Mechanisms of NKT cell-mediated DC licensing and cross-priming

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2.1 Cross-presentation

2.1.1 General mechanisms

$CD8^+$ T cells respond to their specific antigen in the context of Major Histo-Compatibility (MHC)-Class-I molecules, which are expressed by all nucleated cells and allow for a constitutive display of endogenous antigens. However, activation of naïve $CD8^+$ T cells requires more than solely antigen-recognition, referred to as signal 1 (**1**). The second prerequisite is that T cells receive additional costimulatory signals that are provided by dendritic cells (DCs) under inflammatory conditions. These additional signals confer cytolytic capacity to the $CD8^+$ T cell, which can then subsequently destroy infected or altered body cells that display the specific T cell antigen.

As naïve $CD8^+$ T cells depend on both antigen and costimulatory signals for their activation, there has to be a way to induce $CD8^+$ T cell responses if DCs are not infected or altered themselves. A second pathway of antigen processing termed cross-presentation allows the presentation of internalized antigens by professional antigen-presenting cells on MHC-class-I. This pathway is a crucial extension to the classical pathway in preventing the immune escape of viruses that do not infect DCs and thereby might circumvent $CD8^+$ T cell activation due to a lack of synchronous presentation of costimulatory molecules and viral antigens in the context of MHC-class-I. Bevan et al. first described the cross-priming in 1976 (**2**) after they immunized mice with allogenic cells and examined the MHC-restriction of the induced $CD8^+$ T cell response. The immunization generated $CD8^+$ T cells that were restricted to both donor and, surprisingly, host MHC-class-I molecules. The latter was only possible if the host cells acquired donor cellular antigens and processed them on MHC-class-I to $CD8^+$ T cells. This antigen presentation pathway has since been termed cross-presentation. When it results in $CD8^+$ T cell immunity it is referred to as “cross-priming” whereas the induction of tolerance is referred to as “cross-tolerance”.

The cellular mechanisms enabling the cross-presentation pathway are not yet fully understood. It is evident that cross-presentation requires that ingested antigens circumvent lysosomal degradation and MHC-class-II-loading and are instead processed by the MHC-class-I-

loading machinery that is typically located in the endoplasmic reticulum (ER). However, recent studies suggest that loading of soluble and particulate antigen on MHC-class-I molecules does not necessarily occur in the ER, but rather in specialized endocytic and phagocytic compartments (**3**). *Burgdorf et al.* (**4**) described that certain antigens, which are taken up via distinct endocytic receptors (such as C-type lectins and Fc receptors) remain in so-called stable early endosomes that do not develop into late endosomes. Cross-presentation of these antigens is favored by the presence of insulin-regulated aminopeptidase (IRAP), which was found to degrade antigens to a peptide length that is appropriate for loading on MHC-class-I molecules (**5**). Soluble antigens that are taken up by different means are directed into late endosomes where they enter the MHC-class-II-loading machinery. Thus, soluble antigens intended for cross-presentation are spatially separated from other antigens at an early stage (**6**).

For particulate antigens, it has been suggested that cross-presentation occurs in phagosomes, which contain molecules of the MHC-class-I-loading machinery. However, it is currently unclear and a matter of debate how these molecules reach the phagosomes and how this process is regulated (**7**).

2.1.2 Cell types capable of cross-presentation

The cell type best known for its ability to cross-present in mice is the $CD8^+$ DC (**8**). $CD8^+$ DCs represent a tissue-resident DC subset that also express $CD24^+$, $CD205^+$ and $Clec9A^+$, which are useful markers to better define this population (**8**). Human DCs do not express CD8 but the equivalent cell type to mouse $CD8^+$ T cells has been described to be $Clec9A^+$ $DNGR1^+$ $BDCA3^+$ $XCR1^+$ (**9**,**10**).

Although DCs seem to be best suited for cross-presentation, other cell types can also cross-present, including B cells (**11**), human γδ T cells (**12**), neutrophils (**13**), macrophages (**14**) and liver sinusoidal endothelial cells (**15**).

2.2 DC licensing

2.2.1 Requirement for help

It is essential that $CD8^+$ T cell activation is tightly regulated in order to avoid destruction of healthy self-cells. One mechanism that regulates T cell activation is the need for several distinct signals to be provided by the DC in addition to antigen-presentation. The different signals required for T cell activation have been termed Signal 1, 2 and 3 (**1**,**16**,**17**):

Signal 1: Specific antigen in context of MHC-class-I molecules.
Signal 2: Costimulatory molecules of the B7 family for example CD80/86 (bind to CD28 on the T cell) or of the tumor necrosis factor (TNF) receptor family for example CD40 (binds to CD40L)
Signal 3: Cytokines like IL-12 and type-I IFN, proposed to enable optimal effector functions and memory responses.

Under homeostatic conditions, DCs take up antigens and present them on MHC-molecules, but cannot provide costimulatory signals, leading to T cell deletion and cross-tolerance. Only under inflammatory conditions do they become potent inducers of $CD8^+$ T cell immunity by upregulating costimulatory molecules and producing cytokines, thereby reaching a "mature" state (**18**). Stimulation through Pattern Recognition Receptors (PRRs) that indicate the presence of a pathogen can lead to upregulation of costimulatory molecules, but it seems that these signals are not essential to render the DC capable of cross-priming. The DC can become activated in the absence of signals from pathogens or infected cells but instead require signals from antigen-specific $CD4^+$ T cells (**19**,**20**).

2.2.2 Classical licensing via $CD4^+$ T helper cells

Bennett et al. described in 1998 that efficient induction of cytotoxic T cell responses depends on the previous activation of a DC by an antigen-specific $CD4^+$ T cell (**21**). This concept was termed "cognate licensing", indicating that the DC presents the same antigen on MHC-class-II to the $CD4^+$ T cell and on MHC-class-I to the $CD8^+$ T cell. This prerequisite for complete DC maturation functions as a "second opinion" about the relevance of the presented antigen and helps to reduce the risk of activating autoreactive $CD8^+$ T cells. Although autoreactive T cells are present in the periphery despite negative selection in the thymus, an encounter of the antigen-presenting DC with a $CD4^+$ T cell and a $CD8^+$ T cell that are specific for the same self-antigen seems unlikely.

A crucial feature of T cell help seem to be CD40L / CD40 interactions that result in upregulation of adhesion- and costimulatory molecules and induction of cytokines like IL12 by the DC (**22**).

Although the requirement for T cell help has been described in several models, other reports showed help-independent $CD8^+$ T cell responses. This inconsistency has been resolved by studies that showed that primary $CD8^+$ T cell responses can be functional even in the absence of help, but that there is a requirement for $CD4^+$ T cell help for the generation of functional memory $CD8^+$ T cells (**23**,**24**).

2.2.3 Alternative NKT cell mediated licensing

The Natural Killer (NK) T cell ligand α-galactosylceramide (αGalCer) has long been known for its adjuvant effect in enhancing tumor resistance (**25**). Since 2003 several reports described that the mechanism underlying this property might be based upon the fact that NKT cells can substitute for $CD4^+$ help and license DCs (**26**,**27**): αGalCer can be presented by a DC on the MHC-like molecule CD1d, and will be recognized by the invariant T Cell Receptor (TCR) of an NKT cell. The recognition of its antigen activates the NKT cell to upregulate CD40L and to rapidly produce cytokines. The activated NKT cell in turn induces upregulation of maturation markers and cytokines by DCs (**28**,**26**). Similar to classical licensing, CD40 plays a crucial role in the alternative licensing process as CD40-deficient mice were unable to develop enhanced $CD8^+$ T cell responses although DCs upregulated maturation markers due to cytokine signals (**28**).

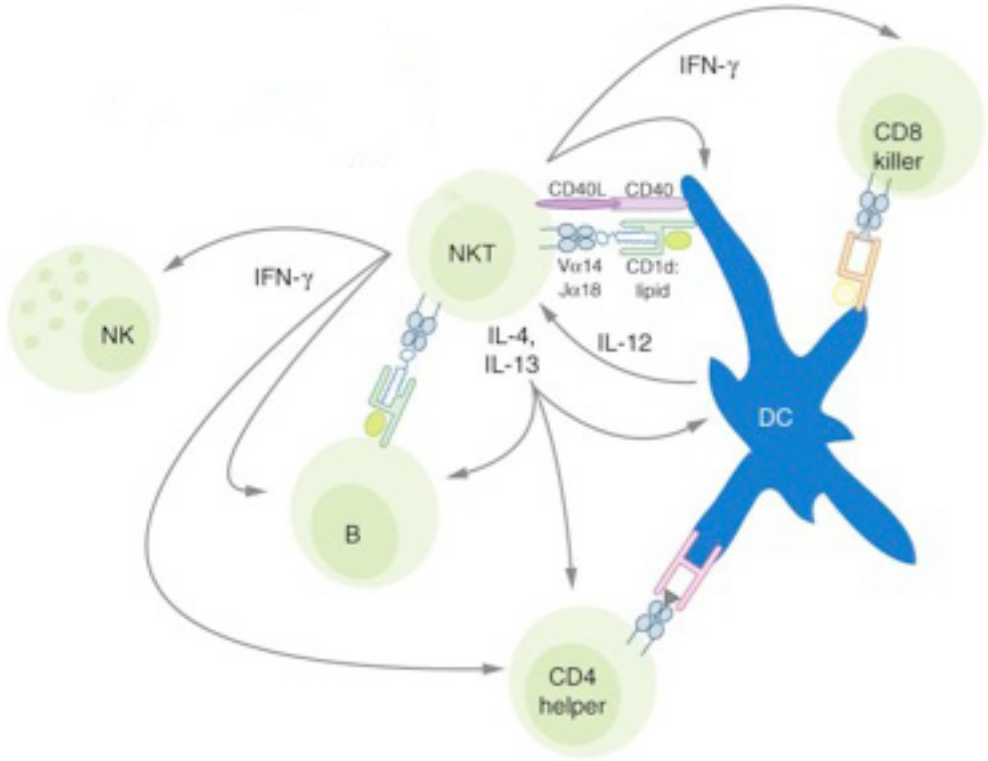

Figure 2.2.3 Cellular network activated by αGalCer. DCs present lipid antigens like αGalCer on the MHC-like molecule CD1d to NKT cells, leading to upregulation of CD40L and production of T_H1 and T_H2 cytokines by NKT cells. In turn, DCs are activated to produce IL12 and express costimulatory molecules. Cytokines can then influence other cell types like NK cells, B cells and T cells. Modified from *Bendelac Ann Rev Immunol 2004*.

2.2.3.1 NKT cells

NKT cells were originally defined as T cells co-expressing an αβ – TCR and NK cell markers such as CD161 (NK1.1) and CD94, but it has become apparent that this is a poor definition as the expression of NK markers differs dependent on both developmental stage and activation state of the NKT cell and some NKT cell subsets entirely lack expression of NK1.1 (**29**).

The NKT cell TCR differs in several ways from the "regular" TCR that αβ- T cells express: NKT cells do not recognize peptide antigens in the context of MHC-class I or II but instead are activated by glycolipids in the context of the MHC-like molecule CD1d (**30**). The "classical" CD1d-restricted NKT cells express a TCR that is composed of Vα14 Jα18 in mice and Vα24 Jα18 in humans and a very limited set of β chains, in mice Vβ8.2 or 7.2, in humans mainly Vβ11 (**29**). Because of this invariability they are also referred to as "invariant" or iNKT cells, or type-1 NKT

cells in contrast to type-2 NKT cells that do not express this semi-invariant TCR. Only type-1 NKT cells are reactive to the model antigen αGalCer and are therefore the focus of this thesis.

αGalCer, which was isolated from the marine sponge *Agelas mauritanius* as a potent adjuvant for cancer therapy (**31**), is the first described and most potent NKT cell antigen. Since its discovery, synthetic αGalCer derivatives have been generated, for example OCH that has a truncated sphingosine chain and induces T_H2-like responses (**32**). Several other glycolipid antigens have since been identified, including bacterial glycolipids like α-galactosyl-diacylglycerol (αGalDAG) derived from *Borrelia burgdorferi*, GSL1´ derived from *Sphingomonas* species (**33,34**), and mammalian glycolipids like isoglobotrihexosylceramide (iGb3) (**35**).

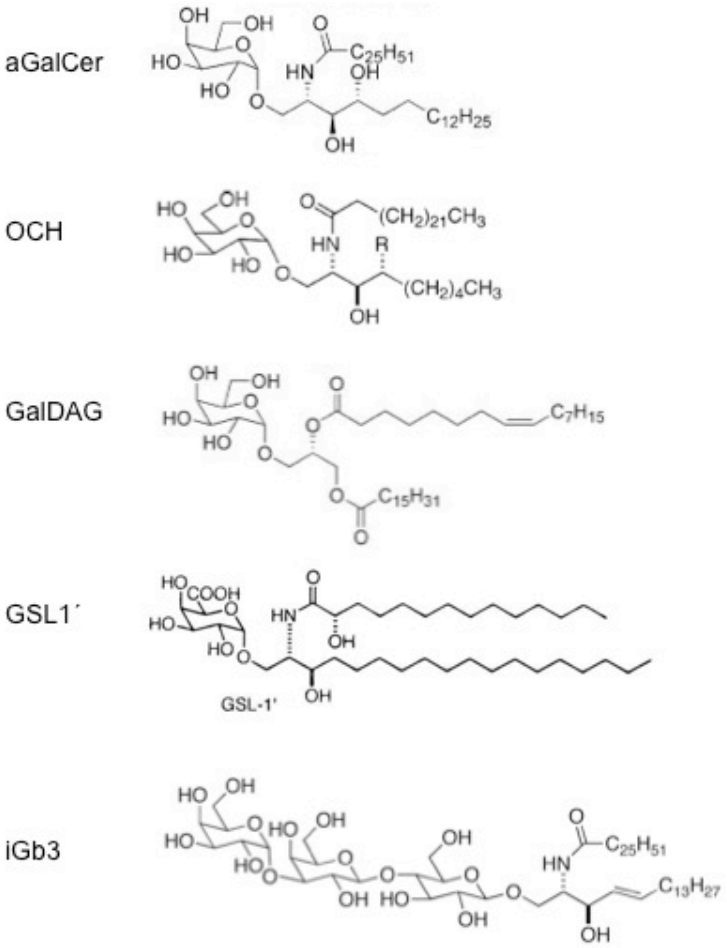

Figure 2.2.3.1 Structure of different NKT cell ligands. Structures of the synthetic NKT cell ligand αGalCer and its analog OCH, the microbial agents aGalDAG (*Borrelia Burgdorferi*) and GSL1´(*Sphingomonas*), and the mammalian glycosphingolipid iGb3.

It has been a matter of some debate how NKT cell activation by self-lipids could be prevented under homeostatic conditions. Recently, *Darmoise et al.* (**36**) demonstrated that the enzyme α-Gal-A inhibits accumulation of self-lipids in lysosomes under steady-state conditions, thereby preventing efficient presentation to NKT cells. Under inflammatory conditions, microbes inhibit α-Gal-A activity, allowing for presentation of self-antigen and activation of NKT cells. These findings are in line with a report by *Mattner et al.* (**37**) that proposed that NKT cells are not directly activated by gram-negative bacteria like Salmonella, but are activated through presentation of iGb3 by LPS-activated DCs.

Several subsets of type-1 NKT cells have been described. Mice have $CD4^+$ and double negative (DN) subsets but lack $CD8^+$ NKT cells, which are found in humans. Additionally, one has to discriminate between $NK1.1^+$ and $NK1.1^-$ NKT cells, the latter including a newly identified subset called NKT-17 cells, which produce large amounts of IL-17 (**38**). NKT cells are known for their ability to secrete many different cytokines rapidly after their activation. The dual production of both T_H1 and T_H2 cytokines, especially IL-4 and IFNγ, is one hallmark of NKT cells enabling them to regulate the quality of immune responses. Therefore, NKT cells are important players in a broad spectrum of diseases including viral infections, malaria, cancer and autoimmunity, and can either promote or suppress immune responses in a particular given setting. Recently it was found that the cytokine profile of NKT cells differs considerably depending upon their CD4 and NK1.1 expression and the organ that the NKT cell was isolated from (**38**). This emerging heterogeneity in functional subsets might shed light on the seemingly contradictory roles that NKT cells can play. Clarifying the role of individual subsets in the particular disease setting might help to understand why NKT cells push immune responses in a certain directions.

2.3 Chemokines

2.3.1 General properties

Chemokines are small, secreted proteins that belong to a molecular superfamily that shares structural similarities: all members possess four conserved cystein residues that form disulfide bonds that are crucial for tertiary structure. In CC-chemokines like CCL17, the first two cystein residues are adjacent whereas in CXC-chemokines like CXCL9, another amino acid is situated between the first two cysteins (**39**).

Chemokines can be grouped in inflammatory / inducible and homeostatic / constitutive mediators based on their expression and function (**39**). Expression of inflammatory chemokines is induced in the periphery at sites of inflammation whereas homeostatic chemokines are constitutively expressed in lymphoid organs. The latter are known for guiding immune cells to the sites of their destination by establishing a chemokine concentration gradient, which cells bearing the appropriate receptor can follow. During immune responses, the expression pattern of chemokine-receptors on a cell and therefore their homing behaviour change. T cells in their naïve state express CCR7 and CXCR4, whose ligands are expressed in lymphoid organs and attract naïve T cells to a restricted area in secondary lymphoid organs. Upon activation, T cells downregulate CCR7, thereby inhibiting further circulation through secondary lymphoid organs, and upregulate other receptors including CCR2, CCR3, CCR4, CCR5, CCR6, CCR8, CXCR5 and CXCR3 (**40**). The ligands for these receptors are expressed in inflamed peripheral organs, thereby directing activated T cells to sites of inflammation.

Chemokines bind to receptors that belong to the superfamily of seven transmembrane domain G protein coupled receptors (GPCR). These receptors are associated with cytosolic heterodimeric G proteins that consist of a Gα, Gβ and Gγ subunit. So far approximately 20 Gα, 6 Gβ and 11 Gγ have been described, indicating the high diversity of GPCR signalling (**41**). Upon ligand binding the Gα subunit dissociates from the Gβ/Gγ complex. The signal is transduced mostly by the Gα subunit but sometimes also through the G$\beta\gamma$ complex. All G proteins engage multiple signaling pathways with various intracellular intermediataries including ion channels, transcription factors and metabolic enzymes. Thereby, a complex network is generated in which signals from GPCRs can be integrated with signals from other receptors.

Chemokines are characterized by a certain degree of redundancy: there can be many ligands binding to the same receptor and vice versa. One example is CCR5, which binds CCL3, CCL4 and CCL5, of which CCL3 and CCL5 can additionally bind to CCR1 (**41**). Another example is CCR4 that has two ligands, CCL17 and CCL22 (**42, 43**).

2.3.2 The chemokine receptor CCR4 and its ligands

Originally, CCR4 expression was associated with T helper cell type 2 (T_H2) responses in allergic conditions in airways or skin (**44**,**45**,**46**). More recently, its expression has been detected on Langerhans cells, monocytes, NK cells, NKT cells, platelets and regulatory T cells (**47**,**48**,**49**).

The best described CCR4 ligand CCL17, also known as thymus- and activation-regulated chemokine (TARC), has been identified in mice as one of few gene products whose expression is highly DC-restricted, with constitutive CCL17 expression in thymus, lymph nodes and lung but complete absence in splenic DCs, even after systemic application of different TLR ligands (**42**,**45**).

The pathophysiological role of CCL17 has been studied *in vivo* in different disease models. CCL17 deficient mice had reduced contact hypersensitivity (**45**), these results being in line with studies on patients suffering from atopic dermatitis that showed increased CCL17 levels, which strongly correlated with disease severity (**46**). *Kawasaki et al.* demonstrated that allergic airway hyper-responsiveness also depended on CCL17 as application of a neutralizing CCL17-antibody could significantly reduce the response in allergen-treated mice (**50**). In these models, disease severity depended on the capacity of CCL17 to recruit T_H2 cells to sites of inflammation, leading to the release of high amounts of cytokines.

A second CCR4-specific ligand has been identified, macrophage-derived chemokine (MDC) or CCL22 (**43**) although this chemokine is less well characterized. Despite the fact that both chemokines bind to CCR4, they have additionally been shown to bind the scavenging non-signaling decoy receptor D6 (**51**). Moreover CCL17 has been described to bind to eosinophils in a CCR4-independent fashion, suggesting the putative existence of a novel unidentified receptor for CCL17 (**52**). *Stutte et al.* (**53**) described a relation between CCL17 and the chemokine receptors CCR7 and CXCR4. They observed a migratory defect in CCL17-deficient but not CCR4-deficient cutaneous DCs in atopic dermatitis and could demonstrate that CCL17 sensitizes DCs for CCR7- and CXCR4-dependent migration.

2.3.3 Chemokines as regulators of immune responses

The concept of DC licensing indicates that the cross-presenting DC has to physically interact with both specific $CD4^+$ and $CD8^+$ T cells. Such interactions need to be regulated to ensure an encounter of rare antigen-specific cells, and chemokines are the molecular agents that fulfill this purpose.

