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Antigen targeting to plasmacytoid dendritic cells - induction of tolerance or immunity

Jakob Loschko

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Vorsitzender: Univ.- Prof. Dr. Bernhard Küster
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2. Priv.- Doz. Dr. Anne Krug
3. Jun.- Prof. Dr. Diana Dudziak
Friedrich-Alexander-Universität Erlangen-Nürnberg

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α	anti
aa	amino acid
Ab	antibody
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
APC	antigen presenting cell
APC	allophycocyanin
APS	ammonium persulfate
BCR	B cell receptor
BST2	bone marrow stromal cell antigen 2
bp	base pair
BSA	bovine serum albumin
°C	centigrade
CD	cluster of differentiation
cDC	conventional dendritic cell
CFA	complete Freund's adjuvant
CFSE	carboxyfluorescein succinimidyl ester
C_H	constant region of the heavy chain
C_L	constant region of the light chain
CTL	cytotoxic T lymphocyte
DC	dendritic cell
ddH_2O	double distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTP	deoxyribonucleotide triphosphate
ds	double stranded
DTx	diphtheria toxin
EAE	experimental autoimmune encephalomyelitis
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
e.g.	for example (*exempli gratia*)

ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Flt3L	FMS-like tyrosine kinase 3 ligand
Fl-DCs	bone marrow derived DCs generated with Flt3L
for	forward
Foxp3	forkhead box P3
g	g force
h	hour(s)
H_C	heavy chain
HEK293T	human embryonic kidney cells expressing the SV40 large T antigen
HEL	hen egg lysozyme
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HRP	horseradish peroxidase
IFN	interferon
IFN I	type I interferon
IgG	immunoglobulin
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
kb	kilobase
kDa	kilodalton
KO	knockout
L_C	light chain
LB	lysogeny broth
M	molar
mAb	monoclonal antibody
MCS	multiple cloning site
µg	microgram
mg	milligram
MHC	major histocompatibility complex
MHC I	major histocompatibility complex class I

MHC II	major histocompatibility complex class II
min	minute(s)
ml	milliliter
mm	millimeter
mM	millimolar
MOG	myelin oligodendrocyte glycoprotein
MW	molecular weight
n	number of replicates
N	normal
NaOH	sodium hydroxide solution
NEAA	non essential amino acids
ng	nanogram
nm	nanometer
nt	nucleotide
OD	optical density
OVA	Ovalbumin
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PEI	polyethylenimine
PFU	plaque forming units
pHEL	HEL peptide
pHEL/I-A^{k}	pHEL/MHC II complex
pMOG	MOG peptide
PolyI:C	polyinosinic-polycytidylic acid
pOVA	OVA peptide
PRR	pattern recognition receptor
PTx	pertussis toxin
PVDF	polyvinyl difluoride
RACE	rapid amplification of cDNA ends
rev	reverse

RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
s.c.	subcutaneous
sec	second(s)
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of mean
Siglec-H	sialic acid binding Ig-like lectin H
ss	single stranded
Strep	streptavidin
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
TBST	tris buffered saline with Tween-20
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-β	transforming growth factor beta
T_H	T helper cell
TLR	Toll-like receptor
Treg	regulatory T cell
U	unit(s)
V_H	variable region of the heavy chain
V_L	variable region of the light chain
vs.	*versus*
VV	vaccinia virus
v/v	volume per volume
w/o	without
wt	wildtype
w/v	weight per volume
w/w	weight per weight

1 INTRODUCTION

1.1 The immune system

Complex multi-cellular organisms like mammals have evolved next to organisms that outlasted millions of years in an almost unaltered way. Simple organisms have an incredible advantage because their short generation time allows them to rapidly evolve to adapt to environmental changes. To keep up with those microorganisms, complex organisms like mammals that have a relatively long generation time had to develop systems that protect them from the invasion of microorganisms. In addition to microbes like bacteria, protozoa, helminths and fungi, viruses are an additional threat that requires an efficient defense system. With the immune system, mammals have developed the most complex and diverse mechanism to defend themselves against different invading pathogens. Because this system is very sensitive and complex it is also susceptible to making errors. The biggest challenge is therefore to provide protection against different invading pathogens and simultaneously avoid self destruction of the host by exaggerated immune responses.

Although every animal and also to some extent plants have many different strategies to defend themselves against invading pathogens that can be called immune system or at least resemble one, this work is focusing on the mammalian immune system. The mammalian immune system is divided into two main branches - the innate immune system that relies on germline encoded receptors and the adaptive immune system that has the ability to evolve with its tasks.

1.1.1 Innate immunity

The innate immune system has long been believed to be an ancient and unspecific defense mechanism against a rather undefined threat. It has then however been shown that a vast number of different receptors sensing diverse signals forms a tight network that might not be as flexible as the adaptive immune response, but compensates this fact with its plurality and sensitivity. The main characteristic of the innate immune system is that its components are germline encoded and highly evolutionary conserved within different species. Ligands for receptors of the innate immune system are so-called pathogen associated molecular patterns (PAMPs).

PAMPs act as danger signal and are molecular structures that are common to many different microbes such as bacterial and viral DNA and RNA as well as components of the bacterial cell wall like lipopolysaccharides. Receptors for these structures are the pattern recognition receptors (PRRs), a large and diverse group of proteins and multi-protein complexes. They all have in common that their aim is to trace pathogenic invaders and activate the defense mechanisms of the host.

Among the best characterized receptors are the membrane bound Toll-like receptors (TLRs), members of the RIG-I like receptor family (RIG-I, MDA5, LGP2) that are all localized in the cytoplasm and the NOD-like receptors that form large multi-protein complexes like the inflammasome (Eisenacher and Krug, 2011; Lemaitre *et al*., 1996; Martinon *et al*., 2002; Medzhitov *et al*., 1997; Pichlmair *et al*., 2006; Takaoka *et al*., 2007). PRRs are differentially expressed by a variety of different cell types including classical immune cells such as dendritic cells (DCs) and macrophages, as well as non hematopoietic cells such as fibroblasts or stromal cells. After a danger signal has been sensed through ligation to its receptor, different signals such as cytokines, chemokines and activating cell surface molecules transmit the danger signal to other cells, especially to those of the adaptive immune system. Ligation of unmethylated CpG motif containing DNA, the ligand for TLR9, is for instance followed by the production of high levels of type I interferon (IFN I) and proinflammatory cytokines such as IL-6 (Hemmi *et al*., 2000). PolyI:C, an artificial analog of double stranded RNA is sensed by TLR3 and MDA5 and also induces the production of high levels of IFN I (Kato *et al*., 2006).

Although the signals that are transmitted by the innate immune system usually result in the initiation of an adaptive immune response, it does not necessarily mean that the innate immune system is not able to clear an infection by its own. Many pathogenic infections can be successfully fought by the innate immune systems without inducing a systemic adaptive immune response. However, involvement of the adaptive immune system is favorable, because an adaptive immune response does not only help to clear an acute infection, but moreover results in long lasting antigen specific immunological memory that provides protection for future contacts with the pathogen.

1.1.2 Adaptive immunity

It has been shown within the last years that the mammalian innate immune system is not the rudimental relict of a primitive past that it has long been believed. Instead, it is a very complex and sensitive system allowing the host to respond efficiently and rapidly to a seeming superiority of pathogens. However, it is still not sufficient to guarantee the survival of the fittest as life is an evolving and dynamic process and new or modified pathogens emerge continuously. To keep up with bacteria and viruses that adapt to changing conditions and requirements very rapidly, germline encoded receptors that recognize highly conserved molecular structures are not always applicable and adequate. Therefore, the adaption to the flexible world of pathogens is covered by the second branch of the immune system - the adaptive immune system.

The main players of the adaptive immune response are B and T cells. In contrast to the receptors of the innate immune system, B cell receptors (BCR) and T cell receptors (TCR) mainly recognize specific peptides derived from protein antigens. The hallmark of the adaptive immune system is that the spectrum of its receptors is not limited in the recognition of antigens. The reason why B and T cell receptors can recognize a vast variety of diverse antigens is their unique composition and development. Both, BCR and TCR are composed of different fragments. Constant regions are similar within all BCRs and TCRs, respectively. Variable regions that account for the specificity of the receptors are however unique for each receptor. Each genome encodes for many different of those variable and constant regions. In a unique process called gene-rearrangement different fragments are randomly fused. This randomized composition ensures that, although all BCRs resemble each other and also every TCR is composed in a very similar way, each BCR and also every TCR has a unique specificity for a defined antigen. Another important attribute of the adaptive immune system is that it can provide immunological memory by preserving distinct B and T cell receptors in specialized memory B and T cells.

1.1.2.1 B cells and antibodies

The main duty of B cells is the production of specific antibodies against distinct antigens. To a smaller extent they also play a role as antigen presenting cells (APC). After a naïve B cell has recognized its specific antigen, it gets activated and eventually develops into a long living memory B or an antibody secreting plasma cell. The specific antigen is recognized by the BCR. The BCR is composed of a membrane bound form of an antibody and a signaling moiety (CD79). The signaling part is uniform for every BCR, whereas the antibody part differs on each B cell. An antibody is composed of two identical light and two identical heavy chains. The sequences encoding for them are located in clusters called the “V” (variable) and the “C” (constant) region. The variable region of the antibody’s heavy chain consists of three segments named V (variable), D (diverse) and J (joining). Each genome encodes for a multitude of V, D and J segments. During B cell development the three segments recombine randomly, a process that is unique for B cells and is called V(D)J rearrangement. This results in the generation of a unique variable domain that is then fused to the constant part of the antibody’s heavy chain. The same processes occur during the generation of the antibody’s light chain with the difference that fewer segments are involved in this recombination. In a second selection step, BCRs of activated B cells are further improved by a process that is called somatic hypermutation. During this selection, only BCRs with high antigen binding affinity mediate the survival of the B cell (positive selection) which then becomes a memory B cell. B cells expressing receptors that recognize self antigen do not survive the selection to prevent autoimmunity (negative selection).

However, recognition of the specific antigen *via* the BCR *per se* is in many cases not sufficient to induce successful and efficient activation of antigen specific B cells. There are some antigens that can induce B cell responses without additional help from T cells. Mice without a thymus which lack T cells can develop B cell responses against those antigens. Nevertheless, for the induction of strong and efficient B cell responses and the subsequent development of plasma cells and memory B cells, help from effector T cells is mandatory. As B cells possess the ability to present antigen on major histocompatibility complex (MHC) II they can present antigens they have acquired to T cells. This antigen presentation might not be sufficient to induce effector T cell responses in naïve T cells, it is however sufficient to drive T cells to

produce effector cytokines when they have been primed with the same antigen by more potent APCs before. These cytokines trigger the proliferation of antigen specific B cells and their differentiation into antibody secreting plasma cells and long living memory B cells. Moreover, T cell mediated B cell activation induces a process called isotype switching. During this step, the approved variable regions of the antibody are maintained, but the constant region is replaced resulting in the production of IgG, IgA and IgE rather than IgM or IgD (Rajewsky, 1996).

1.1.2.2 T cells

In contrast to B cells that promote their immune functions mainly through soluble antibodies, T cells are the key players for cell mediated adaptive immune responses. Common to all subsets of T cells is the expression of the TCR. This receptor complex enables T cells to recognize antigen. It is generated during T cell development by random recombination of different fragments encoding for different parts of the TCR. Similar to B cells, each T cell is specific for a distinct antigen and undergoes many selection steps during its development to avoid the presence of autoreactive T cells.

CD4+ T cells express the co-receptor CD4 that binds to MHC II molecules. CD4+ T cells only recognize antigen that is presented on MHC II, whereas CD8+ T cells can only be activated by antigen that is presented on MHC I molecules.

After antigen has been presented to naïve CD8+ T cells and those cells have been successfully activated, they develop into cytotoxic T lymphocytes (CTL) which can directly mediate the killing of target cells. With the exception of erythrocytes all cells express MHC I. This provides the possibility that every cell can present foreign antigen after it has been infected, for example by a virus. Cells that present foreign antigen on MHC I become target cells for CTLs and can be rapidly removed to limit the spread of an infection. Killing of target cells is mediated by different classes of proteins (perforin, granzyme, granulysin) that act as cytotoxins and induce apoptosis. A second way to induce apoptosis in target cells is the ligation of Fas-ligand on CD8+ T cells and Fas on target cells. Although Fas/Fas-ligand mediated killing of target cells might also contribute for example to the clearance of viral infections its main role presumably is the removal of excessive lymphocytes that are present shortly after an infection has been cleared.

The CD4+ T cell subset is divided in several subtypes. CD4+ T helper cells (e.g. IFN-γ producing T_H1, IL-4 producing T_H2, IL-17 producing T_H17) further activate immune responses such as the differentiation of B cells into plasma cells and memory B cells as well as the activation of macrophages. To efficiently induce effector T helper cells, antigen has to be presented on MHC II in the context of co-stimulatory molecules like CD80 and CD86 and in the presence of proinflammatory cytokines such as IL-6, IL-12 and IFN I.

In contrast to effector CD4+ T helper cells, the main task of CD4+ regulatory T cells (Tregs) is the maintenance of tolerance in the steady state, the suppression of autoreactive T cells that escaped clonal selection in the thymus and the shutdown of adaptive immune responses after an infection has been cleared. The protein forkhead box P3 (Foxp3) has been identified to be one of the key transcription factors that mediate the differentiation of Tregs. Loss of function mutations in this important transcription factor lead to fatal autoimmune disease (Bennett *et al*., 2001a; Bennett *et al*., 2001b). To date, two different origins of Tregs are known. They can develop in the thymus, whereas the exact mechanism of Treg differentiation is still a matter of intense research and debate. It is assumed that self-reactive T cells in the thymus are more prone to become Tregs than T cells that recognize foreign antigens (Klein *et al*., 2009; Wirnsberger *et al*., 2011; Wirnsberger *et al*., 2009). Those thymus derived Tregs are termed naturally occurring Tregs or natural Tregs. Tregs can however not only develop within the thymus, but also arise from naïve T cells in secondary lymphoid organs (e.g. lymph nodes, spleen) during adaptive immune responses. These Tregs are known as adaptive or induced Tregs. The discovery that regulatory T cells can develop in secondary lymphoid organs raised the hope that Tregs could be actively induced either to prevent autoimmunity in patients with certain predispositions for autoimmune disorders or even to reestablish tolerance in ongoing autoimmune reactions to cure patients suffering from autoimmune diseases such as multiple sclerosis. Supporting this idea are findings showing that Tregs mediate tolerance not necessarily in an antigen specific manner (Verginis *et al*., 2008). This is important, because many autoimmune diseases (e.g. multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus) are not restricted to one antigen, but multiple autoantigens activate a multitude of different effector T cells. Hence, induced Tregs could suppress autoreactive T cells even if they do not have the same antigen specificity.

Although the active induction of Tregs could certainly help to treat and/or cure autoimmune diseases, so far, there is no system that enables the *de novo* generation of Tregs in humans *in vivo*. The main focus of influencing adaptive immune responses is to induce protection. However, most of the current vaccines were developed to induce protective antibody responses but are not very efficient in the induction of effector T cell responses. CD4+ as well as CD8+ T cell responses are however required for the prevention or treatment of infections with persisting intracellular pathogens (e.g. *Mycobacterium tuberculosis*) as well as for cell based tumor therapy. One of the main aims in vaccine development is therefore to establish methods that allow to induce strong T cell responses where required (e.g. protective immunity against bacteria and viruses, cell based tumor therapy) or to suppress pathogenic T cell responses with the help of induced regulatory T cells if needed (e.g. autoimmune diseases, organ transplantation).

1.1.3 Dendritic cells link innate and adaptive immune responses

Shortly after DCs have been identified and characterized, their contribution for the initiation of innate and adaptive immune responses was revealed (Steinman *et al*., 1975; Steinman and Cohn, 1973, 1974; Steinman *et al*., 1979; Steinman *et al*., 1974). DCs are now considered to be the most important APCs in the mammalian immune system. These cells have the unique ability to link the innate to the adaptive immune response (Banchereau and Steinman, 1998; Lanzavecchia and Sallusto, 2001). Extensive study of this cell type revealed that DCs are a complex family of professional APCs rather than just one distinct cell type. They fulfill a multitude of important tasks.

Migratory DCs capture antigens in the periphery, the most likely point of pathogen entry, and transport them to secondary lymphoid organs (e.g. lymph nodes, spleen) where subsequent adaptive immune responses can be initiated either directly by antigen presenting migratory DCs or by antigen transfer to resident DCs. Resident DCs capture, process and present blood borne antigens and play an essential role especially for the cross-presentation of antigens (Belz *et al*., 2005; Schnorrer *et al*., 2006). Resident DC subpopulations have been extensively characterized in lymphoid organs of the mouse and consist of plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs) which are further subdivided into two major

subpopulations, CD8α+ and CD8α- cDCs. Table 1 displays the most prominent members of the DC family in the mouse and depicts their phenotypes on the basis of differential expression of cell surface markers.

	Resident CD8α+ DCs	Resident CD8α- DCs	pDCs	Migratory CD11b+ DCs	Migratory CD11b- DCs	Langerhans cells	Monocyte derived DCs
CD11c	++	++	+	++	++	++	+
CD8α	++	-	+/-	+/-	+/-	+/-	-
CD11b	-	++	-	++	-	++	++
CD205	+	-	-	+	+	++	+/-
CD207	+	-	-	-	++	++	ND
CD103	+	-	-	-	++	-	ND
B220	-	-	++	-	-	-	ND
Ly6C	-	-	++	-	-	-	++

Table 1: Phenotype of murine dendritic cells subsets.

-, +/-, +, ++ correspond to the expression level of different surface markers by dendritic cells found in lymphoid organs of mice. ND = not determined. (adapted from Segura and Villadangos, 2009)

In the steady state, immature DCs scan peripheral tissues and act as sentinels ingesting material from their surrounding. In the absence of microbial pathogens, these molecules are mainly self-antigens which usually do not trigger the activation of DCs. Immature DCs in the steady state are responsible for mediating the maintenance of self-tolerance in the periphery. Because DCs express a wide variety of PRRs they can be activated easily by different PAMP containing stimuli such as viral or bacterial nucleic acids and components of bacterial cell walls (Luber *et al*., 2010; Robbins *et al*., 2008). They respond to stimulation with dramatic phenotypic changes. Among these changes are the acquisition of a dendritic morphology, the production of proinflammatory cytokines such as IL-6, IL-12 or IFN I, as well as the upregulation of MHC II and co-stimulatory molecules like CD40, CD80 and CD86 (Reis e Sousa, 2006). Due to a whole machinery of different surface molecules, organelles and enzymes DCs can rapidly ingest and present exogenous antigen very efficiently on MHC molecules. Internalization of exogenous antigen can be non-specific (e.g. macropinocytosis, phagocytosis) or receptor mediated (e.g. C-type lectins, Fcγ receptors, scavenger receptors) (Figdor *et al*., 2002; Trombetta and

Mellman, 2005). The process of antigen uptake and presentation is also affected during DC maturation. Acidification of endosomal and lysosomal compartments leads to activation of proteases resulting in enhanced processing of antigen into peptides that can be loaded on MHC molecules. This is followed by an elevated formation of peptide/MHC complexes on the cell surface (Chow *et al*., 2002; Trombetta *et al*., 2003; Turley *et al*., 2000). Changes in the ubiquitination pattern of MHC molecules result in an increased stability of peptide/MHC complexes and an accumulation of antigen that is presented on MHC molecules on the cell surface (De Gassart *et al*., 2008; Shin *et al*., 2006; van Niel *et al*., 2006; Young *et al*., 2008). This results in long lasting presentation of antigens that were present at the same time as the danger signals that triggered DC maturation.

The DC network is composed of different subsets that share features, but have also some specialized functions (e.g. different tissue distribution, differential expression of PRRs, differences in cytokine production). However, for all DC subpopulations the combination of antigen presentation (self *vs.* non-self), co-stimulation (low *vs.* high levels) and cytokine signaling (anti *vs.* proinflammatory) determines if an adaptive immune response is initiated or tolerance is induced (summarized in Fig. 1) (Macagno *et al.*, 2007).

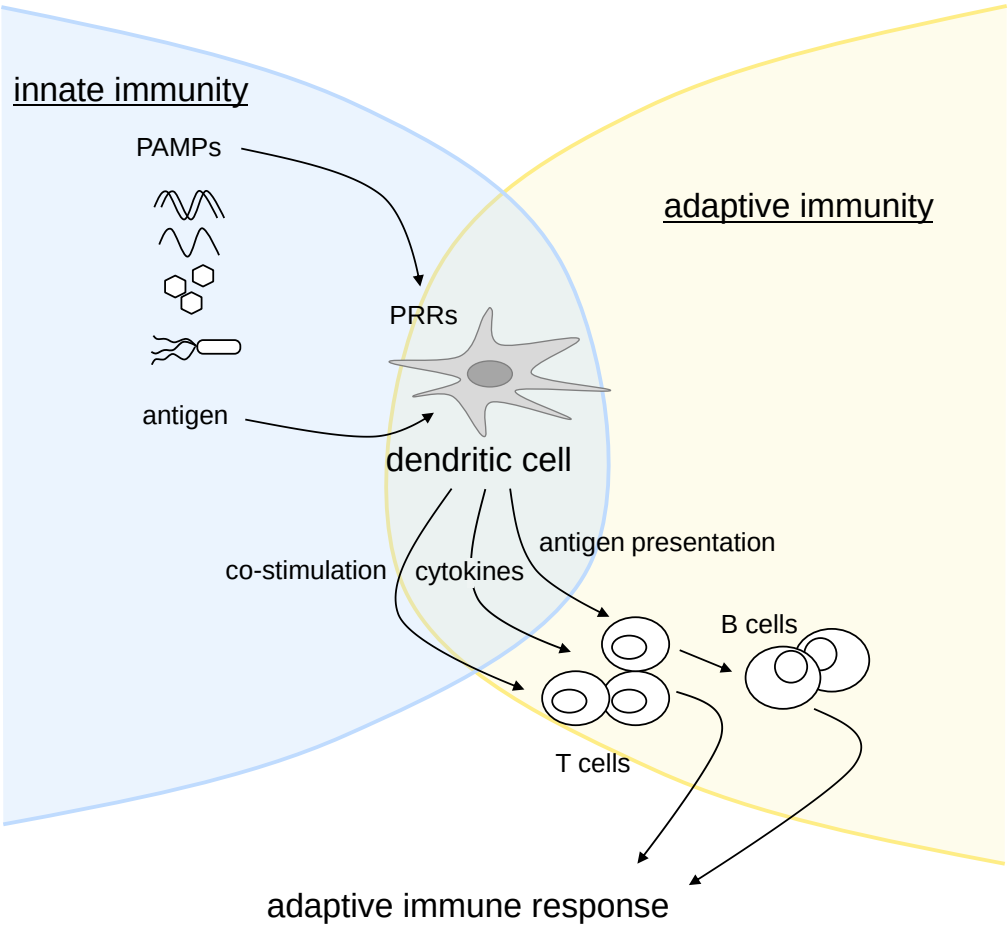

Fig. 1: Dendritic cells link innate and adaptive immunity.
DCs connect the innate to the adaptive immune system. They can detect diverse PAMPs that act as danger signal using different PRRs. Those danger signals drive the maturation of DCs. Activated DCs upregulate co-stimulatory molecules and secrete proinflammatory cytokines. Moreover, DCs can very efficiently capture, process and present antigen on MHC molecules. The combination of antigen presentation, co-stimulation and cytokine signaling determines how successfully T and B cells are activated and whether an adaptive immune response is initiated or if tolerance is induced.

1.1.3.1 Dendritic cell subsets in the mouse

As this study was performed in mice or with murine cells, the description of DC subsets is focusing on findings that have been observed in mice. However, equivalents of murine DC subpopulations with similar functions also exist in humans.
As mentioned, resident DCs can be classified into conventional and plasmacytoid DCs. CDCs consist of a CD8α+ and CD8α- subpopulation. Both cDC subsets express high levels of CD11c and MHC II, but only CD8α+ DCs express CD8α and a cell type specific surface molecule called DEC205 (CD205). In contrast, CD8α- DCs express CD11b and in addition a surface molecule named DCIR2 (Dudziak *et al*.,

2007). PDCs differ from their conventional counterparts in many ways. They only express low levels of the classical DC marker CD11c and lower levels of MHC II, but do express the B cell marker B220. In addition to that, pDCs express sialic acid binding Ig-like lectin H (Siglec-H) and bone marrow stromal cell antigen 2 (BST2), two molecules that are exclusively found on murine pDCs in the steady state (Asselin-Paturel *et al*., 2001; Asselin-Paturel *et al*., 2003; Blasius *et al*., 2006a; Blasius *et al*., 2006b; Zhang *et al*., 2006). Fig. 2 illustrates the different expression of cell surface molecules in distinct murine DC subsets.

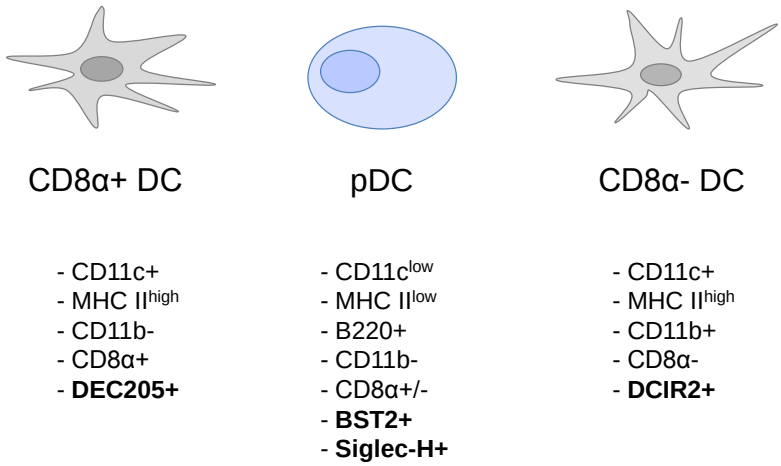

Fig. 2: Murine dendritic cell subsets.
Resident DCs in murine secondary lymphoid organs consist of CD8α+ and CD8α- DCs that are commonly summarized as conventional DCs. Both subsets express high amounts of CD11c and MHC II but differ in the expression of CD8α and CD11b. DEC205 is only expressed on CD8α+ DCs, whereas CD8α- DCs express DCIR2. PDCs differ from cDCs in many ways. Among the differences in the expression of cell surface molecules are Siglec-H and BST2 which are exclusively expressed on pDCs in naïve mice. Bold font indicates cell type specific expression of surface molecules.

As described, the DC network consists of different subtypes that share many features but also differ from one another to some extent and have distinct specialized functions. It has for example been shown that CD8α+ DCs express many proteins that are required for cross-presentation of exogenous antigen on MHC I molecules (Dudziak *et al*., 2007). This specialized machinery enables them to cross-present antigen to CD8+ T cells and makes activated CD8α+ DCs potent inducers of CD8+ effector T cell responses. Due to their constitutive expression of TGF-β immature CD8α+ DCs are also capable of inducing the differentiation of antigen specific naïve T cells into Foxp3+ Tregs and promote peripheral T cell unresponsiveness (Hawiger

et al., 2001; Mahnke *et al*., 2003; Yamazaki *et al*., 2008). The exact opposite is achieved when CD8α+ DCs present antigen in the presence of stimulatory agents such as TLR3/MDA5 ligand PolyI:C or α-CD40. Mature antigen presenting CD8α+ DCs mediate the differentiation of effector CD4+ T cells (mainly T_H1 cells) rather than promoting tolerance by induction of Tregs (Fig. 3) (Bonifaz *et al*., 2004; Boscardin *et al*., 2006; Longhi *et al*., 2009; Pulendran *et al*., 1999; Soares *et al*., 2007; Trumpfheller *et al*., 2008).

Because CD8α- DCs do not express certain proteins that are essential for cross-presentation of exogenous antigens, this DC subset plays only a minor role for the induction of effector CD8+ T cell responses (Dudziak *et al*., 2007). CD8α- DCs promote the differentiation of effector CD4+ T cells, mainly T_H2 cells producing IL-4, rather than mediating the differentiation of Tregs (Fig. 3). However, CD8α- DCs have not been studied as extensively as CD8α+ DCs and their exact role remains to be determined more precisely. (Maldonado-Lopez *et al*., 1999; Maldonado-Lopez *et al*., 2001; Pulendran *et al*., 1999).

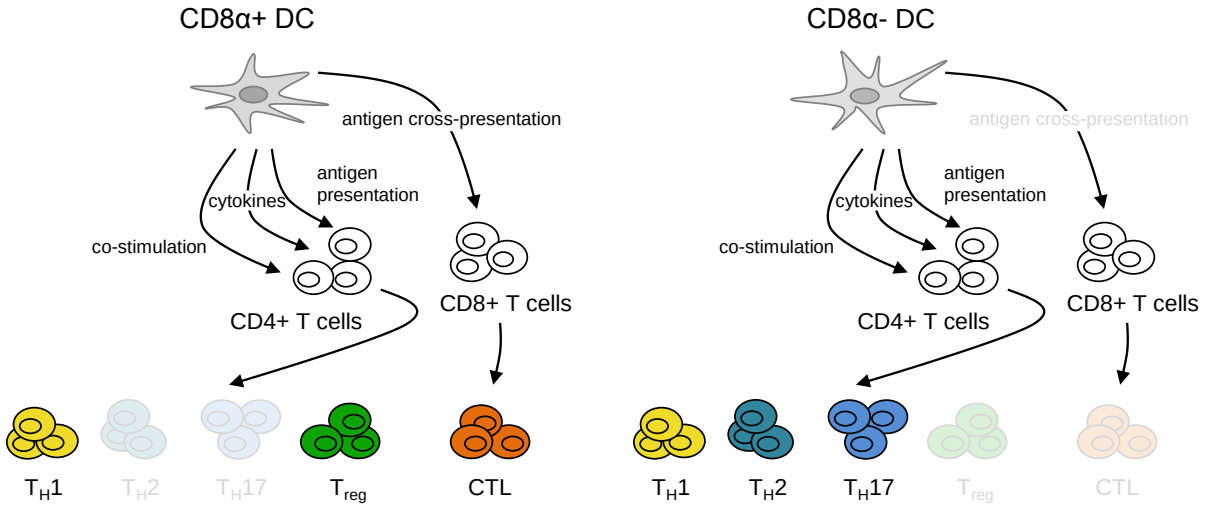

Fig. 3: CDC subsets induce different effector T cells responses.

CD8α+ and CD8α- DCs have different influences on the differentiation of effector T cells. Steady state CD8α+ DCs can induce the expression of Foxp3 in naïve antigen specific T cells and promote the differentiation of Tregs. Activated and mature CD8α+ DCs can break tolerance and induce effector T cell functions (mainly T_H1 responses). Due to their capacity to cross-present exogenous antigen this DC subset is also able to efficiently activate CD8+ T cells. CD8α- DCs fail to induce the expression of Foxp3. Activated antigen presenting CD8α- DCs mainly promote the differentiation of CD4+ effector T cells. They do not cross-present antigen and therefore play no significant role for the induction of CD8+ T cell responses.

In contrast to cDCs that have been studied extensively for many years with the aim to improve DC based vaccination strategies, the role of pDCs in influencing adaptive immune responses is still only poorly understood. PDCs have been regarded in the past as not being very efficient in antigen presentation because they only express low levels of MHC II and were believed to be negligible for adaptive immune responses. PDCs were more regarded as cells that modulate immune responses, mainly known for their ability to secrete large amounts of IFN I during viral infections (Krug *et al*., 2003; Liu, 2005; Swiecki and Colonna, 2010).

Although it has been shown that pDCs can capture, process, and present antigens (Bjorck *et al*., 2008), it still remains unknown to what extent they influence antigen specific T cell responses *in vivo* during ongoing adaptive immune responses. It is still controversial for example, whether pDCs are capable of cross-presenting exogenous antigen to CD8+ T cells and contribute to CTL responses *in vivo* (Mouries *et al*., 2008; Sapoznikov *et al*., 2007). Studies relying on antibody mediated depletion of pDCs indicated that pDCs might be involved in the generation of pathogenic CD4+ T cell responses that lead to autoimmunity (Isaksson *et al*., 2009). In contrast to that, it has also been shown that pDCs can promote T cell tolerance (Bailey-Bucktrout *et al*., 2008; de Heer *et al*., 2004; Hadeiba *et al*., 2008; Irla *et al*., 2010; Jongbloed *et al*., 2009; Ochando *et al*., 2006) even in the presence of activating stimuli (Ito *et al*., 2007). However, many of these *in vivo* studies were based on pDC depletion. Therefore, they could not discriminate between the contributions of innate and adaptive functions of pDCs. Activated pDCs are known to produce high amounts of IFN I and other proinflammatory cytokines, which can have significant impact on T cell responses. It is therefore crucial to further determine to what extent antigen presentation by pDCs influences the outcome of adaptive immune responses *in vivo*.

