T cell activation or memory differentiation during this model was not significantly influenced by the presence of MCs, since MC-deficient mice developed comparable amounts of activated and memory $CD8^+$ T cells after OVA-sensitization and challenge. This result suggests that, although MCs are capable of inducing antigen-specific $CD8^+$ T cell responses *in vivo*, this MC-$CD8^+$ T cell interaction is dispensable for the $CD8^+$ T cell activation observed in the lung and its draining lymph nodes during an allergic airway inflammation. The reason why no significant differences were observed regarding the $CD8^+$ T cell response could be attributed to the fact that only a small proportion of the $CD8^+$ T cells would be OVA-specific. Thus, the use of general activation markers such as CD69 and CD25 and memory markers such as CD44 and CD62L, although provides an accurate evaluation of the overall $CD8^+$ T cell response, however, does not focus on the antigen-specific $CD8^+$ T cells. Therefore, if there were significant differences only in the antigen-specific (OVA-specific) $CD8^+$ T cells, those could be detected only with the use of OVA-specific tetramer staining.

On the other hand, it is possible that MCs do not play a role in the induction of $CD8^+$ T cell response in this model, because other APCs in the lung such as DCs or macrophages induce a sufficient $CD8^+$ T cell response, therefore overtake the role of MCs in this model. Moreover, the total time period in which this sensitization- and challenge-phase occurred lasted for 29 days. It may be an explanation that during this time-period, the role of MCs in inducing $CD8^+$ T cell responses is overtaken by professional APCs, while this would not be the case in a longer-lasting time-frame, since MCs are by far longer-living cells than other APCs. Indeed, some existing evidence suggests that MCs are central participants in late phase immune responses. Thus, MCs promote the development of chronic asthma, as demonstrated in a murine model of chronic asthma lasting for 10 weeks (Yu et al., 2006). Additionally, a recent report demonstrated that MCs are recruited and activated by Treg-produced IL-9 in tolerant allografts and that this interaction between Treg and MCs is essential for the long-term allograft tolerance (Lu et al., 2006). It is therefore suggested that the role of MCs might be more important in the late phase of immune responses rather than in short-term immune reactions. Therefore, a potential physiological role of MC-mediated antigen-specific $CD8^+$ T cell responses in long-term immune reaction should be further investigated.

4.5. The effect of MCs on pre-activated $CD8^+$ T cells

MCs are long-lived, resident cells in the periphery (Marshall, 2004). Therefore, as speculated above, MCs may participate in late-phase immune reactions and in the outcome of a secondary immune response. During a secondary response, MCs encounter $CD8^+$ T cells that have been already primed by the migrating DCs in the lymph nodes. For this reason, the effect of MCs on pre-activated $CD8^+$ T cells was further investigated in this study. First, it was proven that MCs reduce the antigen-specific DC-induced activation of $CD8^+$ T cells. Furthermore, MCs reduced the proliferation of pre-activated $CD8^+$ T cells. These data suggest that MCs exhibit a dual modulatory role in priming $CD8^+$ T cells; on encounter with naïve $CD8^+$ T cells MCs induce antigen-specific activation and proliferation, while on encounter with pre-activated $CD8^+$ T cells, MCs down-regulate the $CD8^+$ T cell responses.

An interaction between MCs-DCs-CD8$^+$ T cells could possibly happen in the lymph nodes, where all the three cell types may encounter each other. MCs have been reported to migrate to the lymph nodes upon infection or inflammatory processes in mice and rats (Wang et al., 1998; Dabak et al., 2004). Moreover, MCs have been detected at increasing concentrations in the lymph nodes in patients with tuberculous lymphadenitis or lung cancer (Tomita et al., 2003; Taweevisit and Poumsuk, 2007). Interestingly, during the progression of infection, MCs in the lymph nodes migrate mostly to the paracortical area and medullary sinuses, where T cells are found in higher proximity (Dabak et al., 2004). These evidence suggest that an interaction between MCs-DCs and CD8$^+$ T cells is indeed a possible physiological phenomenon, that might be mostly relevant for the immune response during infection.

The finding that MCs inhibit the antigen-specific response of DC-mediated primed CD8$^+$ T cells, gives a hint of a role of MCs in participating in tolerance induction, by down-regulating CD8$^+$ T cell responses. Indeed, MCs have been reported to be necessary for the induction of systemic suppression of contact hypersensitivity responses by UVB radiation (Hart et al., 1998). In addition, as mentioned above, MCs were identified as essential mediators of the long-term allograft tolerance, by interacting with regulatory T cells (Lu et al., 2006). It is therefore tempting to hypothesize that the here demonstrated interaction between MCs and pre-activated CD8$^+$ T cells may also play a role in the induction of tolerance in CD8$^+$ T cell-mediated immune responses.