Under homeostatic conditions, an optimal immune surveillance is mediated by CCR7 and CXCR5 expression on T and B cells and secretion of the appropriate ligands in T and B cell zones of secondary lymphoid organs. Lack of CCR7 thus leads to profound morphological alterations resulting in a dissolved microstructure of secondary lymphoid organs (**54**). Chemokine

receptor- and ligand-expression are regulated in a highly sophisticated fashion: for example, upregulation of CCR7 on matured DCs ensures the migration of antigen-experienced, mature DCs along lymphoid vessels towards T cells zones, where endothelial cells express the CCR7 ligand CCL21 (**55**). On the other hand, all naïve T cells express CCR7, which allows them to recirculate within secondary lymphoid organs and make contact with CCL19-producing DCs inside of T cell zones (**56**).

Castellino et al. described the involvement of a different chemokine receptor in the initiation of T cell responses. They could show that $CD8^+$ T cells were preferentially attracted towards mature DCs that had previously been licensed by another T cell (**57**). This attraction depended on CCR5 on T cells and happened in an antigen-independent way. Another report by *Hugues et al.* confirmed the involvement of CCR5 in T cell help by demonstrating that naïve $CD8^+$ T cells were preferentially attracted to mature DCs that had previously interacted with antigen-specific $CD8^+$ T cells (**58**)

2.4 Aims of this study

Besides classical DC licensing, which is mediated by recognition of peptide antigens through a $CD4^+$ T_H cell, DCs can also be licensed after recognition of lipid antigens by NKT cells. Little is known about the mechanisms underlying NKT cell-mediated DC licensing except that DCs mature and upregulate costimulatory molecules. It was my task to clarify the mechanisms underlying NKT cell mediated cross-priming. As classical licensing is regulated by the chemokine receptor CCR5, one obvious question was if the same mechanism applies for NKT cell-mediated DC licensing. The finding that not CCR5 but CCR4 regulates this alternative licensing pathway was surprising as CCR4 and its two ligands have been linked to memory $CD4^+$ T cell responses, especially of the T_H2 type, and to allergic responses of airways and skin. By contrast, little is known about their role in $CD8^+$ T cell responses in the spleen. This led me to investigate the role of CCR4 in more detail. Furthermore, I addressed the question why different licensing events are regulated by distinct chemokines, in order to develop a clearer picture of the chemokine cross-talk in health and disease.

3 Materials and Methods

3.1 Materials

3.1.1 Equipment

Equipment	*Name and company*
Autoclave	Belimed, Cologne
Beakers	5, 10, 25, 50, 100, 250, 500 ml (Schott, Mainz)
Cell counting chamber	Neubauer (Brand, Wertheim)
Centrifuges	Multifuge 3s-r, Biofuge fresco (Heraeus, Braunsschweig)
ELISA reader	SpectraMax 250 (Molecular Devices, Sunnyval, USA)
Flow cytometers	FACS CantoII (BD Biosciences, Heidelberg)
Freezers (-20°C)	Liebherr, Biberach
Freezers (-80°C)	Hera freeze (Heraeus, Braunschweig)
Freezing container	Nalge Nunc Cryo (Nunc, Wiebaden)
Heating block	ThermoStat plus (Eppendorf, Hamburg)
Ice machine	Icematic (Scotsman®, Frimont Bettolinc, Pogliano, Italy)
Incubators	HERAcell (Heraeus, Braunschweig)
MACS cell separator	QuadroMACS (Miltenyi Biotec, Bergisch-Gladbach)
Magnet stirrer	IKA® Laboratory Equipment, Staufen
Measuring cylinders	50 ml, 100 ml, 250 ml, 500 ml, 1l (Schott, Mainz)
Microscopes	IX71 and CKX31 (Olympus, Hamburg)
pH-meter	pH523 (Wissenschaftlich-Techn. Werkstätten, Weilheim)
Pipette-Boy	Pipetus (Hirschmann Labortechnik, Eberstadt)

Pipettes	Eppendorf, Hamburg and Gilson, Heidelberg
Preparation instruments	Labotec, Göttingen
Refrigerators (+4°C)	Bosch, Stuttgart and Liebherr, Biberach
Sieves, steel	University of Bonn, Department “Feinmechanik”
Water bath (37°C)	TW8 (Julabo, Seelbach)
Workbench, sterile	HERAsafe (Heraeus, Hanau)

3.1.2 Software

Software	*Company*
Cell F	Olympus, Hamburg
Corel Draw	Corel Corporation
FACS Diva V6.1.1	BD Biosciences, Heidelberg
Flowjo V8.8.6	Tree star, Inc., USA
Image J	NIH Bethesda, USA
Microsoft Office 2008	Microsoft, USA
Photoshop CS4	Adobe, USA
Prism5 for Macintosh	GraphPad Software, USA
SPF ELISA software	Molecular Devices, USA

3.1.3 Consumables

Consumables	*Name and company*
Cover slides	21x46 mm (Marienfeld, Lauda-Königshofen)
Cryo vials	VWR International, Darmstadt
Cyromolds	VWR International, Darmstadt
ELISA plates	Microlon, 96-wll, flat-bottom (Greiner bio-one, Solingen)
FACS tubes	polystyrene, 12/75 mm (Sarstedt, Nümbrecht)
Injection needles	27G, 25G, 20G (BD Microlance, Heidelberg)
Microtiter plates	96-well, round and flat bottom (Greiner bio-one, Solingen)
Parafilm	Parafilm "M"®(American National Can TM, Greenwich, USA)
Pasteur pipettes	150 mm and 230 mm (Roth, Karlsruhe)
Petri dishes	10 cm (Greiner bio-one, Solingen)
PD10 column	Amersham, Uppsala
Pipette tips	10 µl, 200 µl, 1000 µl (Greiner bio-one, Solingen)
Plastic pipettes	5ml, 10 ml, 25 ml (Sarstedt, Nümbrecht)
Polypropylene tubes	sterile, 15 ml and 50 ml (Greiner bio-one, Solingen)
Reaction tubes	0.5 ml, 1.5 ml, 2 ml (Eppendorf, Hamburg)
Sterile filter	0.2 µm (Schleicher & Schuell)
Syringes	2, 5, 10, 20 ml BD Discardit™ (BD Bioscience, Heidelberg)
Tissue culture plates	12-, 96-well (Sarstedt, Nümbrecht)
Transwell inserts	5µm Corning (Labomedic, Bonn)

3.1.4 Chemicals and reagents

Reagent	*Company*
2-methyl butane	Merck, Darmstadt
α-galactosylceramide	Axxora, Lausen, Switzerland
α-GalDAG	Patrick Perlmutter, Clayton
Ammonium chloride (NH_4Cl)	Merck, Darmstadt
β-mercaptoethanol	Sigma Aldrich, Munich
Bovine serum albumin (BSA)	Roth, Karlsruhe
Carboxyfluorescein succinimidyl ester (CFSE)	Molecular Probes, Leiden, Netherlands
CCL17 recombinant mouse	RnD Systems, Wiesbaden
CpG oligodeoxynucleotides	TIB MolBiol, Berlin
Collagenase A	Roche, Mannheim
Disodium hydrogen phosphate (Na_2HPO_4)	Merck, Darmstadt
DMEM medium	Sigma Aldrich, München
Ethanol, absolute	Merck, Darmstadt
Ethylene diamine tetraacetic acid (EDTA)	Merck, Darmstadt
Far Red fluorochrome	Molecular Probes, Leiden,Netherlands
Fetal calf serum (FCS)	PAA, Cölbe
Glogi Plug/Stop	BD Biosciences, Heidelberg
GSL1´	provided by Paul Savage, Provoh USA
Hoechst 33342	Molecular Probes, Leiden,Netherlands
Hydrogen peroxide (H_2O_2)	Merck, Darmstadt
IFNg, ELISA duoset	BD Biosciences, Heidelberg

IFNγ, recombinant mouse	Biolegend, San Diego, USA
Isoglobotrihexosylceramide (iGb3)	Axxora, Lausen, Switzerland
IL-2, -4, -6, -12, -13 recombinant mouse	Biolegend, San Diego, USA
L-glutamine (200 mM)	PAA, Cölbe
Liquid nitrogen	Linde, Wiesbaden
OCH	Axxora, Lausen Switzerland
o-Phenylenediamine dihydrochloride (OPD)	Sigma Aldrich, München
Ovalbumin (OVA), grade V	Sigma Aldrich, München
Parafomaldehyde	Fluca, Buchs
Penicillin/Streptomycin	PAA, Cölbe
Phosphate buffered saline (PBS)	Biochrom, Berlin
Potassium bicarbonate ($KHCO_3$)	Merck, Darmstadt
RPMI 1640 medium	Invitrogen, Darmstadt
Saponin	Sigma Aldrich, München
SIINFEKL peptide ($OVA_{254-267}$, S8L)	Sigma Aldrich, München
Sodium bicarbonate (Na_2HCO_3)	Sigma Aldrich, München
Sodium hydroxide (NaOH)	Merck, Darmstadt
Sulfuric acid (H_2SO_4)	Merck, Darmstadt
Tissue-Tel® OCT	Sakura, Netherlands
Trypane blue (0.4 %)	Lonza, Köln
Tween20	Roth, Karlsruhe

3.1.5 Buffers, media and solutions

5 mM β-mercaptoethanol

178 µl of 14.3 M β-mercaptoethanol was added to 500 ml PBS and kept under sterile conditions at 4°C.

DC and T cell culture medium

RPMI 1640 medium was supplemented with 10% (v/v) FCS, 1% (v/v) L-glutamine, 1% (v/v) penicillin/streptomycin, 1% (v/v) sodium pyruvate, and 1% (v/v) of 14.3 M β-mercaptoethanol. Kept under sterile conditions at 4°C.

FCS (fetal calf serum)

FCS was heat-inactivated at 56°C for 30 min and stored à 50 ml aliquots at -20°C.

PBS (phosphate buffered saline)

1xPBS was adjusted to pH 7.4, aliquoted à 500 ml, autoclaved and stored at 4°C.

Erythrocyte lysis buffer

16.58 g NH_4Cl, 2 g $KHCO_3$, 74.4 mg Na_2EDTA was dissolved in 2000 ml ultra-pure water and the pH was adjusted to 7.2 - 7.4. Stored under sterile conditions at 4°C.

FACS buffer

1x PBS containing 0,1% (v/v) BSA and 0.1% (v/v) NaN_3. Stored at 4°C.

MACS buffer

1x PBS containing 0,1% (v/v) BSA and 2mM EDTA. Stored under sterile conditions at 4°C.

Saponin buffer

FACS buffer supplemented with 0.5% (w/v) saponin, prepared freshly.

ELISA coating buffer

0.1 M $NaHCO_3$ in ultra-pure water, pH 8.2, autoclaved and stored at 4°C.

ELISA blocking buffer

1% BSA (w/v) in 1xPBS, stored at 4°C.

OPD substrate buffer

15.6 g NaH_2PO4 x 2 H_2O and 14.7 g $Na_3C_6H_5O_7$ x 2 H_2O were dissolved in 500 ml ultrapure water and the pH was adjusted to 5.0. The buffer was stored at room temperature.

1M H_2SO_4 OPD stopping solution

26.5 ml 96% H_2SO_4 was added to 500 ml ultra-pure water and kept at room temperature.

ELISA washing buffer

1x PBS containing 0.01% (v/v) Tween20

4% (w/v) PFA solution

8 g PFA was dissolved in 200 ml 1xPBS by gradual heating. The pH was adjusted to 7.4 and aliquots were stored at -20°C.

3.1.6 Antibodies

Antibodies used in cytokine ELISA

All antibodies used for cytokine ELISAs were purchased from eBioscience or BD Pharmingen. Matching antibody pairs included a primary unlabeled capture antibody and a biotinylated detection antibody. Each antibody was applied in previously titrated amounts. Clones XMG1.2 (IFNγ capture antibody) and RA-6A2 (IFNγ detection antibody) were used.

Antibodies coupled to magnetic beads (MACS® Beads)

Murine anti-CD8a, anti-CD4, anti-CD19 and anti-CD11c antibodies conjugated to magnetic beads (MACS® Beads) were obtained from Miltenyi Biotech, Bergisch Gladbach.

Antibodies for immunohistology and flow cytometric analysis

The following antibodies were purchased from eBioscience, BD Biosciences or Molecular Probes (if not otherwise stated) for flow cytometric analysis of murine molecules expressed at the cell surface or intracellularly. All antibodies were employed at previously determined concentrations, and mostly diluted 1:200 in FACS buffer.

Antigen	*Clone*	*Conjugate*	*Application*
CD1d	3C11	Biotin	Flow cytometry
CD3ε	145-2C11	APC	Cell sorting
	17A2	APC	Flow cytometry
CD4	GK1.5	unconjugated	In vivo depletion
	RM 4-5	Alexa Fluor 405	Flow cytometry
CD8α	53-6.7	APC	Flow cytometry
	53-6.7	PE	Flow cytometry
	53-6.7	PerCPCy5.5	Flow cytometry
CD11c	N418	APC	Flow cytometry
	N418	FITC	Flow cytometry
CD54R (B220)	RA3-6B2	PE	Immunohistochemistry
CD80	MCA1586F	APC	Flow cytometry
CD86	GL-1	APC	Flow cytometry
H2-K^b (MHC class I)	AF6-88.5.5.3	PE	Flow cytometry
I-A^b (MHC class II)	M5/114.15.2	PE	Flow cytometry
NK1.1	PK136	PECy7	Cell sorting,
	PK136	APC	Flow cytometry
Streptavidin		PE	Flow cytometry
		Alexa405	Flow cytometry

3.1.7 Mouse strains

C57BL/6(N) wild type strains (H-2K^b) were purchased from Charles River, Harlan, or Janvier, France. Mice were bred under specific pathogen-free conditions and in accordance to institutional animal guidelines in the animal facility (HET, House of Experimental Therapy) of the University of Bonn. Mice were used at 8-12 weeks of age. Following knock-out (ko) or transgenic animals backcrossed on C57BL/6 were used:

Mouse strain	*Description*
Bm1	This mouse strain has a point mutation in their MHC class I molecule that prevents presentation of the OVA-peptide SIIFEKL to CD8+ T cells.
CCL17-eGFP	This mouse strain expresses an eGFP knock-in construct under the control of the CCL17 promotor and can therefore be used as CCL17 reporter mice. Heterozygous animals can still produce CCL17, albeit at reduced amounts. Homozygous CCL17-eGFP mice can also be employed as CCL17 reporters but do not produce the chemokine, permitting their use as CCL17 ko mice.
CCR4ko	This mouse strain is deficient for the chemokine receptor CCR4.
CCR5ko	This mouse strain is deficient for the chemokine receptor CCR5.
CCR4ko OT-I	This OT-I mouse strain lacks the CCR4 locus.
MHCIIko	This mouse strain is deficient in the H2-A^{b}1 locus and does not have any functional CD4$^+$ T cells due to developmental defects.
CD1dko	This mouse strain is deficient in the CD1d locus and thus lacks NKT cells due to developmental defects.
CD8ko	This mouse strain lacks CD8$^+$ T cells due to developmental defects.
IL-4ko	This mouse strain is deficient for the cytokine IL-4.
OT-I RAG	This mouse strain bears a transgenic T cell receptor that recognizes the OVA 257-264 peptide (SIINFEKL) in H-2K^b molecules. The line is backcrossed on

a RAG-deficient background, leading to a defect in somatic recombination of TCR segments. Hence, this mouse contains T cells that almost exclusively have antigen specificity for the SIINFEKL peptide.

3.2 Methods

3.2.1 Experimental treatment of mice

Cells and reagents were adjusted in PBS for experimental injection. Intravenous (i.v.) and intraperitoneal (i.p.) injections were performed with a volume of 200 µl, subcutaneous injections (s.c.) in the tail basis in a volume of 100 µl.

Soluble ovalbumin (OVA) was injected intravenously (10 µg per gram body weight), accompanied when appropriate by 0.2 µg (1 nmol) of αGalCer, 1 nmol of iGb3, aGalDAG, OCH, GSL1´ or 20 µg of CpG oligodeoxynucleotides. αGalDAG was a gift from Patrick Perlmutter (Monash University, Clayton, Australia) and prepared by an adaptation of a pubished method (**59**). GSL1´was a gift from Paul Savage (Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah, USA) and was synthesized as published (**60**).

3.2.2 Isolation and transfer of primary murine cells

3.2.2.1 Isolation of splenic dendritic cells

Spleens were removed and digested by perfusion with 0,4 mg of collagenase and DNAse per ml of PBS. After incubation for 20 minutes at 37°C, spleens were homogenized through a metal cell strainer and resuspended in cell culture medium or PBS + 0,1% BSA. Cells were centrifuged for 5 min at 1200 rpm and the supernatant was discarded. For DC purification, the cell pellet was resuspended in 600 µl of MACS buffer containing 20 µl of anti-CD11c conjugated magnetic microbeads per spleen and the sample was incubated for 15 min at 4°C. Afterwards, MACS buffer was added and cells were centrifuged for 5 min at 1200 rpm in order to remove unbound magnetic beads. Cells were then resuspended in 3 ml of MACS buffer and purified via magnetic columns, which had been equilibrated with 3 ml of MACS buffer before. Elusion of cells from the columns was performed with 4 ml of MACS buffer.

Depending on the experiment, DCs were left untreated or pulsed with SIINFEKL peptide at a concentration of 20 µg per ml for 20 minutes at 37°C.

3.2.2.2 Isolation of CD8⁺ T cells

For the isolation of $CD8^+$ T cells from spleen and lymph nodes, the respective lymphoid organs were extracted and homogenized through a metal cell strainer. After centrifugation for 5 min at 1200 rpm, $CD8^+$ T cells were isolated by magnetic cell sorting as described above (3.2.2.1) sorting using anti-CD8 conjugated magnetic microbeads.

3.2.2.3 Isolation of splenic NKT cells

A single cell suspension from spleens was prepared by using a metal cell strainer. CD19 positive cells were excluded from suspensions by anti-CD19 conjugated magnetic microbeads as described above (3.2.2.1) before staining NKT cells with anti-CD3 and anti-NK1.1 antibodies for flow cytometry based cell sorting, isolating CD3+ NK1.1+ cells.

3.2.2.4 Cell counting

For determination of cell numbers, cell suspensions were diluted in Trypan Blue solution (10 µl in 190 µl), and 10 µl were applied to a Neubauer counting chamber. Four large squares of the chamber were counted, with only viable (unstained) cells being considered. The total cell number was calculated by using the following formula:

(Counted cell number)/4 x 20 *(dilution factor)* x 10,000 *(chamber factor)*

3.2.3 In vitro cross-priming assay

3.2.3.1 In vitro assay

DCs and $CD8^+$ T cells were isolated from spleen as described above (see 3.2.2.1 and 3.2.2.2). 1,5 x 10^5 T cells were labeled with CFSE (see 3.2.7) and cocultured with DCs at a ratio of 3:1 or anti-CD3 plus anti-CD28 beads in 96-well plates. Before coculture, DCs were pulsed with SIINFEKL peptide at a concentration of 20 µg per ml for 20 minutes at 37°C. Cells were cultured in a total volume of 200 µl at 37°C, with a relative humidity of 90% and a CO_2 content of 5%. After 24 h, 100 µl of supernatant were removed for ELISA analysis

(see 3.2.4). After three days of coculture, activation of $CD8^+$ T cells was assessed by CFSE dilution (see 3.2.7).

3.2.3.2 Detection of IFNγ by enzyme-linked immunosorbent assay (ELISA)

Cytokine production was measured by using an IFNγ-specific sandwich ELISA. Therefore, a 96-well ELISA microtiter plate was coated with 50 µl of the primary unconjugated capturing antibody per well (1:180 in coating buffer) overnight at 4°C. Plates were washed twice with 0.05% Tween20 in PBS before free binding sites were blocked with 100 µl of 1% BSA/PBS per well for one hour at room temperature. Afterwards, 50 µl of each sample were applied to the plate. In addition, a cytokine standard was prepared in order to allow quantification of the cytokine concentration after measurement. Usually, the standard was determined at least in duplicate and a two-fold dilution series was used, starting at a concentration of 8 ng per ml. After incubation at 4°C over night, plates were washed three times with 0.05% Tween20 in PBS and 50 µl of a biotinylated goat anti-mouse IFNγ antibody (1:180 in coating buffer) were added per well. After two hours of incubation at room temperature, plates were washed extensively and then incubated for at least 45 minutes with 50 µl per well of streptavidin-couples horseradish peroxidase (1:5000 in PBS), followed by three washing steps. Afterwards, OPD buffer supplemented with OPD (1 mg per ml) and H_2O_2 (1µl per ml) was added at a volume of 100 µl per well and the reaction was stopped with a 1M solution of H_2SO_4 as soon as colour development had reached saturation in one of the samples. Finally, peroxidase-mediated colour intensity was detected using an ELISA plate reader (wave length: 490 nm).

3.2.4 CFSE proliferation assay of $CD8^+$ T cells

For fluorochrome labeling of $CD8^+$ T cells, OT-I cells or endogenous polyclonal $CD8^+$ T cells were isolated from mice as described (see 3.2.2.2). Single-cell suspensions were then treated with erythrolysis buffer for 5 minutes and isolated via MACS purification. For T cell labeling, 10^6 cells per ml were taken up in PBS and 5 µM (for *in vivo* use) or 1 µM (for *in vitro* use) of CFSE were added for 10 minutes at 37°C. Afterwards, the staining reaction was stopped by adding excess PBS and cells were washed twice with PBS and centrifuged for 5 minutes at 1200 rpm. For transfer into 96-well plates or animals, cell number was adjusted in PBS appropriately.