Irla *et al*. have used a transgenic mouse model in which pDCs lack MHC II expression. In this study, it has been shown that after systemic immunization CD4+ T cell responses were stronger and more prone to mediate autoimmunity in mice with pDCs unable to present antigen on MHC II. Although this study supports previous reports claiming an important role for pDCs in the maintenance and induction of tolerance, it only provides evidence that the failure of pDCs to present antigen on MHC II enhances CD4+ effector T cell responses. A conclusion about whether antigen presentation that is restricted to pDCs could induce antigen specific tolerance

cannot be drawn. Studies investigating the potential of pDCs to induce antigen specific protective adaptive immune responses are even more rare than convincing evidence for the tolerogenic capacity of pDCs. Fig. 4 shows that in contrast to cDCs the role of antigen presenting pDCs for the induction and modulation of adaptive immune responses is not very well understood and remains to be further examined.

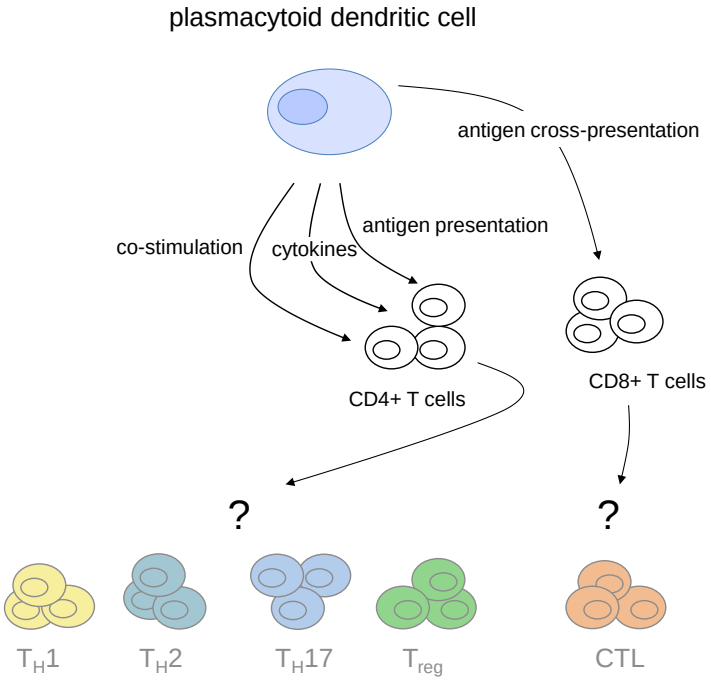

Fig. 4: The role of antigen presenting pDCs for the induction of effector T cell functions remains unclear.

In contrast to cDCs the role of pDCs as antigen presenting cells has not been clarified completely. It is controversially discussed if pDCs are capable to cross-present antigens and also whether pDCs mediate tolerance by inducing the differentiation of Tregs or whether they initiate CD4+ effector T cell responses.

1.1.3.2 Antibody mediated antigen delivery to distinct DC subsets

The idea and the approach to use a recombinant antibody that is directed against an endocytic receptor and is fused to an antigen, to deliver antigen specifically to a DC subset with the aim to induce and influence adaptive immune responses was first described in 2001 for the cell surface receptor CD205/DEC205 (Hawiger *et al*., 2001). By now, several studies showed that antigens can be targeted to different DC subpopulations *in vivo* using antibodies that bind to specifically expressed surface receptors. Table 2 summarizes receptors that have been used for antibody mediated antigen delivery so far. Using specific antibodies for antigen delivery is not only an elegant way to study the role of distinct DC populations, but it has also been shown

that antibody mediated antigen delivery is several orders of magnitude more efficient in inducing T cell activation than free antigen (Bonifaz *et al*., 2004; Corbett *et al*., 2005; Jiang *et al*., 1995).

Although most studies used antibody mediated antigen delivery to investigate the role of a distinct DC population (e.g. DEC205, DCIR2, Dectin-1, CIRE, FIRE, Langerin), several studies have also investigated immune responses after antigen has been delivered to receptors whose expression is more promiscuous (e.g. Clec9A and Clec12A). In addition to targeting endogenous receptors expressed on murine DCs, transgenic mice and mice that were reconstituted with a "human immune system" have been used to test the antibody mediated antigen delivery system and its efficiency with human receptors (e.g. hDC-SIGN and hDEC205).

Receptor	**Target cell**	**First described by**
DEC205	CD8α+ DCs	(Hawiger *et al*., 2001)
DCIR2	CD8α- DCs	(Dudziak *et al*., 2007)
Dectin-1	CD8α- DCs	(Carter *et al*., 2006)
CIRE	CD8α- DCs	(Corbett *et al*., 2005)
FIRE	CD8α- DCs	(Corbett *et al*., 2005)
Langerin	Langerin+ DCs	(Idoyaga *et al*., 2008)
Clec9A	CD8α+ DCs pDCs	(Caminschi *et al*., 2008; Sancho *et al*., 2008)
Clec12A	CD8α+ DCs CD8α- DCs pDCs monocytes macrophages B cells	(Lahoud *et al*., 2009)
hDC-SIGN	CD11c+ DCs (transgenic mice expressing DC-SIGN under the CD11c promotor)	(Singh *et al*., 2009)
hDEC205	DEC205+ cells (in humanized mouse) cDCs pDCs monocytes B cells NK cells	(Gurer *et al*., 2008)

Table 2: List of receptors that have been used for antibody mediated antigen delivery.
Depicted are receptors and their expression on different APC subsets that have been used to deliver antigen with specific antibodies.

In contrast to cDCs, pDCs have so far not been targeted with recombinant antibodies. Different polyclonal and monoclonal antibodies and F(ab')$_2$ fragments have been tested for their ability to introduce antigen to pDCs, however, these studies have several limitations that will be discussed later in this work (Sapoznikov *et al*., 2007; Zhang *et al*., 2006).

Recombinant antibody-antigen fusion proteins were used to elucidate the role of murine CD8α+/DEC205+ and CD8α-/DCIR2+ DC subpopulations for CD4+ and CD8+ T cell activation (Dudziak *et al*., 2007; Mahnke *et al*., 2003; Yamazaki *et al*., 2008). With regard to those findings, antibodies that deliver antigen specifically to pDCs *in vivo* could provide new insights into the function of murine pDCs for the initiation of antigen specific adaptive immune responses (Fig. 5).

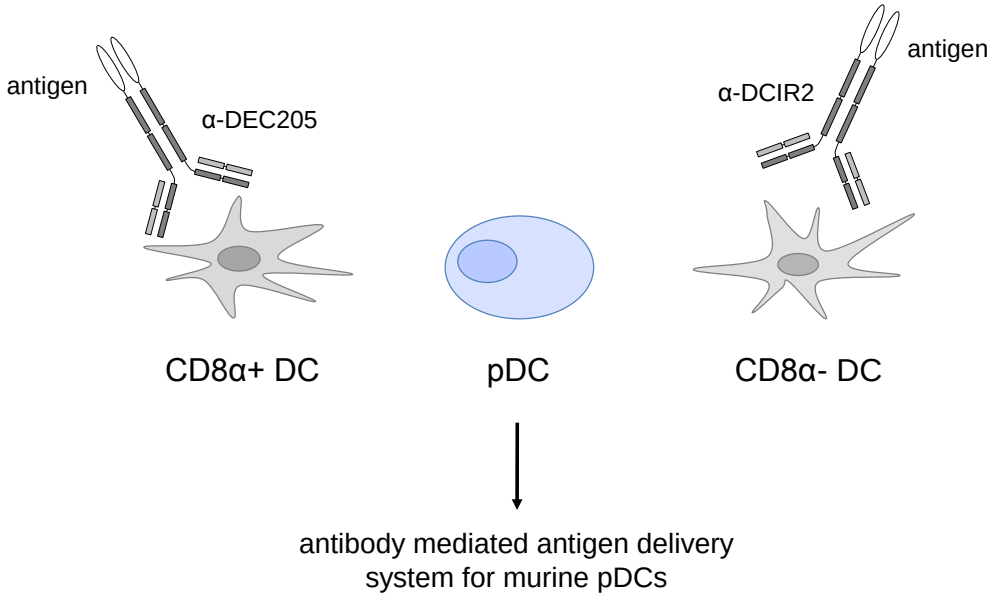

Fig. 5: Antibody mediated antigen delivery using specifically expressed cell surface molecules. Due to the restricted expression of DEC205 and DCIR2, antigen can be delivered specifically to CD8α+/DEC205+ and CD8α-/DCIR2+ DCs with antibodies for those molecules. The specific expression of Siglec-H and BST2 on the surface of pDCs provides the possibility to use this antibody based method to introduce antigen specifically to this distinct DC subpopulation.

Targeting antigen to pDCs is especially appealing as pDCs are a well defined and distinct cell population also in humans. Studying the influence antigen presenting murine pDCs have on the generation of antigen specific adaptive immune responses can therefore provide new and important insights into the function of these cells. This might provide the groundwork for antibody mediated antigen delivery to human pDCs with the purpose to use these cells for vaccination or the induction of tolerance.

2 AIM OF THE STUDY

The aim of this study was to determine the influence of murine pDCs on the initiation of antigen specific adaptive immune responses *in vivo*. Antibody mediated delivery of antigen *in vivo* has been successfully applied to investigate the role of cDC subsets for the induction of protective immunity as well as for mediating antigen specific tolerance. This experimental approach has so far not been used for pDCs.

Murine pDCs express two cell type specific cell surface molecules - Siglec-H and BST2. **The first aim** of this study was to generate recombinant antibody-antigen fusion proteins that allow the delivery of antigen specifically to murine pDCs *via* Siglec-H and BST2. Previous studies suggested an important role for pDCs in inducing antigen specific tolerance. **The second aim** was therefore to investigate whether pDCs are capable of inducing the expression of Foxp3 in naïve T cells and can promote the differentiation of Tregs. As suppression mediated by Foxp3+ Tregs is not the only mechanism to inhibit immune responses, it was also to be tested whether pDCs can mediate tolerance in a Treg independent manner after antigen has been delivered with α-Siglec-H or α-BST2. Experimental autoimmune encephalomyelitis (EAE) is an antigen specific murine model of multiple sclerosis and is a commonly used system to study autoimmunity and was chosen to test the success of tolerance induction after delivery of autoantigen to pDCs. **The third aim** of this study was to examine whether antigen delivery to pDCs in combination with TLR ligands as adjuvants could overcome tolerance and induce a strong antigen specific and protective immune response. To test the efficacy of this vaccination strategy, an antigen specific viral infection model (vaccinia virus encoding Ovalbumin (OVA)) as well as an antigen specific tumor model (B16 melanoma expressing OVA) were chosen. The working schedule to address the described aims is depicted in Fig. 6.

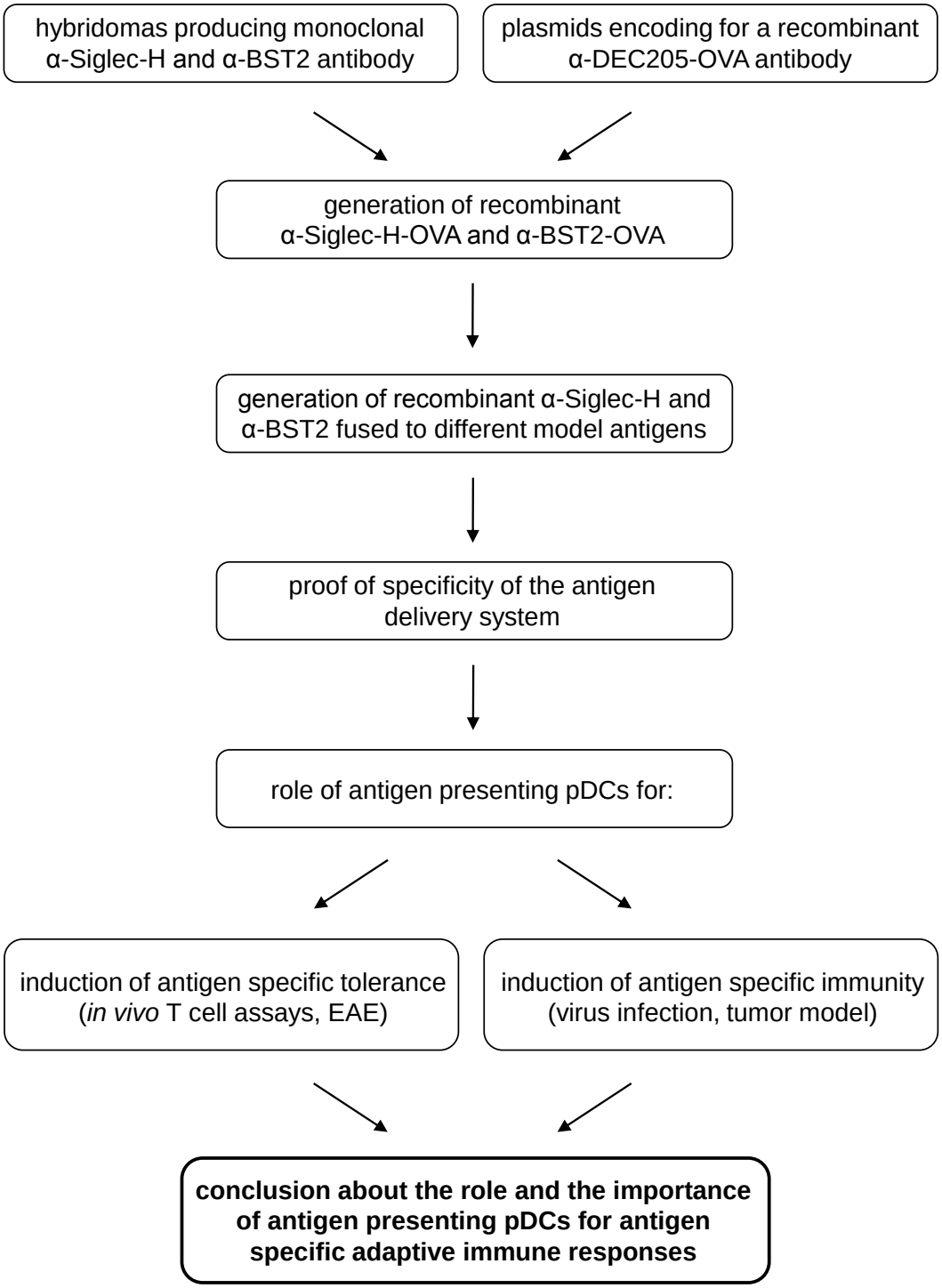

Fig. 6: Working schedule to address the aims of the study.

The flow diagram depicts how the starting materials (monoclonal α-Siglec-H and α-BST2 antibodies and plasmids encoding recombinant α-DEC205-OVA) should be used to generate a system that allows the delivery of antigen specifically to murine pDCs to be able to make conclusions about how pDCs influence the initiation of antigen specific adaptive immune responses.

3 MATERIAL AND METHODS

3.1 Material

3.1.1 Reagents

2-propanol	J.T. Baker (Deventer, Netherlands)
2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)	Roche (Mannheim, Germany)
acetic acid	Merck (Darmstadt, Germany)
agarose	Biozym (Hess.-Oldendorf, Germany)
ammonium persulphate (APS)	Sigma-Aldrich (Seelze, Germany)
ampicillin	Roth (Karlsruhe, Germany)
β-mercaptoethanol	Sigma-Aldrich (Seelze, Germany)
Bacto Tryptone	BD (Franklin Lakes, USA)
bovine serum albumin (BSA)	Sigma-Aldrich (Seelze, Germany)
carboxyfluorescein diacetate succinimidyl ester (CFSE)	Invitrogen (Karlsruhe, Germany)
citric acid	Roth (Karlsruhe, Germany)
complete Freund's adjuvant (CFA)	Difco (Detroit, USA)
Coomassie staining solution	Fermentas (St. Leon-Rot, Germany)
dimethyl sulfoxide (DMSO)	Sigma-Aldrich (Seelze, Germany)
Dulbecco's Modified Eagle's Medium (DMEM)	Invitrogen (Karlsruhe, Germany)
DNA marker (100 bp and 1 kb)	NEB (Frankfurt am Main, Germany)
dNTP mix	Promega (Mannheim, Germany)
EDTA (0.5 M, pH 8.0)	Invitrogen (Karlsruhe, Germany)
ethidiumbromide	Invitrogen (Karlsruhe, Germany)
fetal calf serum (FCS)	Biochrom (Berlin, Germany)
fetal calf serum IgG stripped	PAA (Pasching, Austria)
glacial acetic acid	Roth (Karlsruhe, Germany)

Glutamax-I (100x)	Invitrogen (Karlsruhe, Germany)
glycerol	Roth (Karlsruhe, Germany)
glycin	Roth (Karlsruhe, Germany)
Golgi Plug	BD Biosciences (Heidelberg, Germany)
Golgi Stop	BD Biosciences (Heidelberg, Germany)
HEPES	Sigma-Aldrich (Seelze, Germany)
HiTrap Protein G columns	GE Healthcare (München, Germany)
hydrochloric acid (HCl)	Merck (Darmstadt, Germany)
hydrogen peroxide (30 % (w/w))	Sigma-Aldrich (Seelze, Germany)
Laemmli sample buffer (2x)	Bio-Rad (München, Germany)
LB Agar	Roth (Karlsruhe, Germany)
LysoTracker	Invitrogen (Karlsruhe, Germany)
methanol	J.T. Baker (Deventer, Netherlands)
NEAA (100x)	PAA (Pasching, Austria)
paraformaldehyde	Sigma-Aldrich (Seelze, Germany)
penicillin/streptomycin (100x)	PAA (Pasching, Austria)
pertussis toxin (PTx)	Fluka (Seelze, Germany)
phosphate buffered saline (PBS) (w/o Ca^{2+} and Mg^{2+})	PAA (Pasching, Austria)
polyinosinic-polycytidylic acid (PolyI:C)	GE Healthcare (München, Germany)
polyethylenimine (PEI)	Polysciences (Warrington, USA)
prestained protein ladder	Fermentas (St. Leon-Rot, Germany)
propidium iodide	Sigma-Aldrich (Seelze, Germany)
red blood cell lysis buffer	Sigma-Aldrich (Seelze, Germany)
Rotiphorese Gel 30	Roth (Karlsruhe, Germany)
RPMI 1640	Invitrogen (Karlsruhe, Germany)
saponin	Sigma-Aldrich (Seelze, Germany)

skim milk powder	Roth (Karlsruhe, Germany)
sodium chloride	Roth (Karlsruhe, Germany)
sodium hydroxide solution (NaOH)	Merck (Darmstadt, Germany)
sodium dodecyl sulfate (SDS)	Fluka (Seelze, Germany)
sodium pyruvate solution (100 mM)	Invitrogen (Karlsruhe, Germany)
N,N,N',N'-tetramethylethylenediamine (TEMED)	Fluka (Seelze, Germany)
Tris	Roth (Karlsruhe, Germany)
Trizol	Invitrogen (Karlsruhe, Germany)
Tween-20	Sigma-Aldrich (Seelze, Germany)
yeast extract	BD (Franklin Lakes, USA)

3.1.2 Kits

EndoFree Plasmid Maxi Kit	Qiagen (Hilden, Germany)
intracellular Foxp3 staining kit	eBioscience (San Diego, USA)
MACS cell isolation kits	Miltenyi Biotech (Bergisch Gladbach, Germany)
QIAprep Spin Miniprep Kit	Qiagen (Hilden, Germany)
QIAquick PCR Purification Kit	Qiagen (Hilden, Germany)
QIAquick Gel Extraction Kit	Qiagen (Hilden, Germany)
5' RACE PCR amplification system	Invitrogen (Karlsruhe, Germany)
Super Signal West Pico chemiluminescent detection kit	Pierce (Rockford, USA)

3.1.3 Enzymes

Collagenase D	Roche (Mannheim, Germany)
DNase I	Roche (Mannheim, Germany)
Pfu Ultra Polymerase	Stratagene (La Jolla, USA)
restriction endonucleases	NEB (Frankfurt am Main, Germany) Fermentas (St. Leon-Rot, Germany)

T4 DNA ligase	NEB (Frankfurt am Main, Germany)
Taq DNA Polymerase	Invitrogen (Karlsruhe, Germany)

3.1.4 Antibodies and Streptavidin

Antigen	Conjugate	Application	Manufacturer
OVA	biotin	western blot analysis	Novus Biologicals (Cambridge, UK)
mouse IgG (mIgG)	HRP	western blot analysis	Southern Biotech (Birmingham, USA)
rabbit IgG	HRP	western blot analysis	Jackson Research (Baltimore, USA)
IFN-α	unconjugated	ELISA	PBL (Piscataway, USA)
IL-6	unconjugated, biotin	ELISA	BD Biosciences (Heidelberg, Germany)
IL-12	unconjugated, biotin	ELISA	BD Biosciences (Heidelberg, Germany)
mouse IgG1 (mIgG1)	biotin	ELISA	BD Biosciences (Heidelberg, Germany)
mouse IgG2b (mIgG2b)	biotin	ELISA	BD Biosciences (Heidelberg, Germany)
mouse IgG2c (mIgG2c)	biotin	ELISA	Southern Biotech (Birmingham, USA)
Streptavidin	HRP	ELISA	GE Healthcare (München, Germany)
rabbit IgG	HRP	ELISA	Jackson Research (Baltimore, USA)
Gr-1	unconjugated	pDC depletion	BioXCell (West Lebanon, USA)
CD3	APC-eFluor780	flow cytometry	BD Biosciences (Heidelberg, Germany)
CD4	PerCp-Cy5.5	flow cytometry	BD Biosciences (Heidelberg, Germany)
CD8	eFluor450	flow cytometry	BD Biosciences (Heidelberg, Germany)
CD40	PE	flow cytometry	BD Biosciences (Heidelberg, Germany)
CD69	PE	flow cytometry	BD Biosciences (Heidelberg, Germany)
CD80	PE	flow cytometry	BD Biosciences (Heidelberg, Germany)
CD86	PE	flow cytometry	BD Biosciences (Heidelberg, Germany)
IFN-γ	PE	flow cytometry	BD Biosciences (Heidelberg, Germany)
IL-2	APC	flow cytometry	BD Biosciences (Heidelberg, Germany)
IL-4	PE	flow cytometry	BD Biosciences (Heidelberg, Germany)
IL-10	PE	flow cytometry	BD Biosciences (Heidelberg, Germany)
IL-17	APC	flow cytometry	BD Biosciences (Heidelberg, Germany)

mIgG1	biotin	flow cytometry	BD Biosciences (Heidelberg, Germany)
Vα2 TCR	PE	flow cytometry	BD Biosciences (Heidelberg, Germany)
Vβ4.1 TCR	FITC	flow cytometry	BD Biosciences (Heidelberg, Germany)
CD11c	APC, PE-Cy7	flow cytometry	eBioscience (San Diego, USA)
CD25	PE	flow cytometry	eBioscience (San Diego, USA)
CD45.2	biotin	flow cytometry	eBioscience (San Diego, USA)
B7-H1/PDL-1	PE	flow cytometry	BD Biosciences (Heidelberg, Germany)
B7-H2/ ICOS-L	PE	flow cytometry	eBioscience (San Diego, USA)
B7-H3	PE	flow cytometry	BD Biosciences (Heidelberg, Germany)
B7-H4	PE	flow cytometry	BD Biosciences (Heidelberg, Germany)
CCR9	PE, APC	flow cytometry	eBioscience (San Diego, USA)
DO11.10 TCR (KJ1-26)	PE, APC	flow cytometry	eBioscience (San Diego, USA)
Foxp3	PE, APC	flow cytometry	eBioscience (San Diego, USA)
MHC II ($I\text{-}A^b$)	PE	flow cytometry	eBioscience (San Diego, USA)
B220/CD45R	PE	flow cytometry	Southern Biotech (Birmingham, USA)
BST2 (120G8)	FITC	flow cytometry	own production
Siglec-H (440c)	FITC	flow cytometry	own production
Aw3.18	biotin	flow cytometry	provided by Dr. Diana Dudziak
Streptavidin	PE, APC, PE-Cy7	flow cytometry	eBioscience (San Diego, USA)

Table 3: Antibodies used in this study.

3.1.5 Cell lines, virus strains and bacterial strains

3.1.5.1 Cell lines

Cell line	Species	Application	Source
HEK293T	human	expression of recombinant antibodies	DSMZ (Braunschweig, Germany)
MO5	mouse	OVA specific tumor model	Dr. Carole Bourquin
440c	rat hybridoma	antibody production, RNA isolation	Dr. Marco Colonna
120G8	rat hybridoma	antibody production, RNA isolation	Dr. Giorgio Trinchieri

Table 4: Cell lines used in this study.

3.1.5.2 Virus strains

Virus strain	Application	Source
vaccinia virus encoding OVA (VV-OVA)	virus challenge	Dr. Ingo Drexler

Table 5: Virus strains used in this study.

3.1.5.3 Bacterial strains

Strain	Application	Source
E. coli K12 DH5α	transformation/ cloning	Invitrogen (Karlsruhe, Germany)
E. coli K12 JM109	transformation/ cloning	Promega (Mannheim, Germany)

Table 6: Bacterial strains used in this study.

3.1.6 Media and buffers

3.1.6.1 Media for cell culture

primary cells	RPMI 1640	
(DCs, T cells)	10 % (v/v)	FCS (heat inactivated)
	1 % (v/v)	Glutamax-I
	1 % (v/v)	sodium pyruvate
	1 % (v/v)	NEAA
	1 % (v/v)	penicillin/streptomycin
	0.05 mM	β-mercaptoethanol

HEK293T cells	DMEM	
	10 % (v/v)	FCS (heat inactivated)
	1 % (v/v)	Glutamax-I
	1 % (v/v)	sodium pyruvate
	1 % (v/v)	NEAA
	1 % (v/v)	penicillin/streptomycin
HEK293T cells antibody expression	DMEM	
	4 % (v/v)	IgG stripped FCS (heat inactivated)
	1 % (v/v)	Glutamax-I
	1 % (v/v)	sodium pyruvate
	1 % (v/v)	NEAA
	1 % (v/v)	penicillin/streptomycin
hybridoma cells (440c, 120G8)	RPMI 1640	
	3 % (v/v)	IgG stripped FCS (heat inactivated)
	1 % (v/v)	Glutamax-I
	1 % (v/v)	sodium pyruvate
	1 % (v/v)	NEAA
	1 % (v/v)	penicillin/streptomycin
freezing medium	70 % (v/v)	complete culture medium
	10 % (v/v)	DMSO
	20 % (v/v)	FCS (heat inactivated)

3.1.6.2 Media for bacteria

LB medium	1.0 % (w/v)	Bacto Tryptone
	0.5 % (w/v)	yeast extract
	171 mM	sodium chloride
	in ddH_2O	
	pH 7.3 with 1 N NaOH	
SOC medium	Invitrogen (Karlsruhe, Germany)	
LB agar plates	4 % (w/v)	LB Agar
	in ddH_2O	

For bacterial selection, LB medium and LB agar plates were supplemented with 100 µg/ml ampicillin.

3.1.6.3 Buffers for ELISA

coating buffer	PBS	
blocking buffer	PBS	
	10 % (v/v)	FCS
dilution buffer	PBS	
	10 % (v/v)	FCS
wash buffer	PBS	
	0.5 % (v/v)	Tween-20
ELISA substrate	0.1 M	citric acid pH 4.0
	2 % (v/v)	ABTS (50 mg/ml)
	0.1 % (v/v)	30 % (w/w) hydrogen peroxide
stop solution	1 % (w/v)	SDS
	in ddH_2O	

3.1.6.4 Buffers for antibody purification

equilibration buffer	PBS	
wash buffer	PBS	
elution buffer	0.1 M	glycin
	ddH_2O	
	pH 2.3 with HCl	
neutralization buffer	1 M	Tris
	ddH_2O	
	pH 9.0 with HCl	

3.1.6.5 Buffers and solutions for protein biochemistry

4x stacking gel buffer	0.5 M	Tris
	0.4 % (v/v)	SDS
	in ddH_2O	
	pH 6.8 with HCl	

4x resolving gel buffer	1.5 M 0.4 % (v/v) in ddH_2O pH 8.8 with HCl	Tris SDS
10 % APS	10 % (w/v) in ddH_2O	APS
10x running buffer	1.9 M 250 mM 171 mM in ddH_2O	glycin Tris SDS
1x blotting buffer	21 mM 192 mM 0.2 % (v/v) in ddH_2O	Tris glycin methanol
TBST	1 M 150 mM 0.05 % (v/v) in ddH_2O	Tris-HCl pH 8.0 sodium chloride Tween-20
blocking buffer	5 % (w/v) in TBST	skim milk powder

3.1.6.6 Buffers for molecular biology

50x TAE	2 M 100 mM 5.71 % (v/v) in ddH_2O pH 8.5 with 1 N NaOH	Tris EDTA glacial acetic acid
6x loading dye	25 % (v/v) 0.05 M 0.25 % (w/v) in ddH_2O	glycerol EDTA pH 7.5-8.0 bromophenolblue

3.1.6.7 Buffers for cell staining

FACS buffer	PBS 2 % (v/v)	 FCS

fixation buffer	PBS	
	2% (w/v)	paraformaldehyde (PFA)
permeabilisation buffer	PBS	
	5 % (v/v)	FCS
	0.5 % (w/v)	saponin
	10 mM	HEPES
CFSE staining buffer	PBS	
	0.1 % (w/v)	BSA

3.1.7 PCR primers and DNA oligonucleotides

Nr.	PCR primer	5' Sequence 3'
1	α-Siglec-H L_C #1	GGCGG**GAATTC**GGGATCATCCTCTC
2	α-Siglec-H L_C #2	CCCGTTTCAGTTCCAGCTTGGTCC
3	α-Siglec-H L_C #3	CCAAGCTGGAATTGAAACGGGCTG
4	α-Siglec-H L_C #4	CTCGA**GCGGCCGC**TCAACACTCATTC
5	α-Siglec-H H_C #1	CCGCC**GAATTC**GATAAGATCACTGTCCTC
6	α-Siglec-H H_C #2	GGTGCTGCTGGCCGGGTGGGCAACG
7	α-Siglec-H H_C #3	CCTCCAGCACCTGGCCCAGCGAGACC
8	α-Siglec-H H_C #4	CCTTCTTGGCCATGTC**GCTAGC**TTTACCAGG
9	α-Siglec-H H_C #5	GGCGG**GCGGCCGC**TCAGCTTTCTTC
10	α-BST2 L_C #1	CCGCC**GAATTC**CAGCATGGGCATAAGG
11	α-BST2 L_C #2	GCATCAGCCCGTTTCAATTCCAGCTTGG
12	α-BST2 L_C #3	CCAAGCTGGAATTGAAACGGGCTGATGC
13	α-BST2 L_C #4	CTCGA**GCGGCCGC**TCAACACTCATTC
14	α-BST2 H_C #1	CCGCC**GAATTC**TTGACCATTGATCTCTCCATAGG
15	α-BST2 H_C #2	CCCCGAATGCCTCTTGCACAGAAATAGTATGC
16	α-BST2 H_C #3	GCATACTATTTCTGTGCAAGAGGCATTCGGGG
17	α-BST2 H_C #4	GGTGACGGTCTGGCTGGGCCAGGTGCTGGAGG
18	α-BST2 H_C #5	CCTCCAGCACCTGGCCCAGCCAGACCGTCACC
19	α-BST2 H_C #6	CCTTCTTGGCCATGTC**GCTAGC**TTTACCAGG
20	α-BST2 H_C #7	GGCGG**GCGGCCGC**TCAGCTTTCTTC

21	pMOG #1	CCACTCTCCTGGTAAA**GCTAGC**ATGGAGGTGGGTTGG
22	pMOG #2	CTTGAGAAGGGAGAACGGTACCAACCCACCTCCATGC
23	pMOG #3	CCATTTCGGTAGAGGTGAACCACTCTTGAGAAGGGAG
24	pMOG #4	GGCC**GCGGCCGC**TTACTTGCCATTTCGGTAGAGGTGAAC
25	pHEL #1	CACTCTCCTGGTAAA**GCTAGC**GATGGGAGTACC
26	pHEL #2	ATCTGTAGGATTCCGTAGTCGGTACTCCCATCGCT
27	pHEL #3	CCGCC**GCGGCCGC**TCAGCGGCTGTTGATCTGTAGGATTCC
28	H_C sequencing reverse	GAACTGGACCTCGGGATCATCCTTGC
29	H_C sequencing forward	GCAAGGATGATCCCGAGGTCCAGTTC
Nr.	**Oligonucleotide**	**5' Sequence 3'**
1	CpG 1668	tccatgacgttcctgatgct
2	CpG 2216	ggGGGACGATCGTCgggggg

Table 7: PCR primers and oligonucleotides used in this study.

All PCR primers and oligonucleotides were purchased from MWG Operon (Ebersberg, Germany). Bold font displays the presence of restriction sites (GAATTC *EcoRI*, GCTAGC *NheI*, GCGGCCGC *NotI*). Capital letters (phosphodiester). Small type letters (phosphorothioate).

3.1.8 Proteins and peptides

Protein	**Amino acid sequence**	**Distributor**
OVA	full length	Hyglos (Regensburg, Germany)
Peptide	**Amino acid sequence**	**Distributor**
pOVA 257-264	SIINFEKL	Genscript (Piscataway, USA)
pOVA 265-279	EKLTEWTSSNVMEER	Genscript (Piscataway, USA)
pOVA 323-339	ISQAVHAAHAEINEAGR	Genscript (Piscataway, USA)
pMOG 35-55	MEVGWYRSPFSRVVHLYRNGK	Genscript (Piscataway, USA)

Table 8: Proteins and peptides used in this study.