4.6. Control of MC phenotype by CD8$^+$ T cells

The up to here presented results verify that MCs considerably affect CD8$^+$ T cell responses. In order to investigate whether the MC-activated CD8$^+$ T cells subsequently regulate the activities of their MC counterparts, the effects of CD8$^+$ T cells on MCs in the presence and in the absence of antigen were investigated. Microarray analysis, as well as PCR analysis on MCs isolated after co-culture with CD8$^+$ T cells revealed a differential gene expression profile of MCs after contact with CD8$^+$ T cells. It was therefore concluded that the MC-CD8$^+$ T cell interaction is a dialogue affecting both cell types, rather than a monologue inducing only CD8$^+$ T cell responses.

4.6.1. CD8^{+} T cells control MC phenotype in an antigen-independent manner

Interestingly, CD8^{+} T cells induced up-regulation of several genes in MCs in an antigen-independent manner, showing that CD8^{+} T cells are indeed able to modulate MC responses. A great proportion of those genes are correlated with the signaling pathway of interferons (IFNs). Thus, the signal transducer and activator of transcription 1 (STAT1), which is correlated with IFN-γ production, as well as with IFN-α/β production (Leonard and O´Shea, 1998) was significantly up-regulated in an antigen-independent manner. Similarly, IFN regulatory factor (IRF) 7, which induces IFN-α and IFN-β production, as well as IRF1, responsible for IFN-γ and partially IFN-β production (Taniguchi et al., 2001), were also up-regulated in MCs after contact with CD8^{+} T cells in the absence of specific antigen. Moreover, molecules downstream of IFN-γ signaling, such as Rtp4, induced by IFN-γ, and Igtp, a GTPase induced also by IFN-γ, which regulates innate immune responses to intracellular pathogens (Taylor et al., 2004) displayed enhanced gene expression in MCs after contact with CD8^{+} T cells. These results suggest that CD8^{+} T cells stimulate the IFN-signaling pathway in MCs upon antigen-independent contact. Given the central role of IFNs in the mechanism of host defense, the result that CD8^{+} T cells activate the IFN signaling pathway in MCs, supports the hypothesis that MC-CD8^{+} T cell crosstalk might be crucial in establishing an optimal antiviral response.

Further evidence in the frame of this hypothesis is provided by the almost 10-times up-regulation of the radical S-adenosyl methionine domain containing 2 (Rsad2), an IFN-inducible gene encoding for the antiviral protein viperin (Chin and Cresswell, 2001). Viperin (named for: *v*irus *i*nhibitory *p*rotein, *e*ndoplasmic *r*eticulum-associated, *in*terferon-inducible) inhibits productive human cytomegalovirus (HCMV) infection *in vitro*, by down-regulating several HCMV structural proteins known to be indispensable for viral assembly and maturation (Chin and Cresswell, 2001). Consistent with the present study, Rivieccio et al. (2006) defined viperin as one of the most highly up-regulated genes on human astrocytes upon TLR-3 activation. Therefore, although the protein expression of viperin was not measured, the finding of up-regulated Rsad2 gene expression proposes that MCs induce antiviral responses upon activation by CD8^{+} T cells, as shown in a schematic representation in **Fig. 4.1**.