3.2.5 In vivo cytotoxicity assay

In vivo cytotoxicity assays were performed as previously described (**61**). Briefly, spleen suspensions were pulsed for 15 min at 37 °C with OVA peptide (SIINFEKL; 2 µg/ml) and labeled with 1 µM CFSE (CFSEhi cells) or were not pulsed with peptide and were labeled with 0.1 µM CFSE (CFSElo cells). Both target celltypes ($0{,}5x10^7$ each) were injected intravenously. After 4 h, the survival of target cells in the spleen was analyzed by flow cytometry. Specific lysis was calculated with the following formula: % specific cytotoxicity = 100 − ((CFSEhi / CFSElo) primed / (CFSEhi / CFSElo) control)x100.

3.2.6 Flow cytometry

3.2.6.1 Staining of surface molecules

To assess the expression of surface markers, respective organs were harvested from mice, digested with collagenase and DNAse when appropriate (if DCs were to be analyzed, see 3.2.2.1), and homogenized through a metal cell strainer. After centrifugation for 5 minutes at 1200 rpm, erythrocyte lysis was performed by incubation of erythrolysis buffer for 5 minutes. The reaction was stopped by adding excess PBS and subsequent centrifugation for 5 minutes at 1200 rpm. Subsequently, 50 µl of blocking serum was added and samples were incubated for 15 minutes on ice in order to fill unspecific binding sites and Fc receptors. After further centrifugation, staining was performed for 20 minutes on ice. Antibodies listed in Table 3.1 were diluted in FACS buffer (1:200 if not indicated otherwise). The staining reaction was stopped by adding excess FACS buffer and centrifugation for 5 minutes at 1200 rpm. Finally, the cell pellet was taken up in FACS buffer and analyzed by flow cytometry.

3.2.6.2 Intracellular cytokine staining

Intracellular staining for IFNγ was performed in order to assess T cell activation *in vivo*. Three days after immunization, T cells were isolated from spleen as described above (see 3.2.2.2), taken up in cell culture medium. Afterwards, SIINFEKL peptide was added at a concentration of 20 µg per ml in order to restimulate $CD8^+$ T cells after isolation. Moreover, GolgiPlug reagent was added at a concentration of 1 µl per ml to prevent secretion of newly synthesized cytokines, but rather lead to intracellular cytokine storage. The mixture was incubated for four hours at 37°C. The cells were then washed with PBS and stained with a PE-coupled anti-mouse CD8 antibody (1:200 in FACS buffer), supplemented with blocking serum (1:200) to prevent unspecific binding. After 20

minutes, the suspension was centrifuged for 5 minutes at 1200 rpm and subsequently exposed to 100 µl of fixation reagent containing paraformaldehyde (2% in PBS) for 15 minutes at room temperature. Importantly, exposure to light was avoided as much as possible. Cells were then washed again and taken up in saponin buffer (0.5% of saponin in FACS buffer) and cell membranes were made permeable for 20 minutes at room temperature, followed by centrifugation. Staining was performed with an APC-coupled anti-mouse IFNγ antibody that was diluted 1:200 in saponin buffer and also supplemented with blocking serum. The reaction took place at room temperature for 30 minutes. Afterwards, cells were washed twice in saponin buffer and one more time with FACS buffer before being taken up in 300 µl of FACS buffer for flow cytometric analysis.

3.2.6.3 S8L/H-2K^b-specific tetramer staining

The tetramer staining reagent was used to determine the amount of T cells specific for SIINFEKL peptide and hence their activation after immunization with soluble ovalbumine or the transfer of SIINFEKL-presenting DCs. To this end, splenic CD8$^+$ T cells were isolated as described (see 3.2.2.2) and resuspended in a staining solution that contained APC-coupled S8L/H-2K^b-specific tetramers and blocking serum in FACS buffer. After an incubation time of 20 minutes at room temperature, a PE-coupled anti-mouse CD8 antibody was added (1:200 in FACS buffer) and further incubation was performed on ice for another 20 minutes. Two washing steps with FACS buffer and flow cytometric analysis then followed the staining.

3.2.7 Generation of mixed bone marrow chimeras

Bone marrow cells were obtained from femurs of donor mice. Mice were irradiated with 9 Gy from a ^{137}Cs source and then were injected intravenously with a 1:1 mixture of a total of 1x10^7 bone marrow cells. The ratio of bone marrow types used was always 50%+/-10% of each type at 8 weeks after transplantation.

3.2.8 Immunohistochemistry

3.2.8.1 Isolation and labeling of CD8$^+$ T cells

CCR4-deficient or CCR4-sufficient OT-I T cells or endogenous CD8$^+$ T cells were isolated from spleens as described above (see 3.2.2.2). After magnetic separation, T cells were

resuspended in 5 ml of PBS in a 15 ml tube and 2.5 µl of FarRed staining solution (DDAO-SE, 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)-succinimidyl ester) were added into the lid of the tube, which was carefully inverted thereafter. Due to potential cytotoxicity of the reagent, cells were stained for no longer than 20 minutes at 37°C. The reaction was stopped by adding 7 ml of ice-cold PBS and followd by centrifugation at 1200 rpm for 6 minutes. After resuspension in PBS, the cell number was adjusted to $12.5 \cdot 10^6$ cells per ml of PBS. Finally, 200 µl ($2.5 \cdot 10^6$ cells) were injected intravenously. 16 hours after adoptive transfer, antigen and adjuvant were administered i.v. where appropriate.

3.2.8.2 Preparation of cryosections

10 hours after immunization, spleens were isolated, briefly rinsed in ice-cold PBS and directly embedded in Tissue-Tek®. Afterwards, tissue blocks cooled down to -20°C and could be stored for up to a week or longer if transferred to -80°C. Cryosections of a width of 5 µm were prepared from frozen tissue blocks by using a microtome/cryostat at a working temperature of -18°C. In order to avoid staining of the same cells in different sections, at least two sections between individual slides were discarded.

3.2.8.3 Immunofluorescence staining

Sections were fixed with iced acetone for 10 minutes at 4°C and afterwards air-dried for some minutes. Fixation time did not exceed 10 minutes, in order to avoid a loss of tissue integrity. Blocking solution (1% BSA in PBS) was applied for one hour at room temperature, stained with anti-B220-PE antibody (diluted 1:200 in blocking solution) for one hour and then washed twice with PBS. Finally, sections were covered with mounting medium and cover slides, and could be stored for up to a week if not exposed to light.

3.2.8.4 Quantification of cell numbers

Stained sections were viewed and images were captured with a fluorescence microscope. T cell numbers were counted (utilizing the “Touch count” mode) in T cell areas (determined as “closed polygons”) with Cell F software (Olympus). In addition, as distinct labels for DCs and CTLs were used, intercellular contacts were enumerated. Both cell types were considered to be in direct contact when fluorescence signals partially overlapped or were directly adjacent to one another.

3.2.9 *In vitro* analysis of CD8+ T cell recruitment by DCs

CD8+ T cells and DCs obtained from the spleen were labeled with different fluorochromes and mixed at a ratio of 4:1 in RPMI medium containing 2% (vol/vol) FCS and then were placed on plastic channel slides (μ-slide; Ibidi) coated with fibronectin (Harbour Bio-Products). Time-lapse series were recorded with a fully automated inverted Olympus Fluoview 1000 confocal microscope equipped with motorized *xyz* stage (Märzhäuser) and a climate chamber (37 °C and 5% CO2 with humidity). Up to eight samples were analyzed simultaneously with the multipoint function of the microscope, which allowed use of the same conditions for all probes. Cell motility and cell-cell interactions were monitored over a period of 2 h by capture of fluorescence and differential interference contrast images every 2 min with a 0.75 Plan S Apo 20x objective (Olympus). In each experiment, 100–300 motile CD8+ T cells were tracked with the Manual Tracking plug-in of ImageJ software (National Institutes of Health). The directionality of migrating CD8+ T cells before physical contact with DCs, as well as the duration of cell-cell interactions, was calculated with the Chemotaxis and Migration Tool plug-in (Ibidi) of ImageJ software. For analysis of directionality, the ratio of euclidean to accumulated distance of individual cell tracks was calculated (sample of analysis, **Fig. 3.2.9.1**). CD8+ T cell directionality and CD8+ T cell-DC contact in high resolution was also analyzed as a time series with a 0.9 ultraviolet-transmitting Plan Apo 40x objective (Olympus).

3.2.10 Transwell cell-migration assay

Splenocytes ($1x10^6$) from aGalCer-injected wild-type mice were loaded into the top chamber of Transwell inserts (pore size, 5 μm; Costar). Bottom wells were filled with RPMI medium containing 2% (vol/vol) FCS with or without CCL17 (R&D Systems). Cells in the lower chambers were collected after 6 h at 37 °C and transmigrated CD8+ T cells were counted by flow cytometry with antibody staining and the addition of constant numbers of CaliBRITE beads (BD Biosciences).

3.2.11 Real-time reverse-transcription PCR

RNA was isolated with the RNeasy Micro Kit (Qiagen), then cDNA was synthesized with random hexamer primers. An ABI Prism 7000 Sequence Detection system (Applied Biosystems) was used for RT-PCR with the following settings: 40 cycles of 15 s of denaturation at 95 °C, and 1 min of primer annealing and elongation at 60 °C as described (**62**). RT-PCR was done with 1.5 μl cDNA plus 2.5 μl (0.9 μM) specific primers and 12.5 μl

of 2°— Platinum SYBR Green qPCR SuperMix (Invitrogen), and the following primers: mCCL17 1 (5-TGGTATAAGACCTCAGTGGAGTGTTC-3´) and mCCL17 2 (5-GCTTGCCCTGGACAGTCAGA-3); and mCCL22 1 (5-GAGTTCTTCTGGACCTCAAATCC-3) and mCCL22 2 (5-TCTCGGTTCTTGACGGTTATCA-3); mCCL3 1 (TCTGTCACCTGCTCAACATCAT) and mCCL3 2 (CGGGGTGTCAGCTCCATA); mCCL4 1 (AAACCTAACCCCGAGCAACA) and mCCL4 2 (CCATTGGTGCTGAGAACCCT); mCCL5 1 (GCTGCTTTGCCTACCTCTCC) and mCCL5 2 (TCGAGTGACAAACACGACTGC).

All samples were run in duplicates and results were normalized to those of 18S rRNA or GAPDH.

3.2.12 Statistical analysis

Comparisons were made with the Mann-Whitney or Kruskal-Wallis test and Dunn's post-test, Student´s t-test, or one-way ANOVA and Dunnets, Bonferroni or Tukey post-test using Prism 5 software (Graphpad Software).

4 Results

4.1 NKT cell-mediated DC licensing

4.1.1 Cognate NKT cell-mediated licensing is independent of CD4+ help

Naïve $CD8^+$ T cells need several stimulatory signals provided by a cross-presenting DC for their proper activation. DCs on the other hand only gain their full cross-priming capacity after they have successfully presented antigen to a $CD4^+$ T_H cell and have received activating signals in return. This process has been termed DC licensing (**21**). In addition to classical licensing by $CD4^+$ T_H cells, NKT cells have also been demonstrated to be able to mature DCs that present glycolipid antigens like αGalCer on CD1d (**26-28**). In the present study, the stimulatory effect of NKT cells on cross-priming was confirmed in our model by co-injecting the NKT cell ligand αGalCer with the cross-presented antigen OVA (**Fig. 4.1-1a**). The positive effect of αGalCer is mediated by NKT cells as CD1d-/- mice that lack NKT cells showed no increased T cell response (**Fig. 4.1-1b**). Importantly, the boosting effect is independent of $CD4^+$ T cell help, as MHCII-/- mice that lack classical MHC-II-restricted $CD4^+$ T cells, showed a $CD8^+$ T cell response similar to wild-type mice, demonstrating that classical and NKT cell mediated DC stimulation represent two distinct pathways (**Fig. 4.1-1b**). To investigate whether the same DC that presents αGalCer and is thus licensed by an NKT cell, has to cross-present OVA to a $CD8^+$ T cell for efficient cross-priming, mixed bone marrow-chimeras were generated. As recipients, bm1 mice were used that have a point mutation in their MHC-I molecule and hence cannot present OVA-peptides to $CD8^+$ T cells. These mice were irradiated and reconstituted with a 1:1 mixture of bone marrow from CD1d-/- and bm1 mice, generating chimeras that possess DCs that can only either present to $CD8^+$ T cells via MHC-I or to NKT cells via CD1d (**Fig. 4.1-1c cartoon**). In these mixed bone marrow chimeras, αGalCer did not enhance proliferation of adoptively transferred OVA-specific $CD8^+$ T cells from OT-I transgenic mice, in contrast to the control group that was reconstituted with a 1:1 mixture of wild-type and bm1 bone marrow and thus has DCs that could present to both cell types simultaneously (**Fig. 4.1-1c**). These results demonstrate that NKT cells can license DCs independent of $CD4^+$ T cell help.

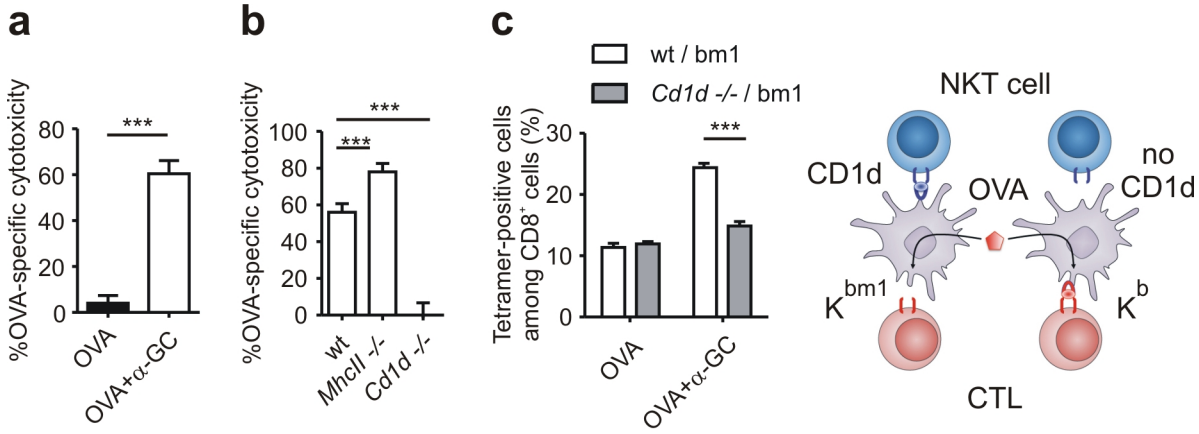

Fig. 4.1-1 Cognate NKT cell-mediated DC licensing. OVA-specific cytotoxicity in the spleen on day 5 after priming wild-type (wt), MHCII-/- or CD1d-/- mice with soluble OVA with or without αGalCer (α-GC) (**a,b**). Flow cytometric analysis of OT-I cells among splenic $CD8^+$ T cells from bm1 mice reconstituted with 50% bm1 bone marrow and 50% wild-type or CD1d-/- bone marrow 8 weeks before, analyzed 3 days after adoptive transfer of $1x10^6$ OT-I cells and priming with OVA with or without αGalCer. Cartoon depicts to which cell types DCs can present antigen in CD1d-/- / bm1 chimeras (**c**). Data are representative of two individual experiments (mean and s.d. of three to four mice per group). *** $P < 0.001$ (Student´s t-test (**a**), one-way ANOVA + Dunnets post test (**b**), one-way ANOVA + Bonferroni multiple comparison post test (**c**)).

4.1.2 NKT cell mediated cross-priming is regulated by CCR4

Classical licensing is regulated by CCR5 and its ligands CCL3, CCL4 and CCL5, which are produced by DC and $CD4^+$ T cells upon successful antigen-presentation under inflammatory conditions (**57**). To investigate whether NKT cell mediated DC licensing is regulated by these same chemokines, CCR5-deficient mice were challenged with OVA and αGalCer. However, CCR5-deficient mice gave the same cytotoxic T cell response as wild-type mice (**Fig. 4.1-2a**). Surprisingly, cross-priming was severely attenuated in CCL17-deficient and CCR4-deficient mice (**Fig. 4.1-2a,b**), although this chemokine receptor has so far been associated with $CD4^+$ T_H cell responses in allergy and hypersensitivity rather than with naïve $CD8^+$ T cell priming. The reduced cross-priming was not due to intrinsic $CD8^+$ T cell activation-defects in the knockout animals as polyclonal $CD8^+$ T cells from these mice had the same response as wild-type $CD8^+$ T cells to stimulation with anti-CD3/anti-CD28 beads (**Fig. 4.1-2c**). Thus, classical and alternative NKT cell mediated cross-priming are regulated by separate chemokines.

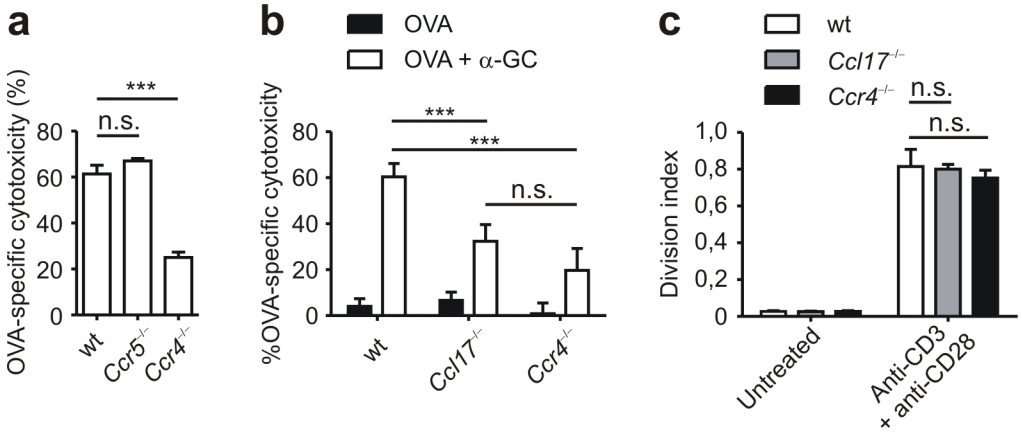

Fig. 4.1-2 NKT cell mediated cross-priming is regulated by CCR4 instead of CCR5. OVA-specific cytotoxicity in the spleen on day 5 after priming wild-type (wt), CCR5-/-, CCR4/- or CCL17-/- mice with soluble OVA with or without αGalCer (**a, b**). Division indices of endogenous $CD8^+$ T cells of wild-type (wt), CCL17-deficient (CCL17-/-) or CCR4-deficient (CCR4-/-) mice, labeled with the cytosolic dye CFSE and left untreated or stimulated with anti-CD3 plus anti-CD28 for 2 days (**c**). Data are representative of three experiments (mean and s.d. of three to four mice per group). *** $P < 0.001$ (one-way ANOVA + Dunnets post test (**a**), one-way ANOVA + Bonferroni multiple comparison post test (**b, c**)).

4.2 Activated NKT cells induce CCL17 expression in splenic DCs

4.2.1 Characterization of CCL17-producing cells in the spleen

The finding that CCL17 regulates NKT cell-mediated cross-priming was surprising as this chemokine had been described to be absent from the spleen even after systemic challenge with various TLR-ligands (**45**). To investigate CCL17 induction after αGalCer treatment, heterozygous (CCL17+/-) and homozygous (CCL17-/-) CCL17-eGFP knock-in mice were used that express GFP under the CCL17 promoter (**45**). These CCL17-reporter mice were challenged with αGalCer and spleen sections were analyzed for CCL17 expression 20 h later by immunohistochemistry. B220-staining enabled discrimination of B cell zones from T cell zones. Upon analysis, the sections demonstrated strong GFP signal in splenic T cell zones of αGalCer-treated mice (**Fig. 4.2-1a**). To further investigate which cells produce CCL17, the phenotype of CCL17-expressing cells was determined by flow cytometry and it could be observed that CCL17 expression is highly DC-restricted as previously described (**45**), with mainly $CD8^+$ (16,4%) but also some $CD8^-$ DC (6,4%) expressing this chemokine (**Fig. 4.2-1b**). Notably, other NKT cell ligands were also able to induce CCL17-eGFP expression in splenic DCs in the following order of potency: aGalCer > OCH > iGb3 > aGalDAG > GSL1´ (**Fig. 4.1-2c**). To investigate whether the same DC that interacts with both NKT and $CD8^+$ T cells (**Fig. 4.1-1b**) also needs to produce CCL17 for efficient cross-priming, mixed bone marrow chimeras were created that received bm1 and CCL17-deficient bone marrow in a 1:1 ratio (**Fig. 4.2-1d cartoon**). In these mice,

αGalCer-treatment failed to enhance cross-priming of adoptively transferred OT-I cells, whereas it did enhance the $CD8^+$ T cell response in control chimeras that received a 1:1 mixture of wild-type and bm1 bone marrow. These control chimeras have DCs that can present to both NKT and $CD8^+$ T cells and can also produce CCL17 (**Fig. 4.2-1d**). These results indicate that CCL17 is rapidly induced by DCs after NKT cell activation, and is only able to enhance cross-priming when it is produced by the cross-presenting DC.