3.1.9 Mice

Specific pathogen free, 6-8 weeks old, female C57BL/6, BALB/c and C3H mice were purchased from Harlan Winkelmann (Borchen, Germany). OT-I, OT-II, OT-II/Rag2-/-, DO11.10/Rag2-/- and MHC II deficient Ax/A0 mice were bred under specific pathogen free conditions according to the guidelines of the Technical University Munich. Experiments were performed in accordance with German animal care and ethics legislation and had been approved by the local government authorities.

Strain	Application
C57BL/6	recipients for OT-I, OT-II and OT-II/Rag2-/- T cells, immunization experiments, vaccinia virus challenge, tumor model, EAE
BALB/c	recipients for DO11.10/Rag2-/- T cells
C3H	*in vivo* antigen presentation on MHC II (I-A^k)
OT-I	isolation of OVA specific CD8+ T cells
OT-II	isolation of OVA specific CD4+ T cells
OT-II/Rag2-/-	isolation of OVA specific CD4+/Foxp3- T cells
DO11.10/Rag2-/-	isolation of OVA specific CD4+/Foxp3- T cells
Ax/A0	MHC II deficient recipients for OT-II/Rag2-/- T cells

Table 9: Mouse strains used in this study.

3.2 Methods

3.2.1 Generation of recombinant pDC specific antibody-antigen fusion proteins

3.2.1.1 Generation of α-Siglec-H-OVA

cDNA was generated from total RNA isolated from α-Siglec-H producing hybridoma cells (clone 440c, monoclonal rat IgG2b) using the 5'-RACE amplification system. This cDNA was subsequently used as template to amplify α-Siglec-H specific sequences. For amplification of sequences encoding the mIgG1-OVA antibody scaffold, plasmids encoding α-DEC205-OVA (Fig. 10) were used as templates. The sequence encoding the variable region of the light chain of the monoclonal α-Siglec-H antibody was amplified with primer 1 and 2. Primer 1 contains an *EcoRI* restriction site and primer 2 binds in the constant region of the light chain (step 1). The constant region of the light chain of the recombinant α-DEC205-OVA antibody was amplified with primer 3 and 4. Primer 3 is the complement of primer 2 and primer 4 contains a *NotI* restriction site (step 2). In step 3, the two PCR fragments generated in step 1 and step 2 were fused using primer 1 and 4. The DNA fragment that arose in this third reaction was then inserted in the plasmid encoding the light chain of the α-DEC205-OVA antibody after the plasmid and the PCR fragment had been digested with *EcoRI* and *NotI*.

Fusion of the Siglec-H specific variable region of the heavy chain to the constant region of the α-DEC205-OVA antibody was performed in a similar way. First, the variable region of the heavy chain of the monoclonal α-Siglec-H antibody was amplified with primer 5 and 6. Primer 5 contains an *EcoRI* restriction site and primer 6 binds to the constant region 1 of the antibody's heavy chain (step 4). During step 5 the constant regions of the heavy chain including the sequence for OVA were amplified using the plasmid encoding the α-DEC205-OVA heavy chain as template. Primer 7 binds to the constant region 1 and is the complement of primer 6. Primer 9 binds to the 3' end of the sequence encoding OVA and contains a *NotI* restriction site. During step 6, the DNA fragments generated in step 4 and 5 were fused and amplified with primer 5 and 9. The PCR product from this reaction was then inserted in the vector for the heavy chain after it had been digested with *EcoRI* and *NotI*. Fig. 7 illustrates the generation of the recombinant α-Siglec-H-OVA antibody.

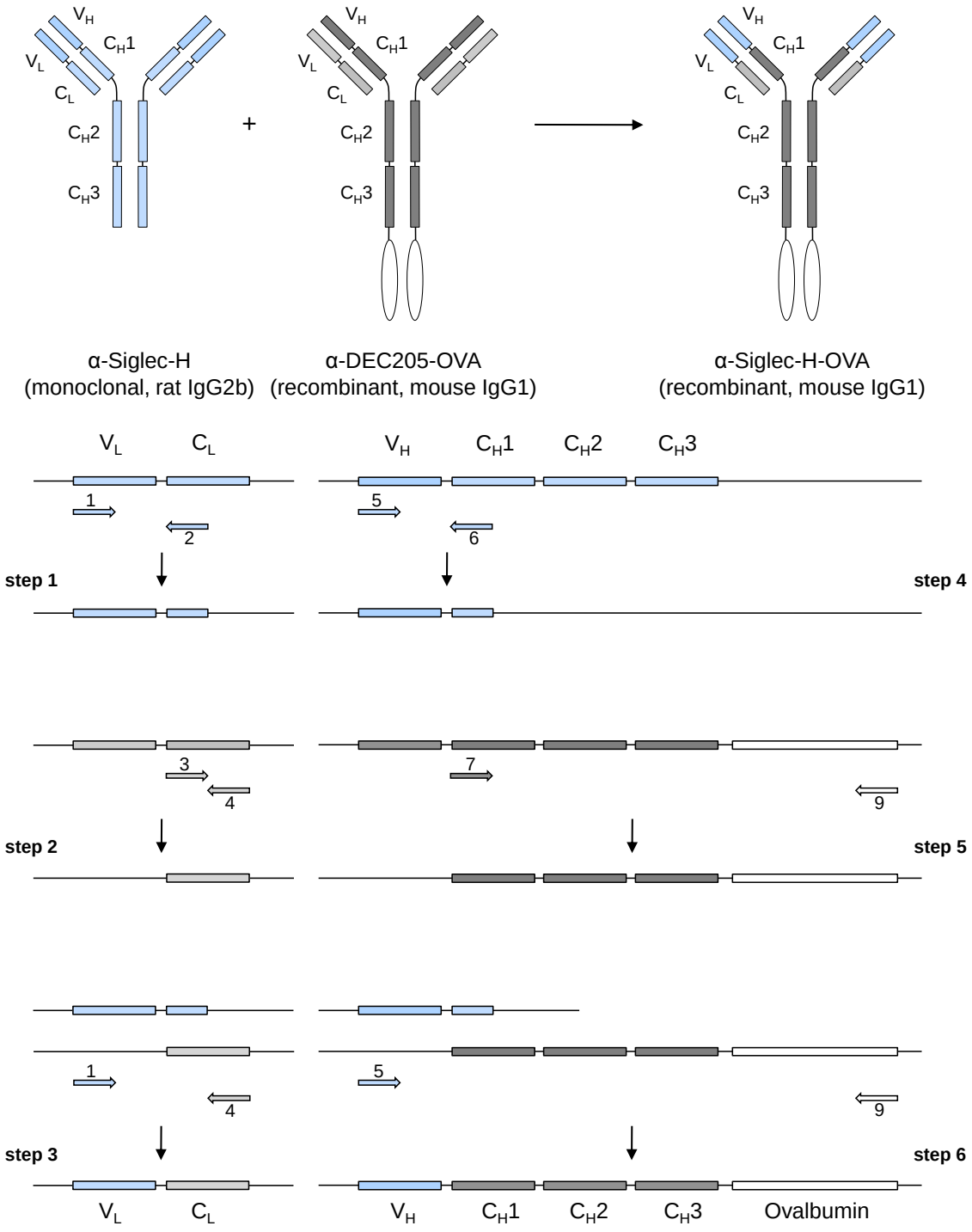

Fig. 7: Generation of the recombinant α-Siglec-H-OVA antibody.

Sequential steps leading to the generation of constructs encoding the light and heavy chain of the α-Siglec-H-OVA antibody-antigen fusion protein are shown. Arrows indicate the position of primers that were used to amplify specific sequences. Antibody parts that originate from the monoclonal α-Siglec-H antibody are depicted in blue, parts that originate from the recombinant α-DEC205-OVA antibody are marked in grey. Indicated primer numbers refer to the list of PCR primers that were used in this project (see Table 7). (V_L variable region of the light chain, C_L constant region of the light chain, V_H variable region of the heavy chain, C_H1, C_H2 and C_H3 constant region 1,2 and 3 of the heavy chain, OVA Ovalbumin)

3.2.1.2 Generation of α-BST2-OVA

The recombinant α-BST2-OVA antibody was generated in a similar way as it has been described for α-Siglec-H-OVA. The only difference in the generation of α-BST2-OVA was that the variable region of the heavy chain of the monoclonal α-BST2 antibody could not be amplified in a single step, but was generated by overlap PCR (step 6) using PCR fragments that were amplified with primer 14 and 15 (step 4) and primer 16 and 17 (step 5), respectively. All other steps were performed as described for α-Siglec-H-OVA. Fig. 8 illustrates the generation of the recombinant α-BST2-OVA antibody.

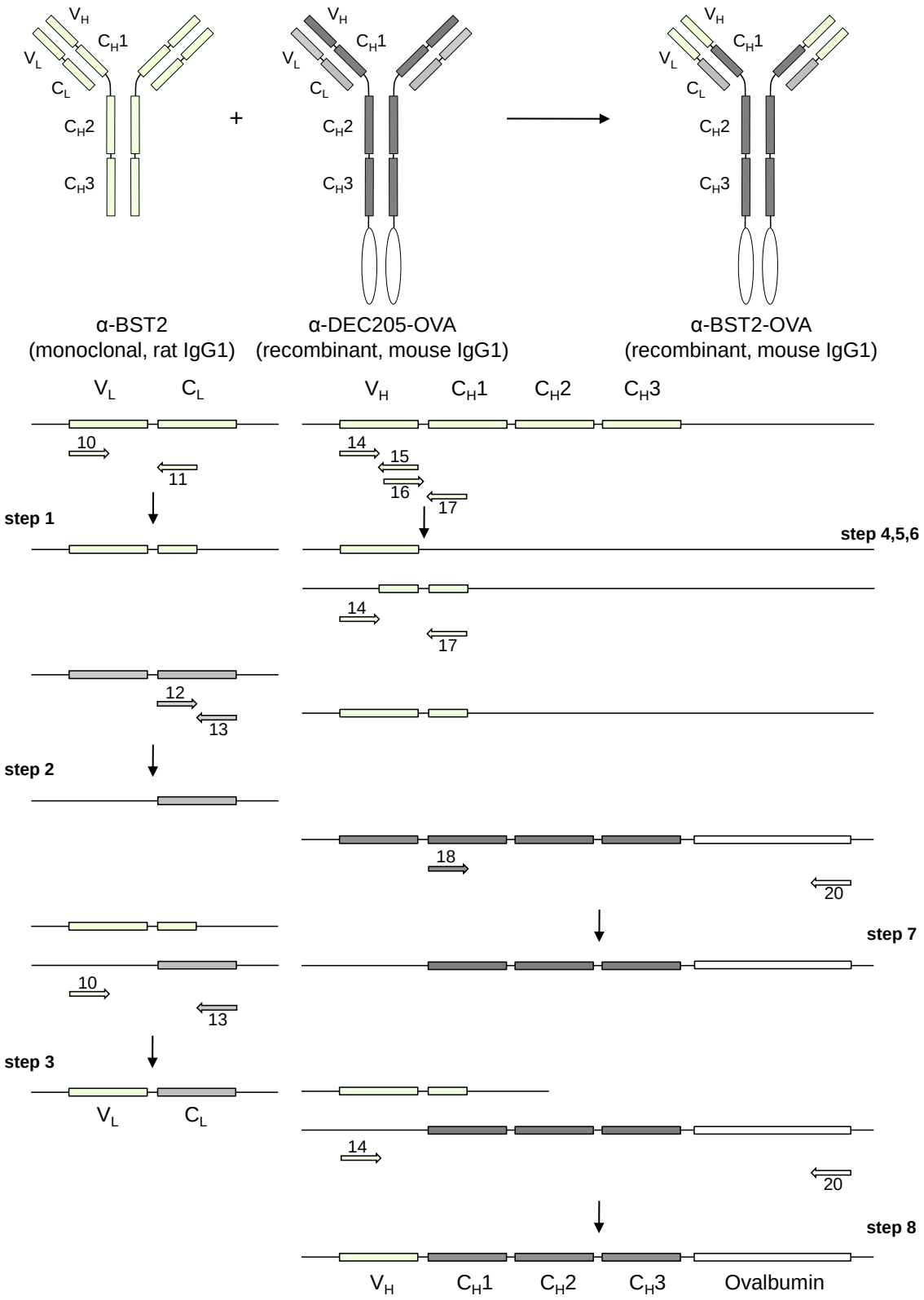

Fig. 8: Generation of the recombinant α-BST2-OVA antibody.

Sequential steps leading to the generation of constructs encoding the light and heavy chain of the α-BST2-OVA antibody-antigen fusion protein are shown. Arrows indicate the position of primers that were used to amplify specific sequences. Antibody parts that originate from the monoclonal α-BST2 antibody are depicted in green, parts that originate from the recombinant α-DEC205-OVA antibody are marked in grey. Indicated primer numbers refer to the list of DNA oligonucleotides that have been used to clone recombinant antibodies (see Table 7). (V_L variable region of the light chain, C_L constant region of the light chain, V_H variable region of the heavy chain, C_H1, C_H2 and C_H3 constant region 1,2 and 3 of the heavy chain, OVA Ovalbumin)

3.2.1.3 Generation of α-Siglec-H and α-BST2 fused to different model antigens

To generate Siglec-H and BST2 specific antibodies that are fused to different antigens, the plasmids encoding the heavy chain of α-Siglec-H-OVA and α-BST2-OVA were digested with *NheI/NotI* and the sequences for other antigens flanked by those restriction sites were inserted (Fig. 9). Thus, antibodies were generated that are fused to a specific peptide from hen egg lysozyme (pHEL, aa 48-62) and a peptide derived from myelin oligodendrocyte glycoprotein (pMOG, aa 35-55). The sequences for pHEL and pMOG were synthesized with overlapping PCR oligonucleotides containing a *NheI* restriction site at the 5' end and a *NotI* restriction site at the 3' end.

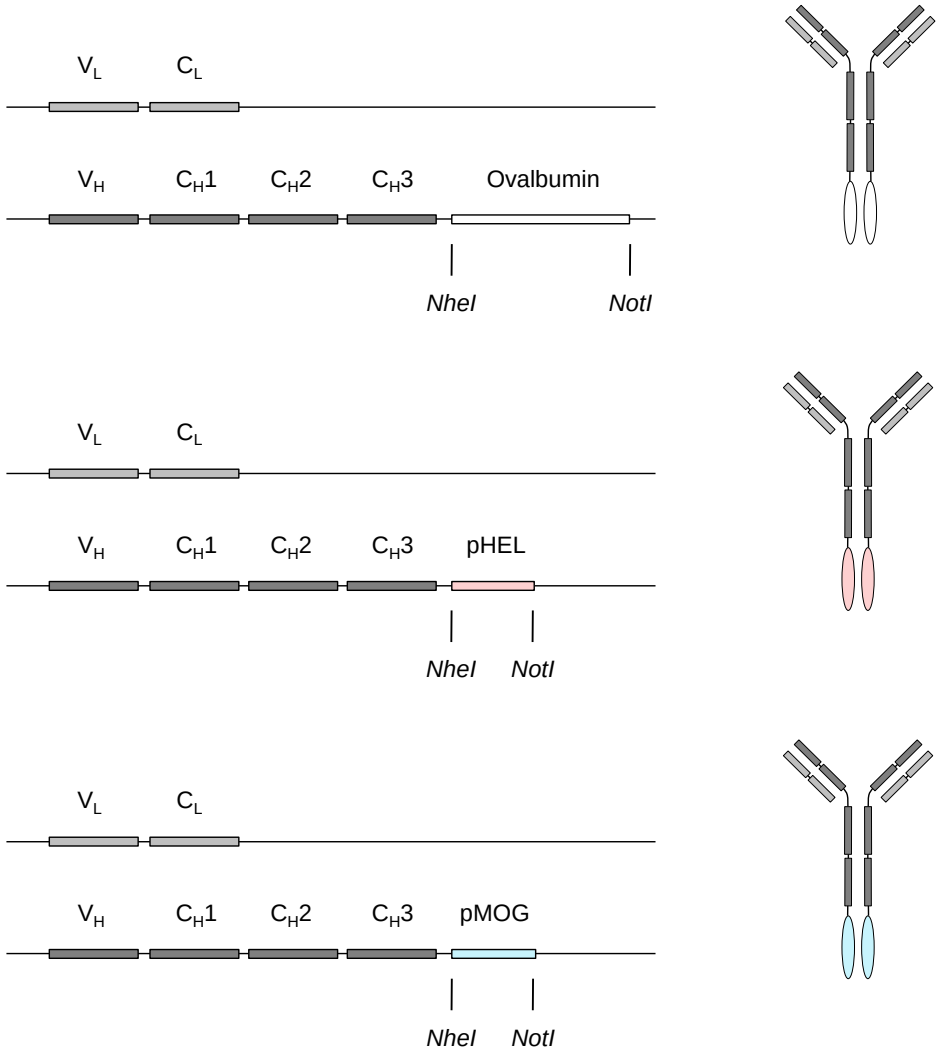

Fig. 9: Fusion of different antigens to recombinant pDC specific antibodies.

pHEL or pMOG were fused to recombinant pDC specific antibodies by replacing the sequence encoding OVA with the sequence encoding pHEL or pMOG. All antigens are flanked by *NheI* and *NotI* restriction sites. (V_L variable region of the light chain, C_L constant region of the light chain, V_H variable region of the heavy chain, C_H1, C_H2 and C_H3 constant region 1, 2 and 3 of the heavy chain, OVA Ovalbumin, pHEL peptide derived from hen egg lysozyme, pMOG peptide derived from myelin oligodendrocyte glycoprotein)

3.2.2 Analysis and cloning of DNA

3.2.2.1 PCR amplification of DNA

Polymerase chain reaction was used to amplify DNA fragments that were subsequently inserted into expression vectors. All DNA fragments that were used in cloning reactions were amplified with proof-reading *Pfu* Ultra DNA polymerase. For other applications (e.g. colony PCR, genotyping) PCR was performed using *Taq* DNA polymerase.

PCR reaction:

10x reaction buffer	5.0 µl
DNA template (20 ng/µl)	5.0 µl
primer forward (10 µM)	2.5 µl
primer reverse (10 µM)	2.5 µl
dNTPs (10 mM)	1.0 µl
ddH_2O	33.0 µl
DNA polymerase	1.0 µl

	50.0 µl

PCR protocol:

1.	95 °C	5 min
2.	95 °C	30 sec
3.	Primer Tm – 5 °C	30 sec
4.	72 °C	1 min/1000 bp
5.	go to step 2 (30 x)	
6.	72 °C	10 min
7.	4 °C	

3.2.2.2 Restriction enzyme digestion of DNA

Digestion of DNA with specific restriction enzymes was performed using the recommended buffer and 10 U/µg DNA of the respective enzyme. If necessary, 2 µl of 10x BSA were added. Digestion was performed for 2 h at 37 °C.

3.2.2.3 Agarose gel electrophoresis

DNA fragments were separated and analyzed by agarose gel electrophoresis. Agarose gels were prepared in 1x TAE buffer with the agarose concentration depending on the size of the DNA fragment to be analyzed. To stain DNA fragments, ethidium bromide was used (final concentration of 100 ng/ml). All gels were run in 1x TAE buffer. For size determination either a 100 bp or 1000 bp DNA ladder was used. DNA fragments were visualized with UV light (254 nm).

3.2.2.4 Extraction of DNA fragments from agarose gels

If separated DNA fragments were used for further cloning steps, the respective band was recovered by gel excision. DNA was purified using the QIAquick Gel Extraction Kit following the manual provided with the kit.

3.2.2.5 Ligation of DNA fragments

Ligation of DNA fragments was performed overnight on ice, using T4 DNA ligase according to the protocol provided with the ligase.

3.2.3 Transformation of competent *E. coli*

Chemically competent *E. coli* K12 strains DH5α and JM109 were used for transformation of plasmid DNA. 20 µl bacteria were thawed on ice and 10-50 ng of DNA were added. Bacteria were then incubated on ice for 30 min and subsequently heat shocked for 45 sec and rapidly returned to ice for 2 min. 200 µl SOC medium were added and cultures were incubated for 1 h at 37 °C in a thermomixer to induce bacterial recovery and growth. 100 µl of the transformation reaction were spread onto LB agar plates containing 100 µg/ml ampicillin for bacterial selection. Plates were incubated for approximately 16 h at 37 °C until single bacterial colonies were clearly visible.

3.2.4 Isolation of plasmid DNA

Small scale (miniprep) and large scale (maxiprep) isolation of plasmid DNA was performed using the respective kits from Qiagen following the manufacturer's instructions.

3.2.5 Tissue culture

3.2.5.1 Cell lines

All cell cultures were maintained at 37 °C in a humified incubator with 5 % CO_2 atmosphere with the respective media listed in 3.1.6.1.

3.2.5.2 Generation of murine bone marrow derived Fl-DCs

Bone marrow derived Fl-DCs were generated using FMS-like tyrosine kinase 3 ligand (Flt3L). To obtain bone marrow cells, hind legs were removed and cleaned from fur and muscle tissue. Cells from *femurs* and *tibiae* were flushed with medium. After centrifugation at 1500 rpm at 4 °C for 5 min, the cell pellet was resuspended in 2 ml red blood cell lysis buffer and incubated for 5 min at RT to lyse erythrocytes. Lysis was blocked by adding 5 ml of primary cell medium to the cells and centrifugation for 5 min at 1500 rpm and 4 °C. Bone marrow cells ($1.5*10^6$/ml) were then incubated in a 6 well plate (3 ml medium/well) for 7 days in the presence of 20 ng/ml Flt3L to obtain Fl-DCs resembling pDCs.

3.2.5.3 Transient transfection of HEK293T cells

For the production of recombinant antibodies HEK293T cells were grown in DMEM + 4 % IgG stripped FCS. Transfection of HEK293T cells with plasmids encoding recombinant antibodies was performed using polyethylenimine (PEI). Culture medium was replaced with fresh and preheated medium 2-4 h prior to the transfection.

Transfection conditions used for a 10 cm (10 ml) dish:

450 µl DMEM
25 µg plasmid heavy chain (1µg/µl)
25 µg plasmid light chain (1µg/µl)
100 µg PEI (1µg/µl)

The transfection mixture was incubated for 10 min at RT before it was added drop wise to the cells. After 4-6 h of incubation at 37 °C, the transfection mixture was removed and fresh, preheated culture medium was added to the cells, which were then further cultured for up to 10 days. During the 10 day culture, medium was

replaced every 2-3 days. The supernatant, containing the secreted antibody, was collected and centrifuged at 12000 g for 30 min to remove cell debris. Usually 40-50 10 cm dishes were transfected to obtain 1.0-1.5 liters supernatant. All supernatants were filtered through a bottle top filter (pore size 0.45 µm) before applying them on protein G columns.

3.2.6 Antibody purification

Recombinant antibodies were purified from cell culture supernatants by protein G affinity chromatography. HiTrap Protein G columns were washed with 5 ml PBS before the supernatant was applied to the column. After the supernatant has passed the column it was washed again with 5 ml PBS. The protein G bound antibody was fractionally (1 ml fractions) eluted with 0.1 M glycin-HCl pH 2.3. The fractions were collected in tubes that contained 60 µl 1M Tris-HCl pH 9.0 to immediately neutralize the antibody containing elution buffer. All purification steps were performed at 4 °C. The protein concentration of each fraction was determined with a spectrophotometer (OD 280 nm, 1 OD = 0.67 mg/ml protein). Fractions with similar protein concentrations were pooled and dialyzed against PBS (overnight at 4 °C, Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, USA)). Each batch was tested for binding to pDCs by FACS analysis and purity was determined by SDS-PAGE and subsequent staining with Coomassie staining solution or western blot analysis.

3.2.7 Internalization and confocal microscopy

To determine the kinetics of Siglec-H and BST2 internalization, splenocytes were stained with biotinylated α-Siglec-H or α-BST2. Cells were incubated at 37 °C for up to 3 h and subsequently placed on ice and stained with Streptavidin-APC. Internalization of Siglec-H and BST2 was measured as the decrease in the percentage of APC+ pDCs. As control, the decrease of fluorescence intensity was measured after splenocytes had been stained with FITC-labeled α-Siglec-H and α-BST2, respectively.

Localization of internalized α-Siglec-H and α-BST2 within the cell was determined by confocal microscopy using a Leica SP5 Microscope (Leica Microsystems, Wetzlar, Germany). Fl-DCs were stained with FITC-labeled α-Siglec-H or α-BST2 and

subsequently incubated at either 4°C or 37 °C for 60 min. Lysosomal compartments were visualized with LysoTracker according to manufacturer's guidelines. Living cells were put on a microscope slide and immobilized with a cover slip.

3.2.8 SDS polyacrylamide gel electrophoresis

Proteins were separated by size using SDS polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions. For gel composition see Table 10. Protein samples were mixed with 2x Laemmli sample buffer containing β-mercaptoethanol and incubated for 5 min at 95 °C prior to loading. For estimation of the protein size, 10 µl of a protein ladder were loaded onto the gel. For analysis of recombinant antibodies, 5 µg of the respective antibodies were loaded per lane.

Component	Resolving Gel (10%)	Stacking Gel (4%)
Rotiphorese gel 30 acrylamide solution	3.3 ml	2.5 ml
4x resolving gel buffer	2.5 ml	—
4x stacking gel buffer	—	3.75 ml
ddH_2O	4.1 ml	9.75 ml
10 % (w/v) APS	100 µl	100 µl
TEMED	3.3 µl	20 µl

Table 10: Composition of SDS-PAGE gels.
The indicated amounts are sufficient to prepare 1 gel (size 6*9 cm).

3.2.9 Immunoblotting

After the separation of proteins by SDS-PAGE, proteins were transferred from the gel to a PVDF membrane (Immobilon-P, Millipore, Schwalbach, Germany) by semidry blotting. After transfer, the membrane was incubated in blocking buffer for 1 h at RT to avoid unspecific antibody binding. Afterwards, the membrane was washed three times for 10 min in TBST. For detection of OVA, the membrane was incubated overnight with an α-OVA antibody in TBS at 4 °C. On the next day, the membrane was washed three times with TBST for 10 min and incubated with HRP-conjugated α-rabbit-IgG antibody in TBS for 1 h at RT. Afterwards, the membrane was washed again three times with TBST, incubated with Super Signal West Pico

Chemiluminescent detection reagent and subjected to autoradiography to visualize protein bands. To directly detect recombinant antibodies, the membrane was incubated with a HRP-conjugated α-mouse IgG antibody after blocking the membrane.

Antigen	Dilution primary antibody	Dilution secondary antibody
mouse IgG	---	1:7500 (α-mouse IgG-HRP)
OVA	1:1000	1:7500 (α-rabbit IgG-HRP)

Table 11: Antibodies for western blot analysis.

3.2.10 Flow cytometry

For FACS analysis, cells were stained with 1:200 dilutions of the respective antibodies for 20 min at 4 °C. To prevent binding of antibodies to Fc receptors antibodies were diluted in the supernatant of a hybridoma cell line producing Fc blocking antibodies (α-CD16/α-CD32). Afterwards, cells were washed twice with FACS buffer. Propidium iodide (2.5 µg/ml) was added to stained cells to exclude dead cells from the analysis. For intracellular cytokine staining, cells were incubated at RT for 20 min with 2 % paraformaldeyde fixation buffer and permeabilized by incubation for 20 min at RT with 0.5 % saponin permeabilisation buffer. Cells were analyzed on a FACS Calibur (BD Biosciences, Heidelberg, Germany) or a FACS Gallios (Beckman Coulter, Miami, USA) flow cytometer. FACS data were analyzed with FlowJo software.

3.2.11 Cell isolation

For isolation of splenocytes, spleens were incubated with DNase I and Collagenase D (100 µg/ml DNase I and 500 µg/ml Collagenase D in RPMI 1640) for 30 min at 37 °C. Single cell suspensions were prepared by straining spleens through 100 µm cell strainers. Red blood cell lysis buffer was used as described in 3.2.5.2 to lyse erythrocytes. Lymph node cells were isolated by straining lymph nodes through 100 µm cell strainers without prior incubation with DNase I and Collagenase D. Bone marrow cells were isolated as described in 3.2.5.2.

3.2.12 Cell separation

CD4+ and CD8+ T cells were negatively separated with the respective MACS isolation kits from spleen cell suspensions according to the manufacturer's protocol. CD11c+ DCs were positively selected with the MACS isolation kit for murine CD11c+ DCs according to the manufacturer's instructions. Cell sorting was performed on a MoFlo cell sorter (Beckman Coulter, Miami, USA).

3.2.13 Labeling of cells with CFSE

For *in vivo* T cell transfer experiments, MACS enriched T cells were labeled with 5 µM CFSE. For *in vitro* co-culture experiments T cells were stained with 1 µM CFSE. For *in vivo* killing assays splenocytes were labeled with either 0.1 µM or 1 µM CFSE. $1*10^7$ cells/ml were incubated in CFSE labeling buffer with the respective CFSE concentration at 37 °C for 10 min. Afterwards cells were washed twice with PBS.

3.2.14 *In vitro* co-culture of T cells and DCs

In vitro co-culture experiments were performed in 96-well round bottom plates. $1*10^5$ CFSE-labeled T cells were co-cultivated for 4 days with $1*10^4$ FACS purified pDCs or graded numbers of CD11c+ DCs. Proliferation was analyzed by CFSE dilution.

3.2.15 *In vitro* stimulation of pDCs

$1*10^6$ Fl-DCs/ml were incubated with the indicated concentrations of α-Siglec-H-OVA or α-BST2-OVA in the absence or presence of 1 µM CpG 1668 or 1 µM CpG 2216. After 24 h, supernatant was collected to detect cytokines and cells were stained to measure expression of cell surface molecules.

3.2.16 *In vivo* mouse experiments

3.2.16.1 In vivo pDC depletion

For *in vivo* depletion of pDCs 300 µg α-Gr-1 were injected intraperitoneally (i.p.) 24 h before mice were immunized with 10 µg α-BST2-OVA and 50 µg PolyI:C. 8 h later, CD11c+ DCs were isolated from splenocytes.

3.2.16.2 In vivo antigen presentation

To investigate antigen presentation *in vivo*, I-A^k expressing C3H mice received 20 µg of the antibody-pHEL fusion proteins (i.p.) without adjuvant or in combination with 10 µg CpG 1668 or 50 µg PolyI:C. After the indicated time periods, splenocytes, inguinal and mesenteric lymph node cells as well as bone marrow cells were isolated and stained with different cell surface antibodies and the Aw3.18 antibody to detect pHEL/MHC II complexes (pHEL/I-A^k).

3.2.16.3 T cell transfer experiments

MACS enriched T cells (OT-I, OT-II, OT-II/Rag2-/-, DO11.10/Rag2-/-) were labeled with 5 µM CFSE and adoptively transferred into recipient mice by intravenous (i.v.) injection (2-4*10^6 cells per mouse in 200 µl PBS). The recipients received the indicated doses of chimeric α-Siglec-H-OVA, α-BST2-OVA or α-DEC205-OVA antibodies or an isotype-OVA control antibody 24 h later (i.p.). Where indicated, mice simultaneously received 10 µg CpG 1668, 50 µg PolyI:C or 100 µg soluble OVA. 4 days after the immunization, T cell responses were analyzed in the spleen and where indicated, in inguinal and mesenteric lymph nodes. Fig. 19 illustrates how T cell transfer experiments were performed in detail.

3.2.16.4 Immunization protocol

C57BL/6 mice were immunized on day 0 and 14 with 10 µg of the indicated antibody-OVA fusion proteins either without adjuvant or in combination with 10 µg CpG 1668 or 50 µg PolyI:C (i.p.). α-OVA antibody titers were detected on day 14 and 21. To detect CD4+ T cell responses, spleens were isolated on day 21 and cytokine production was detected by intracellular staining after OVA restimulation. To determine CD8+ T cell responses *in vivo* killing assays were performed on day 21. Fig. 43 illustrates how immunization experiments were performed in detail.

3.2.16.5 In vivo killing assay

Antigen specific killing was determined as described previously (Hernandez *et al*., 2001). Splenocytes were incubated with SIINFEKL peptide (500 nM) for 3 h and labeled with 0.1 µM CFSE or not pulsed with peptide and labeled with 1 µM CFSE.

$1*10^7$ cells of each population were transferred (i.v.) into immunized recipients or unimmunized control mice and specific lysis was quantified in the spleen 16 h later. Specific killing was calculated according to the formula:

$$\% \text{ killing} = \left(1 - \frac{\% \text{ CFSE}^{high} \text{ (unimmunized)} * \% \text{ CFSE}^{low} \text{ (immunized)}}{\% \text{ CFSE}^{high} \text{ (immunized)} * \% \text{ CFSE}^{low} \text{ (unimmunized)}}\right) * 100$$

$CFSE^{high}$ = cells were incubated with medium and stained with 1 µM CFSE
$CFSE^{low}$ = cells were incubated with 500 nM SIINFEKL and stained with 0.1 µM CFSE
unimmunized: negative control that was not immunized
immunized: mice that were immunized

3.2.16.6 Infection with vaccinia virus encoding OVA

C57BL/6 mice were immunized on day 0 and/or 14 with 10 µg α-BST2-OVA and 50 µg PolyI:C. 8 weeks later, mice were intranasally infected with $3*10^4$ PFU (in 30 µl PBS) of a recombinant vaccinia virus strain encoding OVA (VV-OVA). After the viral challenge mice were weighed daily. 14 days after infection, mice were sacrificed and splenocytes were restimulated *ex vivo* with OVA peptides to determine the frequencies of OVA specific CD4+ T cells producing IFN-γ and IL-2 and CD8+ T cells producing IFN-γ.