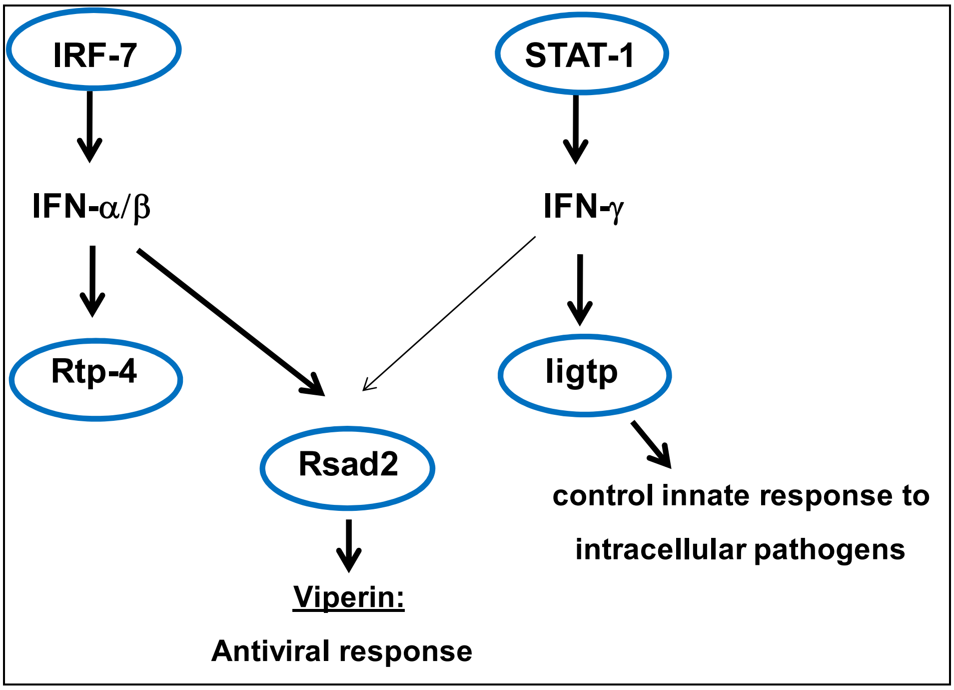

Fig. 4.1. CD8$^+$ T cells stimulate the IFN-signaling pathway in MCs and the induction of an antiviral programme in an antigen-independent manner. *Blue circles highlight the genes that were shown to be up-regulated in MCs after contact with CD8$^+$ T cells in an antigen-independent manner.*

Increasing evidence indicates that MCs can be infected by viruses and respond to viral signals. MCs can detect viral infections via TLR3 and TLR3-activated MCs contribute to host defense by expressing key antiviral response genes (IFN-β, ISG15) and recruiting CD8$^+$ T cells (Orinska et al., 2005). MC activation followed by cytokine and chemokine production was demonstrated upon dengue virus, HIV and encephalomyocarditis virus infection (Bannert et al., 2001; Marone et al., 2001; King et al., 2002; Kitaura-Inenaga et al., 2003). However, the mechanism of MC infection and activation during viral infections as well as their contribution to the establishment of antiviral responses *in vivo* remain to be characterized in detail.

4.6.2. CD8$^+$ T cells control MC phenotype in an antigen-dependent manner

Apart from the antigen-independent gene up-regulation in MC, several genes displayed enhanced expression upon antigen-dependent CD8$^+$ T cell contact. The most prominent of these appeared to be IFN-γ and IFN-related genes, revealing an enhanced stimulation of the IFN-related pathway. Moreover, molecules related to major histocompatibility complex (MHC) class I and II (Qa-2 antigen, H2-Q1, H2-Ab1 and H2-Q8) and the co-stimulatory molecule tumor necrosis factor receptor superfamily member 9 (Tnfrsf9) were significantly up-regulated.

The fact that several genes implicated in the IFN-signaling pathway, both upstream and downstream of IFN, were up-regulated in MCs upon antigen-dependent contact with $CD8^+$ T cells, broadens the conclusions deduced in the previous paragraph. Thus, $CD8^+$ T cells induce the IFN pathway in MCs in the absence of a specific antigen, but this effect is even more potently induced in the presence of an antigen. Moreover, the increased production of IFN-γ resulted in the up-regulation of SOCS-3, which subsequently suppressed TGF-β expression. Given the role of TGF-β as a suppressor factor for the induction of immune responses (Wan and Flavell, 2007), the down-regulation of its expression by MCs upon antigen-dependent contact with $CD8^+$ T cells might provide an essential mechanism for MCs in order to mount the maximal of an antigen-specific $CD8^+$ T cell response.