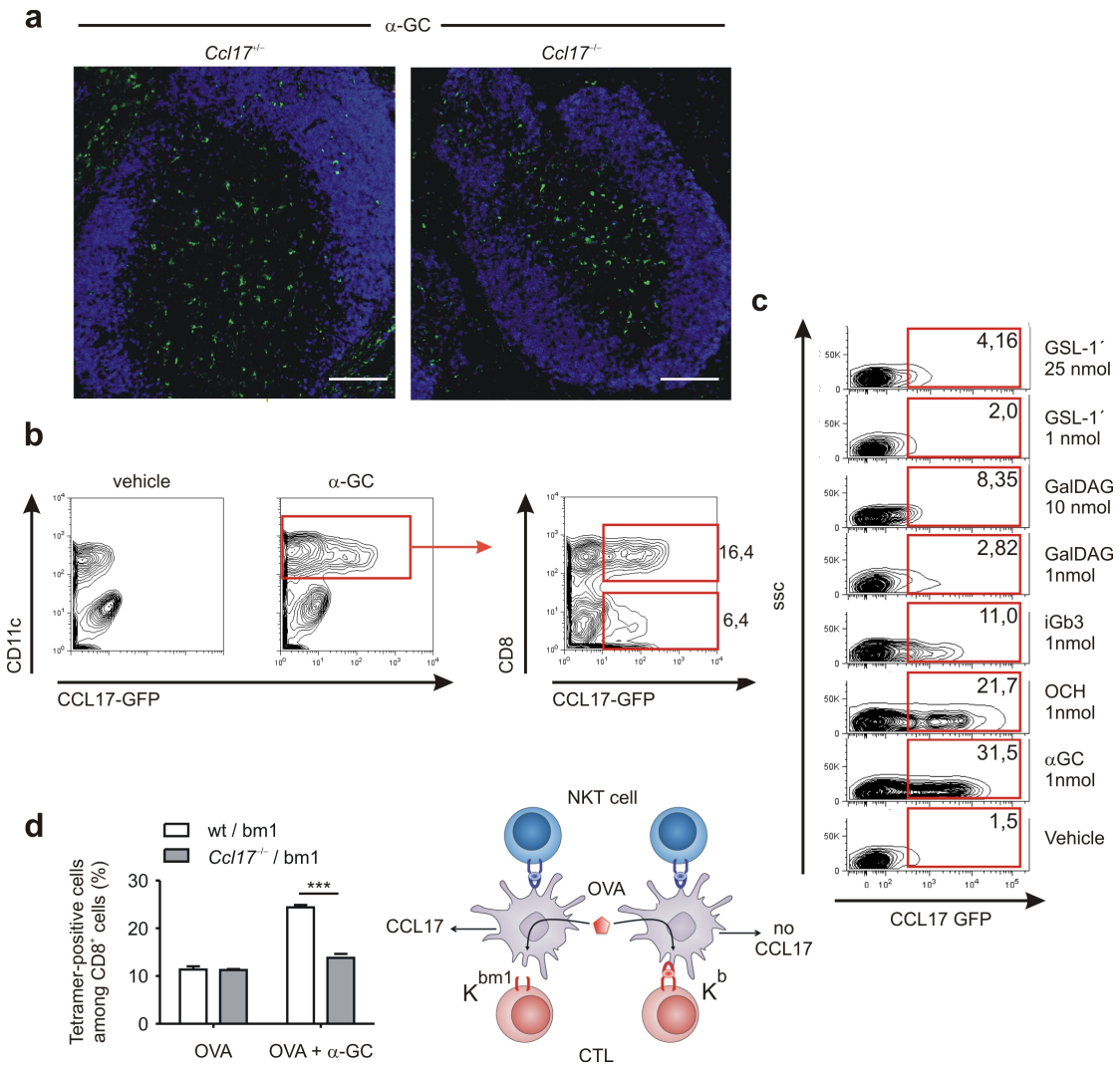

Fig. 4.2-1 CCL17 induction in splenic DCs. Immunofluorescence staining of B cell zones (blue: B220) in heterozygous CCL17-eGFP reporter mice (CCL17+/-) or CCL17-deficient mice (CCL17-/-) after injection of αGalCer (α-GC); scale bars 200µm (**a**). Flow cytometry of spleen cells from CCL17-eGFP reporter mice 5 h after injection of vehicle or αGalCer (α-GC), with gating of $CD11c^+$ cells (red box left) followed by analysis of the expression of CD8 and CCL17 (red boxes right) (**b**). Flow cytometry of spleen cells from CCL17-eGFP reporter mice 20 h after injection of vehicle, αGalCer, OCH, iGb3, GalDAG and GSL1´ (**c**). Flow cytometric analysis of the proportion of OT-I cells in splenic $CD8^+$ cells of bm1 mice reconstituted with 50% bm1 bone marrow and 50% CCL17-deficient (CCl17-/- / bm1) or wild-type bone marrow (wt / bm1) 8 weeks before, analyzed on day 3 after adoptive transfer of $1x10^6$ OT-I cells and priming with OVA plus αGalCer. Cartoon depicts the abilities of DCs to present antigen and produce CCL17 in CCL17-/- / bm1 chimeras (**d**). Data are representative of two experiments (mean and s.d. of three to four mice per group in each). *** $P < 0.001$ (one-way ANOVA + Bonferroni multiple comparison post test (**d**)).

4.2.2 Mechanism of CCL17 induction by NKT cells

As CCL17 production is induced in an NKT cell dependent fashion within three hours of NKT cell ligand injection (data from Veronika Lukacs-Kornek, not shown), I hypothesized that the rapid cytokine production by activated NKT cells might induce CCL17 production in DCs. NKT cells are known to produce high levels of IL-4, IL-13, IFNγ and TNFα and upregulate CD40L. The production of IL-4, IFNγ and TNFα by NKT cells 5 h after injection of αGalCer was confirmed by using a fluorescent bead immunoassay (Bender MedSystems) that allows detection of IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, IFNγ and TNFα (**Fig. 4.2-2a**). Subsequently, the influence of these factors on CCL17 production was tested by culturing DCs that had been obtained from CCL17-eGFP-reporter mice, with different concentrations and combinations of IL-4, IL-13, IFNγ, TNFα and a stimulatory anti-CD40 antibody. Flow cytometric analysis revealed that already low concentrations of IL-4 induced CCL17 production in around 20% of DCs after 15 h of co-culture (**Fig. 4.2-2b**). IL-13 also induced some CCL17 but only at higher concentrations and not to the same degree as IL-4 (**Fig. 4.2-2b**). In comparison IFNγ did not induce CCL17, but inhibited its production induced by IL-4 stimulation (**Fig. 4.2-2b,c**). TNFα alone slightly increased CCL17 expression but to a very low degree and the biological relevance of this effect seems doubtful. However, TNFα did increase the percentage of CCL17-producing DCs induced by NKT cell-derived IL-4 or IL-13 (**Fig. 4.2-2b,c**). Treatment with anti-CD40 did not result in CCL17 expression, and it could not significantly improve IL-13 and TNFα-induced CCL17 production (**Fig. 4.2-2b,c**). In summary, CCL17 production can be induced by IL-4 and to some degree by IL-13, which can be further upregulated by the additional presence of TNFα (**Fig. 4.2-2d**). Nevertheless it seems likely that different cytokines can act redundantly because IL-4, although it is a potent inducer of CCL17, is not essential for its production *in vivo*: IL-4-deficient mice did not display the same cross-priming defect as CCL17-deficient mice but gave a similar response to wild-type mice (**Fig. 4.2-2e**). Additionally, CCL17 mRNA was present in both wild-type and IL-4-deficient DCs after αGalCer-treatment as determined by RT-PCR (**Fig.4.2-2f**). Taken together these results indicate that expression of CCL17 can be induced by IL-4. Nevertheless, CCL17 production seems to be a redundant process in which other cytokines can substitute for IL-4.

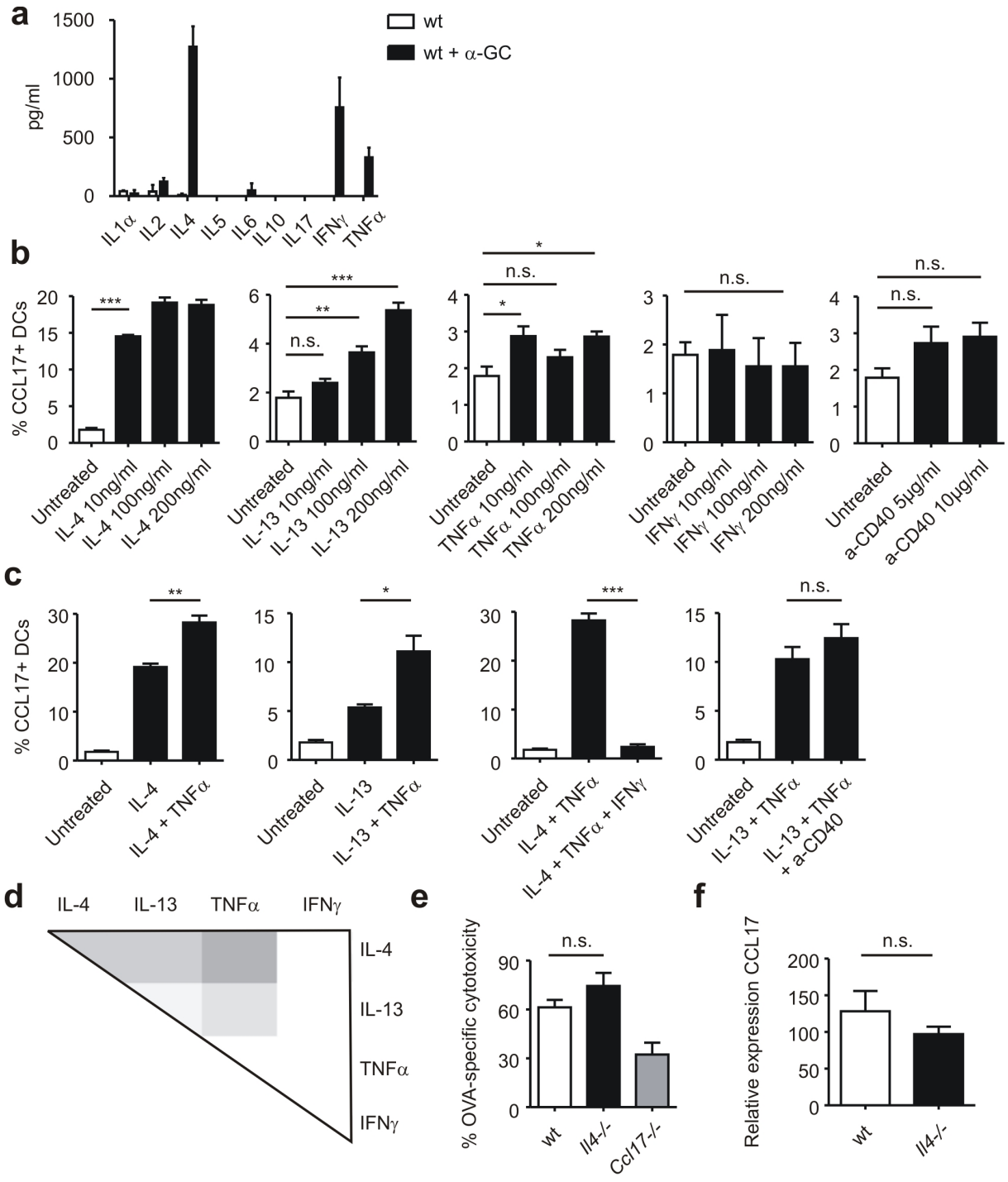

Fig. 4.2-2 Mechanisms of CCL17 induction. Flow cytometric analysis of a fluorescent bead immunoassay (Bender MedSystems) of NKT cell supernatant from 5x10^5 cells isolated from wild-type mice injected or not with 0,2µg αGalCer 5 h previously (**a**). Flow cytometric analysis of CCL17 expression by DCs isolated from CCL17eGFP-reporter mice and cultured for 16 h with IL-4, IL-13, TNFα, IFNγ or anti-CD40 (a-CD40) antibody alone (**b**) or in combinations as indicated in (**c**). Summary of the potential of the indicated cytokines to induce CCL17 in splenic DCs; different levels of gray represent different percentages of CCL17-producing DCs in steps of 5% (**d**). OVA-specific cytotoxicity in the spleen on day 5 after priming wild-type (wt), IL-4-/- or CCL17-/- mice with soluble OVA with αGalCer (**e**). CCL17 mRNA expression in wild-type and IL-4-deficient DCs 20 h after injection of αGalCer, presented relative to GAPDH RNA expression (**f**). Data are representative of three individual experiments (mean + s.d. of three to four mice per group). **P* < 0.05 ***P* < 0.01 *** *P* < 0.001 (one-way ANOVA + Dunnets post test (**b,e**), one-way ANOVA + Tukey post test (**c**), Mann-Whitney (**f**)).

4.3 Effect of CCL17 on DCs and NKT cells

4.3.1 CCL17 does not alter the cross-priming ability of DCs

As the boosting effect of αGalCer on $CD8^+$ T cell responses has so far been associated with the expression of costimulatory molecules on NKT cell-stimulated DCs, I first examined the influence of CCL17- and CCR4-deficiency on the upregulation of these molecules. For this purpose DCs were isolated from wild-type, CCL17-deficient or CCR4-deficient mice that had been injected with αGalCer or vehicle control 14 h previously and the expression of costimulatory molecules was determined using flow cytometry. Expression of CD80, CD86 and CD40 were unaltered in the absence of CCR4 and CCL17 (**Fig. 4.3-1a**), indicating that "signal 2" is not influenced by CCL17. Concerning "signal 3" we knew from previous experiments performed by Veronika Lukacs-Kornek (data not shown), that IL-12 production is indeed dependent on CCR4 signaling as CCL17- and CCR4-deficient DCs produced less IL-12. Nevertheless, p35-/- mice that lack the p35 subunit, which heterodimerizes with the p40 subunit to form functional IL-12 (**63**), displayed a similar T cell response as wild-type mice (data not shown). These results indicate that αGalCer enhances cross-priming independent of IL-12.

To directly investigate if CCL17 influences the stimulatory capacity of DCs, splenic DCs from αGalCer-injected wild-type or CCR4-deficient mice were loaded with the OVA peptide SIINFEKL and co-cultured with OT-I cells. Both DC types induced similar T cell proliferation and IFNγ-production, indicating that CCL17 does not directly alter the stimulatory capacity of DCs (**Fig. 4.3-1b**). To determine if NKT cells might have a CCL17-dependent direct stimulatory effect on $CD8^+$ T cell activation, NKT cells from αGalCer-injected wild-type or CCR4-deficient mice were isolated and cocultured with anti-CD3/anti-CD28-stimulated $CD8^+$ T cells. As the presence of either wild-type or CCR4-deficient NKT cells had no effect on T cell activation (**Fig. 4.3-1c**) it was concluded that NKT cells have no direct stimulatory effect on $CD8^+$ T cell activation but instead influence the cross-priming ability of DCs by inducing expression of costimulatory molecules and CCL17. In summary these data demonstrate that CCL17 does not enhance cross-priming by rendering DCs or NKT cells more stimulatory.

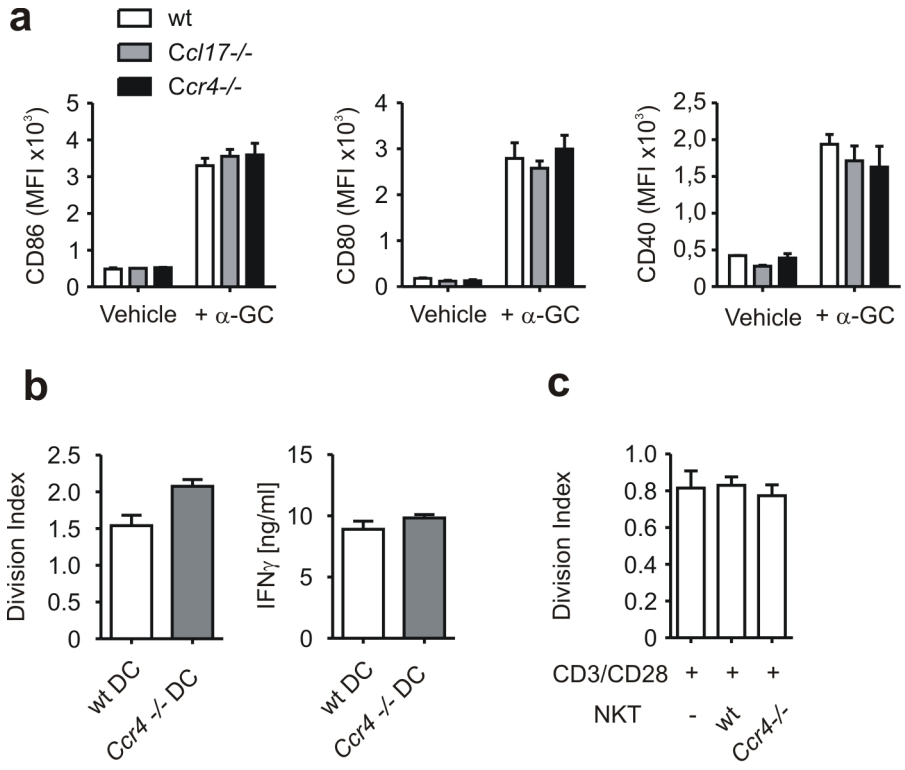

Fig. 4.3-1 CCL17 does not influence DC and NKT cell functions. Flow cytometric analysis of CD86, CD80 and CD40 expression on splenic DCs from wild-type (wt), CCL17-deficient (CCL17-/-) and CCR4-deficient (CCR4-/-) mice injected 14 h previously with αGalCer or vehicle (**a**). Division index (left) and IFNγ-concentration in culture supernatants (right) from 1.5x 10^5 OT-I cells cultured for 2 d together with 0.5x 10^5 wild-type or CCR4-deficient splenic DCs isolated from αGalCer-injected mice and loaded with OVA peptide (SIINFEKL) (**b**). Division indices of splenic $CD8^+$ T cells obtained from αGalCer-injected wild-type mice stimulated with anti-CD3 plus anti-CD28 beads (CD3/CD28) and cultured in the presence or absence of splenic NKT cells from wild-type or CCR4-deficient mice injected with αGalCer 5 h before (**c**). Data are representative of three to four experiments (mean + s.d. of three to four mice per group). MFI, mean fluorescent intensity.

4.3.2 CCL17 does not increase DC or NKT cell recruitment

Another explanation for enhanced cross-priming due to CCL17 could be enhanced numbers of cross-presenting DCs or DC-stimulating NKT cells at the site of T cell priming. Hence, DC and NKT cell numbers were determined in the spleen of mice that had or had not been treated with αGalCer 5 h previously. However, the results showed no significant increase in DC numbers after injection of αGalCer and unchanged DC numbers and subset compositions in CCR4- and CCL17-deficient spleens compared to wild-type mice (**Fig. 4.3-2a**). Similarly, NKT cell numbers were equally high in wild-type and CCR4-deficient spleens (**Fig. 4.3-2b**). Furthermore, similar percentages of mature DCs could be found in the spleens of wild-type and CCR4-deficient mice, indicating that CCL17 does not attract NKT cells to the spleen in order to increase the number of mature DCs (**Fig. 4.3-2c**).

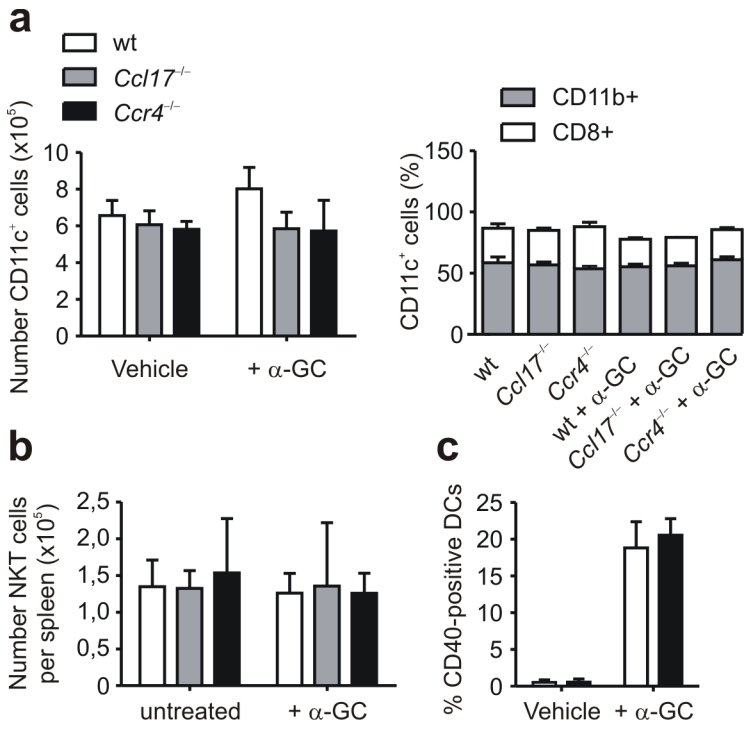

Fig. 4.3-2 CCL17 does not increase DC or NKT cell recruitment. Absolute DC numbers per spleen and proportions of $CD11b^+$ and $CD8^+$ DCs in wild-type (wt), CCL17-deficient (CCL17-/-) and CCR4-deficient (CCR4-/-) mice injected 5 h previously with αGalCer (+ α-GC) or vehicle. Total DC numbers per spleen were defined as 100% (**a**). Absolute NKT cell numbers per spleen in wt, CCL17-deficient or CCR4-deficient mice injected 5 h previously with αGalCer or vehicle (**b**). Flow cytometry of percentage of $CD11c^+$ cells coexpressing CD40 (**c**). Data are representative of three experiments (mean + s.d. of three to four mice per group).