3.2.16.7 Implantation of OVA expressing B16 melanoma cells

C57BL/6 mice were immunized on day 0 with 10 µg α-BST2-OVA and 50 µg PolyI:C. 14 days later, $1*10^6$ MO5 OVA-expressing B16 melanoma cells were injected subcutaneously (s.c.) in the flank. Tumor size was measured at the indicated time points (diameter length multiplied by diameter width). Mice with tumors exceeding 225 mm^2 were sacrificed. For each group, the tumor size was determined until 2 mice died or had to be sacrificed.

3.2.16.8 T cell priming

To induce the expansion and differentiation of endogenous antigen specific T cells mice were immunized (s.c.) with 100 µl of an emulsion containing 100 µg pOVA (aa 323-339) and 250 µg of *M. tuberculosis* H37Ra in Freund's adjuvant oil (complete Freund's adjuvant, CFA) and received 200 ng pertussis toxin (PTx) (i.v.) on the same

day and 2 days after the immunization with pOVA/CFA. To investigate the influence of antigen presenting pDCs on the differentiation of effector T cells, mice received 10 µg α-Siglec-H-OVA 1 day prior to the immunization with pOVA/CFA/PTx. 10 days later, splenocytes were *ex vivo* restimulated with pOVA and the frequency of antigen specific effector CD4+ T cells was determined by intracellular cytokine staining and incorporation of ^{3}H-thymidine.

3.2.16.9 Experimental autoimmune encephalomyelitis

EAE is an inducible and antigen specific autoimmune disease in rodents. To induce EAE, mice were immunized (s.c) with 100 µl of an emulsion containing 100 µg pMOG (aa 35-55) and 250 µg of *M. tuberculosis* H37Ra in Freund's adjuvant oil (pMOG/CFA) and received 200 ng PTx (i.v.) on the same day and 2 days after the immunization with pMOG/CFA. To investigate the influence of antigen presenting pDCs on the progress of the disease, where indicated, mice received 10 µg α-Siglec-H-OVA or α-Siglec-H-pMOG 7 days or 1 day prior to the immunization with pMOG/CFA/PTx. Disease progress and severity were assayed as previously described (Korn *et al*., 2007). Table 12 depicts how clinical symptoms were scored in detail.

Score	Symptoms
0	no symptoms
0.5	initial signs (beginning tail paralysis)
1.0	flaccid tail (tail paralysis)
1.5	flaccid tail and impaired righting reflex
2.0	paraparesis / hind limb weakness
2.5	monoplegia (paralysis of one hind limb)
3.0	paraplegia (paralysis of both hind limbs)
3.5	paraplegia and weak front limb paralysis
4.0	tetraplegia (front and hind limb paralysis)
5.0	moribund

Table 12: Clinical EAE score.

Where indicated, brain and spinal cord were isolated at the peak of disease (day 17) and absolute numbers of infiltrating CD3+/CD4+ T cells were determined. T cells were restimulated *ex vivo* with PMA/Iono and the frequency of cytokine producing T cells was determined by intracellular cytokine staining and flow cytometry.

3.2.17 T cell restimulation assays

3.2.17.1 Protein restimulation

MACS enriched CD11c+ DCs ($1*10^6$ cells/ml) were pulsed with 1 mg/ml OVA for 4 h. An equal number of MACS enriched CD4+ T cells was added to the DCs for another 6 h. Golgi Stop and Golgi Plug were present for the last 4 h and were used according to the manufacturer's protocol.

3.2.17.2 Peptide restimulation

Splenocytes and lymph node cells were restimulated *ex vivo* with 5 µg/ml SIINFEKL (CD8+ T cell responses) or pOVA (aa 323-339) and pOVA (aa 265-279) (CD4+ T cell responses) for 6 h. For restimulation with pMOG 100 µg/ml were used. Golgi Plug and Golgi Stop were present for the last 4 h.

3.2.17.3 ^{3}H-thymidine incorporation assay

To determine the frequency of antigen specific CD4+ T cells in spleen, $1*10^6$ cells/ml ($1*10^5$ cells/well, 96 well plate) were restimulated with different doses of peptide or cultured without peptide for 72 h. 1.25 µCi ^{3}H-thymidine were added to each well and cells were incubated for another 12-18 h. Incorporation of ^{3}H-thymidine was quantified with a scintillation counter after cells had been transferred to a filter mat and were incubated with scintillation fluid. Proliferation of CD4+ T cells in response to stimulation with different amounts of peptide was displayed as stimulation index. The stimulation index depicts the ratio of counts per minute of samples that were restimulated with peptide in comparison to an unstimulated control.

$$\text{proliferation index} = \left(\frac{\text{counts per minute (stimulated sample)}}{\text{counts per minute (unstimulated sample)}} \right)$$

3.2.18 Ovalbumin specific antibody ELISA

To detect OVA specific antibodies, Maxisorp ELISA plates (Nunc, Roskilde, Denmark) were coated with 5 µg/ml OVA in PBS overnight at 4°C. Plates were washed twice and blocked with blocking buffer (1 h at RT). Afterwards, serial dilutions of serum were added to the plates and incubated for 2 h at RT. After washing the plates three times, biotinylated α-mouse IgG1, IgG2b or IgG2c antibodies (1:500 dilutions) were added to the plates and incubated for 1 h at RT. After washing the plates three times, Streptavidin coupled horseradish peroxidase (Strep-HRP 1:3000 dilution) was added to the plates and incubated for 30 min. Plates were washed three times and ABTS containing ELISA substrate was added. The reaction was stopped with SDS containing stop solution. The OD was measured at 405 nm wavelength. Titers represent the highest dilution of serum showing an OD 405 nm ≥ 0.1 (Boscardin *et al*., 2006).

3.2.19 Cytokine ELISAs

ELISAs for murine IL-6, IL-12 and IFN-α were performed using matched antibody pairs. Table 13 briefly depicts how cytokine specific ELISAs were performed.

	IL-6	**IL-12**	**IFN-α**
capture antibody (unconjugated)	1:250 (overnight)	1:1000 (overnight)	1:250 (overnight)
wash	3x	3x	3x
blocking	1 h at RT	1 h at RT	1 h at RT
1st cytokine standard	8000 pg/ml	8000 pg/ml	1000 U/ml
sample dilution	1:10	1:20	1:5
wash	4x	4x	4x
detection antibody (biotinylated or unconjugated)	1:500 (1 h at RT)	1:2000 (1 h at RT)	1:100 (1 h at RT)
wash	4x	4x	4x
HRP	Streptavidin-HRP 1:2000 (1 h at RT)	Streptavidin-HRP 1:2000 (1 h at RT)	α-rabbit IgG-HRP (1:2000) (1 h at RT)
wash	4x	4x	4x
ELISA substrate	ABTS	ABTS	ABTS
stop solution	SDS	SDS	SDS

Table 13: Cytokine ELISAs.

3.2.20 Statistical analysis

Statistical significance was assessed with the unpaired, two-tailed Student's *t*-test, except for Table 14 where the Mann-Whitney U rank sum test was used. p values <0.05 were considered significant (indicated by asterisks).

4 RESULTS

To investigate how antigen presenting pDCs contribute to antigen specific adaptive immune responses, recombinant α-Siglec-H and α-BST2 antibody-antigen fusion proteins that allow the delivery of antigen specifically to murine pDCs were generated.

4.1 Cloning, expression and purification of recombinant α-Siglec-H and α-BST2 antibodies

Recombinant antibodies specific for Siglec-H and BST2 were generated on the basis of a recombinant antibody for DEC205 (Hawiger *et al*., 2001). This DEC205 specific antibody can be used to deliver antigens (e.g. OVA) to CD8α+ DCs and consists of three different domains:

The **first** part consists of the variable regions of a rat monoclonal α-DEC205 antibody that were fused to a murine IgG1 scaffold and account for the specific binding of the recombinant antibody to DEC205.
The **second** part is the mouse IgG1 antibody scaffold that contains mutations in its C_H3 region. These mutations prevent binding of the antibody to Fc receptors.
The **third** part is the antigen (e.g. OVA) that is intended to be delivered to DEC205+ DCs. This antigen is fused to the C-terminus of the antibody's heavy chain.

The described recombinant DEC205-OVA antibody is encoded by two plasmids. One plasmid encodes the light chain of the antibody and the second plasmid is encoding the heavy chain OVA fusion protein (Fig. 10). The sequence for OVA is flanked by specific restriction sites. This provides the possibility to exchange the antigen that is fused to the antibody's C-terminus.

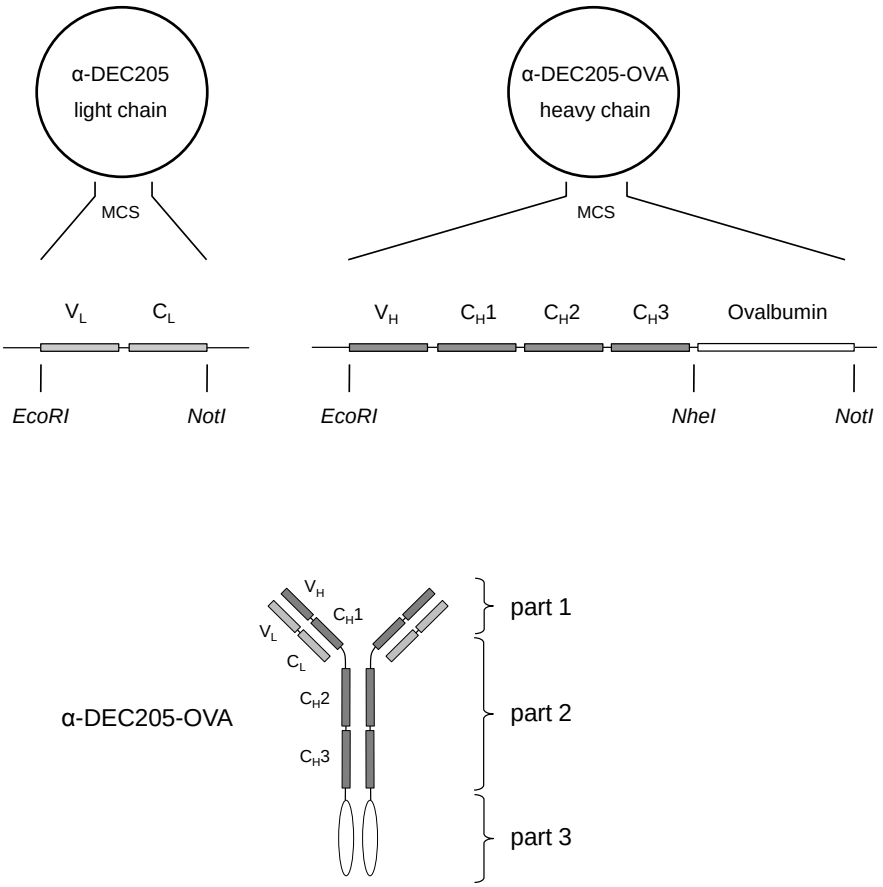

Fig. 10: Recombinant α-DEC205-OVA antibody.

The recombinant α-DEC205-OVA antibody is encoded by two plasmids. The first plasmid encodes the antibody's light chain. The sequence for the light chain is flanked by *EcoRI* and *NotI* restriction sites. The second plasmid encodes the heavy chain OVA fusion protein. The sequence for the heavy chain is flanked by *EcoRI* and *NheI* restriction sites and the sequence encoding OVA is flanked by sequences specific for *NheI* and *NotI*. The recombinant antibody-antigen fusion protein consists of three domains. The first part are the variable regions of a rat monoclonal α-DEC205 antibody that were fused to a mouse IgG1 scaffold (second part). The C-terminus of the antibody's heavy chain is fused to OVA (third part). (V_L variable region of the light chain, C_L constant region of the light chain, V_H variable region of the heavy chain, C_H1, C_H2 and C_H3 constant region 1,2 and 3 of the heavy chain, OVA Ovalbumin, MCS multiple cloning site)

To generate recombinant antibodies that bind to Siglec-H and BST2 the first step was to isolate the sequences that encode the variable regions of the respective antibodies from hybridoma cell lines producing rat monoclonal α-Siglec-H and α-BST2, respectively. For this purpose, total RNA was isolated and cDNA was synthesized using specific primers that mediate amplification of the sequences encoding the antibodies' V_L (variable region of the light chain) or V_H (variable region of the heavy chain). These sequences were then used to replace the DEC205 specific V_L and V_H in the plasmids encoding the light or heavy chain of the α-DEC205-OVA antibody.

Fig. 11 illustrates how recombinant α-Siglec-H-OVA and α-BST2-OVA were generated. For a description explaining how the recombinant α-Siglec-H-OVA and α-BST2-OVA antibodies were cloned in detail see chapter 3.2.1.

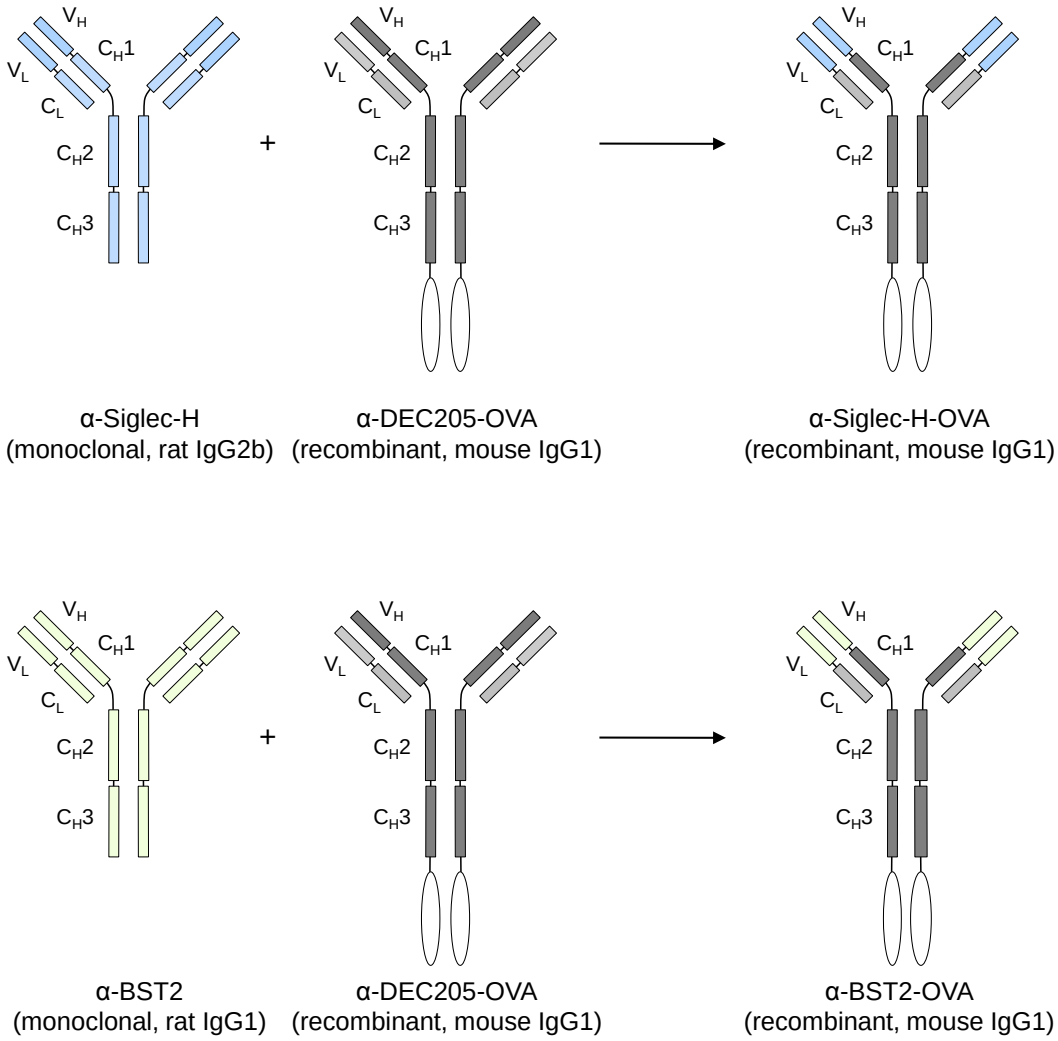

Fig. 11: Generation of recombinant α-Siglec-H-OVA and α-BST2-OVA.

The recombinant α-Siglec-H-OVA and α-BST2-OVA antibodies were generated by replacing the variable regions of the α-DEC205-OVA antibody with the variable regions of a monoclonal rat α-Siglec-H and α-BST2 antibody, respectively. Antibody parts that originate from the monoclonal α-Siglec-H antibody are depicted in blue, antibody parts that originate from the monoclonal α-BST2 antibody are depicted in green and parts that originate from the recombinant α-DEC205-OVA antibody are marked in grey.

To generate Siglec-H and BST2 specific antibodies that are fused to different antigens, the plasmids encoding the heavy chain of α-Siglec-H-OVA and α-BST2-OVA were digested with *NheI/NotI* and the sequences encoding other antigens flanked by those restriction sites were inserted (see chapter 3.2.1.3 Fig. 9). Thus, antibodies were generated that are fused to a specific peptide from hen egg lysozyme (pHEL, aa 48-62) and a peptide derived from myelin oligodendrocyte glycoprotein (pMOG, aa 35-55).

Recombinant Siglec-H and BST2 specific antibodies were expressed in HEK293T cells after transient transfection with the respective antibody encoding plasmids. Due to secretion signals at the N-terminus of the light and heavy chain, the antibody-antigen fusion proteins were secreted into the supernatant of the transfected cells. The antibodies were purified from supernatants by protein G affinity chromatography. Each batch of purified antibody was tested for its purity and integrity by SDS-PAGE and subsequent staining with Coomassie blue. The light chains of both recombinant antibodies have a size of approximately 25 kDa. The size of the heavy chain depends on the size of the antigen that is fused its C-terminus. OVA has a size of ~50 kDa and the heavy chain OVA fusion proteins have a size of ~100 kDa. pHEL consisting of only 15 amino acids (~1.7 kDa) and pMOG consisting of 21 amino acids (~2.3 kDa) only slightly increase the size of the antibodies' heavy chain (~50 kDa) (Fig. 12).

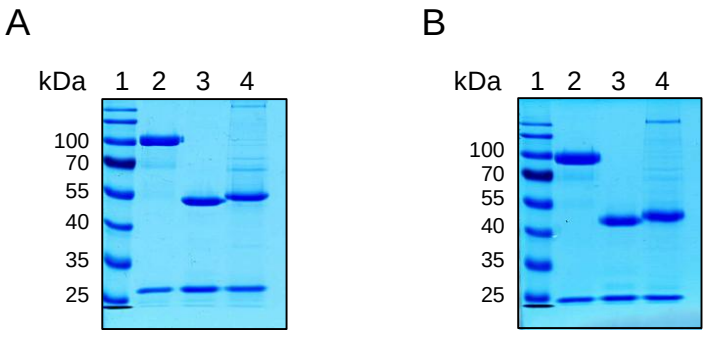

Fig. 12: Purified recombinant α-Siglec-H and α-BST2 antibodies fused with different antigens.
The purity of all antibody preparations was analyzed by SDS-PAGE and subsequent staining with Coomassie blue. 5 µg of **(A)** α-Siglec-H and **(B)** α-BST2 fused to OVA (lane 2), pHEL (lane 3) or pMOG (lane 4) were loaded per lane. Lane 1: protein ladder for estimation of the molecular weight.

To verify the presence of the antigen fused to the antibodies' C-terminus, OVA was detected by western blot analysis. The heavy chain OVA fusion proteins were detectable with α-OVA and α-mIgG antibodies, whereas the light chains were only detectable with the α-mIgG antibody (Fig. 13).

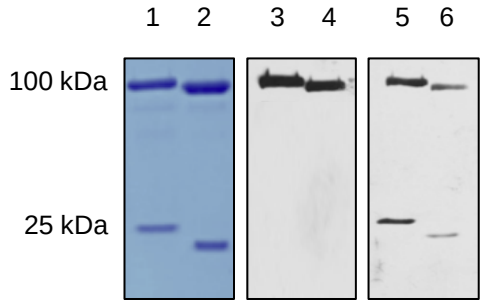

Fig. 13: Western blot analysis of purified α-Siglec-H-OVA and α-BST2-OVA antibodies.
α-Siglec-H-OVA (lane 1, 3, 5) and α-BST2-OVA (lane 2, 4, 6) fusion proteins were analyzed by SDS-PAGE and visualized by staining with Coomassie blue (lane 1 and 2). In addition, heavy chain OVA fusion proteins were detected by western blot analysis using an antibody specific for OVA (lane 3 and 4). Both, light and heavy chains were detected with an α-mIgG antibody (lane 5 and 6).

4.2 Specific binding of recombinant α-Siglec-H and α-BST2 antibodies to murine pDCs

To test whether the purified recombinant antibodies bind to their target cells, α-Siglec-H-OVA and α-BST2-OVA were incubated with splenocytes and binding of the antibodies was detected with a secondary antibody specific for murine IgG1 (mIgG1). Because both surface molecules - Siglec-H and BST2 - are specific markers for pDCs, pDCs were defined either as Siglec-H+ when binding of the recombinant α-BST2 antibody was tested or as BST2+ when preparations of the recombinant α-Siglec-H antibody were tested. As shown in Fig. 14, both recombinant antibodies - α-Siglec-H-OVA and α-BST2-OVA - specifically bound to pDCs. Replacement of OVA by other antigens (pHEL or pMOG) had no impact on the binding specificity of the antibodies (not shown). As previous studies implicated that CCR9 expression defines tolerogenic pDCs (Hadeiba *et al*., 2008), the correlation of the expression of Siglec-H, BST2 and CCR9 was measured. The majority of cells that were bound by recombinant α-Siglec-H and α-BST2 antibodies expressed high levels of CCR9. Only a small fraction lacked expression of CCR9 or expressed low levels (Fig. 14, histograms). Thus, CCR9 expressing pDCs, which have been implicated to have

tolerogenic activity upon adoptive transfer, are targeted using these antibodies. A mIgG1-OVA fusion protein with irrelevant binding specificity served as isotype control for binding of the pDC specific antibodies.

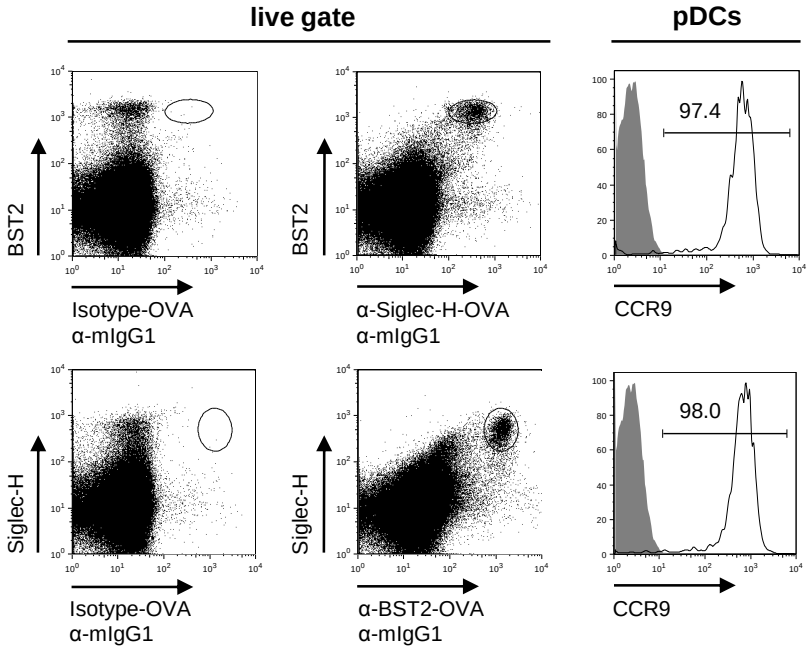

Fig. 14: Specific binding of recombinant α-Siglec-H-OVA and α-BST2-OVA antibodies to pDCs.
Splenocytes were incubated with recombinant α-Siglec-H-OVA (dot blot upper right) and α-BST2-OVA (dot plot lower right), respectively, followed by staining with biotinylated α-mIgG1 and Streptavidin-APC. To identify pDCs, splenocytes were stained with FITC-labeled monoclonal α-Siglec-H and α-BST2, respectively. An isotype-OVA fusion protein served as control (dot plot upper left and lower left). Histograms display CCR9 expression of the cells that are recognized by the recombinant antibodies. (filled grey histograms: w/o CCR9 staining, open histograms: CCR9 staining)

4.3 Internalization of recombinant antibodies

As the aim of this study was to deliver antigen specifically to pDCs, the next step was to investigate whether antibodies that bind to Siglec-H or BST2 on the surface of pDCs are internalized and delivered to compartments where antigen processing occurs and peptides can be loaded onto MHC molecules. To measure internalization, splenocytes were incubated with biotinylated α-Siglec-H or α-BST2 and subsequently incubated at 37 °C. At the indicated time points, remaining antibodies bound to the cell surface of pDCs were detected by staining with Streptavidin-APC. For internalization experiments, pDCs were defined as $CD11c^{low}$/B220+ (Fig. 15 A). For both antibodies, a fast decrease in the percentage of APC+ cells was observed

within the first hour, suggesting that both antibodies were efficiently internalized (Fig. 15 A). In contrast to that, cells that were stained with FITC-labeled α-Siglec-H or α-BST2 did not lose their fluorescence. This excludes the possibility that the loss of the pDC specific biotinylated antibodies that could be detected with Streptavidin was due to dissociation of the antibodies during the incubation phase at 37 °C (data not shown).

To further confirm that the loss of bound antibody at the cell surface was due to internalization, bone marrow derived Fl-DCs were incubated with α-Siglec-H-FITC or α-BST2-FITC and subsequently incubated at 4 °C or 37 °C. Whereas both antibodies exclusively bound to the cell surface after incubation at 4 °C, intracellular localization in vesicular structures overlapping with a marker for lysosomal compartments was observed after incubation at 37 °C for 60 min (Fig. 15 B). Therefore, it can be concluded that α-Siglec-H and α-BST2 antibodies are efficiently internalized and antigens that are fused to these antibodies are delivered to late endosomal compartments where MHC class II peptide loading can occur. Thus, α-Siglec-H and α-BST2 are qualified tools to deliver antigens to pDCs.

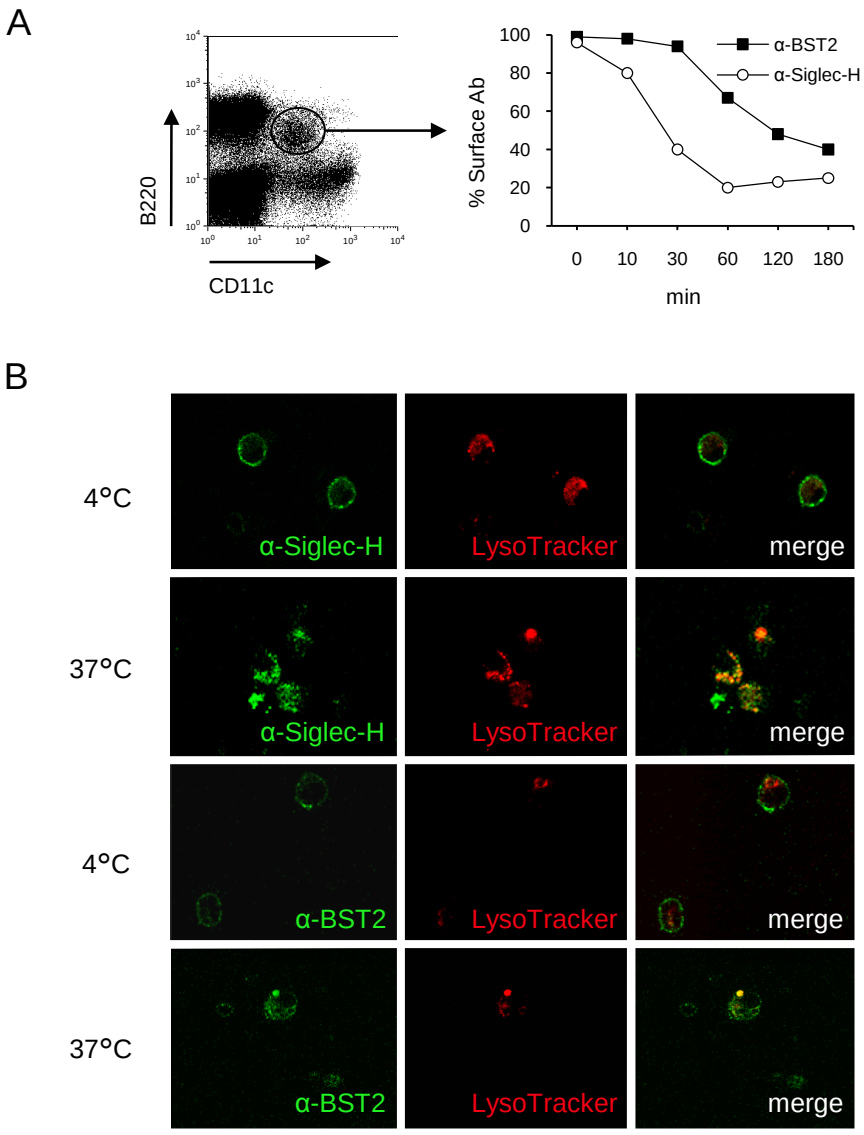

Fig. 15: Internalization of α-Siglec-H and α-BST2.

(A) Splenocytes were stained with biotinylated α-Siglec-H or α-BST2 and were subsequently incubated at 37 °C. At the indicated time points cells were stained with CD11c, B220 and Streptavidin-APC and the percentage of APC+ CD11c^{low}/B220+ pDCs was measured by FACS analysis. **(B)** Bone marrow derived Fl-DCs were incubated with either FITC-labeled α-Siglec-H (upper rows) or FITC-labeled α-BST2 (lower rows) and subsequently incubated at 4 °C or 37 °C for 60 min, stained with LysoTracker (red) and analyzed by confocal microscopy at 64x magnification.

4.4 Presentation of antigen on MHC II after *in vivo* delivery of antigen *via* Siglec-H and BST2

To be able to directly detect antigen presentation on MHC II, α-Siglec-H and α-BST2 fused to pHEL (aa 48-62) were used. Presentation of pHEL on MHC II can be measured with the Aw.3.18 antibody that specifically binds the pHEL/MHC II complex (pHEL/I-A^k).

Other antigen presenting cells than pDCs could ingest the antibody-antigen fusion proteins either by macropinocytosis or endocytosis of dead or dying pDCs leading to unspecific antigen presentation. Therefore, in subsequent experiments the presence of pHEL/I-A^k complexes was analyzed on different APC populations. PDCs were defined as either Siglec-H+/$CD11c^{low}$ or BST2+/$CD11c^{low}$ dependent on whether α-Siglec-H-pHEL or α-BST-pHEL was injected to eliminate competition of recombinant and monoclonal antibody for the antigen. CDCs were defined as $CD11c^{high}$/BST2-/Siglec-H-, B cells as B220+/BST2-/Siglec-H- and non pDCs as BST2-/Siglec-H- (Fig. 16).

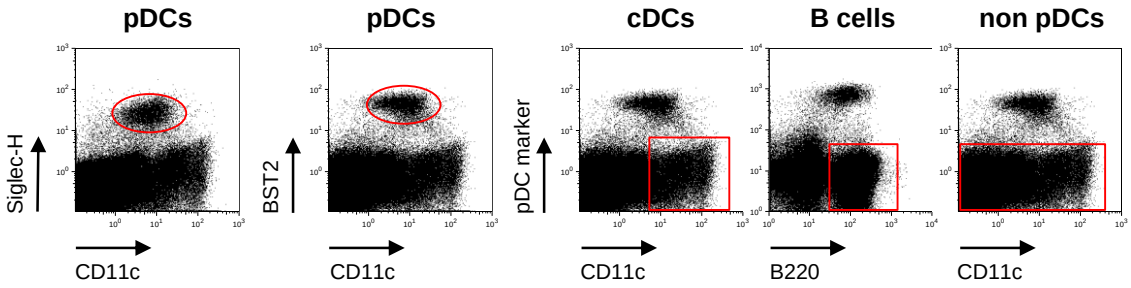

Fig. 16: Definition of antigen presenting cell populations.
Distinct combinations of cell surface markers were used to define specific subpopulations of APCs (here shown for splenocytes). PDCs were defined as $CD11c^{low}$/B220+/BST2+ or $CD11c^{low}$/B220+/Siglec-H+, cDCs as $CD11c^{high}$/Siglec-H-/BST2-, B cells as B220+/Siglec-H-/BST2- and non-pDCs as Siglec-H-/BST2-.