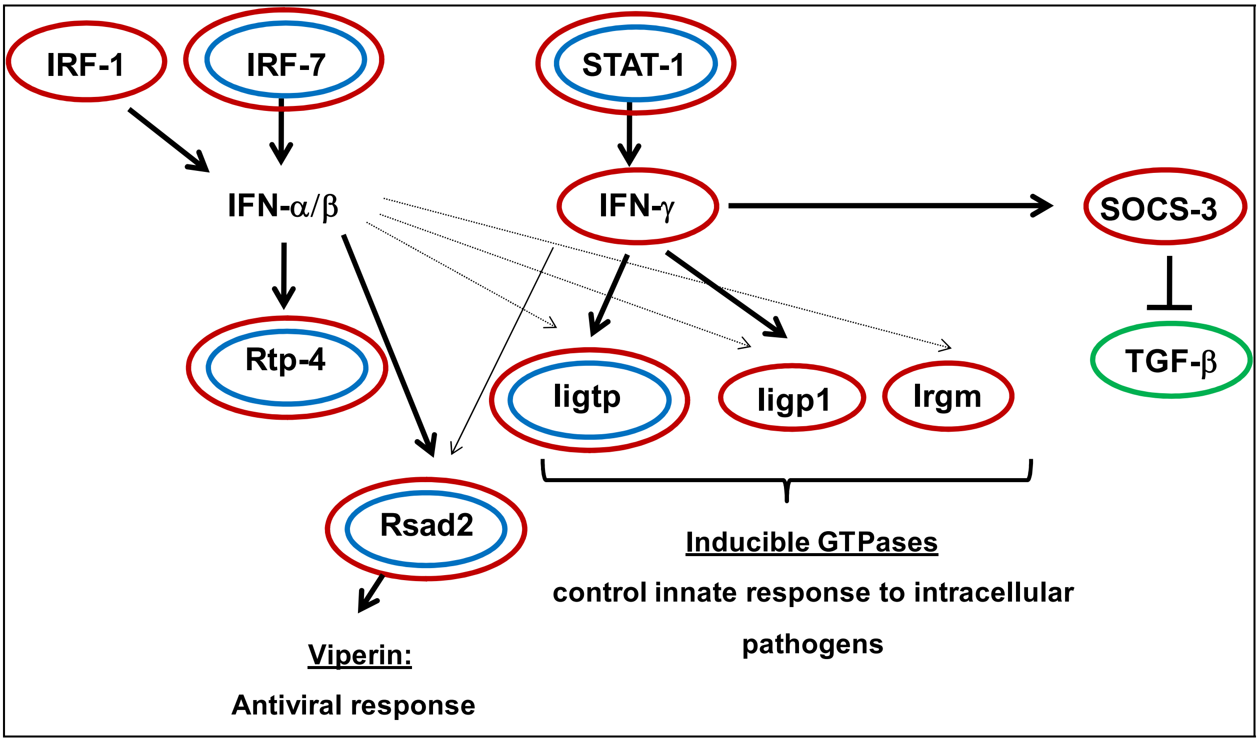

Fig. 4.2. CD8⁺ T cells stimulate the IFN-signaling pathway in MCs and the induction of an antiviral programme in an antigen-dependent manner. *Blue circles highlight the genes that were shown to be up-regulated in MCs after contact with CD8⁺ T cells in an antigen-independent manner. Red circles highlight the genes, which displayed increased up-regulation in the presence of antigen. Green circle shows that TGF-β was down-regulated upon antigen-dependent contact with CD8⁺ T cells.*

The fact that MHC-related antigens were up-regulated on MCs upon antigen-dependent interaction with $CD8^+$ T cells, strongly suggests that MCs are indeed capable of acting as APCs, and this function is significantly promoted after antigen-dependent contact with $CD8^+$ T cells. Thus, MCs initiate antigen-specific activation of $CD8^+$ T cells, which in turn augment the tools for antigen presentation in MCs and therefore promote the ability of MCs to induce antigen-specific responses. It is noteworthy that also MHC class II-related molecules were up-regulated in MCs after antigen-specific contact with $CD8^+$ T cells. This would provide the MCs an enhanced ability to interact also with $CD4^+$ T cells. The ability of MCs to express MHC class II and induce antigen-specific activation of $CD4^+$ T cells has been previously reported (Frandji, et al., 1993; Fox et al., 1994; Love et al., 1996; Poncet et al., 1999). However, the enhanced expression of MHC class II on MCs upon activation by $CD8^+$ T cells sheds additional light to this function: MCs present MHC class I-related antigens to $CD8^+$ T cells, which results not only in $CD8^+$ T cell responses, but also in enhanced MHC class II expression on MCs. Subsequently, MCs possess an enhanced ability to activate $CD4^+$ T cells in an antigen-dependent manner, therefore mediate the crosstalk between $CD8^+$ and $CD4^+$ T cells and regulate an optimal antigen-specific response.

The up-regulation of Tnfrsf9 (also called CD137 antigen or 4-1BB) after antigen-dependent contact with $CD8^+$ T cells, provides evidence of a possible mechanism by which MCs induce $CD8^+$ T cell responses. 4-1BB is a co-stimulatory molecule, promoting proliferation and effector function of T cells (Pollok et al., 1993; Hurtado et al., 1995). Moreover, 4-1BB stimulation induces cytokine production by DCs (Futagawa et al., 2002) and enhances cytokine production and degranulation upon FcεRI- and antigen-stimulation on MCs (Nishimoto et al., 2005). Therefore, the enhanced 4-1BB expression on MCs might be the reason not only for providing the necessary co-stimulation for $CD8^+$ T cells, but also for enhancing the required cytokine and soluble factors production by MCs. Furthermore, the hypothesis of this study that MCs might be crucial regulators of viral defense mechanisms is highly supported by the findings that *in vivo* 4-1BB stimulation enhances and broadens the $CD8^+$ T cell response to influenza virus and can restore the $CD8^+$ T cell response when CD28 co-stimulation is absent (Halstead et al., 2002). Finally, an exciting finding that there is a switch in co-stimulatory requirement from CD28 to 4-1BB during primary versus secondary responses of $CD8^+$ T cells to influenza virus (Bertram et al., 2004), provides further hints that the MC-$CD8^+$ T cell interactions might be more important in secondary immune responses.