4.3.3 CCL17 positively regulates its own production

Another factor that required investigation was chemokine production itself. Indeed, CCL17 mRNA levels were highly reduced in CCR4-deficient DCs as compared to wild-type DCs, indicating that CCL17 positively regulates its own production via CCR4 signaling (**Fig. 4.3-3a**). Interestingly, the alternate CCR4 ligand CCL22 was expressed by αGalCer-activated NKT cells, but not by DCs, in a CCR4-dependent manner (**Fig. 4.3-3b**). Nevertheless, CCL22 seemed to play no critical role in CCR4-mediated cross-priming as T cell cytotoxicity did not differ greatly between CCR4-deficient and CCL17-deficient mice (**Fig. 4.1-2b**). Hence this study concentrated on the role of CCL17. The fact that CCL17 production was the only factor to be altered in CCR4-deficient mice led us to believe that CCL17 might act directly on $CD8^+$ T cells, although CCR4 had so far not been associated with $CD8^+$ T cells.

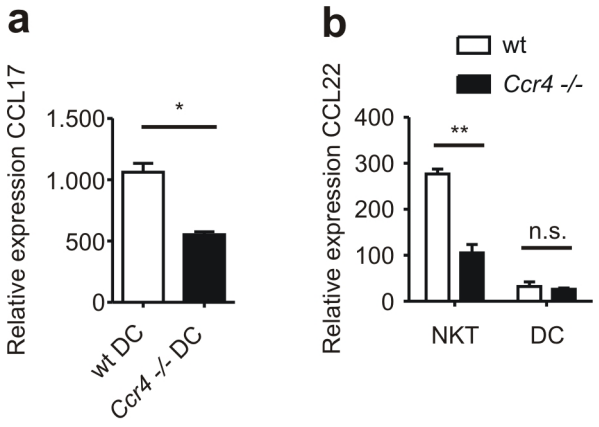

Fig. 4.3-3 CCL17 positively regulates its own production. CCL17 mRNA (**a**) and CCL22 mRNA (**b**) expression in wild-type and CCR4-deficient DCs or NKT cells 5 h after injection of αGalCer, presented relative to 18S RNA expression. Data are representative of two experiments (mean and s.d. of three mice per group) **P* < 0.05 ***P* < 0.01 *** *P* < 0.001 (student´s t-test (**a,b**)).

4.4 Effect of CCL17 on CD8⁺ T cells

4.4.1 CCL17 acts directly on CD8⁺ T cells

Naïve $CD8^+$ T cells are usually associated with the chemokine receptor CCR7, which coordinates positioning of naïve $CD8^+$ T cells in T cell zones. However a potential role for the T_H2-related receptor CCR4 has yet to be documented. Therefore, this issue was addressed through adoptive transfer of CCR4-competent or –deficient OT-I cells into wild-type mice, whereby an influence of CCL17 on $CD8^+$ T cells could selectively be excluded. Indeed, mice that had received CCR4-deficient OT-I cells showed significantly reduced cross-priming compared to mice that had received CCR4-competent OT-I cells (**Fig. 4.4-1a**). On the other hand, transfer of CCR4-competent OT-I cells into CCR4-deficient animals could restore cross-priming to similar levels as observed in wild-type mice (**Fig. 4.4-1a**). This finding did not result from intrinsic activation defects of CCR4-deficient OT-I cells because they were activated to the same degree as wild-type OT-I cells by anti-CD3/anti-CD28 stimulation *in vitro* (**Fig. 4.4-1b**).

To confirm the finding that CCL17 improves cross-priming through a direct effect on $CD8^+$ T cells, mixed bone marrow chimeras were generated in which all cell types except $CD8^+$ T cells can express CCR4. To achieve this, a 1:1 mixture of bone marrow from CCR4-/- and CD8-/- mice was transferred into irradiated CCR4-deficient recipients. As CD8-/- mice lack $CD8^+$ T cells, such mixed chimeras could only generate $CD8^+$ T cells from CCR4-deficient bone marrow (**Fig. 4.4-1c cartoon**). Co-injection of OVA with αGalCer into these mice did not increase the numbers of $CD8^+$ OVA-specific T cells as it did in the control group that received wild-type and CCR4-

deficient bone marrow. Instead, CD8$^+$ OVA-specific T cell numbers were similar to those in chimeras that had received only CCR4-deficient bone marrow (**Fig. 4.4-1c**).

The effect of CCL17 on CD8$^+$ T cell activation was not due to CCL17 acting as a growth factor, as the addition of CCL17 to cultures of anti-CD3/anti-CD28-stimulated CD8$^+$ T cells did not enhance T cell proliferation or IFNγ-production (**Fig. 4.4-1d**). These findings indicate that CCL17 can directly influence CD8$^+$ T cells, however as it does not directly affect T cell activation it may regulate their migration towards licensed DCs.

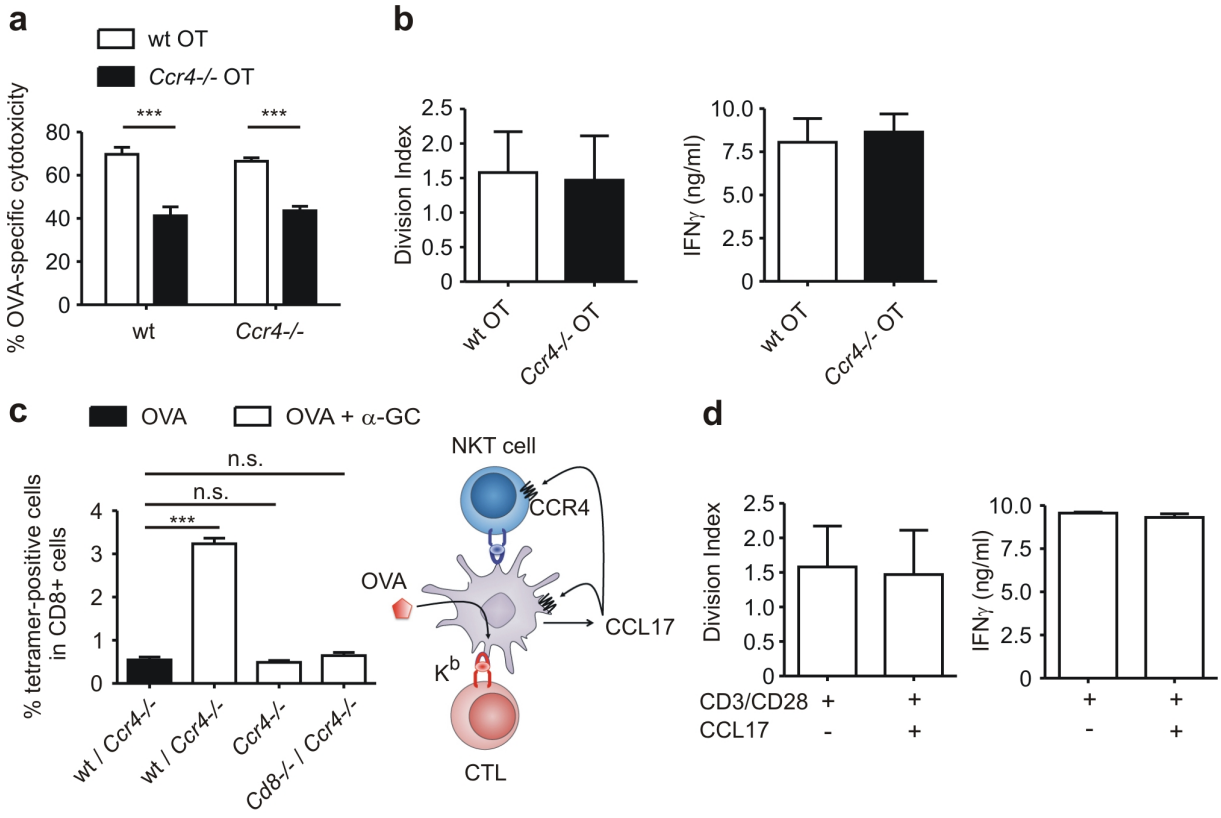

Fig. 4.4-1 CCL17 enhances cross-priming by acting directly on CD8$^+$ T cells. *In vivo* cytotoxicity on day 4 of wild-type and CCR4-deficient mice given 5x 10^3 CCR4-sufficient or CCR4-deficient OT-I cells and primed with OVA plus αGalCer (a-GC) 1 d later (**a**). Division index (left) and IFNγ content in supernatants (right) of 2x 10^5 CCR4-competent or CCR4-deficient OT-I cells stimulated for 2 d with anti-CD3 / anti-CD28 beads (**b**). Flow cytometric analysis of endogenous OVA-specific CTLs among splenic CD8$^+$ cells of CCR4-deficient mice reconstituted with 50% CCR4-deficient and 50% wild-type bone marrow, CCR4-deficient or 50% CD8-deficient and 50% CCR4-deficient bone marrow, assessed 2 d after priming with OVA plus αGalCer or OVA alone. Cartoon depicts on which cells CCL17 can act in CD8-/- / CCR4-/- chimeras (**c**). Division index (left) and IFNγ content in supernatants (right) of 2x 10^5 OT-I cells stimulated for 2 d with anti-CD3 / anti-CD28 beads with or without recombinant CCL17 (600 ng/ml) (**e**); Data are representative of two to three experiments (mean and s.d. of four to five mice per group in each). *** P < 0.001 (one-way ANOVA + Bonferroni multiple comparison post test (**a**), one-way ANOVA + Dunnet´s post test (**c**)).

4.4.2 αGalCer-treatment improves the migration of naïve CD8+ T cells towards CCL17

So far, there have been no reports of CCL17-mediated recruitment of $CD8^+$ T cells. Previous studies had focused on CCR4 recruitment of DCs and $CD4^+$ T cells. Therefore, I aimed at confirming that naïve $CD8^+$ T cells are rendered responsive to CCL17 after injection of αGalCer utilising a transwell assay. For this assay $CD8^+$ T cells were isolated from mice that had received αGalCer at different time points prior to isolation, or from untreated controls, and their migration towards 800ng/ml CCL17 was analyzed after 6 h. The percentage of transmigrated $CD8^+$ T cells increased significantly between 6 and 9 h after αGalCer-injection as determined by flow-cytometry (**Fig. 4.4-2a**), notably only under CCL17-stimulated conditions but not in medium controls. This observation indicated that $CD8^+$ T cells migrate specifically towards CCL17 instead of randomly increasing their motility.

Live-cell imaging of CCR4-competent or –deficient $CD8^+$ T cells in co-culture with CCL17-competent or –deficient DCs from αGalCer-injected or untreated mice was performed to further characterize CCR4-mediated migration of $CD8^+$ T cells. As observed in the transwell assay, $CD8^+$ T cells from untreated mice (- α-GC) showed reduced directionality when migrating towards CCL17-producing DCs from αGalCer-injected mice as compared to $CD8^+$ T cells from αGalCer-injected mice (**Fig. 4.4-2b**). This enhancement of directed migration was dependent on both DC-derived CCL17 and CCR4 on $CD8^+$ T cells, as wild-type $CD8^+$ T cells showed reduced directionality when co-cultured with CCL17-deficient DCs, as did CCR4-deficient $CD8^+$ T cells (**Fig. 4.4-2b**). Interestingly, contact duration between DCs and $CD8^+$ T cells was also increased in a CCR4-dependent manner (**Fig. 4.4-2c**). These findings were confirmed by using tripartite cultures where the directionality and contact duration of CCR4-competent and –deficient $CD8^+$ T cells in culture with CCL17-producing DCs were compared (**Fig. 4.4-2d,e**), or of CCR4-competent $CD8^+$ T cells in culture with both CCL17-competent and –deficient DCs (**Fig. 4.4-2f,g**). CCR4-competent $CD8^+$ T cells migrated more accurately towards CCL17-producing DCs and established longer contacts of approximately 50 minutes, while in comparison CCR4-deficient $CD8^+$ T cells had an average contact duration of 25 minutes (**Fig. 4.4-2d,e**). Similarly, CCR4-competent $CD8^+$ T cells migrated more accurately towards CCL17-producing DCs and established longer contacts than with CCL17-deficient DCs (**Fig. 4.4-2f,g**). In summary these results demonstrate that $CD8^+$ T cells are rendered responsive to CCL17 after injection of αGalCer, which enhances their directional CCR4-dependent migration towards CCL17-producing DCs.

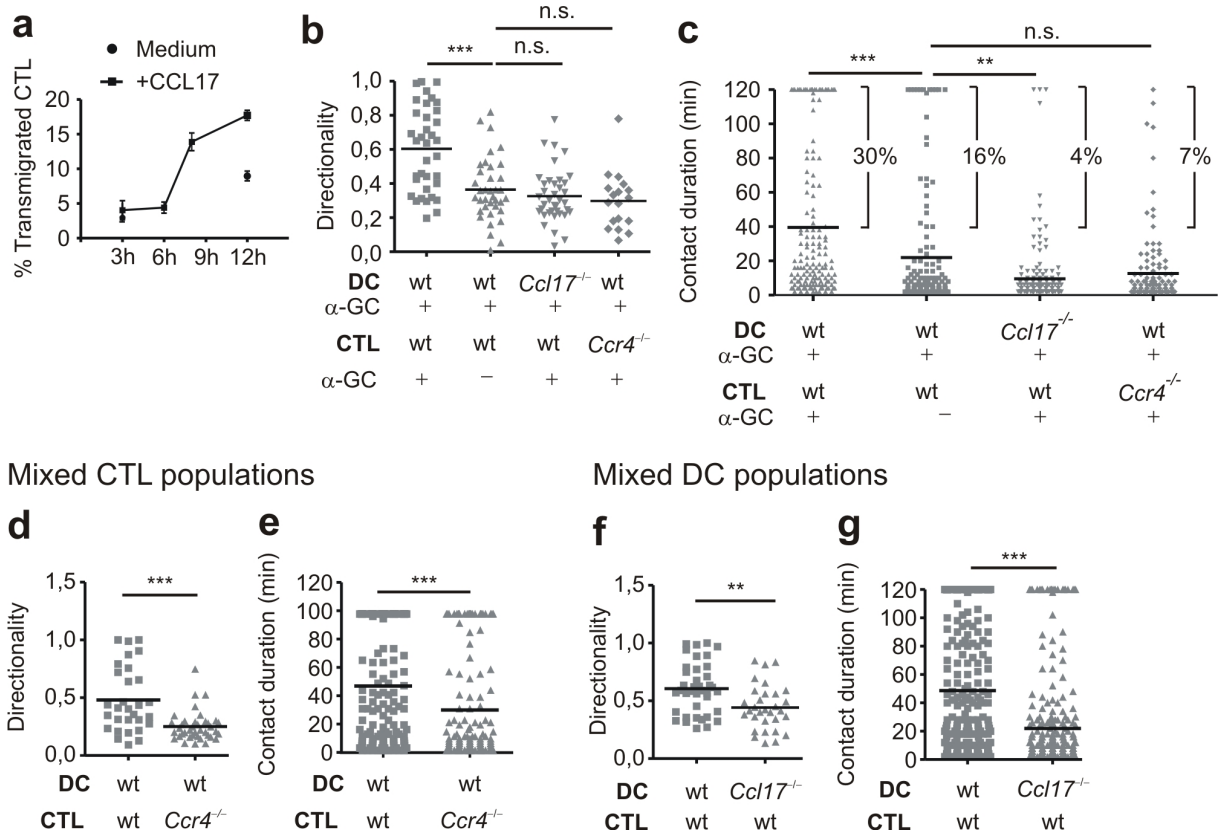

Fig. 4.4-2 αGalCer-treatment induces CCR4-dependent migration of CD8$^+$ T cells. Transwell assay of the migration of polyclonal CD8$^+$ T cells towards CCL17 (800 ng/ml); cells were isolated from mice injected with αGalCer 3, 6, 9 or 12 h prior to analysis (**a**). *In vitro* migration of CD8$^+$ T cells with or without CCR4 expression towards DCs with or without CCL17 production, recorded by time-lapse videomicroscopy over 2–3 h and presented as CD8$^+$ T cell directionality before physical contact with DCs (**b**) or subsequent duration of CTL-DC contact (**c**). Below graphs: α–GC indicates DCs or CD8$^+$ T cells from donor mice injected with αGalCer 14 h before (+) or not (–). Numbers adjacent to vertical brackets (**c**) indicate percent contacts lasting longer than 40 min. CD8$^+$ T cell directionality (**d**) and contact duration (**e**) of mixed populations of CD8$^+$ T cells with or without CCR4 expression, recorded by time-lapse videomicroscopy over 2–3 h. CD8$^+$ T cell directionality (**f**) and contact duration (**g**) of mixed populations of DCs with or without CCL17 production, recorded by time-lapse videomicroscopy over 2–3 h. In **b**–**g**, each symbol represents an individual cell (*n* = 30–40 cells (directionality) or *n* = 100–300 cells (contact duration)); small horizontal lines indicate the mean. Data are representative of three experiments (mean and s.d. of three to four mice per group). n.s., not significant, ** $P < 0.01$ *** $P < 0.001$ (Kruskal-Wallis and Dunn's post-test (**b**,**c**) or Mann-Whitney (**d**–**g**)).

4.4.3 CD8+ T cells accumulate in splenic T cell zones following αGalCer-injection

To determine the physiological relevance of the above findings, I wished to confirm the *in vitro* results on CCR4-dependent $CD8^+$ T cell migration *in vivo*. To this end, immunofluorescence microscopy of spleen sections was performed where the numbers of $CD8^+$ T cells present in T cell zones of mice treated with or without αGalCer were compared. FarRed-labeled OT-I cells were adoptively transferred into CCL17-eGFP reporter mice in order to allow identification of OVA-specific $CD8^+$ T cells and CCL17-producing DCs. 10 h after challenging the mice with OVA, αGalCer, or OVA plus αGalCer, spleen sections were stained for B220 to discriminate between splenic B cell and T cell zones. Counting the number of FarRed-labeled OT-I cells in T cell zones revealed the presence of more $CD8^+$ T cells in T cell zones in mice that had been co-injected with OVA and αGalCer compared to mice injected with OVA alone (**Fig. 4.4-3a**). Importantly this effect was antigen-independent as T cell numbers were similar in mice that received OVA plus αGalCer and those that were injected with αGalCer alone (**Fig. 4.4-3b**). The increase in T cell numbers depended upon both CCR4 expression on $CD8^+$ T cells and CCL17 production by DCs as transfer of CCR4-competent OT-I cells into CCL17-deficient mice or transfer of CCR4-deficient OT-I cells resulted in reduced numbers of $CD8^+$ T cells accumulating in T cell zones as compared to controls (**Fig. 4.4-3c**). To ensure that the observed effects were indeed antigen-independent, the same experiments were performed with polyclonal $CD8^+$ T cells instead of OT-I cells and indeed also these cells accumulated in a CCR4-dependent fashion (**Fig. 4.4-3d**). The same was true when NKT cells were activated by another ligand, namely iGb3 (**Fig. 4.4-3e**), demonstrating that CCR4-dependent attraction and accumulation of $CD8^+$ T cells is a general mechanism that is induced upon activation of NKT cells.