α-Siglec-H-pHEL, α-BST2-pHEL or an isotype-pHEL control were injected into C3H mice. 16 h after the injection, pHEL/I-A^k complexes were detected on the surface of splenocytes (Fig. 17 A), lymph node cells (Fig. 17 B) and bone marrow cells (Fig. 17 C).

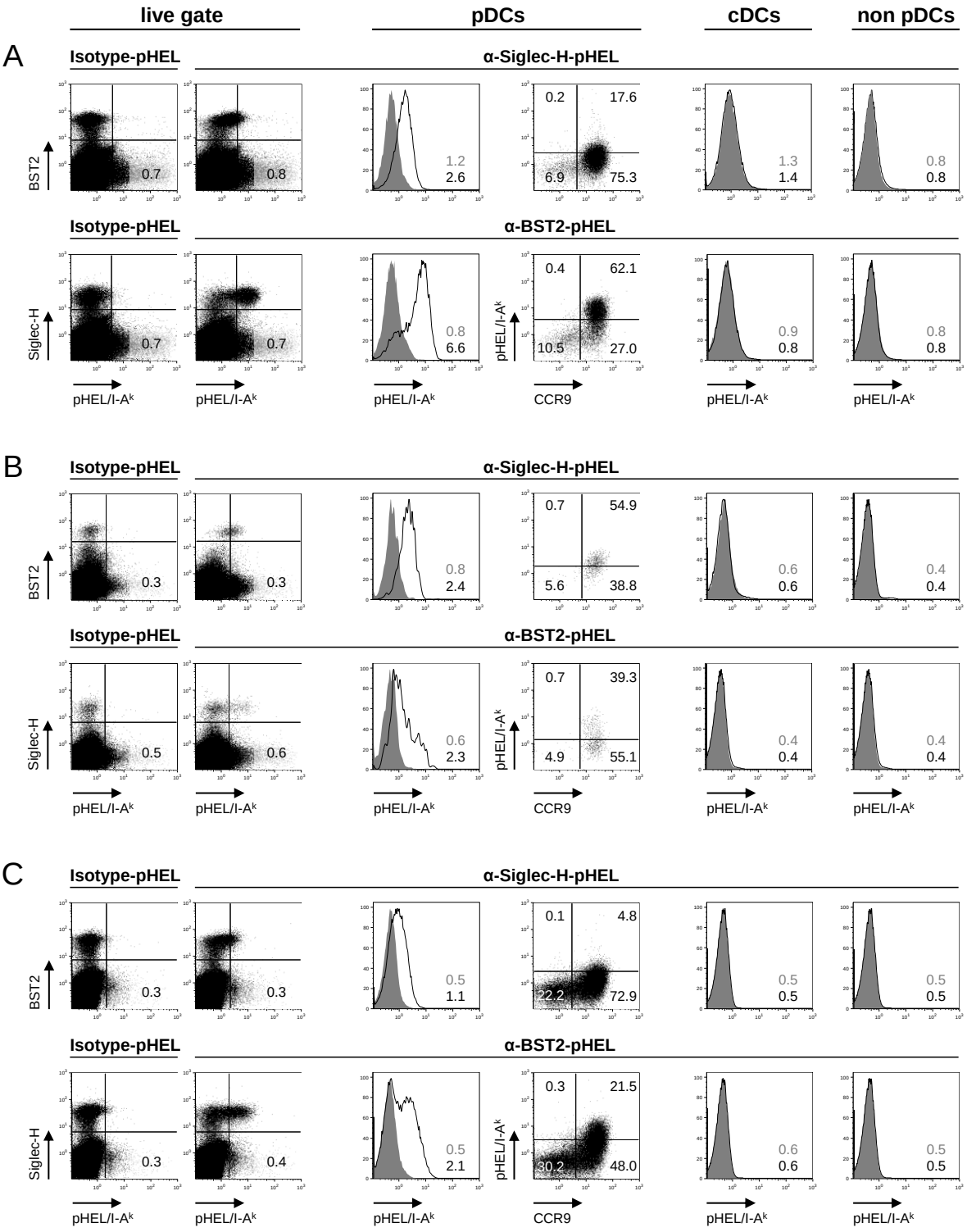

Fig. 17: Antigen presentation on MHC II after antibody mediated antigen delivery *in vivo*.

20 µg α-Siglec-H-pHEL or α-BST2-pHEL were injected (i.p.) into C3H mice. pHEL/MHC II complexes (pHEL/I-A^k) were detected 16 h after the injection. Presence of pHEL/I-A^k complexes on the surface of pDCs, cDCs and remaining cells after treatment with α-Siglec-H-pHEL or α-BST2-pHEL (open histograms) or application of an isotype-pHEL control (filled grey histograms) was traced on **(A)** splenocytes, **(B)** lymph node cells and **(C)** bone marrow cells. Numbers in histograms indicate the mean fluorescence intensity (grey: isotype-pHEL control, black: α-Siglec-H-pHEL or α-BST2-pHEL). Dot plots show correlation of pHEL/I-A^k formation and CCR9 expression for pDCs. The percentages are indicated in the quadrants.

Administration of the isotype-pHEL fusion protein did not result in the formation of pHEL/I-A^k complexes beyond background levels (Fig. 17, dot plots left row). After injection of α-Siglec-H-pHEL and α-BST2-pHEL, respectively, pHEL/MHC II complexes were specifically presented only by pDCs, but not by cDCs or any other cell type (Fig. 17, histograms). Therefore, it can be concluded that antigen that is fused to α-Siglec-H or α-BST2 is specifically delivered to pDCs and these cells are the only cells that present the antigen on MHC II after injection of α-Siglec-H- and α-BST2-antigen fusion proteins.

CCR9 expression has been described to be a hallmark of pDCs (Segura *et al*., 2009) and may define pDCs with tolerogenic functions (Hadeiba *et al*., 2008) within the $CD11c^{low}$/B220+ cell population. Therefore, the correlation of antigen presentation and CCR9 expression was determined for pDCs. The majority of pDCs in the spleen and peripheral lymph nodes expresses CCR9 and pHEL/I-A^k complex formation was restricted to the CCR9+ subpopulation. The small fraction of CCR9- pDCs did not present antigen on MHC II. The percentage of CCR9-/$CCR9^{low}$ pDCs in the bone marrow is much higher. However, also in the bone marrow only CCR9+ pDCs presented the antigen, whereas CCR9- cells did not. Therefore, it can be excluded that CCR9- pDCs that were shown to be precursors of pDCs with the ability to differentiate into cDC like cells (Schlitzer *et al*., 2011; Segura *et al*., 2009) present the antigen that is delivered *via* Siglec-H or BST2. Thus, it can be concluded that both - α-Siglec-H and α-BST2 - deliver antigen specifically to CCR9+ pDCs. Furthermore, it can be excluded that antigen is delivered to pDC precursors that have the ability to differentiate into cDC like cells.

TLR9 ligand CpG 1668 was used as stimulus in subsequent studies to discriminate between immature/unstimulated and mature/stimulated pDCs and to investigate the influence of TLR signaling on the outcome of pDC mediated adaptive immune responses. To exclude that TLR9 ligation affects the specificity of the antibody mediated antigen delivery, presentation of pHEL on MHC II was also determined after antigen had been delivered to pDCs *in vivo* in the presence of CpG 1668. As shown in Fig. 18, TLR9 ligand CpG 1668 did not influence antigen presentation on MHC II after the antigen had been delivered to pDCs *via* Siglec-H or BST2. Similar amounts of antigen were detected on pDCs after antibody mediated antigen delivery, irrespective of whether α-Siglec-H-pHEL and α-BST2-pHEL were injected in the

absence or presence of CpG 1668. No other cell population in the spleen did present antigen on MHC II molecules 16 h after administration of the antibody-antigen fusion protein. This was also confirmed at later time points (24, 48, 72 h, data not shown)

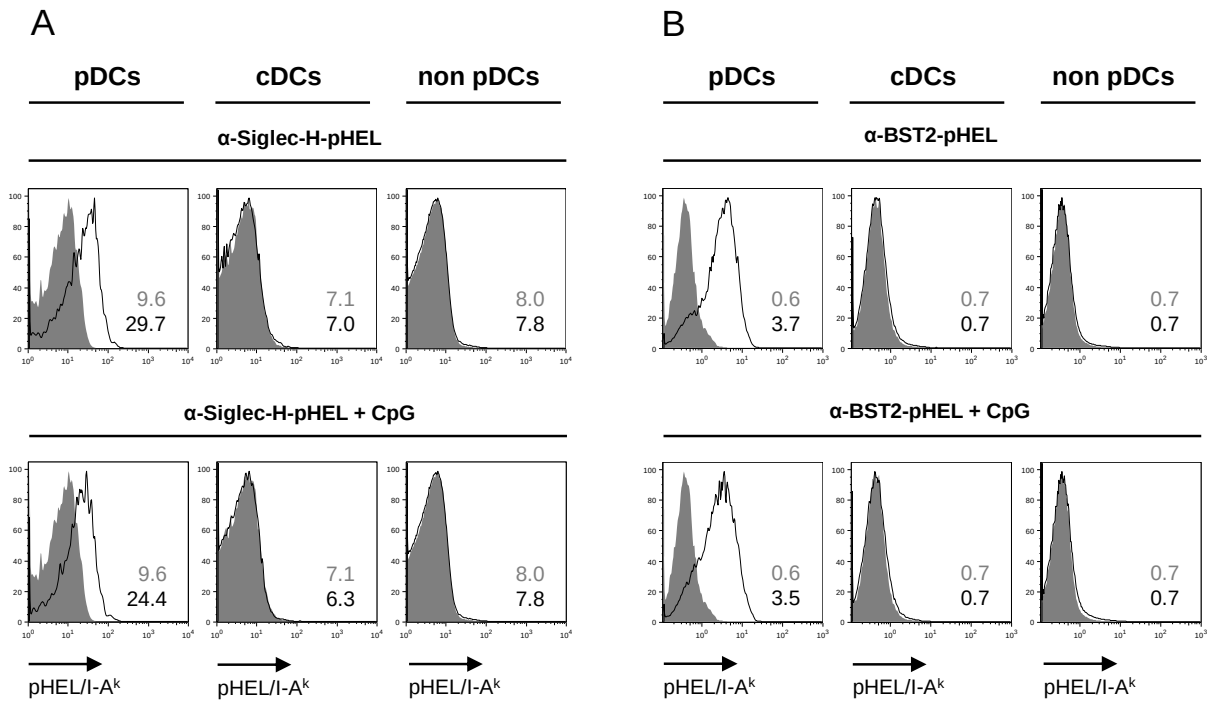

Fig. 18: Antigen presentation on MHC II after antibody mediated antigen delivery *in vivo* in the presence of TLR9 ligand CpG 1668.

20 µg α-Siglec-H-pHEL or α-BST2-pHEL were injected (i.p.) into C3H mice in the absence or presence of 10 µg CpG 1668. pHEL/MHC II complexes (pHEL/I-A^k) were detected on splenocytes 16 h after the injection. Presence of pHEL/I-A^k complexes was measured on the surface of pDCs, cDCs and remaining cells after treatment with **(A)** α-Siglec-H-pHEL or **(B)** α-BST2-pHEL (open histograms) or application of PBS (filled grey histograms). Numbers in histograms indicate the mean fluorescence intensity (grey: PBS, black: α-Siglec-H-pHEL or α-BST2-pHEL).

4.5 Lack of Foxp3 expression in adoptively transferred T cells after pDC specific antigen presentation

Naturally occurring Tregs in naïve mice develop during clonal selection in the thymus. One of the major mechanisms for the induction of active antigen specific tolerance in peripheral tissues is the extrathymic *de novo* generation of Foxp3 expressing Tregs from naïve precursors. Since pDCs have been shown to be involved in the induction of antigen specific tolerance upon adoptive transfer, the capability of antigen presenting pDCs to induce Foxp3 expression in naïve antigen specific T cells and the subsequent differentiation into Tregs *in vivo* was investigated.

To distinguish between *de novo* generation of Tregs and expansion of natural Tregs, OVA specific T cells from DO11.10/Rag2-/- mice, which lack naturally occurring Tregs, were used. Detection of Foxp3 expression in this experimental setup was therefore a true evidence for *de novo* expression of the Treg specific transcription factor Foxp3. To visualize proliferation of antigen specific T cells, CFSE was used to stain DO11.10/Rag2-/- T cells and proliferation was measured as the decrease in CFSE intensity. CFSE-labeled DO11.10/Rag2-/- T cells were transferred into wildtype (wt) BALB/c mice and proliferation as well as *de novo* induction of Foxp3 expression were analyzed in DO11.10-TCR+ T cells 4 days after immunization with OVA-fused pDC specific antibodies in the presence or absence of CpG 1668 (Fig. 19).

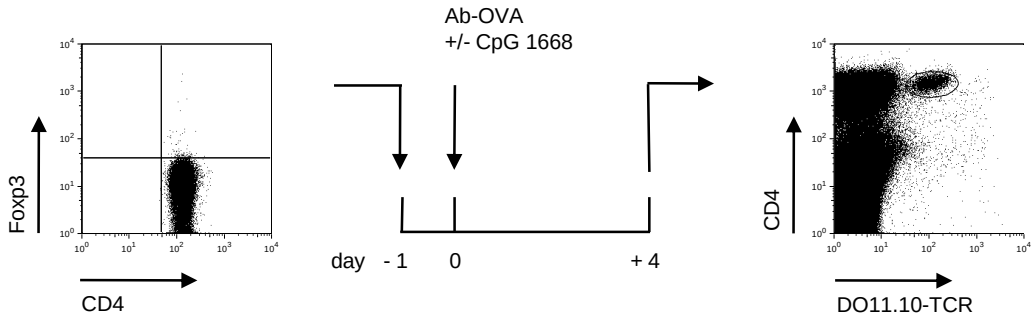

Fig. 19: Adoptive transfer of OVA specific naïve CD4+ T cells.

MACS enriched CD4+/Foxp3- OVA specific DO11.10/Rag2-/- T cells were labeled with CFSE and transferred into wt BALB/c recipient mice (i.v.) 24 h prior to injection (i.p.) of antibody-OVA fusion proteins in the absence or presence of 10 µg CpG 1668. After 4 days, pooled splenocytes and lymph node cells were analyzed by flow cytometry. Transferred T cells were identified by their unique expression of the DO11.10-TCR.

α-DEC205-OVA which delivers antigen to CD8α+ DCs and is known to induce *de novo* generation of Tregs (Yamazaki *et al*., 2008) was used as positive control. A non-binding isotype-OVA antibody was used as negative control.

Injection of α-Siglec-H-OVA and α-BST2-OVA both induced proliferation of the transferred naïve OVA specific CD4+ T cells in a dose dependent manner. This T cell response was further enhanced when the antibody-antigen fusion proteins were injected in the presence of TLR9 ligand CpG 1668. Injection of the isotype-OVA control antibody did not result in proliferation of OVA specific T cells, neither in the absence nor in the presence of TLR9 ligand CpG 1668. As expected, immunization with 1 µg α-DEC205-OVA resulted in *de novo* induction of Foxp3 expression in a small fraction of the adoptively transferred DO11.10/Rag2-/- T cells after 4 days. In

contrast to antigen delivery to CD8α+ DCs *via* DEC205, antibody mediated antigen delivery to pDCs *via* Siglec-H or BST2 failed to induce significant *de novo* expression of Foxp3 and the differentiation of Tregs. Neither titration of the antigen nor stimulation with TLR ligand CpG 1668 had any influence on that result (Fig. 20).

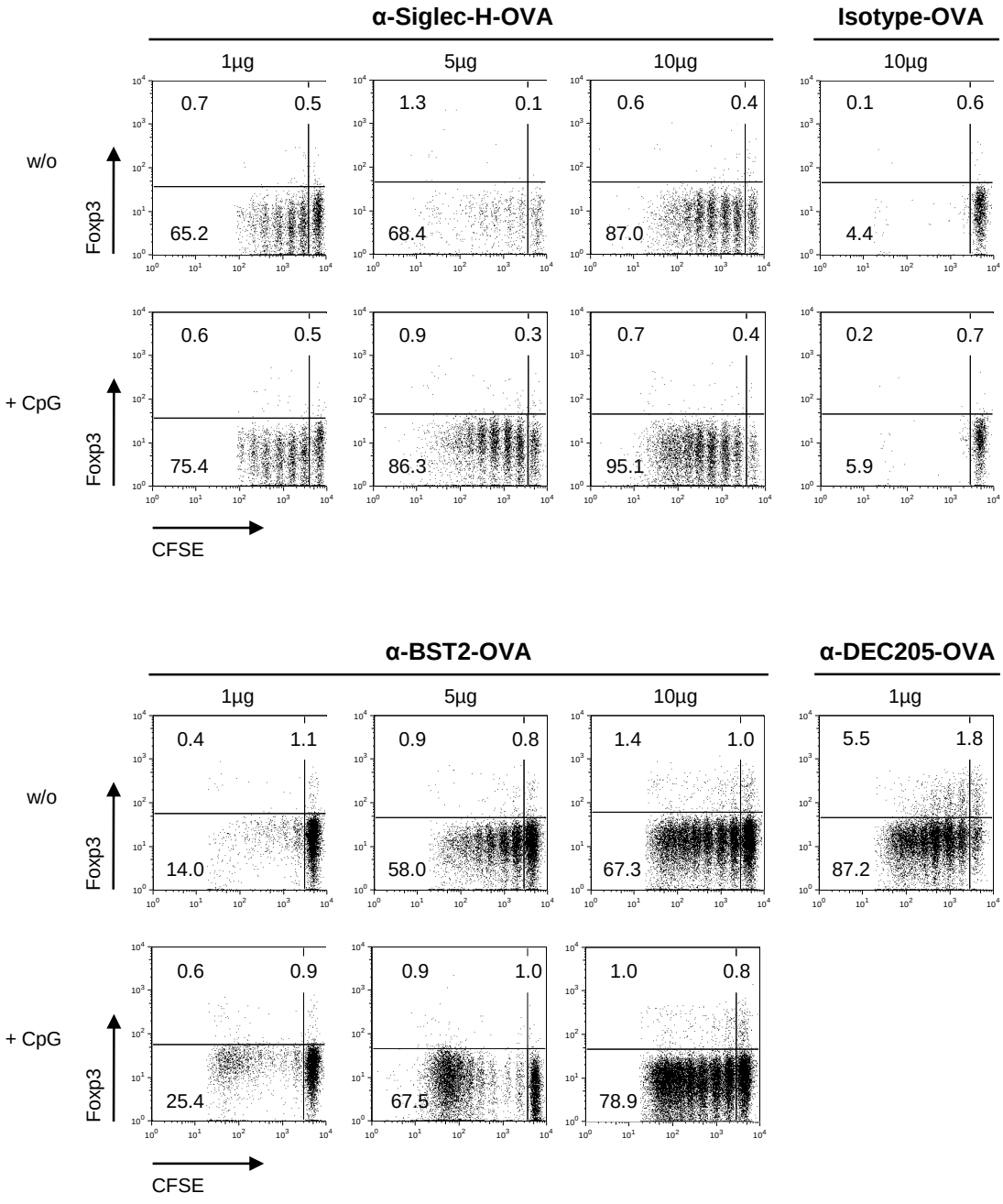

Fig. 20: Foxp3 expression and proliferation of adoptively transferred OVA specific CD4+ T cells after antigen targeting to pDCs.

CFSE dilution and Foxp3 expression were measured in DO11.10-TCR+ T cells 4 days after different doses of OVA had been delivered to pDCs with α-Siglec-H (upper rows) or α-BST2 (lower rows) or to CD8α+ DCs with α-DEC205, respectively. An isotype-OVA fusion protein was used as control. Where indicated, 10 µg CpG 1668 were used to activate dendritic cells.

After immunization with α-DEC205-OVA, more than 20% of the undivided transferred T cells expressed Foxp3 (Fig. 21). Foxp3 expression decreased progressively with the number of cell divisions. In mice in which antigen presentation was restricted to pDCs, below 5% of the undivided DO11.10-TCR+ T cells expressed Foxp3, confirming that Foxp3 induction was inefficient (Fig. 21).

These results revealed that antigen presenting pDC are not able to induce Foxp3 expression in naïve T cells and fail to promote the differentiation of Foxp3+ Tregs. However, it can be concluded that delivery of antigen to pDCs with antibodies specific for Siglec-H or BST2 leads to the priming and proliferation of naïve antigen specific CD4+ T cells, demonstrating the ability of pDCs to provide a sufficient TCR stimulus to activate naïve T cells to enter the cell cycle.

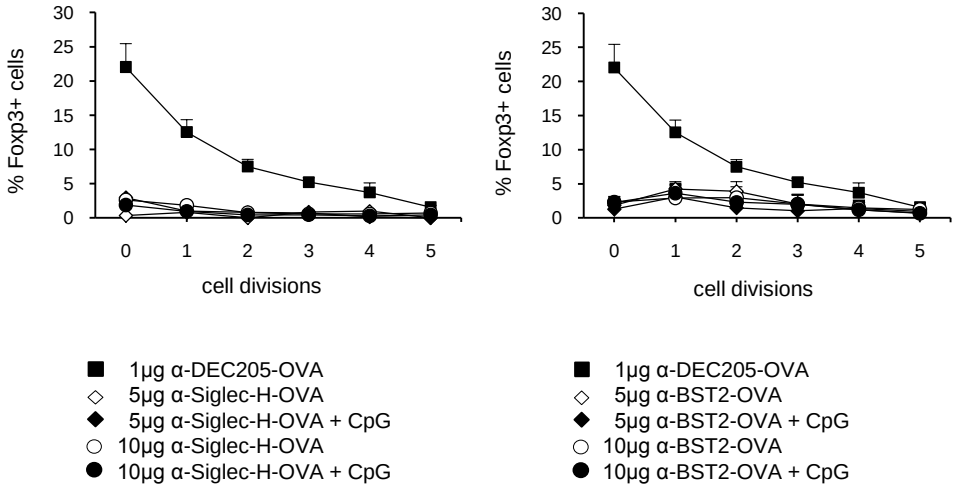

Fig. 21: Correlation of Foxp3 expression and cell division.
As in Fig. 20. Displayed is the percentage of Foxp3 expressing cells for each cell division (mean ± SD, *n*=3).

To confirm these results and to exclude that the unique method of antigen delivery using pDC specific antibodies has any influence on the outcome of T cell responses and the differentiation of Foxp3+ Tregs, an alternative method of pDC specific antigen presentation was used. PDCs were sorted to high purity from splenocytes and pulsed *in vitro* with either a high or low dose of OVA peptide (pOVA, aa 323-339). pOVA pulsed pDCs were then transferred into wt BALB/c mice that had received CFSE-labeled DO11.10/Rag2-/- T cells the day before.

In accordance with the results obtained after introducing antigens to pDCs *via* the cell surface receptors Siglec-H and BST2, pDCs loaded with pOVA also induced proliferation of the transferred T cells (Fig. 22). This response was dependent on the amount of peptide that was bound to MHC II on pDCs. However, also in this experimental setup, antigen presenting pDCs failed to induce *de novo* expression of Foxp3, irrespective of whether high or low amounts of antigen were loaded on pDCs (Fig. 22 A).

To prove that the observed T cell responses were not influenced by transfer of the antigen from pDCs to any other APCs, MHC II deficient mice were used as recipients. After transfer of wt pDCs into MHC II deficient recipient mice, the transferred pDCs are the only cell population that is able to present antigen on MHC II molecules and all observed T cell responses are therefore mediated by pDCs. PDCs were isolated from wt C57BL/6 mice and pulsed *in vitro* with pOVA. These pOVA pulsed pDCs were then transferred into MHC II deficient recipients that had received OVA specific CD4+ OT-II/Rag2-/- T cells the day before. Also in this experimental setting that excludes the influence of any other APC population on the differentiation of Tregs, pDCs failed to induce Foxp3 expression in naïve antigen specific T cells (Fig. 22 B).

It can therefore be excluded that the T cell responses elicited by pDCs (Fig. 20 and Fig. 22 A) were due to transfer of antigen from antigen pulsed pDCs to recipient cDCs. These results clearly show that antigen presentation by pDCs *in vivo* does not lead to relevant *de novo* induction of Foxp3+ Tregs, irrespective of the method used for antigen loading.

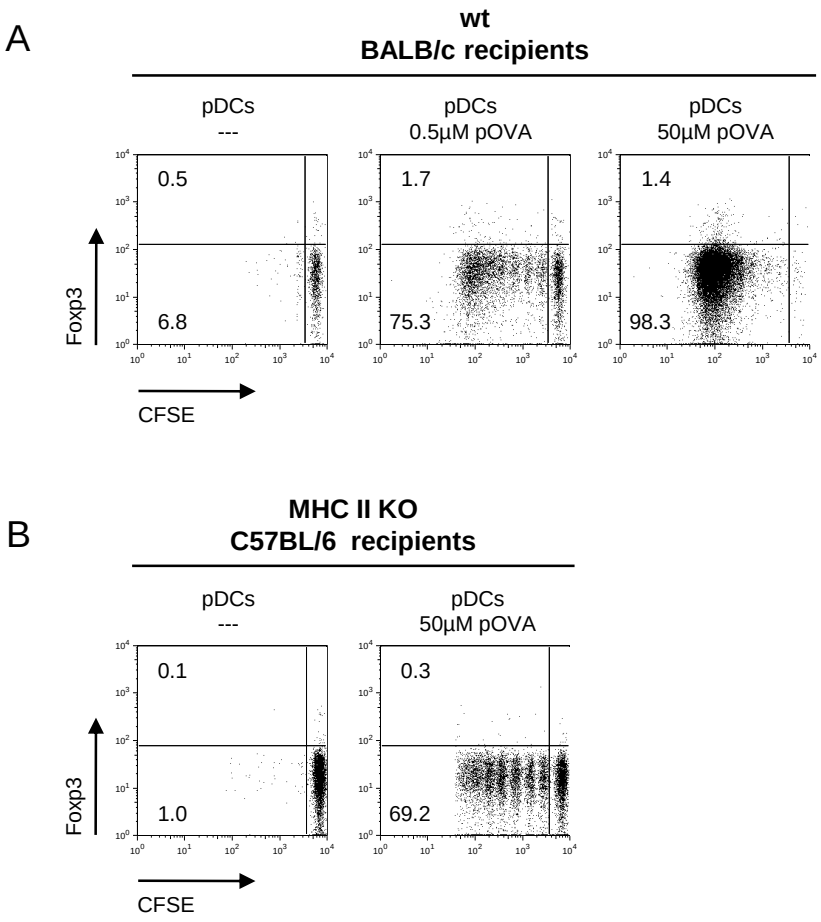

Fig. 22: T cell proliferation and Foxp3 expression in adoptively transferred OVA specific CD4+ T cells after transfer of pDCs loaded with pOVA.

(A) PDCs were isolated from BALB/c splenocytes and were incubated with either 0.5 µM or 50 µM pOVA (aa 323-339) for 3 hours at 37 °C, washed and transferred (i.v.) into BALB/c mice that had received CFSE-labeled OVA specific DO11.10/Rag2-/- T cells 24 h earlier. Unpulsed pDCs were used as negative control. **(B)** As in (A) but pDCs were isolated from wt C57BL/6 mice and loaded with 50 µM pOVA and were transferred (i.v.) into MHC II deficient C57BL/6 mice that had received CFSE-labeled OVA specific T cells from OTII/Rag2-/- mice. Proliferation and expression of Foxp3 in transferred OVA specific T cells were analyzed 4 days after pDC transfer in pooled splenocytes and lymph node cells. Numbers in quadrants indicate the percentages of proliferating and Foxp3 expressing T cells.

4.6 Opposing T cell responses after co-administration of soluble OVA with α-Siglec-H-OVA and α-BST2-OVA, respectively

Although antigen specific Foxp3+ Tregs play a pivotal role in the maintenance and establishment of peripheral tolerance they are not the only cell population and their action is not the only mechanism that is able to attenuate or inhibit adaptive immune responses. Other mechanisms of tolerance such as T cell anergy or deletion may also contribute to the reported tolerogenic activity of pDCs.

To investigate whether antigen presenting pDCs are able to influence or impair T cell responses that are triggered by other APCs, α-Siglec-H-OVA and α-BST2-OVA were administered simultaneously with soluble OVA. As cDCs are much more efficient in absorbing soluble antigen and presenting antigen on MHC II compared to pDCs (Young *et al*., 2008), systemically administered OVA is predominantly presented on cDCs. Mice that had received CFSE-labeled DO11.10/Rag2-/- T cells 1 day before, were immunized with soluble OVA in the absence or presence of CpG 1668 as stimulus and simultaneously received α-Siglec-H-OVA or α-BST2-OVA.

Administration of soluble OVA in combination with α-Siglec-H-OVA resulted in slower proliferation of the transferred T cells leading to a considerably reduced expansion of OVA specific T cells after 4 days compared to control mice which had received soluble OVA only (Fig. 23 A). While the majority of T cells in mice that were immunized with soluble OVA alone underwent at least 6-8 cell divisions, most T cells in mice that were treated with α-Siglec-H-OVA and soluble OVA divided only 2-4 times (Fig. 23 B). Reduced T cell proliferation in mice treated with α-Siglec-H-OVA correlated with lower percentages of IL-2 producing T cells (Fig. 23 A). Treatment with α-Siglec-H-OVA reduced T cell expansion in response to soluble OVA even in the presence of TLR9 ligand CpG 1668 (Fig. 23 C). In contrast to α-Siglec-H-OVA, α-BST2-OVA did not inhibit, but rather enhanced T cell expansion in response to soluble OVA and higher levels of DO.11.10-TCR+ CD4+ T cells were found in mice that were immunized with OVA and α-BST2-OVA compared to mice that were immunized with soluble OVA alone (Fig. 23 A). The attenuated T cell response after administration of soluble OVA and α-Siglec-H-OVA and the enhanced T cell expansion after administration of soluble antigen in combination with α-BST2-OVA resulted in a significantly different T cell response in the absence as well as in the presence of TLR9 ligand CpG 1668 (Fig. 23 D, E). Thus, depending on the receptor that was used for antigen delivery, pDCs are able to inhibit as well as support T cell responses to soluble antigen.

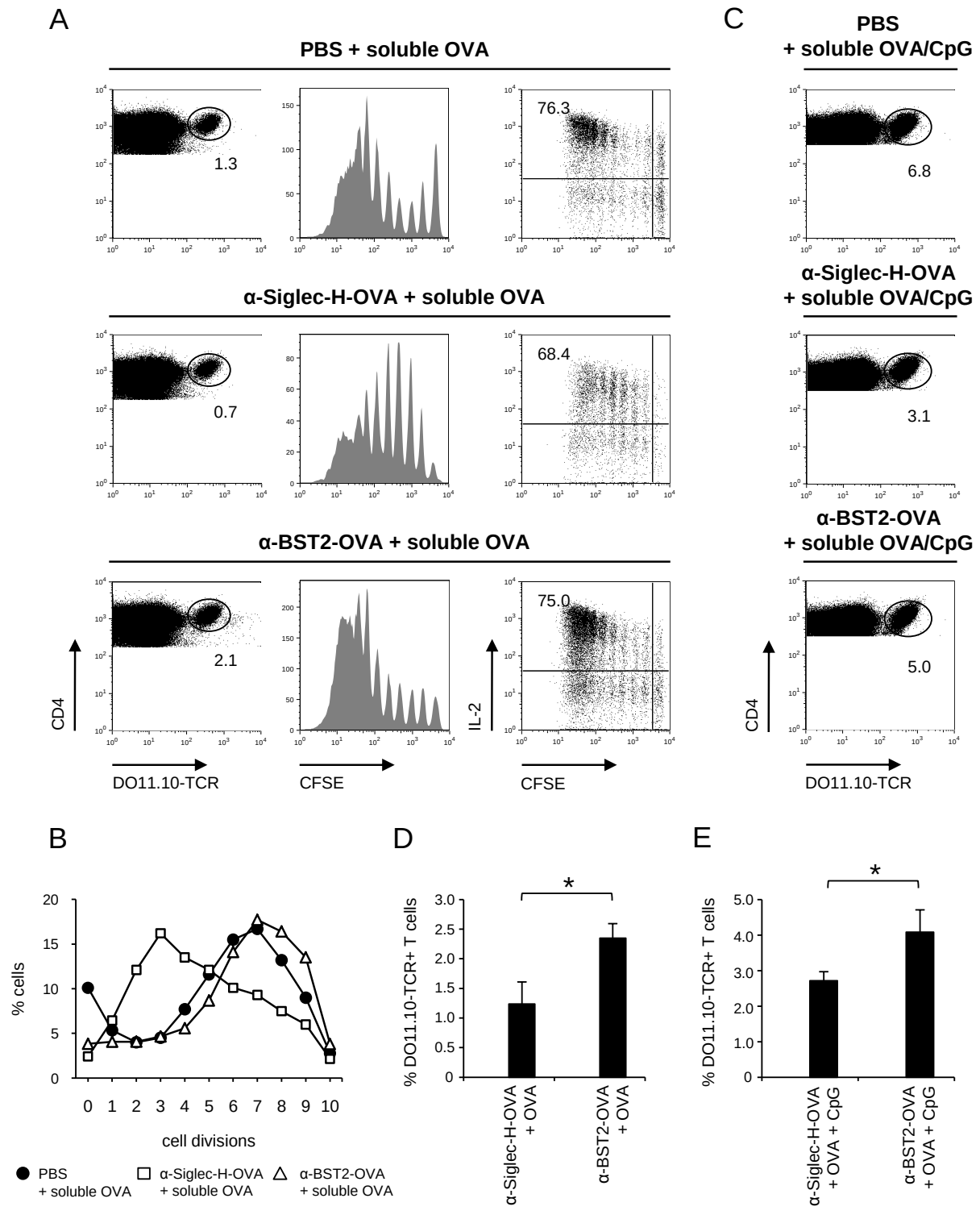

Fig. 23: CD4+ T cell response after co-administration of soluble OVA and pDC specific antibody-OVA fusion proteins.