The analysis of MC gene expression after $CD8^+$ T cell contact revealed that only 3 genes were significantly down-regulated in MCs after contact with $CD8^+$ T cells; except TGF-β, as discussed above, lysozyme and lipase were also down-regulated in an antigen-independent manner. The fact that enzymes, such as lysozyme and lipase, localized in the granules of MCs were down-regulated provides evidence of a possible regulatory mechanism used by MCs; upon $CD8^+$ T cell encounter, molecules related with adaptive immunity and a possible crosstalk with T cells become up-regulated, while the potential for degranulation becomes reduced. This would direct MCs to instruct specific responses to T cells, while the possible boost of inflammation through MC degranulation would be minimized.

In conclusion, this study has demonstrated that there is a bi-directional crosstalk between MCs and $CD8^+$ T cells, which on one hand enhances effector $CD8^+$ T cell functions and on the other hand instructs MCs to regulate an adaptive immune response and mediate an anti-viral reaction. Such a role of MCs in inducing antiviral responses *in vivo* should be further investigated.

5. Conclusions and Perspectives

In conclusion, this study demonstrated that MCs act as potent regulators of CD8$^+$ T cell responses. Thus, it was shown that MCs are powerful antigen-presenting cells of MHC class I-related antigens *in vitro* and *in vivo*. On the other hand, the antigen-specific interaction between MCs and CD8$^+$ T cells affects also MC activities. MCs increasingly express the antiviral genes as well as co-stimulatory molecules and MHC class I and class II molecules after contact with CD8$^+$ T cells. Briefly, the findings of this study are summarized in **Fig. 5.1**.