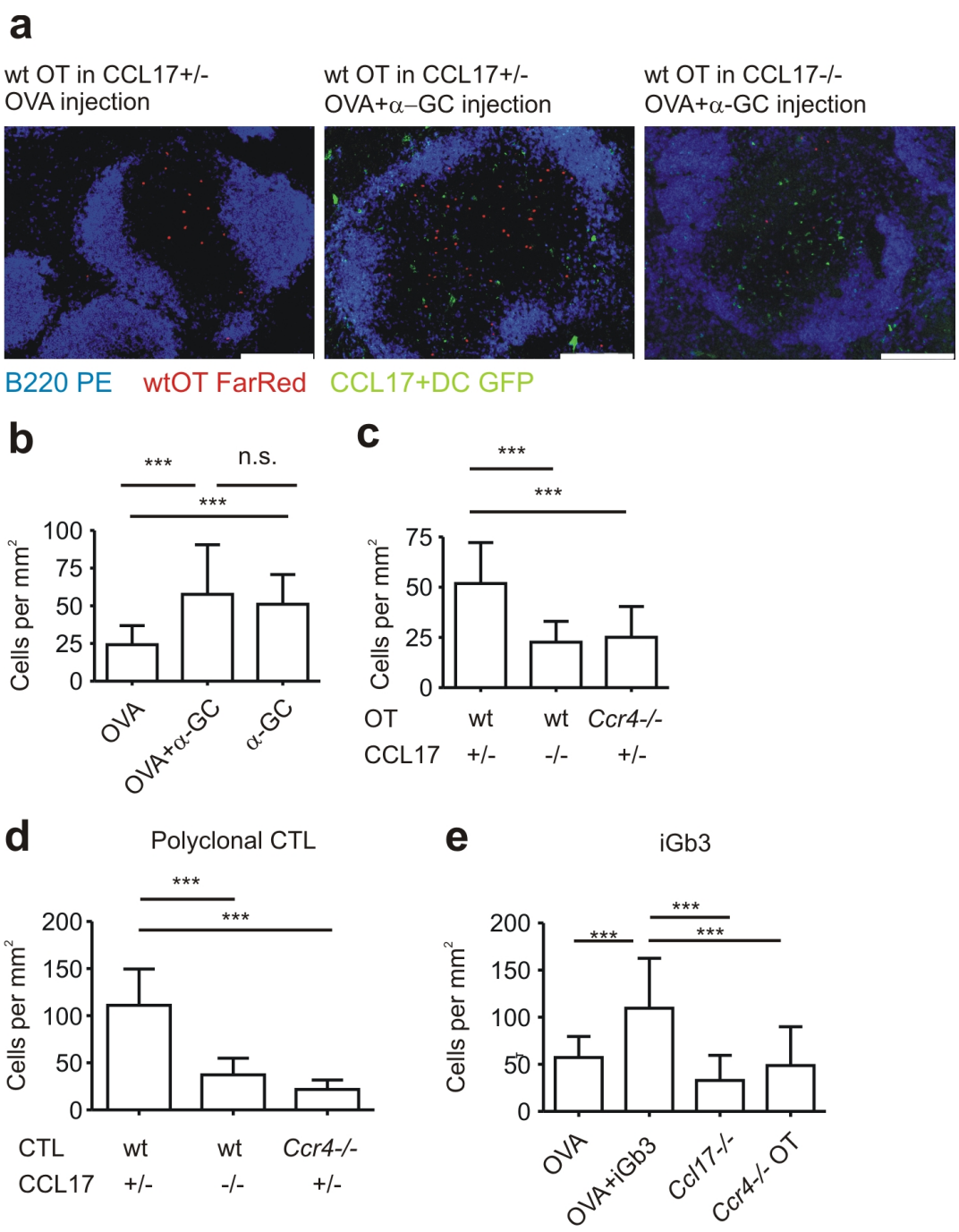

Fig. 4.4-3 CD8$^+$ T cells accumulate in splenic T cell zones in a CCR4-dependent fashion. Immunofluorescence staining of spleen cryosections from CCL17-eGFP reporter mice or CCL17-deficient mice injected with 2.5x 10^6 FarRed fluorochrome–labeled CCR4-sufficient OT-I cells on day -1 and then injected with OVA with or without αGalCer on day 0. Blue staining indicates B220$^+$ cells (defines B cell zones). Scale bars 200 µm (**a**). Absolute numbers of CCR4-sufficent or CCR4-deficient OT-I cells in CCL17-deficient or CCL17-eGFP reporter mice 10 h after injection with OVA with or without αGalCer, presented as cells per mm^2 of the T cell–DC zone enclosed by the (blue) B cell zone (**b**,**c**). Absolute numbers of FarRed fluorochrome–labeled polyclonal wild-type or CCR4-deficient CD8$^+$ T cells transferred into αGalCer-injected CCL17-deficient mice or CCL17-eGFP reporter mice as described in **a-c** (**d**). Absolute numbers of CCR4-sufficent or CCR4-deficient OT-I cells in CCL17-deficient or CCL17-eGFP reporter mice 10 h after injection of iGb3, presented as described in **b**,**c** (**e**). Data are representative of three individual experiments with at least 25 T cell–DC zones per group derived from three to four nonconsecutive sections from three mice each (mean and s.d.). n.s., not significant, *** $P < 0.001$ (Kruskal-Wallis and Dunn's post-test (**b-e**)).

4.4.4 Mechanism of CCR4 induction

As mentioned previously (section 4.1-2), naïve $CD8^+$ T cells are usually not responsive to CCR4 ligands. However as currently demonstrated, the injection of αGalCer and the accompanying activation of NKT cells not only induced the production of CCL17 in DCs but also enabled the responsiveness of $CD8^+$ T cells to CCL17 (**Fig. 4.4-2, 4.4-3**). To discriminate between the expression of CCR4 being upregulated or the receptor simply being rendered responsive on $CD8^+$ T cells, $CD8^+$ T cells from wild-type mice were injected with αGalCer at different time points prior to analysis or from untreated controls for expression of CCR4 by flow cytometry. Indeed, CCR4 signal was observed in αGalCer-injected mice but not in untreated mice (**Fig.4.4-4a**), with levels of CCR4 increasing between 8 h and 14 h after injection and slowly declining at 16 h. These results suggest that CCR4 is upregulated by certain signals that occur after NKT cell activation and DC licensing. A fluorescent bead immunoassay (Bender MedSystems) was used to identify which cytokines are present in the spleen after αGalCer-injection that could be involved in upregulation of CCR4. The assay was performed with supernatants of splenocytes isolated from untreated or αGalCer-injected wild-type mice and cultured for 24 h in 96 well plates. These supernatants revealed the presence of IL-4, TNFα and IFNγ, as would be expected after activation of NKT cells, and the upregulation of IL-6 (**Fig. 4.4-4b**). IL-6 was probably produced by endothelial cells or DCs but not NKT cells as isolated NKT cells did not produce high levels of IL-6 (**Fig. 4.2-2a**). As regards other DC-derived cytokines that could be involved in CCR4 induction, high levels of IL-12 were produced by DCs after injection of αGalCer (data from Veronika Lukacs-Kornek, not shown), thus IL-12 was included in the analysis.

To investigate the effect of individual factors on upregulation of CCR4, an *in vitro* assay was used in which naïve $CD8^+$ T cells were cultured in the presence of the particular reagent(s) for 14 h before analyzing CCR4 expression by flow cytometry. 14 h was chosen as time point as CCR4 expression peaked around 14 h (**Fig.4.4-4a**) First, $CD8^+$ T cells were cultured with serum from mice that had been injected with αGalCer 5 h previously, to determine if a soluble factor is responsible for CCR4 expression. Indeed, addition of serum induced CCR4 expression in $CD8^+$ T cells (**Fig. 4.4-4c**). Subsequently, the influence of the individual cytokines at different concentrations (10 – 200 ng/ml) was determined, but no effect on CCR4 expression with any of these cytokines was found (**Fig. 4.4-4c**). Neither the combination of all cytokines, nor the combination of IL-4 and TFNα, which is able to induce CCL17, induced CCR4 expression on $CD8^+$ T cells (**Fig. 4.4-4d**). Since the presence of certain cytokines might have an inhibitory effect on CCR4 expression, different combinations with 2 or more cytokines were investigated,

but upregulation of CCR4 was not seen (**table 4.4-1**). Furthermore, the effect of the CCR4 ligands CCL17 and CCL22 themselves was analyzed, but neither CCL17, CCL22, nor the two combined could induce expression of CCR4 (**Fig. 4.4-4e**). In addition, other cytokines that have been implicated in T_H2-related responses (**64**, **65**) were tested, namely IL-33 and TSLP, but they too had no effect (**Fig. 4.4-4d**). **Table 4.4-1** summarizes all the combinations tested for CCR4 induction. Thus the question remains which signals besides the classical NKT cell and DC-related cytokines are essential to induce upregulation of CCR4, and further investigation will be needed to clarify this question.

Cytokine stimulus	Cytokine stimulus	Cytokine stimulus
IFNγ 10/100/200 ng/ml	IL4+TNFα	IL4+IL12+IL2+IL33
IL4 10/100/200 ng/ml	TNFα+IL2	IL6+IL12+IL2
IL13 10/100/200 ng/ml	TNFα+IL6	IL6+IL2+IL4
TNFα 10/100/200 ng/ml	TNFα+IL12	IL2+IL4+TNFα+IFNγ+IL6
IL2 10/100/200 ng/ml	TNFα+IFNγ	IL2+IL4+TNFα+IFNγ+IL12
IL12 10/100/200 ng/ml	IL2+IFNγ	IL2+IL4+TNFα+IL12+IL6
IL6 10/100/200 ng/ml	IL2+IL6	IL2+IL4+IL12+IFNγ+IL6
TSLP 10/100/200 ng/ml	IL2+IL12	IL2+IL12+TNFα+IFNγ+IL6
IL15 10/100/200 ng/ml	IL2+TSLP	IL12+IL4+TNFα+IFNγ+IL6
IL33 10/100/200 ng/ml	IL2+IL15	IL2+IL4+TNFα+IFNγ+IL6+IL12+TSLP
CCL17200/600/1000 µg/ml	IL2+CCL17	IL2+IL4+TNFα+IFNγ+IL6+IL12+CCL17
CCL22 200/600/1000 µg/ml	IL2+IL33	IL2+IL4+TNFα+IFNγ+IL6+IL12+CCL22
GM-CSF	IL6+IL12	IL2+IL4+TNFα+IFNγ+IL6+IL12+IL33
IL4+IFNγ	IL6+IFNγ	IL2+IL4+TNFα+IFNγ+IL6+IL12+IL15
IL4+IL2	IFNγ+IL12	IL2+IL4+TNFα+IFNγ+IL6+IL12
IL4+IL12	IL4+TNFα+IL12	TSPL+CCL17
IL4+IL6	IL4+IFNγ+IL12	TSPL+CCL22
IL4+TSLP	IL4+TNFα+IL2	TSLP+IL4+TNFα
IL4+IL33	IL4+IL2+CCL22	IL4+IL2+TNFα+IL12
IL4+CCL17	IL4+IL2+TNFα+CCL17	

Table 4.4-1 Cytokine combinations tested for CCR4 expression in $CD8^+$ T cells. Single cytokines were applied at different concentrations for 14 h on $2x10^5$ naïve $CD8^+$ T cells isolated from wild-type spleens. Cytokine concentrations in combinations were used at the highest concentrations tested for each cytokine.

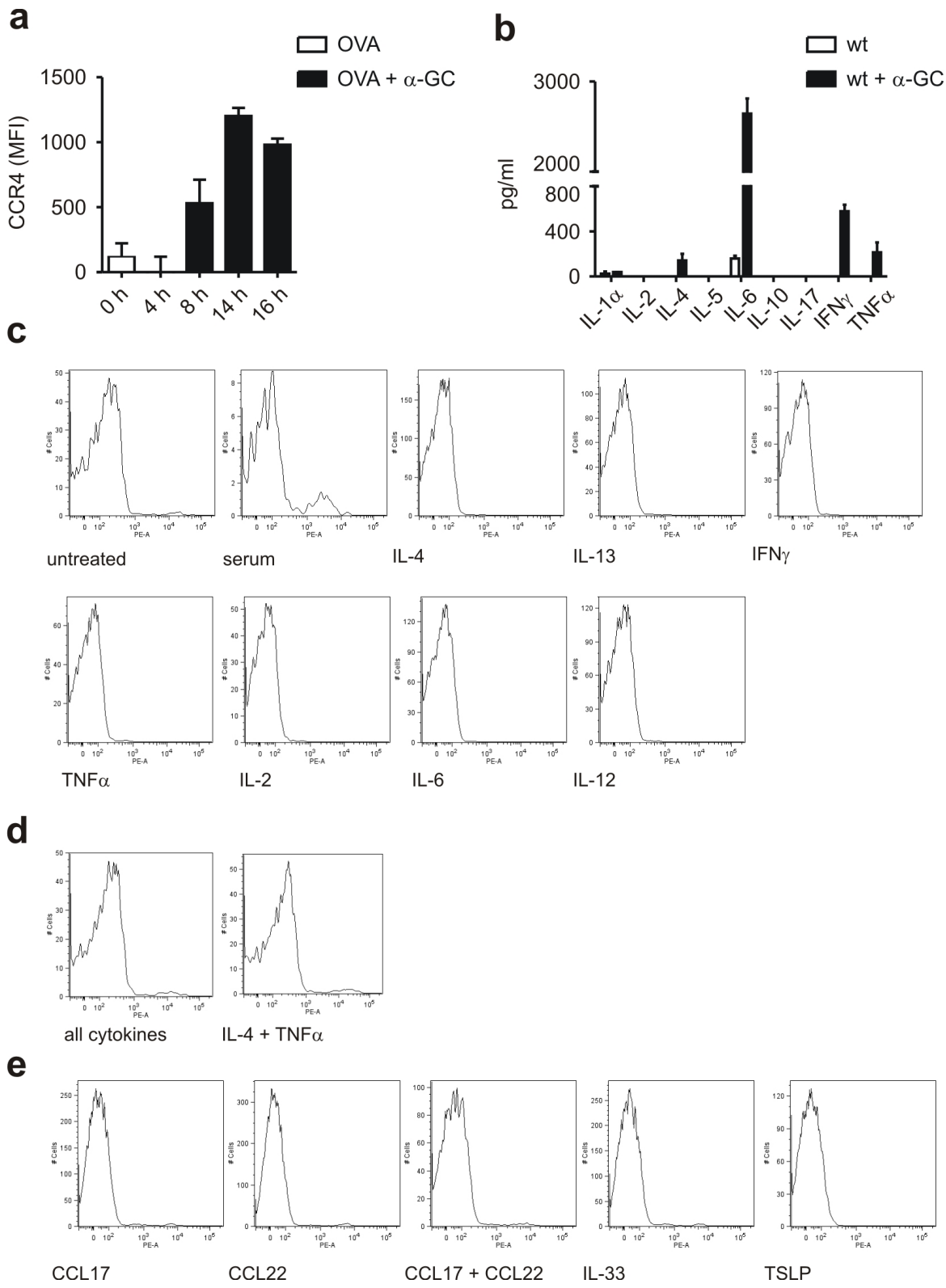

Fig. 4.4-4 Regular NKT cell and DC cytokines do not induce CCR4 expression. Flow cytometric analysis of CCR4 expression on splenic $CD8^+$ T cells from wild-type mice injected 4,8,14 or 16 h previously with αGalCer (**a**). Flow cytometric analysis of a fluorescent bead immunoassay (Bender MedSystems) of NKT cell supernatant from $5x10^5$ cells isolated from wild-type mice injected or not with 0,2µg αGalCer 5 h previously (**b**). CCR4 expression of $CD8^+$ T cells isolated from untreated wild-type spleens and cultured with serum from αGalCer-treated wild-type mice or different cytokines for 14 h before analysis by flow cytometry (**c-e**). MFI: mean fluorescence intensity.

4.5 Synergistic effect of classical and alternative DC licensing

4.5.1 Combination of classical and alternative DC licensing boosts cross-priming

The data presented so far suggested that NKT cell-mediated DC licensing is a pathway that operates separately from classical DC licensing and is regulated by distinct chemokine signals. To confirm that CCR4 regulates $CD8^+$ T cell attraction after NKT cell-mediated DC licensing, but not attraction after classical licensing, histological analyses were performed. Indeed, CCR4-deficient T cells failed to accumulate after injection of OVA plus αGalCer but not after injection of OVA plus CpG (**Fig. 4.5-1a**), confirming the involvement of CCR4 in attraction of $CD8^+$ T cells in NKT cell-mediated cross-priming but not in classical cross-priming. On the other hand, CCR5-deficient T cells accumulated in spleens after injection of OVA plus αGalCer but not after OVA plus CpG (**Fig.4.5-1b**), indicating that CCR5 regulates classical cross-priming rather than NKT cell-mediated cross-priming.

These findings raise the question how a combination of classical and NKT cell mediated DC licensing would affect cross-priming. This question was addressed in an *in vivo* cytotoxicity assay to compare the T cell response after DC licensing in the presence of glycolipids, TLR ligands or both. Wild-type mice that were challenged with OVA plus maximally effective doses of αGalCer plus CpG displayed a significantly better cytotoxic T cell response compared to mice that received OVA plus αGalCer or CpG alone. This was true for T cell responses in both the spleen after intravenous injection of αGalCer (**Fig. 4.5-1c**) and in the draining lymph node after subcutaneous injection (**Fig. 4.5-1d**). These results demonstrate that the combined effect of DC licensing by NKT cells and T_H cells synergize and result in an even better T cell activation.

To analyze how chemokine-signals are involved and if the augmented $CD8^+$ T cell response is accompanied by augmented attraction of $CD8^+$ T cells, T cell numbers in the splenic T cell zone were dissected as described in **Figure 4.4-3**. When adoptively transferred OT-I cells that are competent for both CCR4 and CCR5 were transferred, they accumulated in higher numbers after co-injection of OVA plus αGalCer plus CpG compared to injection of OVA plus αGalCer or CpG alone (**Fig. 4.5-1e**). These data indicate that the synergy of classical and NKT cell-mediated cross-priming is at least in part achieved by improved attraction of $CD8^+$ T cells.

To clarify how the enhanced attraction of $CD8^+$ T cells is mediated, the chemokine production by DCs was investigated in more detail. Histological results had shown that OVA plus CpG-induced DC licensing is independent of CCL17- CCR4 signals (**Fig. 4.5-1a**). Nevertheless, a possible influence of co-injection of CpG and subsequent activation of TLR9 on CCL17 levels

had to be excluded. Thus, CCL17eGFP-reporter mice were analyzed for the percentage of DCs expressing CCL17 after injection of OVA plus αGalCer and of OVA plus αGalCer plus CpG, but similar percentages of DCs upregulating CCL17 were found (**Fig. 4.5-1f**). Also the amount of CCL17 produced per DC was similar with or without co-injection of CpG (**Fig. 4.5-1g**).

To clarify if CCL17 and CCR5 ligands are produced by two distinct DC subsets and therefore more DCs might attract $CD8^+$ T cells, DCs were isolated from CCL17eGFP-reporter mice that had been injected with OVA plus αGalCer plus CpG, and sorted DCs expressing CCL17 (= CD11c GFP) or not (= CD11c GFP^-). When these two subsets were examined for expression of CCR5 ligands by RT-PCR, CCL17-expressing DCs were found to co-express high levels of CCL3 and especially CCL5 (**Fig. 4.5-1h**). These results indicate that similar numbers of DCs are able to attract T cells after classical DC licensing, NKT cell mediated DC licensing and the combination of both. Thus remains to be investigated whether CCL17 and CCR5 ligands attract different subsets of $CD8^+$ T cells. The combined attraction of two different subsets might explain higher T cell numbers after the combined induction of both chemokine pathways.

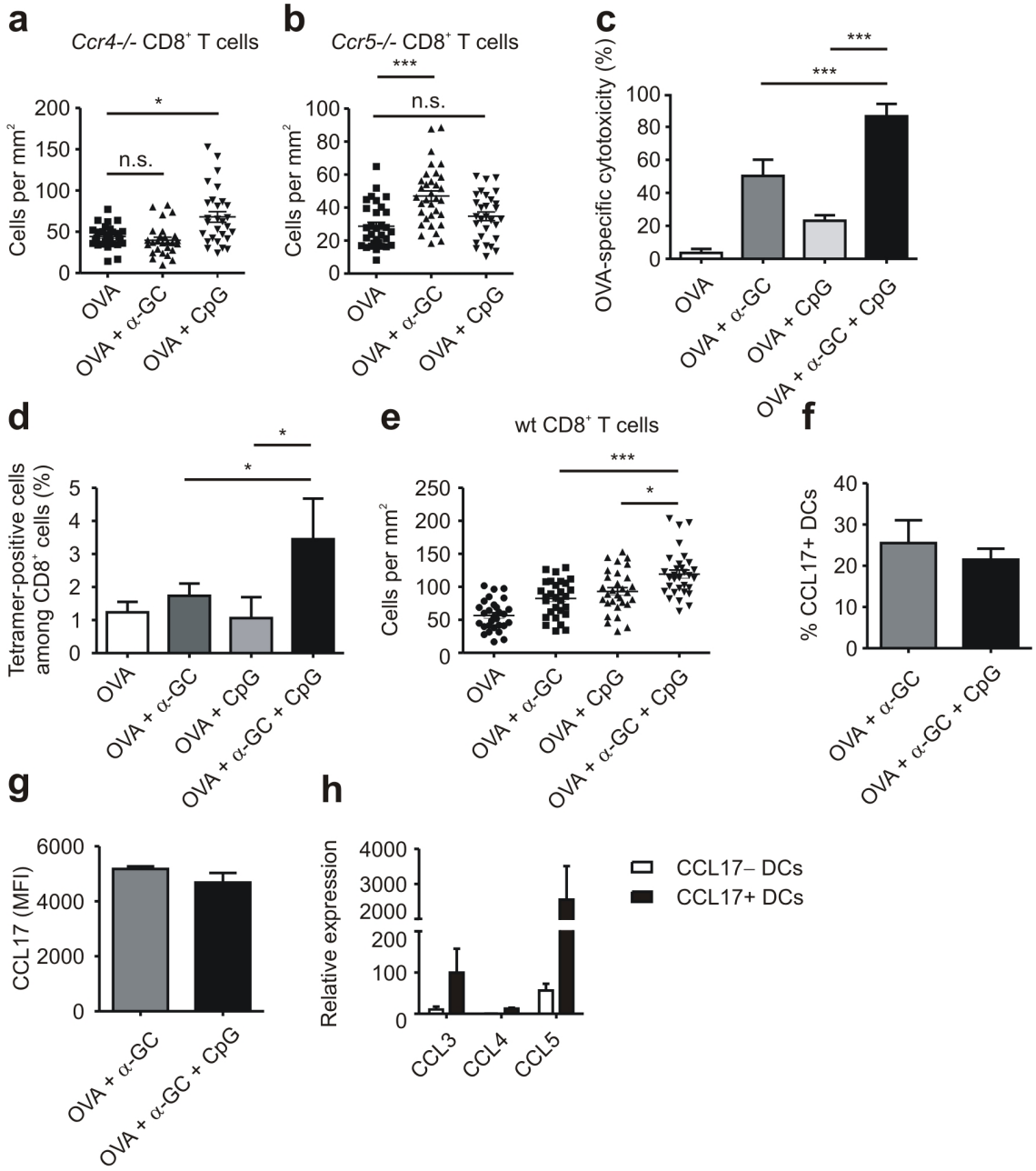

Fig. 4.5-1 Combination of classical and NKT cell mediated cross-priming induce even better T cell responses. Absolute number of adoptively transferred CCR4-deficient (**a**) or CCR5-deficient (**b**) in OVA-primed wild-type mice coinjected with αGalCer, CpG or both, assessed by histology as described in **Figure 4.4-3** (presented as cells per mm^2 of the T cell–DC zone). OVA-specific cytotoxicity in the spleen 5 d after priming wild-type mice with soluble OVA plus αGalCer and/or CpG intravenously in spleen (**c**). Flow cytometric analysis of the proportion of OT-I cells in splenic $CD8^+$ cells in the draining lymph node 3 d after subcutaneous injection of soluble OVA plus αGalCer and/or CpG (**d**). Absolute number of adoptively transferred wild-type OT-I T cells (**e**) in OVA-primed wild-type mice coinjected with αGalCer, CpG or both, assessed by histology as described in **Figure 4.4-3** (presented as cells per mm^2 of the T cell–DC zone). Flow cytometry of spleen cells from CCL17-eGFP reporter mice 20 h after injection of OVA plus αGalCer with or without CpG, with gating on $CD11c^+CCL17^+$cells (**f,g**). CCL3, CCL4 and CCL5 mRNA expression in CCL17-negative (CCL17-) and CCL17-producing (CCL17+) DCs 20 h after injection of αGalCer, presented relative to GAPDH RNA expression (**h**); Data are representative of two experiments (mean and s.d. of three to four mice per group in each). *$P < 0.05$ *** $P < 0.001$ (One-way ANOVA + Tukey post test (**c,d**), Kruskal Wallis + Dunn´s selected pairs (**a,b,e**).