BALB/c mice received CFSE-labeled DO11.10/Rag2-/- T cells and were immunized (i.p.) 24 h later with 100 µg soluble OVA (A, B, D) or soluble OVA and 10 µg CpG 1668 (C, E) and simultaneously received PBS or 10 µg α-Siglec-H-OVA or α-BST2-OVA. After 4 days proliferation and the percentage of DO11.10-TCR+ T cells of all CD4+ T cells was measured. **(A)** Displayed are the percentages of OVA specific T cells, the proliferation and IL-2 production after *ex vivo* restimulation with 5 µg/ml pOVA (aa 323-339). **(B)** Diagram displays the percentage of transferred T cells that divided 1-10 times. **(C)** Displayed are the percentages of OVA specific T cells. **(D)** and **(E)** Bar graphs depict the percentage of OVA specific T cells of all CD4+ T cells after immunization with α-Siglec-H-OVA/OVA ± CpG or α-BST2-OVA/OVA ± CpG. (mean ± SD, *n*=3, * p<0.05 Student's *t*-test).

To exclude that binding of α-Siglec-H or α-BST2 to pDCs influences the T cell response in an antigen unspecific manner, antibodies fused to an irrelevant antigen (pHEL) that is not recognized by OVA specific DO11.10-TCR+ T cells were used as controls. However, T cell proliferation in response to soluble OVA and soluble OVA/CpG was not affected by administration of α-Siglec-H-pHEL or α-BST2-pHEL (Fig. 24). This clearly demonstrates that the observed attenuated T cell response to soluble OVA after administration of α-Siglec-H-OVA and the enhanced T cell responses to soluble OVA after injection of α-BST2-OVA are antigen specific effects.

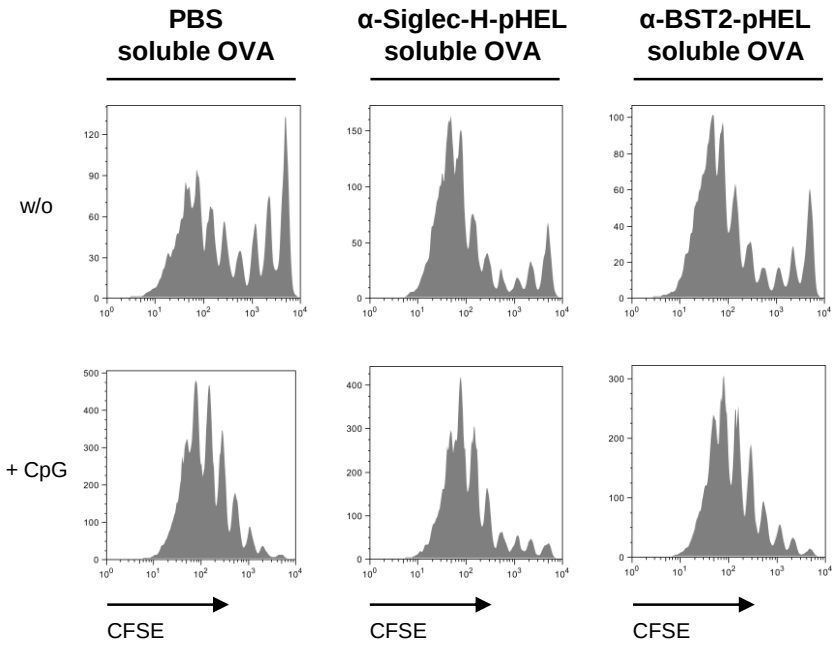

Fig. 24: CD4+ T cell response after co-administration of soluble OVA and pDC specific antibody-pHEL fusion proteins.

BALB/c mice which had received CFSE-labeled DO11.10/Rag2-/- T cells 24 h earlier were immunized (i.p.) with 100 µg soluble OVA (upper row) or 100 µg soluble OVA and 10 µg CpG 1668 (lower row) and simultaneously received PBS or 10 µg α-Siglec-H-pHEL or α-BST2-pHEL (i.p.). After 4 days, proliferation was determined by CFSE dilution.

4.7 Differences between antibody mediated antigen delivery to pDCs *via* Siglec-H and BST2

Antigen delivery to pDCs *via* Siglec-H and BST2 elicited different effects on adaptive T cell responses. α-Siglec-H mediated antigen delivery resulted in antigen specific attenuation of T cell responses, whereas antigen fused to α-BST2 enhanced T cell responses to soluble antigen. This was unexpected and surprising, as both antibodies specifically deliver antigen to pDCs. Fig. 25 illustrates the different and

opposing influence of α-Siglec-H and α-BST2 mediated antigen delivery on antigen specific T cell responses.

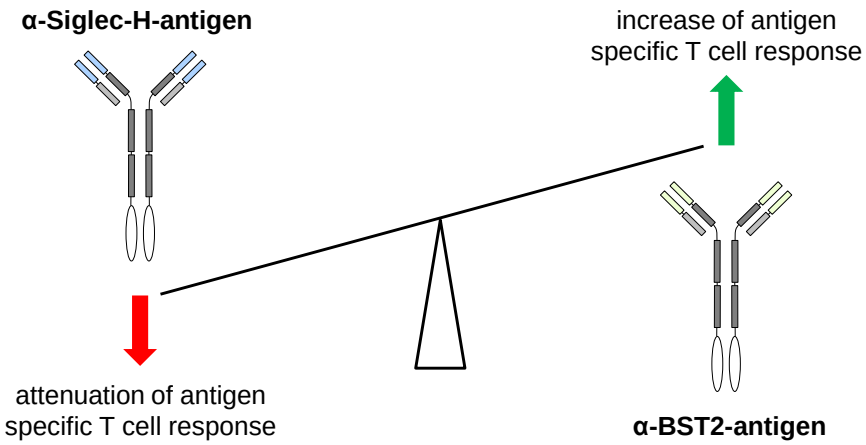

Fig. 25: Antigen delivery to pDCs *via* Siglec-H and BST2 influences an antigen specific T cell response in contrary ways.
Antigen delivery to pDCs with α-Siglec-H is able to attenuate a T cell response to soluble antigen whereas α-BST2 mediated delivery of antigen further increases this T cell response.

Because of the different and opposing results that were obtained after delivery of antigen to pDCs with α-Siglec-H and α-BST2, it was examined whether binding of α-Siglec-H or α-BST2, which may crosslink the receptors and may induce signaling, influences the phenotype of pDCs in a way that could explain this disparities.

In vitro generated pDCs were incubated with α-Siglec-H-OVA or α-BST2-OVA either in the absence or presence of TLR9 ligand CpG 1668. After 24 h, expression levels of co-stimulatory molecules (CD80, CD86), activation markers (CD40, CD69) and members of the B7-H family (B7-H1/PDL-1, B7-H2/ICOS-L, B7-H3, B7-H4) were analyzed on the surface of pDCs by flow cytometry. As depicted in Fig. 26 A, binding of α-Siglec-H-OVA or α-BST2-OVA to pDCs did not affect or change the phenotype of naïve pDCs. Stimulation with CpG 1668 induced the maturation of pDCs and promoted the expression of co-stimulatory molecules and activation markers (Fig. 26 B). Ligation of the recombinant antibodies to their receptors did not influence this maturation process and similar levels of activation markers and co-stimulatory molecules were observed on cells that were incubated with CpG 1668 alone and cells that were incubated with α-Siglec-H-OVA/CpG and α-BST2-OVA/CpG, respectively (Fig. 26 B).

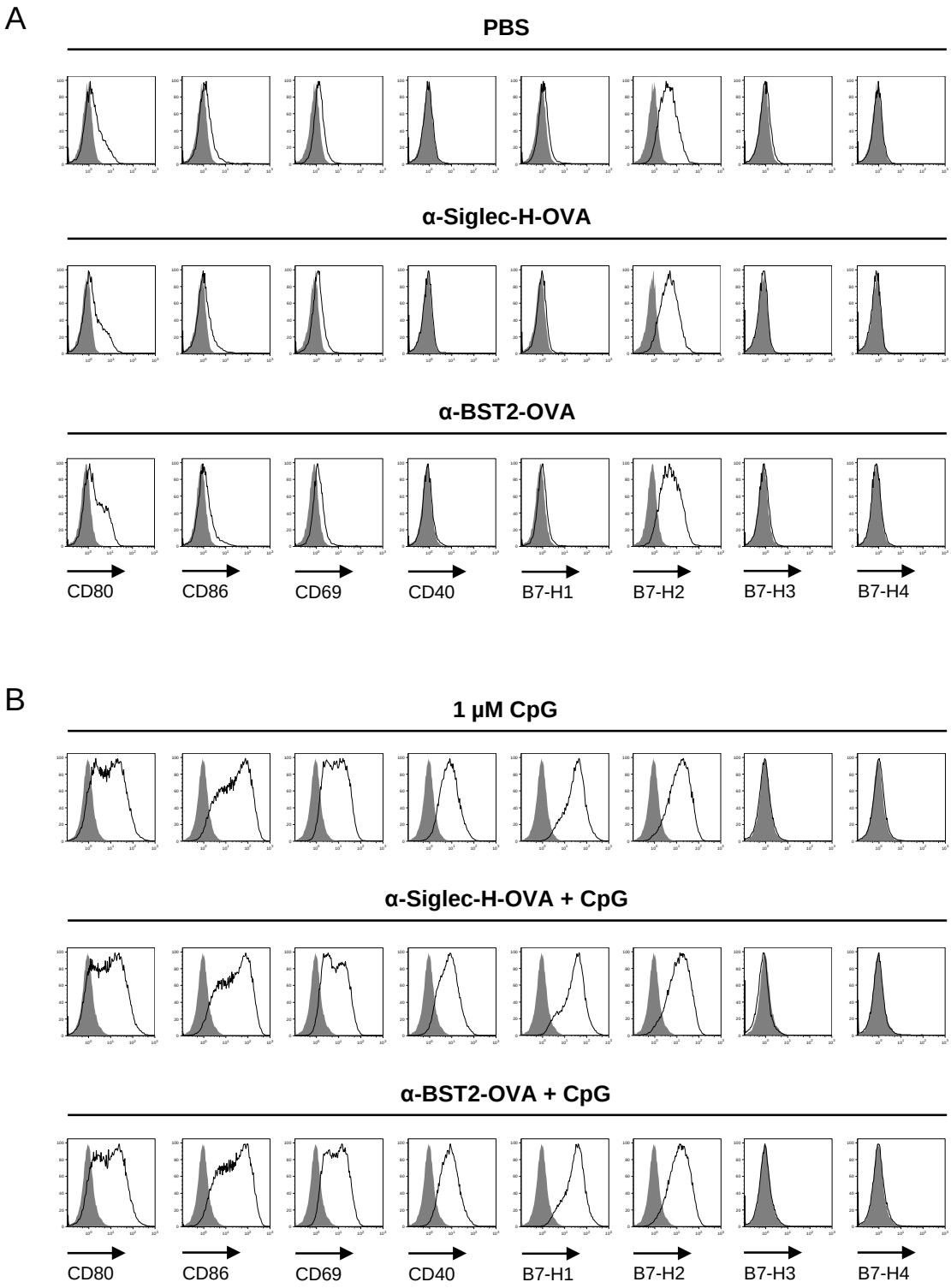

Fig. 26: Expression of inducible cell surface molecules on pDCs *in vitro*.
$1*10^6$ cells/ml were incubated with 1 µg/ml α-Siglec-H-OVA or α-BST2-OVA either in the absence **(A)** or presence **(B)** of 1 µM CpG 1668. After 24 h, expression of cell surface markers was analyzed by flow cytometry. (filled grey histograms: without staining, open histograms: antibody staining)

In addition to the expression of cell surface molecules, also the capacity to produce cytokines in response to stimulation with TLR ligands was analyzed. *In vitro* generated pDCs were incubated with different doses of α-Siglec-H-OVA or α-BST2-

OVA in the absence or presence of TLR9 ligands CpG 1668 and CpG 2216. CpG 2216 was used in this experimental setup, because stimulation with CpG 2216 results in the production of higher levels of IFN I compared to stimulation with CpG 1668. Fig. 27 displays that binding of α-Siglec-H-OVA or α-BST2-OVA did not interfere with cytokine production upon stimulation with TLR9 ligands. Similar levels of IL-6, IL-12 and IFN-α were detected in the supernatant of stimulated cells, irrespective of whether α-Siglec-H-OVA or α-BST2-OVA bound to the cells.

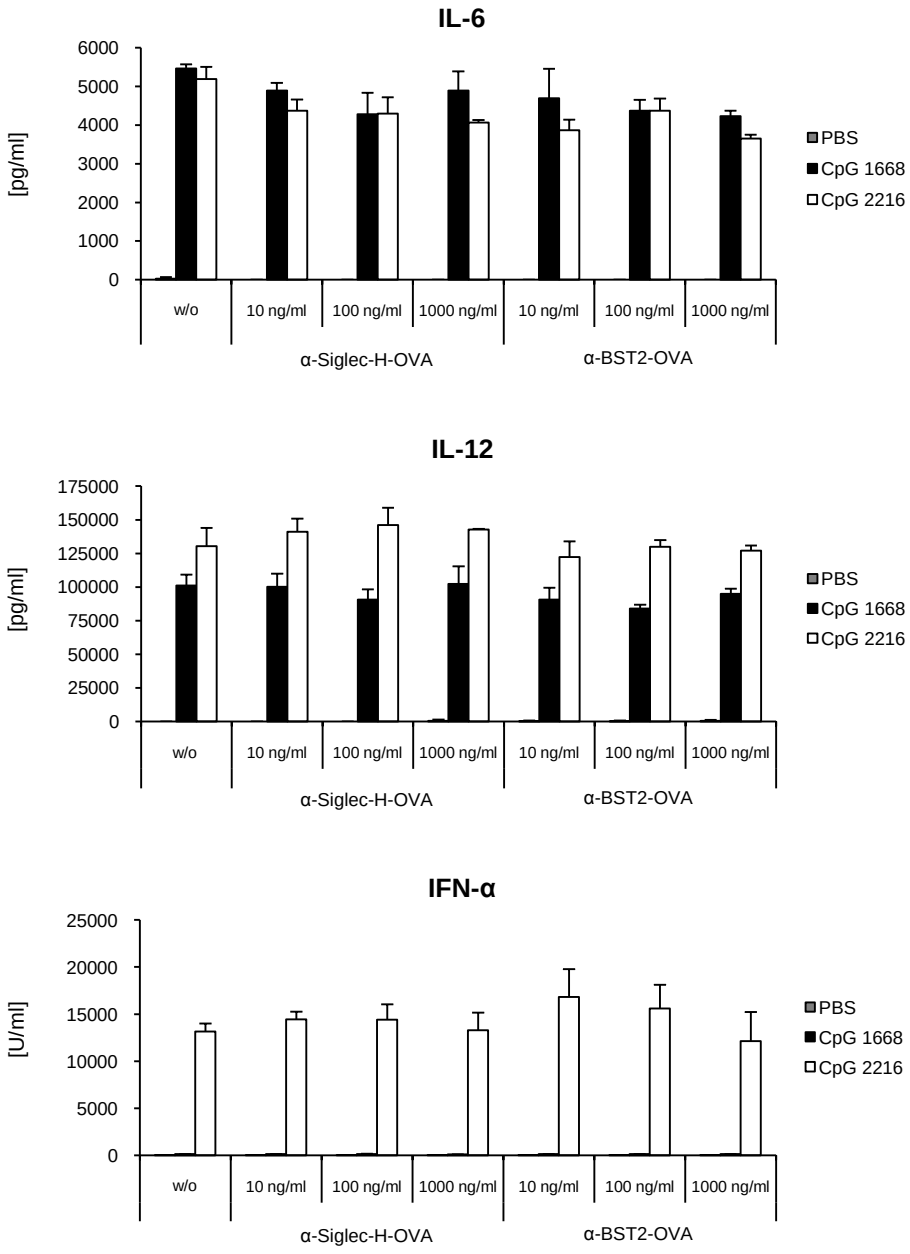

Fig. 27: Cytokine production of pDCs *in vitro*.

$1*10^6$ cells/ml were incubated with the indicated concentrations of α-Siglec-H-OVA or α-BST2-OVA either in the absence or presence of 1 µM CpG 1668 or 1 µM CpG 2216. After 24 h cytokine levels were analyzed in the supernatant. (mean ± SD, *n*=3)

In addition to the *in vitro* incubation of pDCs and recombinant pDC specific antibodies, a potential effect of administration of α-Siglec-H-OVA and α-BST2-OVA was also analyzed *in vivo*. Similar to the findings obtained *in vitro*, constitutive expression of CD80 and CD86 was not affected by ligation of α-Siglec-H and α-BST2. Expression levels of MHC II or the activation marker CD69 were not affected either. CCR9 and B7-H2/ICOS-L have both been linked to tolerance induction mediated by pDCs (Hadeiba *et al*., 2008; Ito *et al*., 2007). However, expression of both surface molecules was not influenced by binding of antibodies to Siglec-H or BST2 (Fig. 28). Thus, changes in the activation status of pDCs after binding of antibodies to Siglec-H and BST2 do not explain the differences in pDC mediated T cell responses after delivery of antigen with α-Siglec-H and α-BST2.

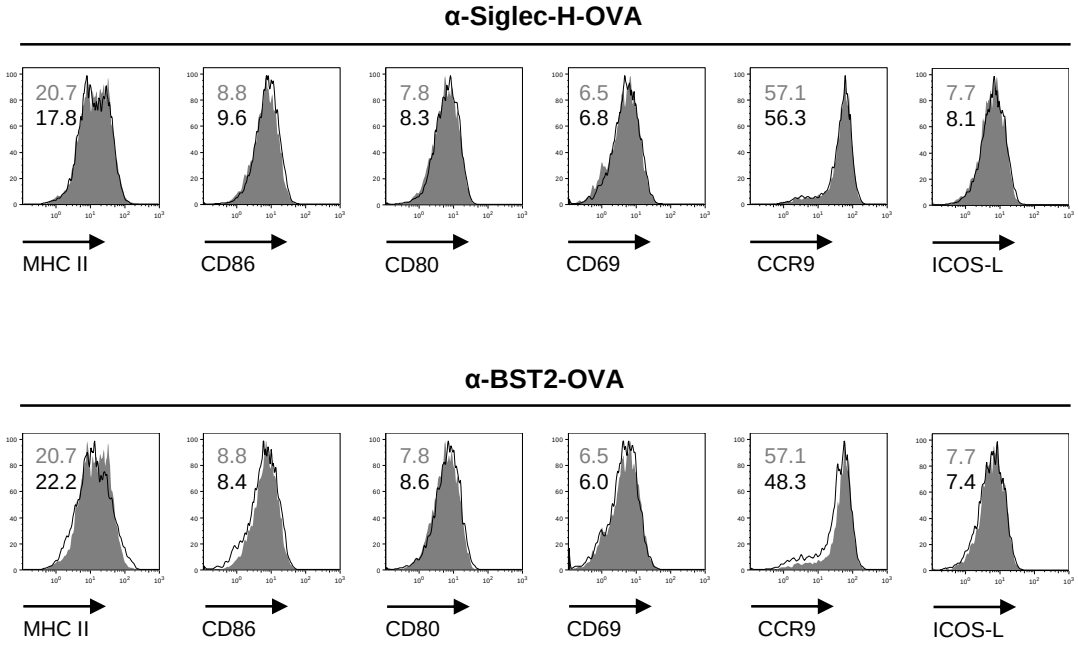

Fig. 28: Activation state of pDCs after binding of α-Siglec-H and α-BST2 *in vivo*.
10 µg α-Siglec-H-OVA or α-BST2-OVA were injected (i.p.) into C57BL/6 mice. Expression of MHC II, CD86, CD80, CD69, CCR9 and B7-H2/ICOS-L was analyzed 24 h after injection of antibody-OVA fusion proteins (open histograms) or application of PBS (filled grey histograms) on pDCs in the spleen. Numbers in histograms indicate the mean fluorescence intensity (grey: PBS, black: α-Siglec-H-OVA or α-BST2-OVA).

As no evidence for a change in the activation status of pDCs or the capacity to produce cytokines was found after binding of α-Siglec-H and α-BST2, it was assumed that differences in the presentation of the antigen that is fused to these antibodies may account for the converse immune response. It had already been

shown in Fig. 17 that higher levels of pHEL/MHC II complexes were detectable on the surface of pDCs after the antigen had been delivered *via* BST2 compared to Siglec-H. Analysis of pHEL/MHC II complexes on the surface of pDCs at different time points after injection of α-Siglec-H-pHEL or α-BST2-pHEL revealed that antigen that is introduced to pDCs with α-Siglec-H is stably presented on MHC II for 6-8 days after only one injection of α-Siglec-H-pHEL. In contrast, antigen that was delivered to pDCs with α-BST2 was presented at much higher levels, but antigen presentation was not very stable and peptide/MHC II complexes disappeared about 48 h after administration of α-BST2-pHEL (Fig. 29). Although α-Siglec-H and α-BST2 both deliver antigen specifically to pDCs the density of peptide/MHC II complexes on the cell surface of pDCs and the duration of antigen presentation is substantially different. This difference in MHC II restricted antigen presentation may account for the contrary adaptive immune responses that were elicited by α-Siglec-H and α-BST2 mediated antigen delivery.

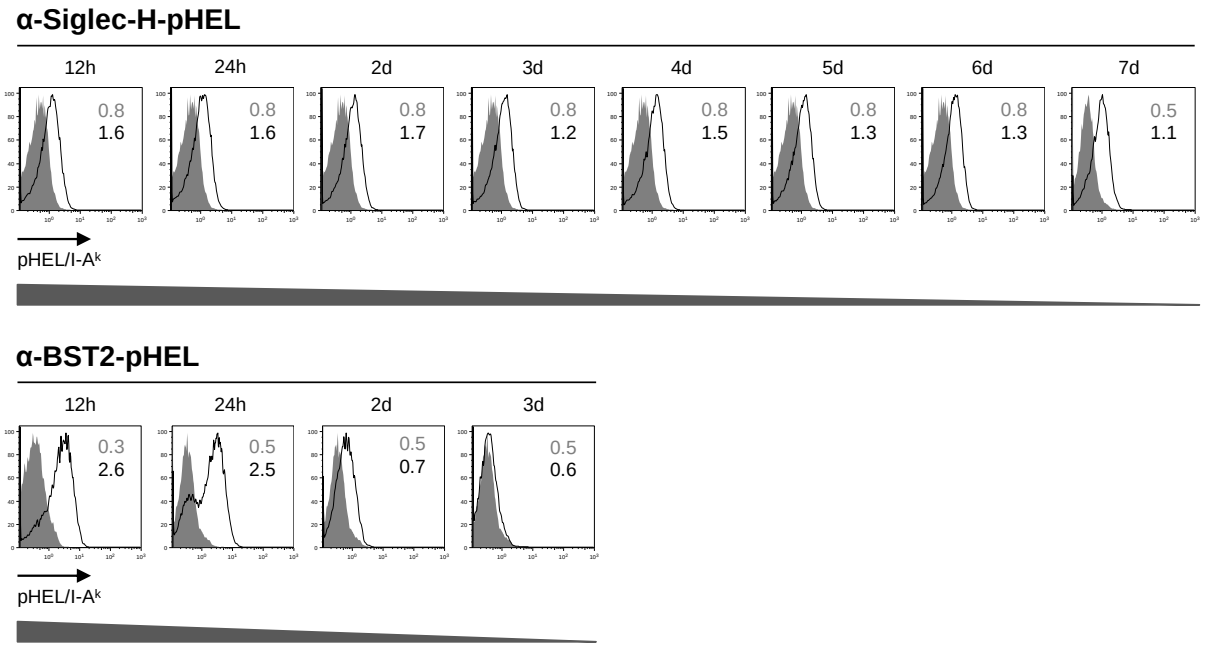

Fig. 29: Differences in antigen presentation after antigen delivery *via* Siglec-H and BST2.
20 µg α-Siglec-H-pHEL or α-BST2-pHEL were injected (i.p.) into C3H mice. Presence of pHEL/I-A^k complexes on the surface of splenic pDCs was measured at different time points after injection. Filled grey histograms: PBS, open histograms: α-Siglec-H-pHEL (upper row) or α-BST2-pHEL (lower row). Numbers in histograms indicate the mean fluorescence intensity (grey: PBS, black: α-Siglec-H-pHEL or α-BST2-pHEL).

4.8 PDC mediated attenuation of immune responses

As co-administration of soluble OVA and α-BST2-OVA enhanced rather than attenuated T cell expansion, subsequent experiments with the purpose to induce antigen specific tolerance were only performed with the α-Siglec-H antibody. Further studies investigating whether antigen that is fused to α-BST2 can induce protective T cell responses and if α-BST2-antigen fusion proteins can be used as vaccine are described from chapter 4.9 on.

4.8.1 Antigen delivery to pDCs *via* Siglec-H inhibits antibody responses to soluble OVA and PolyI:C

To test whether antigen delivery to pDCs *via* Siglec-H is able to inhibit endogenous immune responses, OVA specific antibody titers were determined after mice were immunized with a combination of α-Siglec-H-OVA, soluble OVA and PolyI:C or soluble OVA/PolyI:C alone. PolyI:C was chosen because it has been used as adjuvant in many previously published studies to induce strong adaptive immune responses including high titers of antigen specific antibodies (Boscardin *et al*., 2006).

In the serum of mice that were immunized with OVA/PolyI:C high levels of OVA specific antibodies could be detected 14 days after immunization, indicating that a strong adaptive immune response was initiated. Especially the presence of isotype switched IgG2b and IgG2c antibodies suggested the involvement of T_H1 cells (Fig. 30). In contrast to that, co-administration of α-Siglec-H-OVA almost completely abrogated the antibody response triggered by soluble OVA/PolyI:C. Only very low levels of OVA specific antibodies were detected. Thus, antigen delivery to pDCs *via* Siglec-H is able to inhibit antigen specific antibody responses.

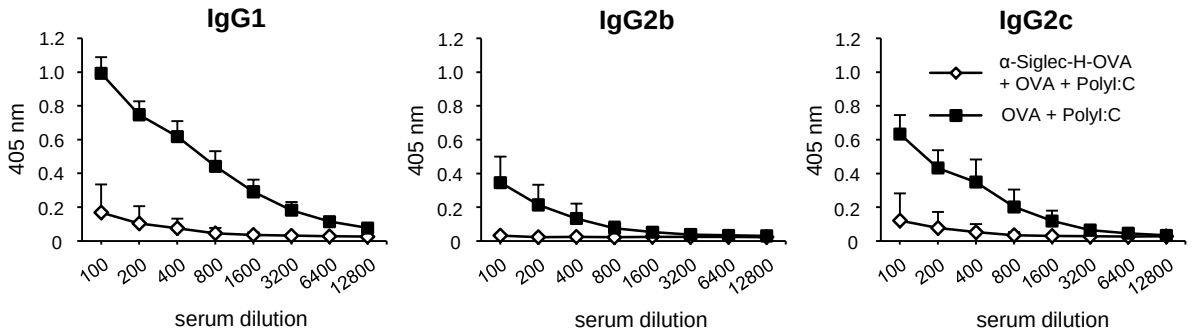

Fig. 30: OVA specific antibody titers after immunization with soluble OVA/PolyI:C and α-Siglec-H-OVA.

C57BL/6 mice were immunized with 100 µg soluble OVA and 50 µg PolyI:C (i.p.). Where indicated mice simultaneously received 10 µg α-Siglec-H-OVA (i.p.). Antibody titers were determined in the serum of immunized mice 14 days after the immunization. Diagrams depict absorbance at 405 nm of serial serum dilutions. (mean ± SD, *n*=6 for α-Siglec-H-OVA/OVA/PolyI:C, *n*=7 for OVA/PolyI:C)

4.8.2 Antigen delivery to pDCs *via* Siglec-H inhibits priming of OVA specific effector T cells

The absence of high titers of OVA specific antibodies after co-administration of soluble OVA and α-Siglec-H-OVA even in the presence of the strong adjuvant PolyI:C indicated that antigen delivery to pDCs *via* Siglec-H can suppress CD4+ T cell help that is required for an effective antibody response (Boscardin *et al*., 2006). To investigate whether antigen delivery to pDCs *via* Siglec-H is indeed capable of attenuating endogenous T cell responses, naïve mice were treated with α-Siglec-H-OVA 1 day before they were immunized with pOVA (aa 323-339) in complete Freund's adjuvant (CFA). In addition, pertussis toxin (PTx) was administered systemically to promote T_H1/T_H17 responses (Hofstetter *et al*., 2007; Hofstetter *et al*., 2003). Mice were pretreated with α-Siglec-H-OVA and were not treated simultaneously on the same day they were immunized with pOVA/CFA/PTx to assure that antigen was already presented by pDCs at the time mice were immunized. T cell responses in the spleen were analyzed 10 days after immunization with pOVA/CFA/PTx by *ex vivo* restimulation with pOVA. Expansion of antigen specific T cells was measured by ^{3}H-thymidine incorporation and acquisition of effector functions was determined by intracellular cytokine staining.

In mice that did not receive any pretreatment, immunization with pOVA/CFA/PTx resulted in a strong proliferative response of splenocytes to peptide restimulation reflecting a high frequency of OVA specific CD4+ T cells in the spleen (Fig. 31 A). Furthermore, CD4+ T cells responded to restimulation with pOVA with the production of helper T cell cytokines such as IL-2, IL-17 and IFN-γ. Pretreatment of mice with α-Siglec-H-OVA 1 day prior to immunization with pOVA/CFA/PTx greatly reduced the proliferative response and the frequency of cytokine producing CD4+ T cells after peptide restimulation (Fig. 31 B). A shift to IL-4 producing T_H2 or IL-10 producing Tr1 cells was not observed, since the frequency of IL-4+ or IL-10+ CD4+ T cells was very low in all conditions tested. These results demonstrate that pDC specific antigen presentation after Siglec-H mediated antigen delivery is capable of inhibiting the generation of antigen specific T_H1 and T_H17 cells and that antigen delivery to pDCs *via* Siglec-H can be used to inhibit antigen specific effector T cell responses.

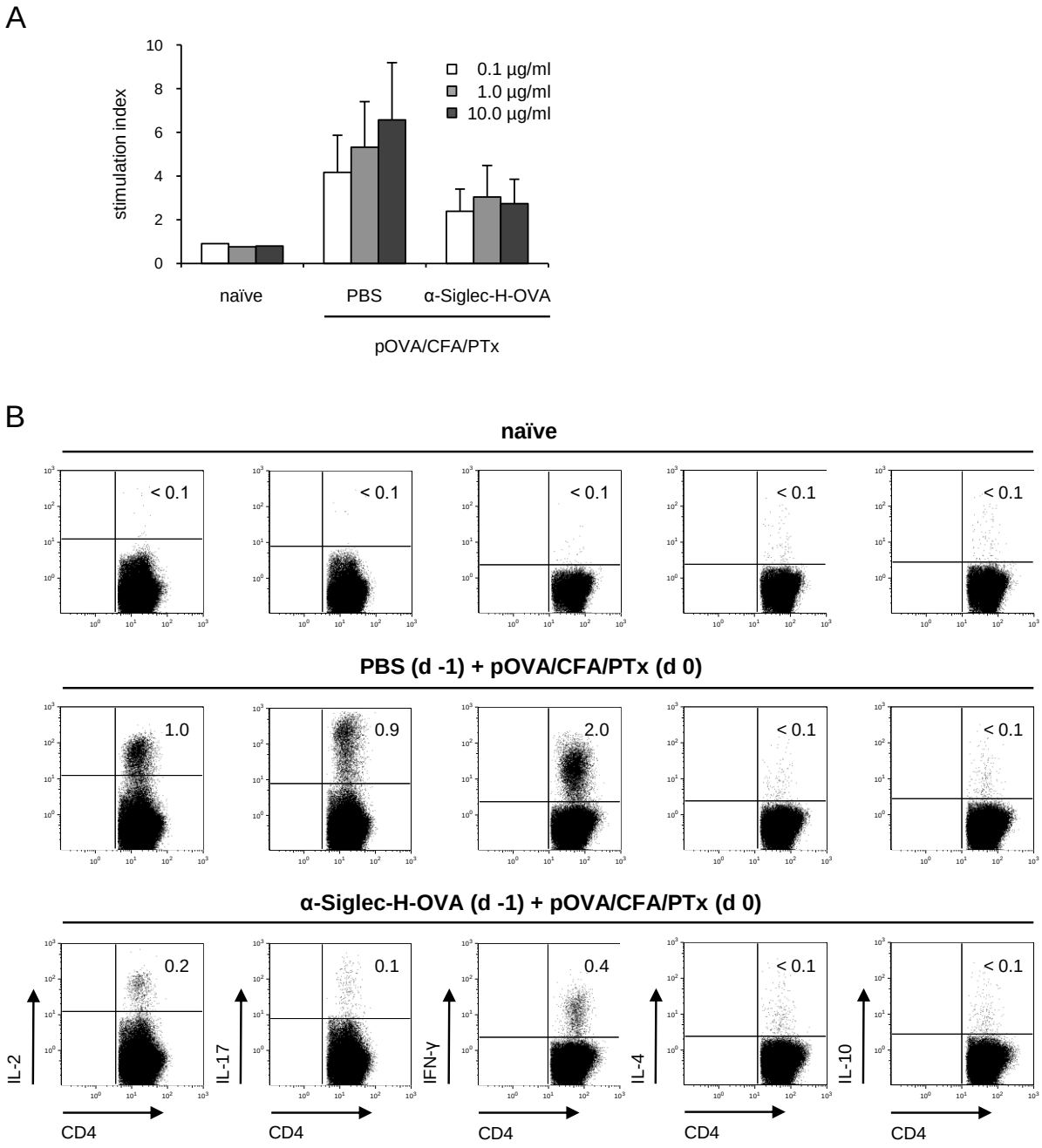

Fig. 31: Effect of α-Siglec-H-OVA on priming of OVA specific CD4+ helper T cells.