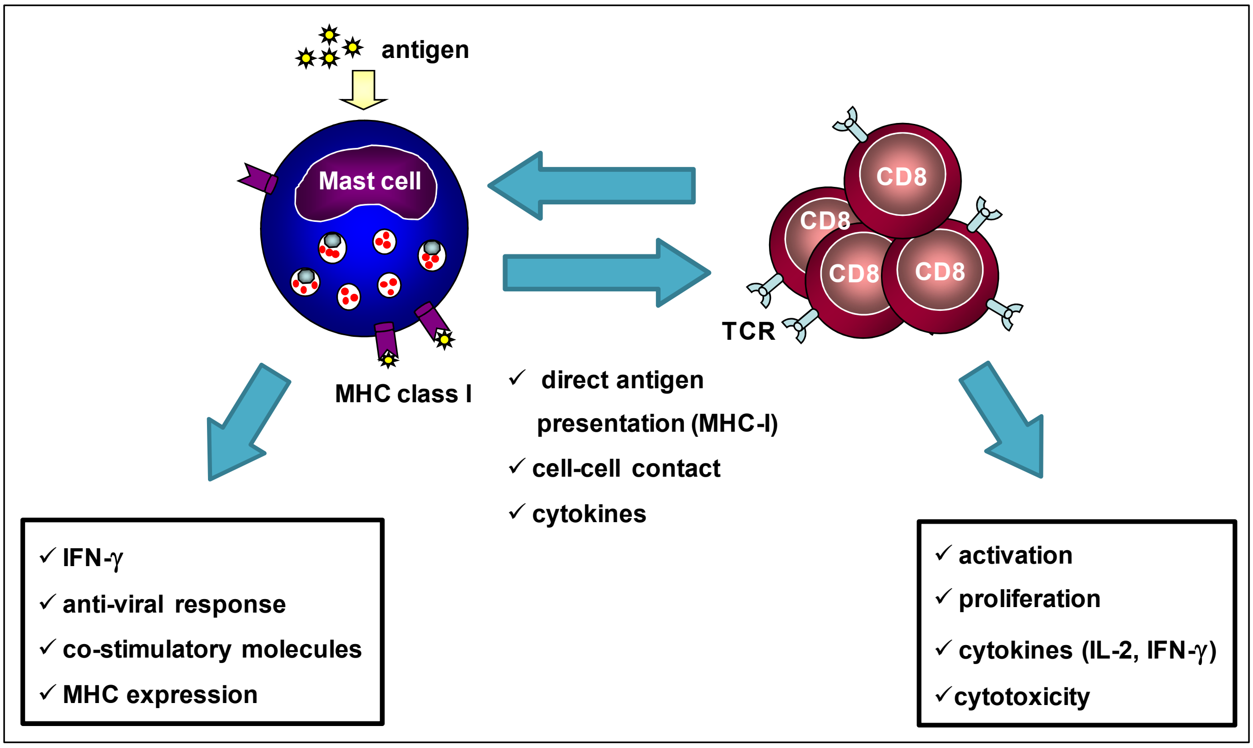

Fig. 5.1. The main conclusions of the present study

The here demonstrated ability of MCs to induce antigen-specific CD8$^+$ T cell responses upon presentation of MHC class I-related antigens *in vitro* and *in vivo* supports the hypothesis that MCs are efficient antigen presenting cells (APCs). The MC-induced proliferation of naïve primary CD8$^+$ T cells *in vivo* introduces further questions regarding the (patho)physiological situations in which this MC-CD8$^+$ T cell interaction is relevant.

The fact that MCs did not significantly influence the CD8$^+$ T cell responses in an *in vivo* model of allergic airway sensitization should direct further research of MC-CD8$^+$ T cell interactions to longer-term pathological situations. Since MCs are long living cells in peripheral tissues, it is highly speculated that their interaction with $CD8^+$ T cells may contribute to the induction of memory $CD8^+$ T cell differentiation at peripheral sites. Consistent with this hypothesis, the co-stimulatory molecule 4-1BB, which was highly up-regulated in MCs after antigen-specific interaction with $CD8^+$ T cells, is important in the induction of secondary rather than primary $CD8^+$ T cell responses (Bertram et al., 2004). Consequently, the role of MCs as modulators of $CD8^+$ T cell responses should be elaborated in secondary immune reactions, such as late phase allergic responses, memory cell differentiation or induction of peripheral tolerance.

On the other hand, the MC-induced augmentation of the cytotoxic potential of $CD8^+$ T cells introduces further topics to be investigated: Do MCs contribute to the induction of cytotoxic $CD8^+$ T cells upon MHC class I-dependent antigen presentation *in vivo*? And is this MC-$CD8^+$ T cell interaction essential for host defense mechanisms? Very recent findings (Heib et al., 2007), support the requirement of MCs for the induction of CTL responses after TLR-7 activation *in vivo.* However, the significance of this cross-talk for an *in vivo* pathological situation has not yet been explored.

Finally, a novel direction in MC research is innovated by the fact that several anti-viral genes in MC were up-regulated after interaction with $CD8^+$ T cells. In addition to the available evidence of MCs being activated upon different viral infections, this result proposes an important role of MCs for the outcome of viral infections. The investigation of the role of MCs in inducing anti-viral responses *in vivo* is regarded to be of great biological as well as medical interest and remains to be examined in detail.

MCs remain enigmatic cells, despite the attempts of several investigators over the past decades to unravel their different roles in health and disease. The contribution of the present study further underline the biological importance of MCs in pathological situations. Biological research will always be splendid; we may keep on discovering exciting information about the cell and possibly unexpected functions of particular cell types, however the currently available information will always be a step towards understanding the biological functions and manipulate them for the human benefit.

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Summary

A precisely regulated crosstalk between innate and adaptive immunity is a prerequisite for an optimal immune response and successful survival strategy. Important players of innate immunity are the mast cells (MCs). These are long-living cells at sites of host-environment interface and important effector players during allergic responses. Recently, MCs have been described as central regulatory cells not only in innate but also in adaptive immune responses. MCs interact with cells of the adaptive immune system and recruit $CD8^+$ T cells upon different stimuli. The purpose of this study was to investigate the interaction between MCs and $CD8^+$ T cells, identify the factors that modulate this interaction and examine its downstream effects.

By using murine bone marrow-derived MCs, this study demonstrated that MCs promote the survival of naïve, primary $CD8^+$ T cells in an antigen-independent and cell-cell contact-dependent manner. The investigation of the antigen-dependent interaction between MCs and $CD8^+$ T cells showed that MCs induce antigen-specific activation, proliferation and cytokine secretion by TCR-transgenic $CD8^+$ T cells *in vitro*. Furthermore, the increased intracellular content of granzyme B and enhanced $CD8^+$ T cell degranulation indicated an increase in the cytotoxic potential of $CD8^+$ T cells. This antigen-driven communication between MCs and $CD8^+$ T cells required both direct cell-cell contact and the release of soluble factors by MCs. TLR-mediated activation of MCs augmented their capacity to activate $CD8^+$ T cells, partially due to enhanced surface expression of MHC class I molecules. Remarkably, the adoptive transfer of antigen-pulsed MCs induced proliferation of antigen-specific $CD8^+$ T cells *in vivo*, in wild-type as well as in β_2-microglobulin-deficient mice, which lack functional MHC class I expression. Thus, MCs promote $CD8^+$ T cell responses, inducing effector $CD8^+$ T cells *in vitro* and *in vivo*.