5 Discussion

The NKT cell ligand α-galactosylceramide (αGalCer) has long been known for its adjuvant effect in enhancing tumor immunity (**25**). Initial studies demonstrated that injection of αGalCer could promote the survival of mice with melanoma (**66**), and NKT cell-deficient mice showed an increased susceptibility to methylcholanthrene-induced tumors (**67**). The exact mechanisms how NKT cells control anti-tumor responses remain unclear. However, direct recognition of tumor cells by NKT cells is not crucial for tumor rejection as CD1d-deficient tumors can be rejected in wild-type mice (**68**). Instead, NKT cells enhance tumor resistance indirectly by positively regulating functions of other cells, for example DCs. Several studies demonstrated that NKT cell activation by αGalCer resulted in DC maturation and in a subsequent boost of T cell responses. Thus, coinjection of αGalCer with a relevant antigen can be used to expand antigen-specific responses (**26**,**69**,**70**,**71**). These results provided the foundation for the design of DC-based immunotherapeutic protocols to potentiate immune responses against tumors and pathogens. Indeed, injection of αGalCer-loaded DCs improved T cell responses and attenuated tumor growth in clinical trials (**72**,**73**,**74**). However, the results obtained are not ideal as the beneficial effects of DC transfer on the disease severity could be observed in only few patients whereas most patients did not benefit from the improved T cell response. Further understanding the mechanisms how NKT cells expand immune responses might help to improve tumor therapy as well as vaccination strategies against a range of pathogens like malaria and other intracellular pathogens (**71**). Thus, the present study wanted to clarify the events underlying NKT cell-mediated DC maturation.

5.1 Role of CCL17 and CCR4 in NKT cell-mediated cross-priming in the spleen

5.1.1 Adjuvant effect of NKT cell activation on cross-priming

Co-injection of the glycolipid αGalCer with the cross-presented antigen OVA has been demonstrated to have an adjuvant effect on $CD8^+$ T cell responses (**26-28, 71**), which was confirmed in the present study. The events underlying this adjuvant effect have only been partially described in the literature. Presentation of αGalCer by DCs leads to activation of NKT cells, resulting in rapid cytokine production and upregulation of CD40L by NKT cells. In turn, the activated NKT cells induce upregulation of costimulatory molecules on the antigen-presenting DC via cytokine signals and CD40 signaling (**26-28**). *Fujii et al.* (**28**) attributed the

adjuvant effect of αGalCer to the mature state of the DCs, as costimulation is believed to initiate and amplify T cell priming. In line with this interpretation, *Hermans et al.* suggested that the adjuvant effect depended on simultaneous presentation of αGalCer and OVA by the same DC, as the mature state of the cross-presenting DC could increase $CD8^+$ T cell responses (**26**). They addressed this issue by analyzing $CD8^+$ T cell responses after transfer of antigen-loaded DCs. DCs loaded with both αGalCer and OVA induced highly increased T cell responses, whereas transfer of a mixture of αGalCer-loaded and OVA-loaded DCs did not enhance T cell activation (**26**). To clarify this issue in the present study, mixed bone marrow chimeras were generated that possessed DCs that could present antigen to either NKT or $CD8^+$ T cells but not to both cell types simultaneously due to a lack of functional MHC-I or CD1d respectively. These mice were unable to enhance $CD8^+$ T cell responses after co-injection of OVA with αGalCer. These data confirm that NKT cell activation results in cognate DC licensing. However, not only the expression of costimulatory molecules but the additional expression of the chemokine CCL17 by the cross-presenting DC efficiently enhanced $CD8^+$ T cell responses as demonstrated again in mixed bone marrow chimeras. These chimeras possessed both DCs that could present simultaneously to both NKT and $CD8^+$ T cells but could not produce CCL17, as well as DCs that could produce CCL17 but could not cross-present peptide-antigens to $CD8^+$ T cells. These mice displayed a highly reduced $CD8^+$ T cell response, demonstrating that CCL17 expression by cross-presenting cells highly increases $CD8^+$ T cell responses.

Thus, the present study presents a new aspect of DC licensing by showing that activated NKT cells not only induce expression of costimulatory molecules by DCs, but also expression of the chemokine CCL17. Moreover, it demonstrates that the induction of this chemokine is a crucial component of DC licensing and is critical for the efficiency of $CD8^+$ T cell responses. Thus not only the expression of costimulatory molecules but also the expression of chemokines by cross-presenting DCs strongly influences $CD8^+$ T cell responses and accounts for the majority of the adjuvant effect of αGalCer.

5.1.2 CCL17 expression in the spleen

CCL17 is expressed as a homeostatic chemokine in the thymus, lymph nodes, lung and intestine, and its expression can be augmented in these organs by treatment with microbial agents such as TLR ligands. In contrast, splenic DCs have been demonstrated to lack CCL17 expression, even after systemic microbial challenge (**45**). The data presented here show that CCL17 can be induced in splenic DCs by activation of NKT cells through injection of glycolipid

antigens like αGalCer. Expression of the second CCR4 ligand CCL22 was found in NKT cells but not DCs after αGalCer-treatment. As reported for CCL5, which amplifies its own expression (**75**), both CCL17 and CCL22 enhanced their own production in a CCR4-dependent fashion. The individual role of the less well characterized CCL22 remains unclear, as does the question whether the two CCR4 ligands interact in shaping the immune response in NKT cell-mediated cross-priming. In fact, it seems that CCL17 is the major mediator of CCR4-dependent attraction of $CD8^+$ T cells as the cytotoxic T cell response was mostly reduced in CCL17-deficient mice and the differences between CCR4- and CCL17-deficient mice were not significant. It has been described previously that chemokines can have differential effects although they signal through the same receptor. For example CCL19 and CCL21, the two CCR7 ligands, are known to exert differential signaling effects, as CCL19 induces CCR7 phosphorylation and internalization, thereby causing receptor desensitization, which is not the case for CCL21 (**76**). In this way CCL19 desensitizes cells in their responsiveness towards CCL21 signals. As DCs express CCL19, this effect could be crucial to stop T cells at antigen-presenting cells rather than to allow continued migration towards CCL21-producing endothelial cells. The expression by different cell types suggests that CCL17 and CCL22 also act differentially, but their individual roles and how they might interact needs to be clarified.

Several studies have investigated the prerequisites for CCL17 induction, with results varying considerably depending on the investigated cell type: Langerhans cells were described to produce CCL17 in response to the cytokines IL-4 and TNFα, whereas IFNγ inhibited its expression (**77**,**78**). In contrast, studies with the human keratinocyte cell line HaCaT found CCL17 induction after the combined application of TNFα and IFNγ, which could be inhibited by IL-4 (**79**,**80**). The fibroblast cell line NG1RGB responded again differently: the combination of IL-4 and TNFα induced CCL17 expression and could be further increased by IFNγ (**80**). The present study demonstrates that IL-4 potently induced CCL17 expression in splenic DCs and that TNFα further increased this effect, although TNFα alone had only a minor effect on CCL17 expression. IFNγ had an inhibitory effect on CCL17 expression and could completely suppress CCL17 expression induced by the combination of IL-4 and TNFα.

Regarding IFNγ, it seems counter intuitive that a cytokine, which is usually associated with the progression of $CD8^+$ T cell responses, has an inhibitory effect on chemokine expression and the subsequent initiation of cross-priming. Nevertheless, *Xiao et al.* (**77**) described a similar inhibitory effect of IFNγ on CCL17 expression in Langerhans cells. As CCL17 is a chemokine that is involved in T_H2 diseases of the skin, they suggested that CCL17 inhibition by the T_H1 cytokine

IFNγ and stimulation through the T_H2 cytokine IL-4 could be an amplification circuit that increases CCL17 production in the T_H2 cytokine microenvironment of the skin in diseases like atopic dermatitis. As NKT cells are able to secrete both T_H1 and T_H2 cytokines simultaneously, the polarization of immune responses is more complex in NKT cell-mediated cross-priming in the spleen, and a classification into T_H1 and T_H2 cytokines might not be adequate.

Furthermore, NKT cell-derived IFNγ might affect cross-priming differently than $CD8^+$ T cell-derived IFNγ as it is present during the early licensing events. Thus, it might influence other cellular functions, for example the cross-priming ability of DCs rather than $CD8^+$ T cell proliferation.

However, the *in vivo* relevance of NKT cell-derived IFNγ in DC licensing remains unclear and various aspects like IFNγ-receptor expression or delayed IFNγ production might regulate its impact. To address these questions, it would be necessary to further characterize both the kinetics of expression of NKT cell-derived IFNγ and of the IFNγ receptor. Regarding IFNγ expression, several studies previously demonstrated an early burst of IL-4 that is followed by delayed production of IFNγ (**81**,**32**). From these data it could be hypothesized that IFNγ might act by down-modulating CCL17 expression after the initial CCL17 induction through IL-4 and TNFα. However, this hypothesis requires testing by the experiments outlined above and additional experiments using IFNγ-deficient mice.

Although I found IL-4 to be the most potent inducer of CCL17, it was not essential for CCL17 expression, as DCs isolated from αGalCer-treated wild-type or IL4-deficient mice produced similar levels of CCL17 mRNA. Furthermore, IL-4-deficient mice did not show the same cross-priming defect observed in CCL17-deficient mice. Interestingly OCH, a synthetic derivate of αGalCer that is known to selectively induce IL-4 as opposed to IFNγ, induced lower levels of CCL17 expression as compared to αGalCer. This was unexpected, as higher concentrations of IL-4 were anticipated to increase CCL17 expression, and reduced IFNγ production should further enhance CCL17 induction due to reduced inhibition. However, the *in vivo* situation is certainly much more complex than the conditions generated *in vitro*, and thus we might have failed to detect the contribution of certain factors that shape CCL17 expression *in vivo*. Although certain cytokines can induce CCL17 production *in vitro*, they might not be the critical agents *in vivo*, for example due to an inhibitory effect of another factor. Thus, these data indicate that regulation of CCL17 expression is a process that involves various cytokine signals and understanding it in more detail will need further investigation. One approach to further the understanding of CCL17 induction in DCs by NKT cells could be to investigate NKT cell

subsets *in vivo*. The mouse has αGalCer-reactive $CD4^+CD8^-$ and $CD4^-CD8^-$ NKT cell subsets that can additionally differ in their expression of NK1.1, and even the presence of a $CD4^-CD8^+$ subset has been suggested (**82**). Recently it has become more and more apparent that the different NKT subsets can carry out different functions. For instance, cytokine production differs highly depending on expression of CD4 and NK1.1, and on the organ the cells were isolated from (**29**, **38**, **83**). Defining the NKT cell subset that is responsible for CCL17 induction and analyzing its cytokine profile might give a clearer picture of the factors that regulate CCL17 expression in the spleen.

Besides cytokines, the mast cell mediators histamine and prostaglandin E_2 have been described to have an influence on expression of CCR4 ligands, as they upregulate the production of CCL17 and CCL22 in immature human DCs (**84**). These results are especially relevant for allergic responses in skin or airways where DCs are in close contact with mast cells. Whether these mediators also play a role in CCL17 induction in the spleen needs to be tested.

In addition to soluble factors, cell surface molecules might be involved in CCL17 induction as DCs establish close contacts with NKT cells. Signaling through CD40 has been described to play an important role in DC licensing and is required for the development of immunity (**26**). So far, it has not been determined by which pathway CD40L – CD40 interactions exert this effect. *Fujii et al.* analyzed the influence of CD40 signaling on the expression of costimulatory molecules and cytokines, however CD40-deficient mice upregulated CD80 and CD86 to the same degree as wild-type mice in response to αGalCer (**28**). Instead, upregulation of CD80/86 depended on cytokine signals such as TNFα. Although CD40 signaling induced IL-12 production (**28**, **85**), this factor was not essential for an efficient NKT cell-mediated cross-priming (**28**, data generated by Veronika Lukacs-Kornek, not shown). Hence, CD40 signaling must induce other signals apart from signals 2 and 3. As additional chemokine signals are required for the development of immunity, the influence of CD40 stimulation on CCL17 expression was addressed in an *in vitro* assay. However, DC-stimulation through CD40 had no effect on CCL17 expression *in vitro*, though one has to consider that untreated DCs were used that had not yet fully upregulated CD40. To fully resolve this issue, one would need to investigate CCL17 expression *in vivo* in CD40- or CD40L-deficient mice, or in mice treated with a blocking anti-CD40-antibody.

Taken together, these results demonstrate that the expression of CCL17 in splenic DCs is induced by IL-4, whereas IL-13, especially in combination with TNFα, can perform the same function and might be able to substitute for IL-4. IFNγ on the other hand inhibits CCL17 expression. If additional factors are involved in CCL17 induction could be further investigated

by blocking both IL-4 and IL-13 *in vivo*. As this study focused on the role of CCL17, it remains unclear by which signal CCL22 expression is induced in NKT cells. Future studies should address the question of induction and function of CCL22 to obtain a complete picture of the functions of CCR4 ligands in cross-priming.

5.1.3 Effect of CCL17 on the cross-priming ability of DCs

Although chemokines are best known for their role in regulating cell migration, it is evident that they can also modulate T cell responses independent of their chemoattractant activities. Thus, several studies have described an involvement of chemokines in DC maturation. The CCR7 ligands CCL19 and CCL21 have been demonstrated to induce maturation in DCs migrating from peripheral organs to secondary lymphoid organs where they present their captured antigen. This process ensures that antigen is presented to naïve T cells in an optimal context. Similarly, CCL3 (**86**) and CCR2 (**87**) have been implicated in the upregulation of the maturation markers CD80, CD86 and CD40. CCL5 has been demonstrated to induce production of cytokines such as TNFα and IL-6, and of chemokines like CXCL2, CCL3, CCL4 and CCL5 in immature bone marrow derived DCs (BMDCs) (**75**). So far, there are no reports on CCL17-mediated induction of DC maturation markers, but there is a striking connection between CCL17 expression and upregulation of costimulatory molecules, in that IL-4 and TNFα do not only play an important role in induction of CCL17 but also in the induction of costimulatory molecules such as CD80 and CD86 (**26**, **77**). These data led to the hypothesis that IL-4 and TNFα might induce the expression of costimulatory molecules indirectly through induction of CCL17 expression, which in turn might upregulate CD80/86 expression. However, I found that expression of costimulatory molecules was unaltered in CCR4- and CCL17-deficient mice, demonstrating that CCL17 does not influence the expression of costimulatory molecules in cross-presenting DCs.

However, the overall cross-priming ability of DCs might be influenced by factors other than CD80/86 and CD40 expression, hence the cross-priming capacity of DCs that had been licensed in αGalCer-injected CCR4-competent or CCR4-deficient mice was analyzed *ex vivo*. As both cell types induced similar $CD8^+$ T cell responses, it could be concluded that CCL17 has no direct influence on the cross-priming ability of DCs and is likely to increase cross-priming efficiency by affecting cell migration.

To investigate if CCL17 enhances $CD8^+$ T cell responses by attracting higher numbers of DCs to the spleen in NKT cell mediated cross-priming, the number and subset composition of DCs in

the presence or absence of CCL17 was compared. However, there was no difference in DC numbers and subsets, and also the attraction of higher numbers of NKT cells into the spleen could be excluded. Nevertheless, these experiments cannot exclude an altered distribution of cells inside the spleen, as more resident DCs or NKT cells might be recruited to the place of NKT cell – DC interactions. However, similar percentages of mature DCs were present in CCR4-competent and CCR4-deficient mice, arguing against a CCR4-dependent increase of DC – NKT cell interactions and a subsequently increased number of licensed DCs. These results demonstrate that the frequency of NKT cell – DC interaction is unaltered in CCR4-deficient mice, as is the overall cross-priming capacity of licensed DCs. Furthermore, NKT cells might directly activate $CD8^+$ T cells as suggested by *Matsuda et al.* (**88**). One pathway through which NKT cells could influence cross-priming directly may be type-I IFN as it can be produced by NKT cells and has been described to have a direct effect on $CD8^+$ T cell activation (**89**). However, the presence of NKT cells from αGalCer-treated CCR4-competent or CCR4-deficient mice did not affect $CD8^+$ T cell activation *in vitro*.

Taken together, these results argue against a CCL17-mediated alteration of NKT cell functions or the cross-priming capacity of DCs, and show that CCL17 does not influence the recruitment of DCs or NKT cells into the spleen.

5.1.4 CCL17-responsiveness of $CD8^+$ T cells

Naïve $CD8^+$ T cells are known to express CCR7 and to upregulate CCR5 under inflammatory conditions (**54,56**). The data presented in this thesis demonstrate that naïve $CD8^+$ T cells can become responsive to ligands of the chemokine receptor CCR4 after injection of αGalCer. In general there are two major mechanisms for chemokine receptor regulation. First, the responsiveness of a chemokine receptor can be regulated by sensitization as demonstrated for CCR7 and CXCR4 that can be sensitized by CCL17 signals (**53**), or desensitization, where receptors are internalized after ligand binding (**90**). Second, responsiveness can be regulated by up- or down-regulation of a certain receptor (**90**). Flow-cytometric analysis of $CD8^+$ T cells revealed that CCR4 is present on αGalCer-treated but not untreated $CD8^+$ T cells, suggesting that CCR4 is upregulated by a signal that is produced during αGalCer-mediated NKT cell activation and DC licensing.

Several studies have described that microenvironmental signals, especially pro- and anti-inflammatory cytokines, are responsible for down- or upregulation of chemokine receptors (**91**). As high amounts of cytokines are produced rapidly after NKT cell activation, we

hypothesized that cytokines produced by activated NKT cells or licensed DCs might be responsible for CCR4 induction. Soluble signals seemed obvious candidates to induce CCR4 expression, as they could influence chemokine receptor expression at a distance and facilitate cellular attraction towards CCL17-producing DCs. Indeed, CCR4 expression could be induced in naïve $CD8^+$ T cells after coculture with serum from αGalCer-treated mice, indicating that a soluble mediator that is released into the circulation of injected mice can influence CCR4 production. However, no upregulation of CCR4 was detected on naïve $CD8^+$ T cells after treatment with any conventional cytokine produced by activated NKT cells or DCs, or with a combination of these cytokines.

Although the involvement of the CCR4 ligands themselves seemed unlikely, the effect of CCL17 and CCL22 on CCR4 induction was investigated as they might act independently of CCR4 expression on $CD8^+$ T cells by binding to an as yet unidentified receptor (whose existence has been suggested previously (**52**, **53**)). However, neither the individual chemokines nor their combination altered CCR4 expression *in vitro*. Yet, these results cannot exclude an indirect effect of CCL17 or CCL22 *in vivo*, as they might bind to CCR4-expressing cells like NKT cells or DCs to induce expression of unidentified mediators that could in turn influence CCR4 expression in $CD8^+$ T cells. Analysis of CCR4 induction in CCL17-deficient mice could clarify if CCL17 has an indirect impact on the expression of CCR4.