Mice received PBS or 10 µg α-Siglec-H-OVA (i.p.) 1 day (d -1) before immunization with 100 µg pOVA (aa 323-339) in CFA (s.c.). In addition to that mice received 200 ng PTx (i.v.) on day 0 and day 2. T cell responses were analyzed 10 days after immunization in the spleen. **(A)** Proliferation of splenocytes in response to restimulation with different doses of pOVA was analyzed by incorporation of ^{3}H-thymidine. Stimulation indices display the increase of cells responding to antigen restimulation with incorporation of ^{3}H-thymidine compared to unstimulated samples. A naïve mouse that was not immunized with pOVA/CFA/PTx served as negative control. (mean ± SD, *n*=2-4). **(B)** Splenocytes were restimulated with 5 µg/ml pOVA and cytokine production was analyzed by intracellular staining for IL-2, IL-4, IL-10, IL-17 and IFN-γ. The percentages of CD4+ T cells producing the respective cytokine are depicted in the quadrants.

4.8.3 Delivery of autoantigen to pDCs *via* Siglec-H inhibits severe experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is an inducible and antigen specific autoimmune disease that resembles multiple sclerosis in humans. Immunization of mice with a CD4+ T cell epitope from myelin oligodendrocyte glycoprotein (pMOG, aa 35-55) in CFA in the presence of PTx results in the development of a pathogenic CD4+ T cell response that causes severe neurological defects. The disease that follows immunization with pMOG/CFA/PTx is predominantly mediated by CD4+ T cells. Therefore, EAE was chosen to test whether delivery of pMOG to pDCs *via* Siglec-H is able to inhibit pathogenic CD4+ T cell responses and can influence the progress of this autoimmune disease. Mice were pretreated with α-Siglec-H-pMOG on day -1. On the next day (day 0), mice were immunized with pMOG/CFA/PTx.

As shown in Fig. 32, immunization of mice with pMOG/CFA/PTx resulted in the development of severe signs of illness (score > 2.5) between day 13 and day 24. Pretreatment of mice with α-Siglec-H-pMOG on day -1 resulted in a significantly less severe course of the disease between day 14 and day 20 (Fig. 32 A). In the experiment displayed in Fig. 32, only 1 mouse out of 6 developed severe signs of illness, whereas the other mice showed only a mild disease (score 1 - 2.5) or did not develop any signs of illness (score < 1) (Fig. 32 B). For a detailed description of the EAE score see chapter 3.2.16.9.

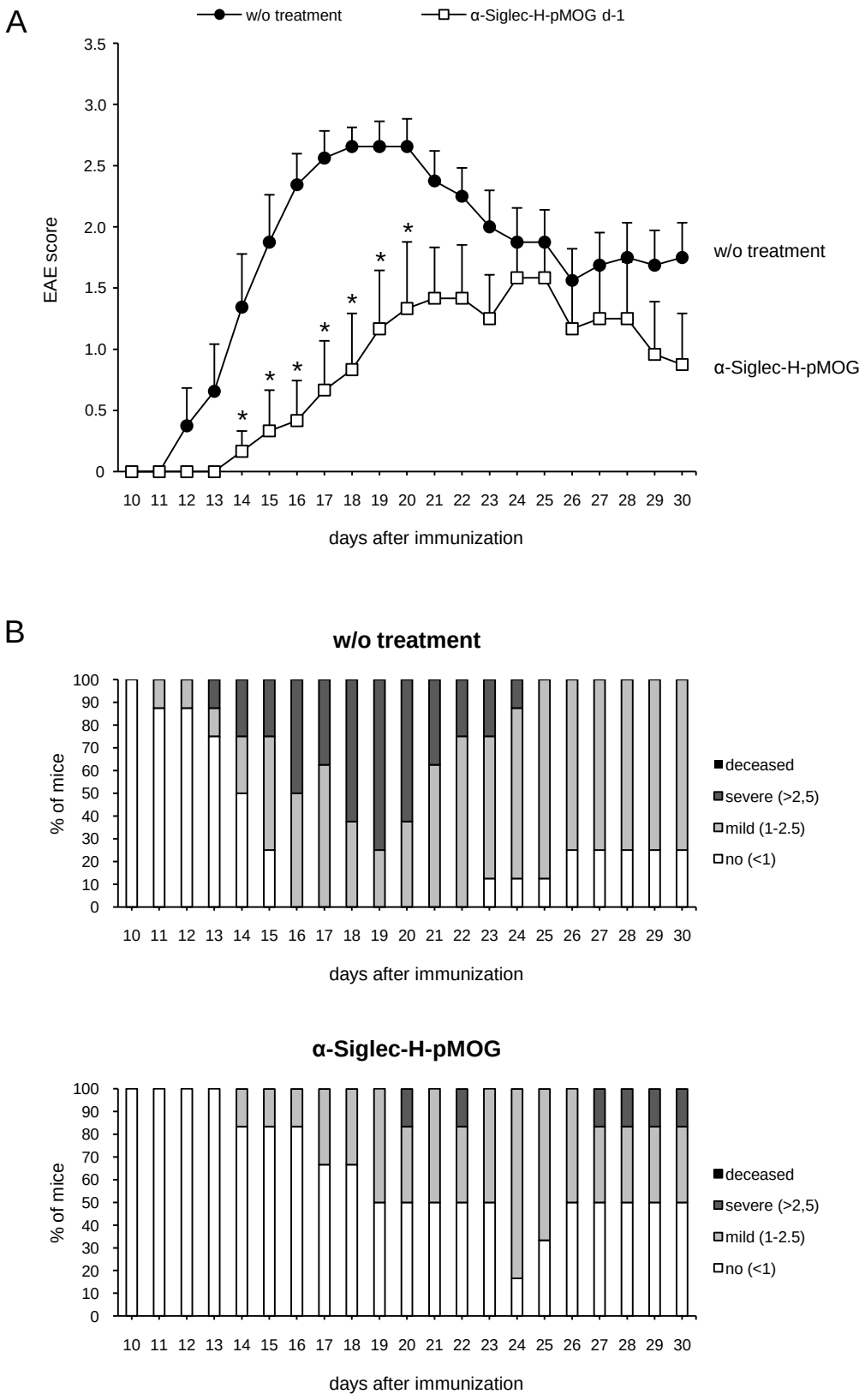

Fig. 32: Administration of α-Siglec-H-pMOG on day -1 and its effect on the progress of pMOG induced EAE.

Mice were pretreated with 10 µg α-Siglec-H-pMOG on day -1 or did not receive any treatment. On the next day, all mice were immunized with 100 µg pMOG in CFA (s.c.) and received 200 ng PTx (i.v.) on day 0 and day 2. Mice were scored daily for signs of illness. **(A)** Mean EAE score (mean ± SEM, *n*=6 for α-Siglec-H-pMOG, *n*=8 for w/o treatment, * p<0.05, Student's *t*-test). **(B)** Percentage of mice that developed no, mild or severe signs of illness or deceased.

Correlating with the alleviated disease, the number of CD3+/CD4+ T cells infiltrating the central nervous system (CNS) was significantly reduced at the peak of the disease (day 17) in mice which had received α-Siglec-H-pMOG 1 day before EAE induction compared to untreated mice (Fig. 33 A). The percentage of CD4+ T cells producing IL-2, IFN-γ or IL-17 was unaltered by pretreatment with α-Siglec-H-pMOG, only the percentage of IFN-γ/IL-17 double producing T cells was slightly reduced (Fig. 33 B right diagram). Accordingly, the absolute numbers of CD4+ T cells producing IFN-γ, IL-17 or IL-2 alone or producing both IFN-γ and IL-17 were significantly reduced in mice treated with α-Siglec-H-pMOG compared to controls (Fig. 33 B left diagram). No significant increase in absolute numbers or the percentage of Foxp3+ Tregs was observed in the CNS of mice that were treated with α-Siglec-H-pMOG compared to mice that did not receive any pretreatment (Fig. 33 C).

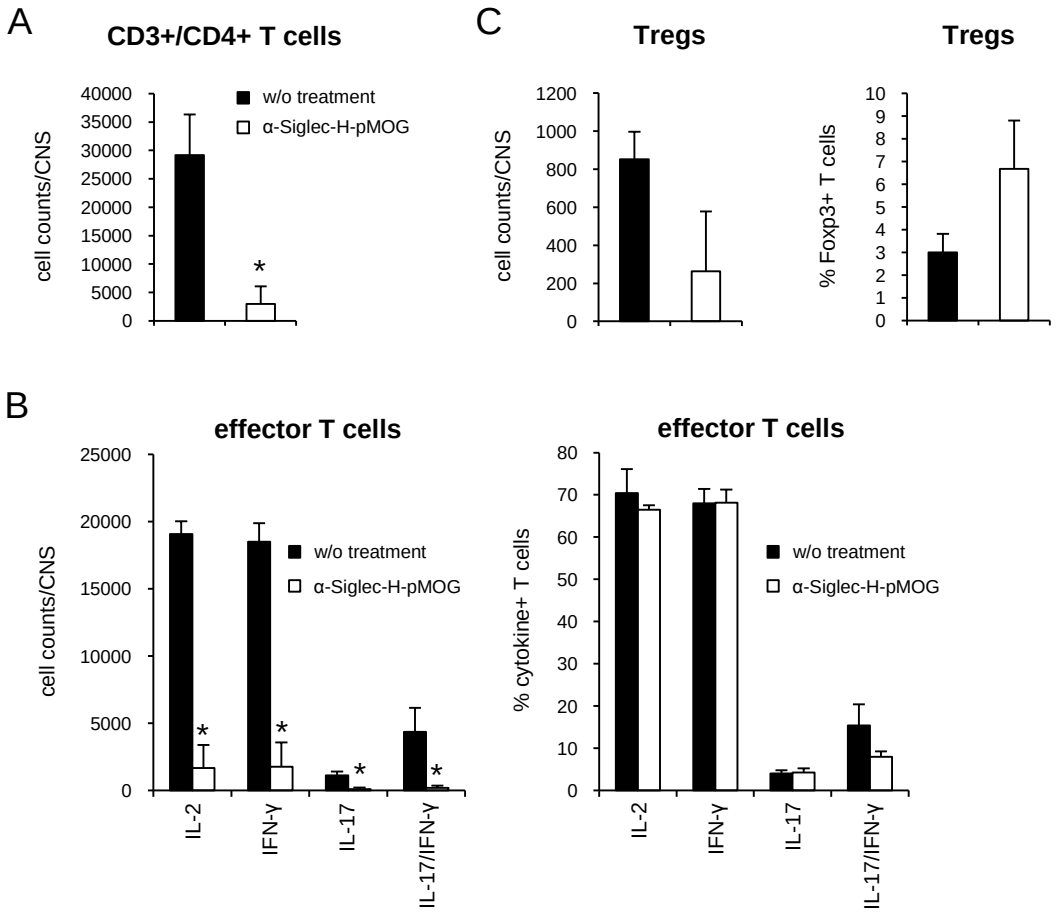

Fig. 33: CD4+ T cell infiltration in the CNS after induction of EAE.

Mice were pretreated with 10 µg α-Siglec-H-pMOG on day -1 or did not receive any treatment. On the next day, all mice were immunized with 100 µg pMOG in CFA (s.c.) and received 200 ng PTx (i.v.) on day 0 and day 2. At the peak of the disease (day 17), T cell infiltration was analyzed in the CNS. **(A)** Absolute numbers of CD3+/CD4+ T cells/CNS. **(B)** Absolute numbers of cytokine producing T cells /CNS and percentage of cytokine producing T cells of all CD3+/CD4+ T cells. **(C)** Absolute numbers of Foxp3+ Tregs/CNS and percentage of Foxp3+ Tregs of all CD3+/CD4+ T cells. (mean ± SD, *n*=3, * p<0.05 Student's *t*-test).

Because antigen delivery to pDCs *via* Siglec-H was followed by the formation of peptide/MHC II complexes that were stable for 6-8 days (Fig. 29), it was investigated whether pretreatment of mice with α-Siglec-pMOG 7 days before the immunization would also be able to mediate the observed protective effect of α-Siglec-H-pMOG. Mice that received α-Siglec-H-pMOG on day -7 also showed a delayed onset of the disease compared to untreated mice. However, pretreatment on day -7 had a weaker effect compared to administration of α-Siglec-H-pMOG on day -1. Mice did only differ significantly from mice that did not receive any treatment on day 16-18 (Fig. 34). Moreover, the mean maximal EAE score was considerably higher (mean max. EAE score 2.4, cumulative results of 2 individual experiments) compared to mice that received α-Siglec-H-pMOG on day -1 (mean max. EAE score 1.7, cumulative results of 3 individual experiments).

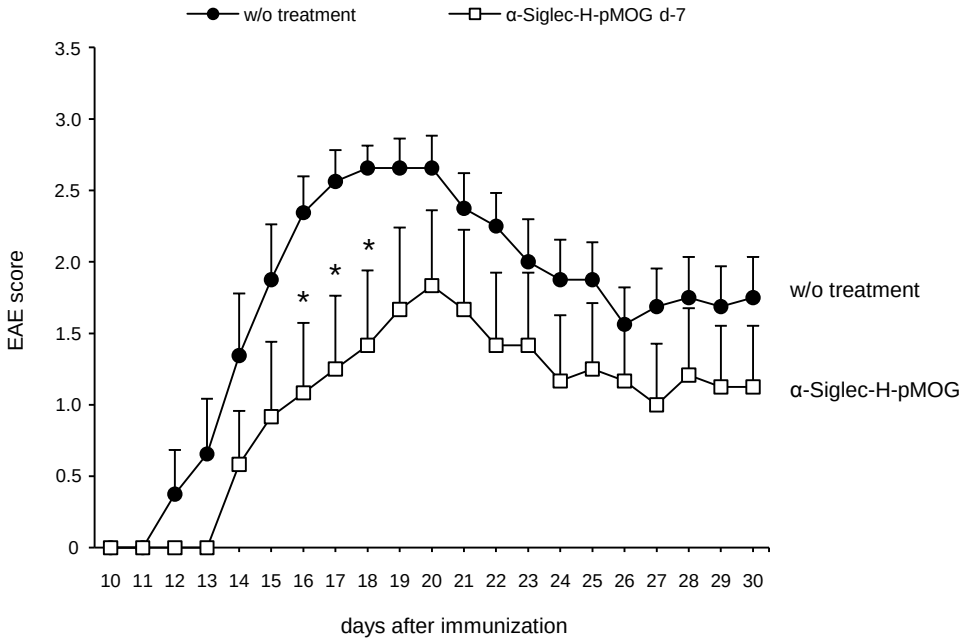

Fig. 34: Administration of α-Siglec-H-pMOG on day -7 and its effect on the progress of pMOG induced EAE.

Mice were pretreated with 10 µg α-Siglec-H-pMOG on day -7 or did not receive any treatment. On day 0, all mice were immunized with 100 µg pMOG in CFA (s.c.) and received 200 ng PTx (i.v.) on day 0 and day 2. Mice were scored daily for signs of illness. (mean ± SEM, *n*=6 for α-Siglec-H-pMOG, *n*=8 for w/o treatment, * $p<0.05$, Student's *t*-test).

To exclude that binding of α-Siglec-H influences innate immune functions of pDCs that in turn could influence the progress of the disease, α-Siglec-H-OVA (irrelevant antigen control) was used. However, as shown in Fig. 35, pretreatment of mice with α-Siglec-H-OVA on day -1 or day -7 had only little influence on disease development. Thus, the observed protective effect of α-Siglec-H-pMOG was not due to an antigen unspecific effect mediated by binding of the antibody to pDCs.

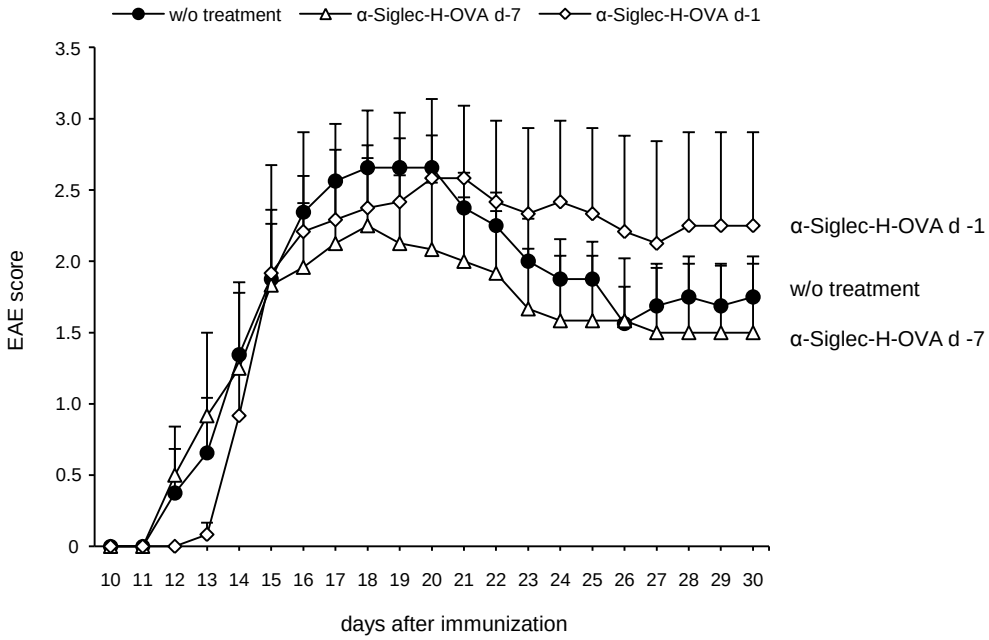

Fig. 35: Administration of α-Siglec-H-OVA on day -1 or day -7 and its effect on the progress of pMOG induced EAE.

Mice were pretreated with 10 µg α-Siglec-H-OVA on day -1 or day -7 or did not receive any treatment. On day 0, all mice were immunized with 100 µg pMOG in CFA (s.c.) and received 200 ng PTx (i.v.) on day 0 and day 2. Mice were scored daily for signs of illness. (mean ± SEM, *n*=6 for α-Siglec-H-OVA, *n*=8 for w/o treatment).

Table 14 summarizes the results from 3 independent experiments. Delivery of pMOG to pDCs *via* Siglec-H 1 day before EAE was induced results in a much lower percentage of mice that developed disease after immunization with pMOG/CFA/PTx (incidence) compared to mice that did not receive any pretreatment or received α-Siglec-H-OVA. Moreover, mice that developed symptoms showed a significantly delayed onset of the disease (first day score ≥ 1) and also developed significantly less severe signs of illness (mean max. EAE score) compared to untreated mice or mice that received α-Siglec-H-OVA.

Treatment	Number of mice	Incidence † [%]	Mortality [%]	Mean day of EAE onset † ± SEM	Mean day of EAE peak † ± SEM	Mean max. EAE score ± SEM
w/o	24	95.8 (23/24)	16.7	14.0 ± 0.5	16.6 ± 0.6	3.1 ± 0.2
α-Siglec-H-OVA day -1	14	92.9 (13/14)	7.1	14.8 ± 0.6	16.8 ± 0.8	2.6 ± 0.3
α-Siglec-H-pMOG day -1	21	61.9 (13/21)	0	17.0 ± 1.1 *	18.5 ± 1.1	1.7 ± 0.2 **

Table 14: Summary of EAE scores: cumulative results of 3 individual experiments.

The incidence depicts the percentage of mice that developed any signs of illness (score ≥1).
The mortality depicts the percentage of mice that died or had to be sacrificed.
The onset of disease is defined as the first day where any signs of illness (score ≥1) can be observed.
For a detailed description of the EAE score see chapter 3.2.16.9. († score ≥1, * p = 0.0284 compared to w/o control, ** p = 0.0005 compared to w/o control, p = 0.0422 compared to α-Siglec-H-OVA, Mann-Whitney U rank sum test)

From these experiments it can be concluded that the attenuation of effector CD4+ T cell responses after antigen targeting to pDCs (Fig. 31) correlates with a delayed onset and a reduced severity of an antigen specific CD4+ T cell mediated autoimmune disease. Effector T cell responses could not be inhibited successfully when antigen was delivered to pDCs 7 days ahead of the immunization. This indicates that pDCs have to actually present the antigen on MHC II to inhibit antigen specific CD4+ T cell responses.

4.9 PDC mediated immunity

Attenuation of effector CD4+ T cell responses was only achieved by antibody mediated antigen delivery to pDCs *via* the cell surface molecule Siglec-H. In contrast, antigen delivery *via* BST2 did not result in an attenuated effector T cell response, but was followed by an enhanced T cell response to soluble antigen (Fig. 23). This result demonstrates that antigen delivery to pDCs *per se* is not tolerogenic and also provides the opportunity to use pDC targeting for the induction rather than the inhibition of T cell responses. The second part of this work therefore addresses the question whether BST2 is a suitable target for antibody mediated antigen delivery to pDCs with the aim to induce protective adaptive immune responses.

4.9.1 Immunization with α-Siglec-H-OVA and α-BST2-OVA in combination with adjuvant

Because maturation and activation of DCs is a prerequisite for breaking tolerance and inducing effective immune responses, different adjuvants were used in subsequent immunization experiments. TLR9 ligand CpG 1668 directly activates TLR9 expressing pDCs. The receptors sensing PolyI:C (TLR3 and MDA5) are not expressed in murine pDCs. The strong adjuvant activity of PolyI:C is mediated indirectly by induction of IFN I in non pDCs (e.g. monocytes, stromal cells). PolyI:C was used, because it has been described as strong adjuvant and was used in many studies that used α-DEC205 to deliver antigen to CD8α+ DCs with the aim to induce protective immunity (Longhi *et al*., 2009; Trumpfheller *et al*., 2008; Trumpfheller *et al*., 2006).

To determine the success of antigen delivery *via* Siglec-H and BST2 to induce adaptive immune responses, mice were immunized with α-Siglec-H-OVA or α-BST2-OVA either without adjuvant or in combination with CpG 1668 or PolyI:C. OVA specific antibodies and their respective isotype were detected in the serum of immunized mice as evidence for a successful and efficient immunization. As shown in Fig. 36, only the immunization with α-BST2-OVA in combination with adjuvant resulted in the generation of high α-OVA antibody titers, whereas immunization with α-Siglec-H-OVA, even in the presence of CpG 1668 or PolyI:C, did not result in elevated levels of OVA specific antibodies. α-Siglec-H was therefore not further

tested in subsequent experiments for its capacity to deliver antigen to pDCs to induce adaptive immunity. Compared to CpG 1668, PolyI:C is a stronger adjuvant and immunization with α-BST2-OVA/PolyI:C resulted in higher levels of OVA specific antibodies in the serum of immunized mice.

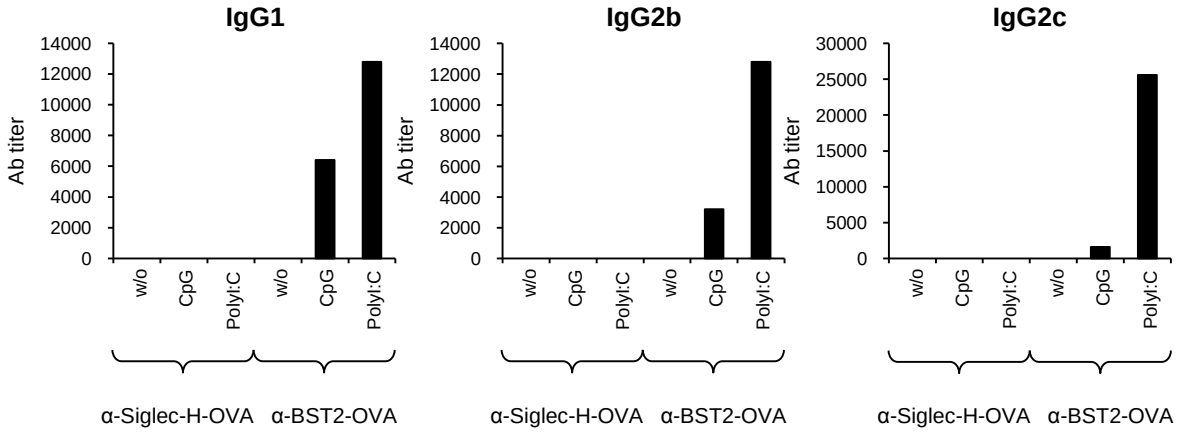

Fig. 36: OVA specific antibody titers after immunization with α-Siglec-H-OVA and α-BST2-OVA.
C57BL/6 mice were immunized (i.p.) with 10 µg α-Siglec-H-OVA or α-BST2-OVA without adjuvant or in combination with 10 µg CpG 1668 or 50 µg PolyI:C. OVA specific antibody titers were analyzed in the serum of immunized mice 14 days after immunization. Diagrams display the levels of different isotypes of OVA specific antibodies.

4.9.2 Binding of α-BST2-OVA in the presence of adjuvant

Because BST2 is known to be upregulated upon IFN I signaling on other cells than pDCs (Blasius *et al*., 2006b), it had to be demonstrated that the observed immune response was in fact due to delivery of antigen to pDCs and was not mediated by other APCs that may have upregulated BST2 following administration of CpG 1668 or PolyI:C.

To examine whether α-BST2-OVA that is injected in combination with CpG 1668 or PolyI:C specifically binds to pDCs *in vivo*, the recombinant antibody was detected *ex vivo* at different time points after injection. Fig. 37 A shows that 4 h after injection of recombinant α-BST2-OVA, the antibody was only detectable on pDCs but not cDCs, B cells or any other cell population in the spleen, irrespective of whether it was administered in combination with adjuvant or not. 4 h after administration of α-BST2-OVA/CpG 1668 and α-BST2-OVA/PolyI:C, BST2 was not or only slightly expressed

on other cells than pDCs (Fig. 37 A, histograms on the right side). 16 h after administration of α-BST2-OVA/CpG 1668 and α-BST2-OVA/PolyI:C, when BST2 was considerably expressed on other cells than pDCs (Fig. 37 B, histograms on the right side) injected recombinant α-BST2-OVA was not detectable anymore, neither on pDCs nor on other cell types (Fig. 37 B). Binding of α-BST2-OVA to pDCs therefore precedes upregulation of BST2 on other cell types. This prevents binding of α-BST2-OVA to other APCs even after co-administration of IFN I inducing agents such as CpG 1668 or PolyI:C.

Therefore, it can be concluded that α-BST2 can be used to deliver antigen specifically to pDCs even in the presence of adjuvants that promote expression of BST2 on other cells than pDCs.

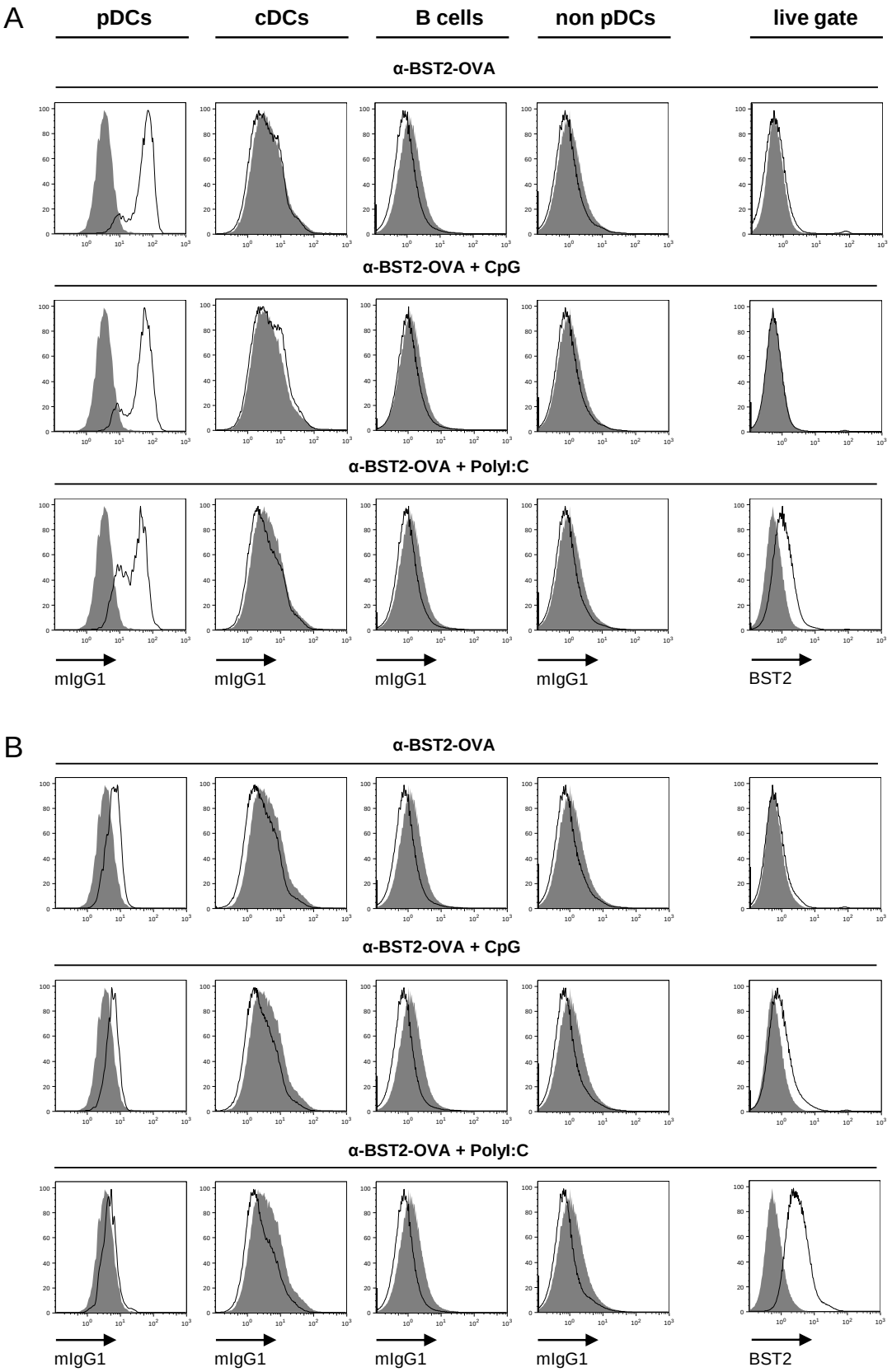

Fig. 37: Binding of α-BST2-OVA in the presence of adjuvant.

10 µg α-BST2-OVA were administered (i.p.) to C57BL/6 mice without adjuvant or in combination with 10 µg CpG 1668 or 50 µg PolyI:C. Presence of recombinant α-BST2-OVA antibody on the surface of splenocyte subpopulations was detected after 4 h **(A)** and 16 h **(B)** by staining with α-mIgG1. Expression of BST2 after administration of α-BST2-OVA +/- CpG 1668 and PolyI:C was determined after 4 and 16 h on living splenocytes.

4.9.3 Presentation of antigen on MHC II after administration of α-BST2-pHEL in the presence of adjuvant

It was shown that binding of α-BST2 is restricted to pDCs even in the presence of adjuvant. Next, it was investigated whether antigen presentation on MHC II is also limited to pDCs in conditions where CpG 1668 or PolyI:C are present. Therefore, C3H mice were immunized with α-BST2-pHEL without adjuvant or in combination with CpG 1668 or PolyI:C. After 16 h, the presence of pHEL/MHC II complexes on the surface of pDCs, cDCs, B cells and non pDCs was measured in the spleen by FACS analysis. As shown in Fig. 38, administration of α-BST2-pHEL in combination with CpG 1668 or PolyI:C was followed by pHEL/I-A^k complex formation that was detectable exclusively on pDCs but not on any other cell population 16 h post injection. With regard to MHC II restricted antigen presentation it can be concluded that BST2 is a suitable target to deliver antigen specifically to pDCs even in the presence of CpG 1668 or PolyI:C that promote upregulation of BST2 on non pDCs.