Furthermore, $CD8^+$ T cells enhanced the expression of several genes in MCs, in an antigen-dependent as well as antigen-independent manner, as demonstrated by differential gene expression analysis of MCs. Many of these genes are implicated in the signal transduction pathway of interferons, suggesting that the MC-$CD8^+$ T cell interaction may contribute significantly to host defense mechanisms. Additionally, upregulation of major histocompatibility complex-related molecules and of the co-stimulatory molecule 4-1BB suggests that the contact with $CD8^+$ T cells enhances the potential of MCs to modulate adaptive immune responses.

In conclusion, this study adds new insights into the physiological role of MCs in the context of adaptive immune responses, such as a $CD8^+$ T cell-driven antiviral immune response. This novel understanding of MC biology foresees new promising approaches for a therapeutic manipulation of antiviral immunity.

Zusammenfassung

Ein exakt regulierter Dialog zwischen angeborener und adaptiver Immunität ist eine wesentliche Voraussetzung für eine optimale Immunantwort und somit für eine erfolgreiche Überlebensstrategie. Zu den bedeutenden Zellen der angeborenen Immunität zählen unter anderen die Mastzellen (MZ). MZ sind langlebige Zellen, welche überwiegend an Umwelt-exponierten Körperflächen lokalisiert sind und wichtige Effektorzellen während einer allergischen Reaktion darstellen. Kürzlich wurden MZ als zentrale, regulatorische Zellen sowohl der angeborenen, als auch innerhalb der adaptiven Immunantwort beschrieben. MZ interagieren mit Zellen des angeborenen Immunsystems und vermögen nach unterschiedlicher Stimulation $CD8^+$ T-Zellen zu rekrutieren. Absicht der vorliegenden Studie war es, die Interaktion zwischen MZ und $CD8^+$ T-Zellen zu untersuchen, die diese Interaktion modulierenden Faktoren zu identifizieren und deren weiterführende Auswirkungen zu bestimmen.

Unter Verwendung aus murinem Knochenmark generierter MZ zeigte diese Studie, dass MZ das Überleben naiver Primär-$CD8^+$ T-Zellen Antigen-unabhängig und Zell-Zell-Kontakt-abhängig unterstützen. Untersuchungen der Antigen-abhängigen Interaktionen zwischen MZ und $CD8^+$ T-Zellen zeigten, dass MZ eine Antigen-spezifische Aktivierung, Proliferation und Zytokinproduktion TCR-transgener $CD8^+$ T-Zellen *in vitro* induzieren. Desweiteren deuten ein erhöhter intrazellulärer Gehalt an Granzym B und ein Anstieg der $CD8^+$ T-Zell-Degranulation auf ein gesteigertes zytotoxisches Potential der $CD8^+$ T-Zellen hin. Diese Antigen-gesteuerte Kommunikation zwischen MZ und $CD8^+$ T-Zellen benötigte sowohl Zell-Zell-Kontakt als auch die Freisetzung löslicher Faktoren durch Mastzellen. Eine Aktivierung der MZ durch die Toll-like-Rezeptoren erhöhte deren Fähigkeit $CD8^+$ T-Zellen zu aktivieren, teilweise vermittelt durch eine gesteigerte Zelloberflächenexpression der MHC Klasse I Molküle. Bemerkenswerter Weise induzierte der direkte Transfer Antigen-stimulierter MZ die Proliferation Antigen-spezifischer $CD8^+$ T-Zellen *in vivo*, sowohl in wildtypischen als auch in β_2-Mikroglobulin-defizienten Mäusen, welchen eine funktionale MHC Klasse I Expression fehlt. Somit wurde deutlich, dass MZ die $CD8^+$ T-Zelle-Antwort fördern und dabei Effektor-$CD8^+$ T-Zellen *in vitro* und *in vivo* induzieren.

Zudem waren $CD8^+$ T-Zellen in der Lage die Expression verschiedener Gene in MZ Antigen-abhängig als auch Antigen-unabhängig deutlich zu verstärken, wie mittels differentieller Genexpressionsanalyse gezeigt werden konnte. Viele dieser Gene haben eine wichtige Funktion innerhalb der Signaltransduktionswege von Interferonen, was zu der Annahme führte, dass die MZ-$CD8^+$ T-Zell-Interaktion wesentlich zu Abwehrmechanismen beitragen könnte. Zusätzlich lässt die Hochregulation des Major Histocompatibility Complex-verwandten Moleküls 4—1BB vermuten, dass der Kontakt mit $CD8^+$ T-Zellen das Potential der MZ, die adaptive Immunantwort zu modulieren, erhöhen könnte.