Furthermore, the investigated classical cytokines might be indirectly involved in regulation of CCR4 expression as they might affect other cell types, which in turn could produce the critical agent for CCR4 induction. This agent could be a factor expressed by non-hematopoietic cells inside of T cell zones or at the place of T cell entry, the marginal zone bridging channels. Thus, soluble mediators related to T_H2 responses that can be expressed by non-hematopoietic cells were investigated for their influence on CCR4 expression. Thymic stromal lymphopoietin (TSLP) can be produced by epithelial cells in asthma, but this factor did not affect CCR4 expression in our setting. Also the T_H2-related cytokine IL-33, which can be expressed by several cell types including endothelial cells (**92**), had no effect on CCR4 expression. Many cell types could function as mediators in CCR4 induction as NKT cell-derived cytokines have been described to be able to influence a variety of cell types, including NK cells, neutrophils and eosinophils (**89**). Also cells that are situated at the site of T cell entry into splenic T cell zones could be involved, as they would be at the ideal position to induce CCR4 expression on immigrating $CD8^+$ T cells. These sites are the marginal zone bridging channels (MZBC), which represent breaks in the marginal sinus. Various cell types, including T cells, are thought to enter the splenic white pulp at these sites through interactions with Fibroblastic Reticular Cells (FRC) that line the MZBC

and function as a kind of access road (**93**). FRCs but also other cell types in this area might be able to directly interact with naïve $CD8^+$ T cells. Additionally there is a strong correlation between NKT cells, mast cells and CCL17 expression, which are all major mediators of asthma development (**94**, **95**). Hence, it would be interesting to investigate the effect of mast cell-derived mediators on CCR4 expression, especially as histamine and prostaglandin E_2 were described to upregulate expression of the CCR4 ligands CCL17 and CCL22 (**84**).

Taken together, these results suggest that CCR4 expression on $CD8^+$ T cells is regulated by a factor or a combination of factors apart from the traditional NKT cell-associated cytokines. What factor it is and which cell it is expressed by requires clarification in further experiments in order to fully understand the regulation of chemokine signals in cross-priming.

5.1.5 Chemokine-dependent regulation of cellular interactions in DC licensing

Naïve $CD8^+$ T cells need to receive several stimulatory signals provided by DCs to become activated. Thus, a rare antigen-specific $CD8^+$ T cell needs to encounter a mature DC presenting its specific antigen. Such cellular interactions are typically regulated by chemokines. The best-described example is CCR7, which coordinates attraction of both antigen-presenting DCs and naïve T cells into the T cell zone of secondary lymphoid organs (SLOs) (**54**). By attracting both cell types to the same compartment inside of SLOs, CCR7 highly increases the likeliness of an encounter between a naïve T cell and its cognate antigen on a DC.

The correct positioning of immune cells is particularly important in classical and NKT cell-mediated cross-priming that depend on the interaction of three different cell types. In these cross-priming events, $CD8^+$ T cells are attracted in a second step following the successful presentation of antigen to a $CD4^+$ T_H cell or an NKT cell. This “secondary” attraction of $CD8^+$ T cells is regulated by CCR5 in classical cross-priming, as the antigen-specific interaction between a $CD4^+$ T_H cell and a DC results in expression of the CCR5 ligands CCL3, CCL4 and CCL5 (**57**) . By a yet unknown mechanism, naïve $CD8^+$ T cells upregulate CCR5 and are thus attracted towards the licensed DCs.

The present study identified CCR4 as the chemokine receptor regulating NKT cell-mediated cross-priming, as it attracted naïve $CD8^+$ T cells towards CCL17-producing DCs that had been licensed by NKT cells. Thus, CCR5 and CCR4 not only locate DCs and $CD8^+$ T cells to the same compartment inside of SLOs, they also actively recruit $CD8^+$ T cells to sites of DC licensing where mature, antigen-presenting DCs can readily prime naïve $CD8^+$ T cells. This directed

migration highly enhances the efficiency of $CD8^+$ T cell responses, probably due to a faster attraction and activation or the attraction and activation of higher numbers of $CD8^+$ T cells.

However, this idea also poses conceptual questions: The fact that $CD8^+$ T cells are attracted in an antigen-independent fashion implies that T cells of random antigen specificity are recruited towards licensed DCs. This suggests an overabundance of naïve $CD8^+$ T cells accumulating at a single DC, which would hamper access to the antigen-presenting DC for the relevant antigen-specific $CD8^+$ T cell. $CD8^+$ T cells would have to leave as quickly as they arrive to make room for other cells. In contrast, the results from *in vitro* migration experiments suggest that CCL17 signaling increases contact duration even in the absence of antigen. The absence of either chemokine receptor expression or CCL17 production reduced contact duration significantly. One scenario that could explain the discrepancy between the *in vitro* data and the proposed *in vivo* problem could be the expression of a certain signal, which was not present in *in vitro* cultures, that decreases contact duration when the presented antigen is not recognized by the T cell. This signal might be induced by an additional cell type that interacts with DCs and / or T cells *in vivo*, one obvious candidate being the NKT cell that licenses the DC. One could envisage a scenario where NKT cells induce the expression of a certain factor by DCs that inhibits T cell adhesion, until the DC receives signals from antigen recognition by a specific TCR, for example via MHC-I. This factor could for example act by modulating the function of the T cell adhesion molecule LFA-1. This hypothesis could be tested by FACS staining for the active form of LFA-1 or microscopically analysis of LFA-1 clustering. However, the *in vitro* experiments did not compare antigen-specific and antigen-unspecific interactions, and interactions in the presence of antigen might be even longer. Visualizing the *in vivo* events by two-photon microscopy could help to clarify the dynamics of DC – $CD8^+$ T cell contacts.

However, it is known that chemokines influence $CD8^+$ T cell priming not only by guiding $CD8^+$ T cells to the site of antigen-presentation, but also by modulating the interaction between T cells and DCs. *Friedman et al.* demonstrated that surface-bound CCL21 prolonged cell contacts (**96**), during which T cells can scan the DC surface. This long-lasting contact was necessary to ensure the sustained signaling that maintains gene transcription and promotes T-cell-cycle progression (**97**). Thus, prolonged contact duration due to chemokine signals also has positive implications, and might nevertheless be involved in mediating efficient T cell responses *in vivo*. The need for rapid dissociation from the CCL17-producing DC might not be as profound as one might assume, as the amount of T cells accumulating at a given time point might be limited by the fact that only 15-20% among all naïve $CD8^+$ T cells upregulated CCR4. However, if upregulation of CCR4 was limited due to the concentration of the factor that upregulates this

chemokine receptor or due to $CD8^+$ T cell intrinsic properties that allow upregulation in only a subset of T cells needs to be further investigated and might provide insights into the regulation of chemokine receptors in T cell subsets. Determining the factors that induce expression of CCR4 on $CD8^+$ T cells would be helpful to address this question, as the responsiveness of $CD8^+$ T cells for these particular factors could be tested.

The mechanistic events behind the chemokine-dependent increase in contact duration have been partially characterized. *Friedman et al.* suggested that chemokine contacts transiently tether $CD8^+$ T cells to chemokine-presenting DCs in a LFA-1 dependent fashion (**96**). It is well established that chemokine receptor activation can increase the affinity of LFA1 for ICAM1 (**98**, **99**), which would augment the stability of cell-cell contacts. Besides integrin-mediated adhesiveness, *Friedman et al.* observed that the $CD8^+$ T cell acquired a polarized morphology and the T cell receptor (TCR) localized towards the contact site, whereby the hyperpolarized T cell gained enhanced sensitivity to antigen at the leading edge (**96**). This might be a mechanism that has a costimulatory effect on the induction of $CD8^+$ T cell responses and may also play a role in CCL17-mediated contact duration. They found no influence of CCL17 on tethering of $CD8^+$ T cells, but this could be due to the fact that they used untreated $CD8^+$ T cells that do not express CCR4 and could therefore not respond to CCL17. An additional mechanism that could increase contact duration is the recruitment of certain chemokine receptors to the immunological synapse (IS). CCR4 belongs to the so-called subordinate receptors that are recruited to the IS and thus become ignorant to chemokine gradients (**100**, **101**). By this mechanism, more stable contacts between DC and $CD8^+$ T cell might be formed and this would permit more extensive scanning of the DC surface. This CCL17-dependent increase in $CD8^+$ T cell – DC contact duration might also account for higher numbers of $CD8^+$ T cells in splenic T cell zones evidenced by histology, as $CD8^+$ T cells might accumulate due to a prolonged interaction with DCs.

In summary, CCL17 may increase the efficiency of $CD8^+$ T cell responses on the one hand by mediating the preferential scanning of “relevant” DCs that provide both antigenic and costimulatory signals, and are therefore more likely to induce an efficient immune response than unlicensed DCs. On the other hand, CCL17 prolongs $CD8^+$ T cell - DC contact duration. As CCL17 signals can be considered as indicators of the presence of mature, antigen-presenting DCs, it is reasonable that they increase the accuracy with which $CD8^+$ T cells scan these DCs for the presence of their specific antigen. Thereby CCL17 expression further enhances the likeliness of efficient T cell activation. *In vivo* cytotoxicity assays clearly demonstrate the

impact of these chemokine signals on cross-priming, as the cytotoxic capacity of $CD8^+$ T cells was reduced by 50-60% in CCL17- and CCR4-deficient mice.

5.2 Synergistic effect of classical and NKT cell-mediated DC licensing

5.2.1 Distinct regulation of classical and NKT cell-mediated cross-priming

The data presented in this study identify CCL17 and CCR4 as the second chemokine – chemokine receptor pair that regulates cross-priming besides CCR5 and its ligands CCL3, CCL4 and CCL5. DC licensing by $CD4^+$ T_H cells in the presence of the TLR9 ligand CpG induces expression of CCR5 ligands (**57**) whereas expression of CCR4 and its ligands is induced by the presence of glycolipid antigens through activation of NKT cells. Moreover, $CD4^+$ T_H cells were dispensable for NKT cell-mediated cross-priming as demonstrated in MHCII-deficient mice, which were able to generate a $CD8^+$ T cell response as efficient as were wild-type mice after immunization with OVA plus αGalCer, suggesting that classical and NKT cell-mediated cross-priming operate separately. Histological analysis revealed that $CD8^+$ T cells are attracted in a CCR4-dependent fashion in NKT cell-mediated but not classical cross-priming. On the other hand, CCR5 attracted $CD8^+$ T cells in classical but not NKT cell-mediated cross-priming. Furthermore, the cytotoxic T cell response of CCR5-deficient mice was as efficient as that of wild-type mice after injection of OVA plus αGalCer, confirming that CCR5 is not involved in NKT cell-mediated cross-priming. Additionally, CCL17 expression is not influenced by CpG-induced TLR9 stimulation, as similar percentages of DCs expressed comparable levels of CCL17 after injection of OVA plus αGalCer with or without CpG. These results indicate that the chemokines, which are induced by classical and NKT cell-mediated DC licensing do not influence each other's expression. Thus one can state that classical and NKT cell-mediated cross-priming are regulated by distinct chemokine pathways that do not influence each other and that both ensure improved attraction and / or retention of $CD8^+$ T cells.

5.2.2 Synergistic effect of classical and NKT cell-mediated cross-priming on $CD8^+$ T cell responses

The fact that classical and NKT cell-mediated cross-priming are regulated by different chemokines raises the question what advantage might lie in the induction of two distinct chemokine pathways. One possible explanation could be that they might act in a synergistic manner to further enhance T cell activation, as successful antigen-presentation to both $CD4^+$ T_H

and NKT cells would emphasize the relevance of the presented antigen. To address this hypothesis, I analyzed the cytotoxic T cell response after the simultaneous activation of both classical and NKT cell-mediated cross-priming by co-injecting of OVA with αGalCer and CpG. Interestingly, the $CD8^+$ T cell response was highly improved compared to coinjection with αGalCer or CpG alone, and this could be observed both after intravenous injection in the spleen as well as in draining lymph nodes after subcutaneous injection, which is especially relevant for hypothetic application for vaccination.

These results might be relevant for application in vaccination strategies and tumor therapy. So far, both TLR ligands and NKT cell ligands alone have been identified as valuable adjuvants for vaccinations against pathogens as well as tumors (**102**,**103**,**104**). αGalCer is already being employed as an adjuvant in tumor immunotherapy (**105, 72-74**), in which the transfer of αGalCer-loaded DCs has so far provided the best results. However, the results are not yet optimal in terms of disease amelioration and may be further improved by the simultaneous application of CpG. Indeed, some reports have indeed described a beneficial effect of the combined application of αGalCer and TLR ligands (**70, 106**). For other diseases in which NKT cells are involved like malaria, *Borrelia burgdorferi* or *Sphingomonas* infections, it seems likely that pathogens provide a range of microbial agents and would activate both pathways.

Thus, to be able to optimize treatment strategies, the events underlying enhanced $CD8^+$ T cell responses after the combination of αGalCer and CpG injection were investigated. Obvious factors that could be involved are increased levels of costimulatory molecules, cytokines and chemokines. Concerning signal 2, it has been described that the expression of costimulatory molecules is further increased after co-injection of αGalCer with various TLR ligands, including CpG, compared to injection of one adjuvant alone (**106**). In that way, DCs could provide more costimulatory signals and improve $CD8^+$ T cell activation. Nevertheless, the results presented in this study demonstrate that chemokine signals are at least as important for efficient $CD8^+$ T cell responses as costimulatory signals, as cytotoxic T cell responses were reduced by around 60% due to lack of chemokines. The exact contribution of costimulatory signals versus chemokine signals to cross-priming has not yet been investigated, but experiments could be performed using CD80/86-deficient mice to address this question.

However, the present study focused on the contribution of chemokines to enhanced $CD8^+$ T cell responses after the combined activation of classical and NKT cell-mediated DC licensing. First, the attraction and accumulation of $CD8^+$ T cells was investigated. Indeed, increased numbers of $CD8^+$ T cells accumulated in splenic T cell zones after injection of OVA with αGalCer

plus CpG compared to coinjection with either adjuvant alone, confirming the importance of chemokine signals for the synergistic effect. As a first approach to answer the question how this enhanced migratory effect is mediated, chemokine production by DCs was investigated. The results revealed that DCs produced CCL17, CCL3 and CCL5 after injection of OVA plus αGalCer plus CpG. Interestingly, the same DCs that produce CCL17 co-expressed the CCR5 ligands whereas the remaining DCs produced only low levels of CCL3 and CCL5. These results raised the question how chemokine production is orchestrated *in vivo*. As shown in the present study, the induction of chemokine expression is linked to cell-cell interactions during DC licensing, but soluble factors such as IL-4, IL-13 and TNFα were able to induce chemokine expression *in vitro*. Thus, cell-cell contact between a $CD4^+$ T_H or an NKT cell with a DC might induce chemokine expression through the upregulation of cytokines rather than in a cell-cell contact-dependent fashion. In this case, all secreted cytokines would act on the same DCs, leading to the uniform production of several chemokines by the same cells. However, only a subset of DCs expresses these chemokines. One possible explanation could be that the effect of the secreted cytokines could be restricted to a certain area, so that only DCs in that area would respond by expressing chemokines. Another explanation could be that only a subset of DCs is responsive to the factors that induce chemokine expression. This question could be addressed by staining for IL-4- and TNFα-receptors on CCL17-expressing or non-expressing DCs. Furthermore, DCs might be rendered responsive to the critical signals during licensing, which could be investigated by staining for the relevant receptors before and after licensing. Furthermore, it would be necessary to test the requirement of cell-cell contact for CCL17 expression by analyzing the CCL17 expression of licensed or unlicensed DCs. To this end, CD1d-deficient and –sufficient DCs from CD1d-/- / bm1 mixed bone marrow chimeras could be sorted after injection of OVA plus αGalCer, and they could be analyzed for CCL17 expression by real-time PCR. CCL17 expression by CD1d-deficient DCs would argue against the requirement for cell-cell contact in inducing chemokine expression. Furthermore, it will be interesting to investigate chemokine production by the licensing cells, namely $CD4^+$ T_H and NKT cells. *Castellino et al.* described the expression of CCL4 in classical licensing. As the present study did not detect the expression of CCL4 by DCs, the source of this chemokine could be $CD4^+$ T_H cells. In parallel, NKT cells produced the second CCR4 ligand CCL22. Thus, not only DCs but also the licensing T cells seem to contribute to the attraction of naïve $CD8^+$ T cells.

My results suggest that $CD8^+$ T cells expressing CCR4 and CCR5 are attracted towards the same DCs. As mentioned above, this concept is problematic in a way that an overabundance of attracted T cells could inhibit T cell activation rather than enhancing $CD8^+$ T cell responses.

However, production of both CCR4 and CCR5 ligands by the same DC might facilitate T cell attraction as competing chemokine signals could distract $CD8^+$ T cells and even impair their directional migration. *Ricart et al.* (**107**) demonstrated that DCs stopped their directional migration when they were exposed to two equipotent perpendicular chemokine gradients of CCL19 and CXCL12 and limited their migration to a zone between the gradients. Nevertheless, whether this is also true for $CD8^+$ T cells remains unclear.

However, it will be interesting to characterize the expression of chemokine receptors on $CD8^+$ T cells. As chemokine receptor expression seems to be upregulated by soluble signals, it can be expected that $CD8^+$ T cells coexpress both receptors. Nevertheless, only a small percentage of $CD8^+$ T cells upregulated CCR4, raising the question if the critical signal for CCR4 induction only reaches a small number of $CD8^+$ T cells, or if some $CD8^+$ T cells are not responsive to this agent. Whether this is also the case for CCR5 expression and by which cells CCR4 and CCR5 are expressed needs to be determined by flow-cytometric analysis. These experiments might clarify whether their synergistic effect is mediated by attraction of higher numbers of $CCR4^+CCR5^+$ $CD8^+$ T cells, or if a mixture of $CCR4^+$ and $CCR5^+$ $CD8^+$ T cell subsets accumulates. The latter would raise questions about the prerequisites for the expression of chemokine receptors by $CD8^+$ T cells, and these require further investigations.

In summary, the results obtained during my thesis work suggest that the synergistic effect of classical and NKT cell-mediated cross-priming is at least in part mediated by migratory cues. However, the exact mechanisms underlying the enhanced $CD8^+$ T cell responses require further investigations, and the contributions of antigenic, costimulatory, cytokine and chemokine signals need to be determined. Clarifying these mechanisms could help to improve vaccination strategies and will further the understanding of chemokine-regulated cell interactions.

6 Summary

The detection and destruction of virus-infected or tumor cells is the main task of $CD8^+$ T cells. Their activation needs to be tightly regulated to avoid the misguided killing of healthy cells. To this end, activation of $CD8^+$ T cells requires not only antigenic signals but also additional costimulatory signals provided by mature DCs. The process that renders DCs capable of cross-priming has been termed DC licensing and can be induced by antigen-specific interactions with $CD4^+$ T_H cells. As the frequencies of specific antigen-bearing DCs and the relevant antigen-specific naïve $CD4^+$ and $CD8^+$ T cells are low, the involvement of chemokines as regulators of cell – cell interactions seems likely. Indeed, CCR5 has been described to be upregulated on naïve $CD8^+$ T cells, thereby attracting these cells to the site of DC – $CD4^+$ T_H cell interactions, where the CCR5 ligands CCL3 and CCL4 are produced. Recently it has been established that not only $CD4^+$ T_H cells but also NKT cells can provide help for DC maturation, but the exact mechanisms remain unclear.

The present study characterizes NKT cell-mediated DC licensing and presents a new aspect of DC licensing by demonstrating that the presentation of glycolipid antigens by DCs to NKT cells resulted not only in DC maturation but also in the expression of the chemokine CCL17 by the antigen-presenting DC. Moreover, it establishes that the induction of this chemokine is a critical component of DC licensing and is crucial for the generation of efficient $CD8^+$ T cell responses, as the lack of CCL17 or its receptor CCR4 strongly impaired cross-priming. Several possible mechanisms for impaired cross-priming were investigated. Published findings of others made an effect on DCs and NKT cells most likely, but this was not the case. Instead, CCL17 increased the efficiency of cross-priming by acting directly on $CD8^+$ T cells. Several experimental approaches revealed that $CD8^+$ T cells that had upregulated CCR4 in response to a yet unknown factor were attracted by CCL17 towards licensed DCs. Additionally, CCL17 increased the DC – $CD8^+$ T cell contact duration, thus promoting efficient scanning of the DC surface and enhancing the chances of efficient cross-presentation.

These results identify CCL17 – CCR4 as a second chemokine – chemokine receptor pair that regulates cross-priming. Furthermore, they uncovered a previously unrecognized role of CCL17 and CCR4 in cytotoxic T cell responses. Finally, these results demonstrated that the two chemokine mechanisms that regulate classical and NKT cell-mediated cross-priming act in a synergistic manner by further increasing the efficiency of $CD8^+$ T cell responses. Understanding the molecular mechanism of this synergistic effect may help improving vaccination strategies.

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8 Abbreveations

αGalCer	α-galactosylceramide
APC	antigen-presenting cell
BM	bone marrow
Bp	basepair
BSA	Bovine serum albumin
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CFSE	5,6-Carboxy-Succinimidyl-Fluoresceine-Ester
CTL	cytotoxic T lymphocyte
DC	Dendritic cell
FACS	Fluorescence activated cell sorting
FCS	Fetal Calf Serum
FITC	Fluorescein-5-isothiocyanate
FRC	Fibroblastic reticular cell
HEV	High endothelial venules
ICAM	Intracellular adhesion molecule
IFN	Interferone
IL	Interleukine
NKT cell	Natural killer T cell
i.p.	intraperitoneal
IS	Immunological Synapse
i.v.	intravenous
LFA-1	Leukocyte functional antigen 1
LN	Lymphnode

LPS	Lipopolysaccharide
MACS	Magnetic activated cell sorting
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
OPD	O-phenylendiamin
OVA	Ovalbumin
PFA	Paraformaldehyde
RAG	Recombination activating gene
RT PCR	real-time polymerase chain reaction
s.c.	subcutaneous
SLO	Secondary lymphoid organ
TARC	Thymus and activation-regulated chemokine
TCR	T cell receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T_H	T helper cell