Zusammenfassend ist zu sagen, dass diese Studie neue Einsichten in die physiologische Rolle der MZ im Kontext der adaptiven, wie einer $CD8^+$ T-Zelle-gesteuerten anti-viralen Immunantwort gibt. Dieses neue Verständnis der MZ-Biologie birgt vielversprechende Ansätze für eine Manipulation der anti-viralen Immunantwort im therapeutischen Sinne.

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Curriculum Vitae

Name:	Erietta
Last name:	Stelekati
Date of birth:	19-05-1980
Place of birth:	Thessaloniki, Greece
Nationality:	Greek
Current address:	Parkallee 22, 23845, Borstel, Germany

EDUCATION

05/2004 - 12/2007	Doctoral student Department of Immunology and Cell biology, Division of Immunobiology, Research Center Borstel, Germany *The role of mast cells in $CD8^+$ T cell-mediated immune responses*
02/2002 - 12/2002	Diploma in Immunobiology Department of Immunopharmacology, Faculty of Pharmaceutical Sciences, Utrecht University, The Netherlands *Investigation of the role of macrophages in occupational asthma*
1998 - 2002	Studies of Biology Faculty of Biology, School of Positive Sciences, Aristotle University of Thessaloniki, Greece Grade of graduation diploma: 8.4 / 10, “very good”
1995 - 1998:	Secondary School 2^{nd} High School of Harilaou, Thessaloniki, Greece Grade of graduation diploma: 19.4 / 20, “excellent”

AWARDS

2002	Erasmus/Socrates scholarship from the EU
2000-2001	Award of progress from the IKY (Greek Institution State Scholarships) (success series: 3rd)
1999-2000	Award of progress from the IKY (Greek Institution State Scholarships) (success series: 2nd)
1998-1999	Award of progress from the IKY (Greek Institution State Scholarships) (success series: 3rd)

PUBLICATIONS

A. Papers

- **Stelekati, E.**, Orinska, Z., Bollinger, A., Paus, R., Bulfone-Paus, S., Mast cells induce $CD8^+$ T cell activities in an antigen-specific manner *in vitro* and *in vivo*. **(in preparation)**
- **Stelekati, E.**, Orinska, Z., Bulfone-Paus, S., 2007. Mast cells in allergy: innate instructors of adaptive responses. Immunobiology 212, 505-519.
- Valstar, D.L., Schijf, M.A., **Stelekati, E.**, Nijkamp, F.P., Bloksma, N., Henricks, P.A., 2006. Trimellitic anhydride-conjugated serum albumin activates rat alveolar macrophages *in vitro*. J. Occup. Med. Toxicol. 1, 13-20.

B. Poster presentations

- **Stelekati, E.**, Orinska, Z., Brandt, K., Bulfone-Paus, S., 2006. Mast cells induce antigen-specific $CD8^+$ T cell responses *in vitro*. 2^{nd} MASIR Conference: Measuring Antigen-Specific Immune Responses, Santorini, Greece
- **Stelekati, E.**, Orinska, Z., Brandt, K., Bulfone-Paus, S., 2006. Mast cells induce antigen-specific T cell responses. 2^{nd} Spring School on Immunology of the German Society for Immunology, Ettal, Germany
- **Stelekati, E.**, Orinska, Z., Brandt, K., Bulfone-Paus, S., 2006. Mast cells as potential modulators of T cell responses. EAACI-GA^2LEN Summer School: Mouse models of allergy and asthma, Antalya, Turkey

C. Oral presentations

- **Stelekati, E.**, Orinska, Z., Bollinger, A., Brandt, K., Bulfone-Paus, S., 2007. Mast cells as modulators of $CD8^+$ T cell responses. 2^{nd} International Conference on Crossroads between Innate and Adaptive Immunity, Crete, Greece
- **Stelekati, E.**, Orinska, Z., Bollinger, A., Brandt, K., Rückert, R., Bulfone-Paus, S., 2006. Mast cells as modulators of T cell responses. 29^{th} Meeting of the North German Immunologists, Borstel, Germany
- **Stelekati, E.**, Orinska, Z., Bulfone-Paus, S., 2005. The role of mast cells in the activation of T cells. 9^{th} Meeting of the Society for Dermatological Research on Mast Cells / Basophils, Berlin, Germany

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbständig verfasst und keine weiteren als die darin angegebenen Quellen und Hilfsmittel verwendet habe. Diese Arbeit hat weder in gleicher noch in ähnlicher Form an anderer Stelle im Rahmen eines Prüfungsverfahrens vorgelegen. Auzüge dieser Arbeit wurden bereits zur Veröffentlichung eingereicht bzw. sind bereits veröffentlicht.

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Erietta Stelekati