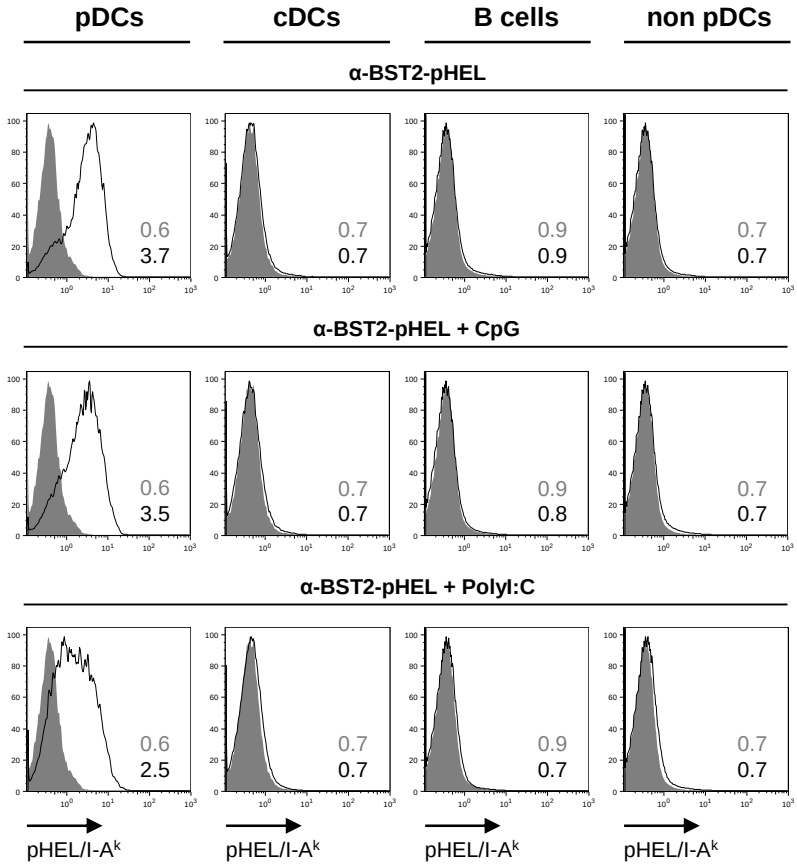

Fig. 38: Antigen presentation on MHC II after injection of α-BST2-pHEL with adjuvant.

20 µg α-BST2-pHEL were injected (i.p.) into C3H mice with or without 10 µg CpG 1668 or 50 µg PolyI:C. pHEL/I-A^k complexes were detected on splenocyte subpopulations 16 h after injection. Displayed is the formation of pHEL/I-A^k complexes on the surface of pDCs, cDCs, B cells and non-pDCs after treatment with α-BST2-pHEL (open histograms) or application of PBS (filled grey histograms). Numbers in histograms indicate the mean fluorescence intensity (grey: PBS, black: α-BST2-pHEL).

4.9.4 Cross-presentation of antigen on MHC I after delivery to pDCs *via* BST2

It could be demonstrated that binding of α-BST2 and presentation of antigen that is fused to α-BST2 on MHC II is restricted to pDCs even in the presence of adjuvant. In contrast to presentation of antigen on MHC II, it has so far not been completely clarified whether murine pDCs can cross-present antigen. A commercially available antibody that detects the OVA specific CD8+ T cell epitope SIINFEKL on MHC I was not sensitive enough to detect antigen that was delivered to DC subsets *in vivo*. SIINFEKL/MHC I complexes were neither detected after antigen delivery to CD8α+ DCs with α-DEC205-OVA nor after systemic administration of high amounts of soluble OVA (data not shown). Therefore, the capability of murine pDCs to cross-present antigen was examined by co-cultivating FACS purified pDCs and CFSE-labeled OVA specific CD8+ OT-I T cells. Fig. 39 clearly demonstrates that pDCs that were incubated with α-BST2-OVA are able to trigger CD8+ T cell proliferation. This result indicates that pDCs must have presented the antigen on MHC I. Stimulation of pDCs with TLR9 ligand CpG 1668 further increased CD8+ T cell proliferation.

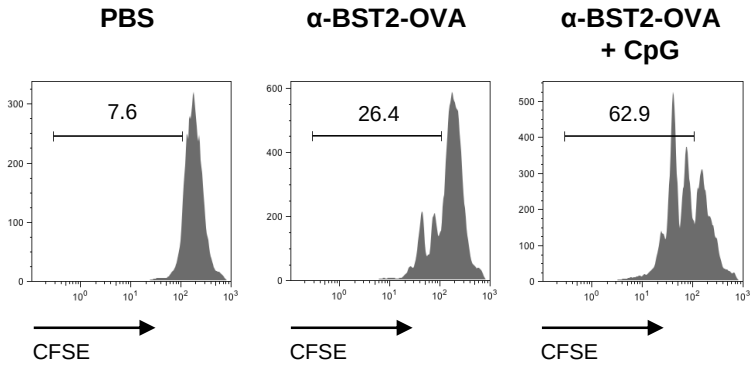

Fig. 39: Priming of CD8+ T cells after α-BST2 mediated antigen delivery.
$1*10^4$ FACS sorted pDCs were incubated with $1*10^5$ CFSE-labeled CD8+ OT-I T cells in the presence of 500 ng/ml α-BST2-OVA either without stimulus or in the presence of 1 µg/ml CpG 1668. T cell proliferation was analyzed by CFSE dilution after 4 days of co-culture. Numbers in the histograms display the percentages of OT-I T cells that proliferated. Co-culture of pDCs and OT-I T cells in the absence of α-BST2-OVA served as control.

To further demonstrate that BST2 mediated antigen delivery *in vivo* leads to cross-presentation by pDCs and that CD8+ T cell responses after injection of α-BST2-OVA were mediated exclusively by pDCs and not cDCs even in the presence of an adjuvant such as PolyI:C, pDC depletion was performed.

To deplete pDCs *in vivo*, α-Gr-1 was used that has been shown to bind to pDCs and is able to mediate depletion of these cells (Asselin-Paturel *et al*., 2001; Asselin-Paturel *et al*., 2003; Nakano *et al*., 2001).

Fig. 40 A demonstrates the successful depletion of pDCs with α-Gr-1 and shows that CD8α+ DCs that are very efficient in cross-presenting antigen were not depleted by α-Gr-1 treatment. CD11c+ DCs isolated from a mouse that was immunized with α-BST2-OVA/PolyI:C induced the proliferation of CD8+ OT-I T cells *in vitro* (Fig. 40 B). This T cell response was abrogated by depletion of pDCs with α-Gr-1 before the immunization demonstrating that pDCs are required for the initiation of CD8+ T cell responses after antigen targeting to BST2 (Fig. 40 B). Furthermore, it can be excluded that CD8+ T cell responses that were observed after administration of α-BST2-OVA were mediated by CD8α+ DCs, as those cells were not affected by α-Gr-1 treatment, but CD11c+ DCs isolated from a mouse that was treated with α-Gr-1 were unable to prime OT-I T cells.

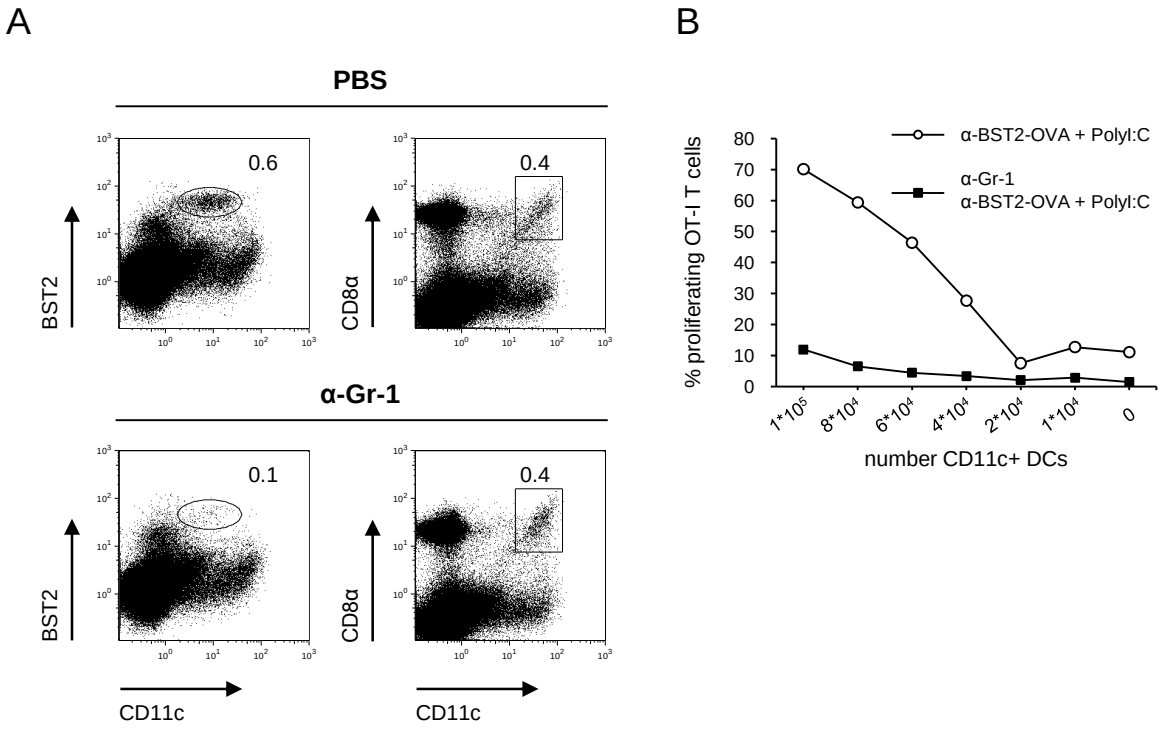

Fig. 40: CD8+ T cell responses after *in vivo* delivery of antigen to pDCs *via* BST2.

C57BL/6 mice were immunized with 10 µg α-BST2-OVA in combination with 50 µg PolyI:C. Where indicated, pDCs were depleted by injection (i.p.) of 300 µg α-Gr-1 24 h prior to the immunization. 8 h after immunization, CD11c+ DCs were enriched with MACS beads and graded doses of CD11c+ DCs were co-cultivated with $1*10^5$ CFSE-labeled CD8+ OT-I T cells. T cell proliferation was assayed by CFSE dilution 4 days later. **(A)** Percentages of pDCs and CD8α+ DCs in the spleen of an untreated (upper dot plots) and an α-Gr-1 treated (lower dot plots) mouse. **(B)** The percentages of proliferating OT-I T cells in response to co-culture with graded doses of CD11c+ DCs were measured and are displayed in the graph.

4.9.5 Influence of adjuvants on T cell responses mediated by antigen delivery to pDCs *via* BST2 *in vivo*

In vitro experiments were able to demonstrate that antigen delivery to pDCs *via* BST2 results in specific presentation of antigen on MHC I and MHC II, even in the presence of CpG 1668 and PolyI:C. To further investigate the impact of adjuvants on the strength of T cell responses that are mediated by antigen presenting pDCs *in vivo*, CFSE-labeled OVA-TCR-transgenic CD8+ OT-I and CD4+ OT-II T cells were adoptively transferred into C57BL/6 mice. These recipients were subsequently immunized with α-BST2-OVA with or without CpG 1668 and PolyI:C. Proliferation of OT-I and OT-II T cells was measured by CFSE dilution 4 days later.

Almost 100 % of the CD8+ OT-I T cells proliferated in response to injection of α-BST2-OVA regardless of whether the antibody was injected without adjuvant or in combination with CpG 1668 or PolyI:C (Fig. 41 A). In mice that were immunized with α-BST2-OVA without adjuvant a 7-fold increase in the percentage of OT-I T cells of all CD8+ T cells was observed compared to mice that did not receive any antigen. Administration of α-BST2-OVA in combination with CpG or PolyI:C increased the expansion significantly compared to immunizations without adjuvant (Fig. 41 B). Immunization with α-BST2-OVA also efficiently induced proliferation and expansion of CD4+ OT-II T cells, which again was significantly increased by administration of CpG 1668 or PolyI:C (Fig. 41 C).

Thus, the use of adjuvants does not interfere with the specificity of BST2 mediated antigen delivery, but significantly enhances CD4+ and CD8+ T cell responses *in vivo* and can therefore be used to strengthen pDC mediated adaptive immune responses.

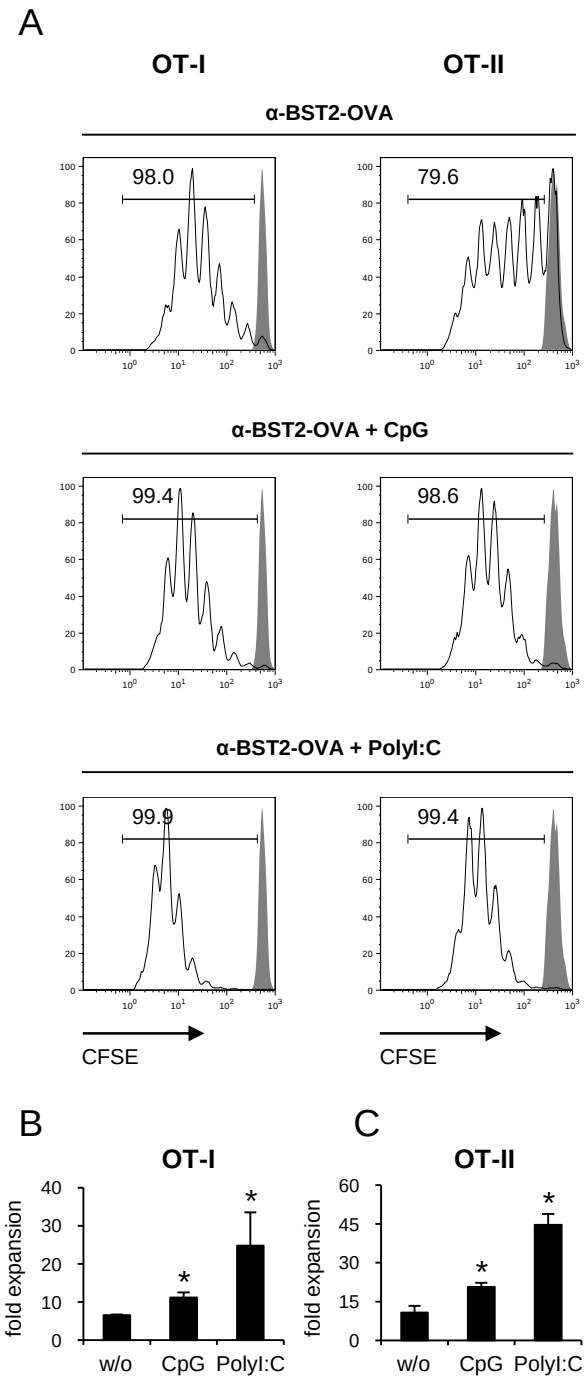

Fig. 41: CD4+ and CD8+ T cell responses after antibody mediated antigen delivery to pDCs in the presence of adjuvants.

C57BL/6 mice which had received CSFE-labeled CD8+ OT-I or CD4+ OT-II T cells (i.v.) were immunized (i.p.) with 10 µg α-BST2-OVA with or without 10 µg CpG 1668 or 50 µg PolyI:C. T cell responses were analyzed in the spleen 4 days after immunization. **(A)** T cell proliferation was assayed by CFSE dilution. Numbers in the histograms indicate the percentage of transferred T cells that had divided (open histograms: α-BST2-OVA, grey filled histograms: PBS). Bar graphs show the expansion of **(B)** OT-I and **(C)** OT-II T cells after immunization with α-BST2-OVA without adjuvant or in combination with CpG 1668 or PolyI:C as fold expansion in relation to mice which had received PBS as control. (mean ± SD, *n*=3, * p<0.05 Student's *t*-test)

4.9.6 Endogenous T and B cell responses after immunization with α-BST2-OVA

Successful vaccines induce expansion of endogenous antigen specific T and B cells from a polyclonal pool of naïve T and B cells and above that promote their differentiation and the acquisition of effector functions as well as the generation of long living memory T and B cells. Because the frequency of antigen specific T and B cells in a naïve organism is very low, common immunization protocols are composed of two immunization rounds. The first immunization primes antigen specific naïve T and B cells and the second antigen pulse provides a fresh stimulus that prevents deletion of expanded antigen specific cells due to the lack of TCR or BCR stimuli (death by neglect).

4.9.6.1 Presentation of antigen on MHC II after repeated injections of α-BST2-pHEL in combination with adjuvant

It has been shown in Fig. 38 and Fig. 40 that neither the use of CpG 1668 nor injection of PolyI:C has any influence on the specific delivery of antigen to pDCs even though BST2 is upregulated on other cells than pDCs after administration of those IFN I inducing agents. However, specificity needed to be verified also for a prime boost immunization protocol, because it could not be excluded that upregulation of BST2 after the first immunization leads to binding of α-BST2 to other cells when α-BST2 fused antigen is injected a second time to boost the immune response. Therefore, α-BST2-pHEL was administered with or without CpG 1668 or PolyI:C on day 0 and day 14 and formation of pHEL/MHC II complexes was determined on splenocyte subpopulations 16 h after the second injection.

As depicted in Fig. 42, antigen presentation was restricted to CCR9+ pDCs and not detectable in CCR9- pDCs or any other cells than pDCs even in conditions that promote the expression of BST2 on non pDCs. The use of CpG 1668 and PolyI:C as adjuvants increased the expression of the co-stimulatory molecule CD86 on pDCs. Because pDCs do not express MDA5 or TLR3 which are sensors for PolyI:C, indirect activation of pDCs by IFN I is sufficient to induce the expression of co-stimulatory molecules on pDCs.

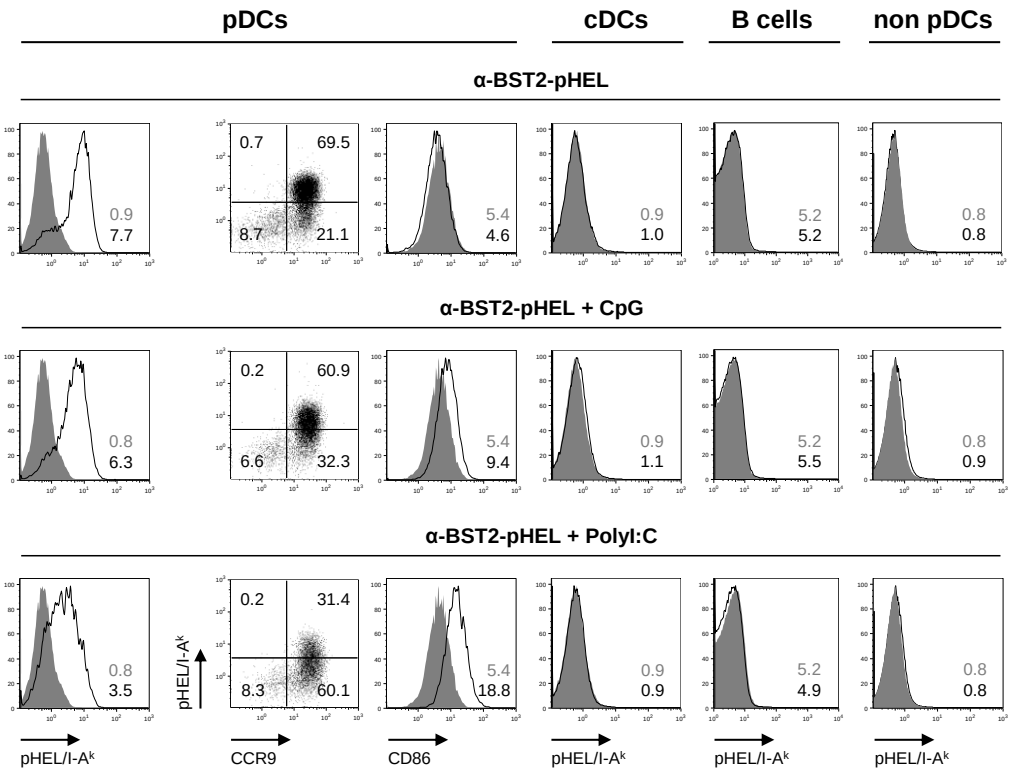

Fig. 42: Antigen presentation on MHC II after repeated injections of α-BST2-pHEL and adjuvant.
20 µg α-BST2-pHEL were injected into C3H mice (i.p.) with or without 10 µg CpG 1668 or 50 µg PolyI:C on day 0 and 14. pHEL/MHC II complexes (pHEL/I-A^k) were detected on splenocyte subpopulations 16 h after the second injection. Formation of pHEL/I-A^k was measured on the surface of pDCs, cDCs, B cells and non pDCs after treatment with α-BST2-pHEL (open histograms) or application of PBS (filled grey histograms). For pDCs, expression of CD86 is also displayed as histogram. Numbers in histograms indicate the mean fluorescence intensity (grey: PBS, black: α-BST2-pHEL). Dot plots show correlation of pHEL/I-A^k formation and CCR9 expression in pDCs. The percentages are indicated in the quadrants.

Taken together, these results show that the specific delivery of antigen to pDCs *via* BST2 is preserved in a prime boost immunization protocol using CpG 1668 or PolyI:C as adjuvant.

4.9.6.2 Induction of endogenous antigen specific T cell and antibody responses after α-BST2 mediated antigen delivery

To determine endogenous OVA specific adaptive immune responses, mice received antibody-OVA fusion proteins on day 0 in the presence of either CpG 1668 or PolyI:C and the same combination of antibody-OVA and adjuvant again on day 14 (Fig. 43). To be able to track the development of the immune response, serum was collected on day 14 before mice received the boost and also on day 21 when in addition T cell responses were analyzed. For experiments that aimed to investigate the CD8+ T cell

response, SIINFEKL pulsed and CFSE labeled target cells were transferred to immunized mice 16 h prior to analysis.

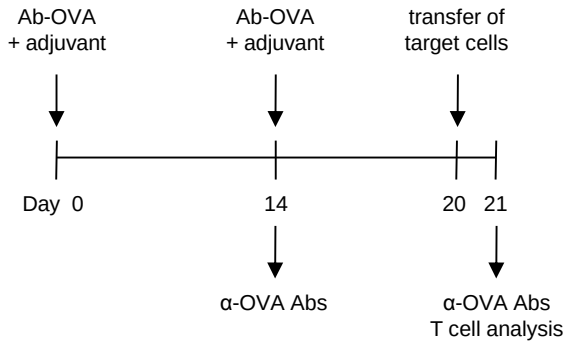

Fig. 43: Immunization protocol.

C57BL/6 mice were immunized (i.p.) twice with a 14 day interval with 10 µg of the respective antibody-OVA fusion protein and either 10 µg CpG 1668 or 50 µg PolyI:C. To determine antibody titers, serum was collected on day 14 (before the boost) and day 21 (one week after the boost). T cell responses were analyzed on day 21. If CD8+ T cell responses were analyzed, target cells were transferred at day 20 and efficiency of killing was determined the next day.

As the aim of immunizing mice with α-BST2-OVA was to investigate the efficiency of initiating endogenous T and B cell responses, α-DEC205-OVA in combination with PolyI:C was used as a positive control for the induction of a strong antigen specific CD4+ and CD8+ T cell response as well as for humoral immune responses. The high efficiency and quality of this immunization strategy has been demonstrated previously in several studies (Longhi *et al*., 2009; Trumpfheller *et al*., 2008; Trumpfheller *et al*., 2006).

After *ex vivo* restimulation of splenic CD4+ T cells with OVA pulsed CD11c+ DCs the highest frequency of IFN-γ producing CD4+ T cells was found after immunization with α-BST2-OVA/PolyI:C (Fig. 44 A). Immunizing with α-BST2-OVA/CpG 1668 or α-DEC205-OVA/PolyI:C resulted in comparable frequencies of IFN-γ producing CD4+ T cells. The frequency of CD4+ T cells producing IL-4 after restimulation was very low in all conditions tested. Thus, immunization with α-BST2-OVA together with adjuvant is very efficient in inducing a strong antigen specific T_H1 response.

To investigate, if immunization with α-BST2-OVA in combination with adjuvant can also efficiently activate and expand CD8+ T cells with cytolytic function *in vivo*, a combination of SIINFEKL pulsed and unpulsed splenocytes labeled with different

concentrations of CFSE was transferred as target cells on day 20. Specific lysis of SIINFEKL pulsed cells was determined 16 h after the transfer. CTLs that were induced by immunization with α-BST2-OVA/PolyI:C were very efficient in specific killing of target cells and similarly potent as CTLs that were induced by immunizing with α-DEC205-OVA/PolyI:C (Fig. 44 B). In contrast to α-BST2-OVA/PolyI:C, α-BST2-OVA/CpG 1668 failed to induce strong CTL responses.

In addition to the described effector T cell responses, delivery of OVA to pDCs *via* BST2 in combination with CpG 1668 or PolyI:C also led to the production of high levels of OVA specific antibodies that were detectable in the serum of immunized mice. These OVA specific antibodies were already present at day 14 after only one injection of α-BST2-OVA. Boosting mice with the same combination of antibody fused OVA and adjuvant further increased the antibody titers (Fig. 44 C). Different IgG isotypes were detectable including IgG2b and IgG2c antibodies reflecting the efficient T_H1 response that was achieved by this immunization strategy and is required for isotype switching.

With regard to these findings it can be concluded that in combination with PolyI:C as adjuvant antigen delivery to pDCs *via* BST2 is as efficient as antigen delivery to CD8α+ DCs *via* DEC205 in inducing strong T_H1 responses, efficient CTL responses and high titers of antigen specific antibodies with a broad range of isotypes.

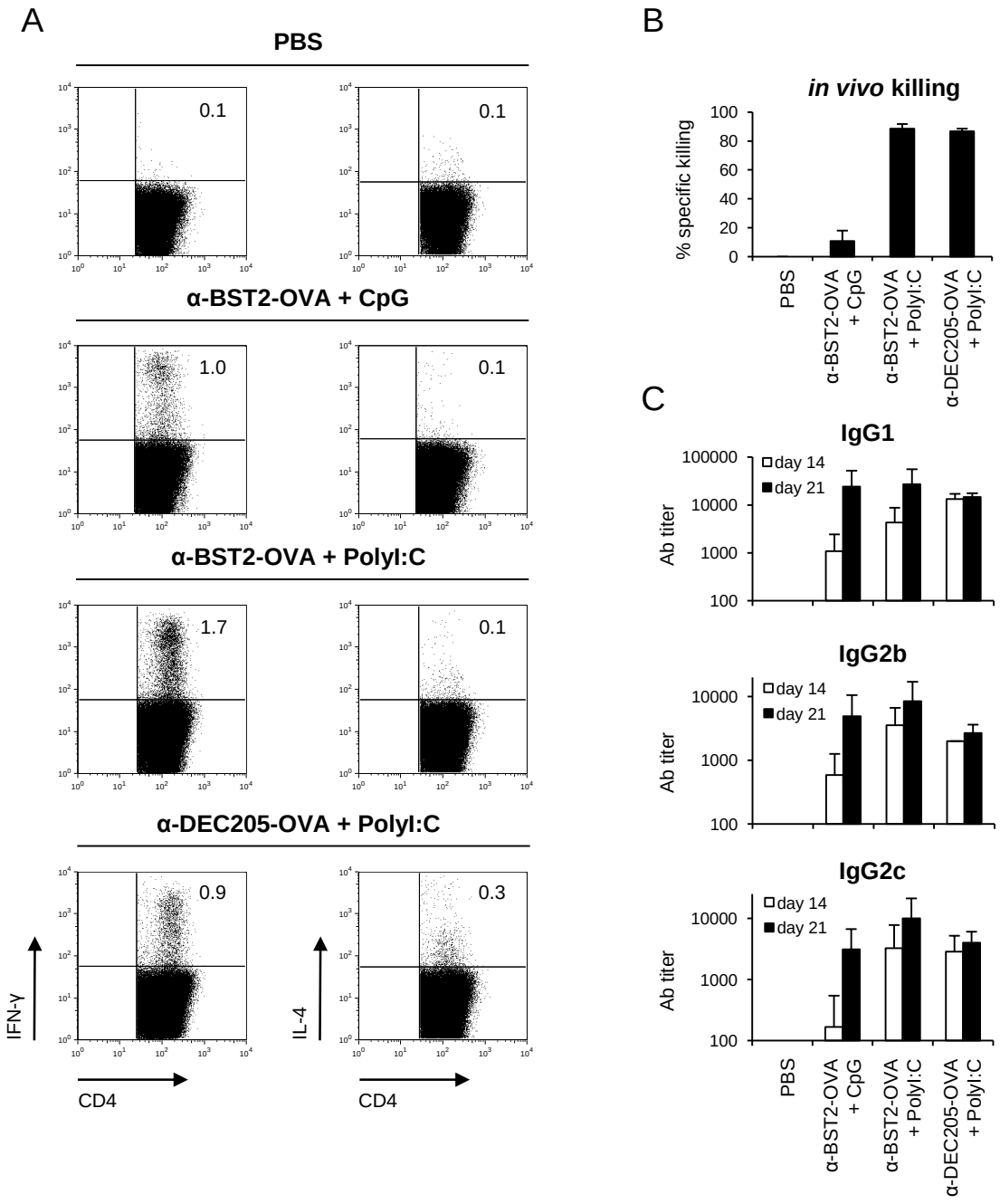

Fig. 44: Endogenous CD4+ and CD8+ T cell responses and α-OVA antibody titers after immunization with α-BST2-OVA in combination with adjuvant.

C57BL/6 mice were immunized with the indicated combinations of Ab-OVA and adjuvant as described in Fig. 43. **(A)** MACS-enriched CD4+ T cells were restimulated with OVA pulsed CD11c+ DCs. Dot plots show frequencies of IFN-γ and IL-4 producing CD4+ T cells. **(B)** *In vivo* killing of antigen loaded target cells was assayed on day 21. Syngeneic splenocytes were pulsed with SIINFEKL peptide (500 nM) for 3 h and labeled with 0.1 µM CFSE. Unpulsed cells were labeled with 1 µM CFSE. $1*10^7$ cells of each population were injected together (i.v.) and specific lysis was quantified 16 h later in the spleen. (mean ± SD, *n*=4-5) **(C)** Titers and isotypes of OVA specific antibodies were determined on day 14 and 21 (logarithmic scale). (mean ± SD, *n*=5-10)

4.9.7 Immunization with α-BST2-OVA together with PolyI:C protects mice in an antigen specific viral infection model

To investigate whether the CD4+ and CD8+ T cell responses that can be induced by targeting antigen to murine pDCs in combination with PolyI:C are sufficient to provide protection in an antigen specific infection model, a recombinant vaccinia virus strain that encodes OVA (VV-OVA) was used. This infection model has been used in previous studies to test the efficiency of effector T cells that were induced by antigen targeting to CD8α+ DCs to mediate protection (Bonifaz *et al*., 2004). After infection with VV-OVA, infected cells express OVA and can therefore be detected and fought by OVA specific T cells. Mice were immunized with α-BST2-OVA and PolyI:C as previously described. Because elevated α-OVA antibody titers indicated that already after one injection of α-BST2-OVA/PolyI:C a significant immune reaction occurred, it was also tested whether priming naïve mice with α-BST2-OVA/PolyI:C would be sufficient to mediate protection.

As shown in Fig. 45 A, a single immunization with α-BST2-OVA/PolyI:C was indeed sufficient to protect mice from severe body weight loss after intranasal infection with VV-OVA eight weeks after the immunization. In contrast, mice that had received PolyI:C but no antigen lost significantly more body weight and did not completely recover either, excluding the possibility that the adjuvant alone and its IFN I mediated immune responses contributed to the protection in an antigen independent manner. Mice immunized with α-BST2-OVA/PolyI:C regained their initial body weight already between day 8 and 9 after the infection. Mice that were immunized as described in Fig. 43 using the prime boost protocol also showed significantly less body weight loss compared to mice that received the adjuvant without antigen (Fig. 45 B). However, immunizing following the prime boost protocol had no additional advantage compared to a single immunization. The observed protection correlated with rapid expansion of OVA specific CD4+ and CD8+ effector T cells producing IFN-γ after viral challenge. The high frequencies of OVA specific effector T cells suggest that sufficient T cell memory had been induced by the immunization protocol including IFN-γ/IL-2 double positive multifunctional CD4+ T cells (Fig. 45 C, D). Studies using wt vaccinia virus had previously shown that both CD4+ and CD8+ effector T cells contribute to resistance (Xu *et al*., 2004), whereas the CD4+ T cell response appears to dominate the protective immune response in this model (Trumpfheller *et al*., 2006). It can be concluded that α-BST2 mediated delivery of OVA was translated into a long

lasting protective antiviral immunity at a mucosal surface when α-BST2-OVA was administered together with PolyI:C as adjuvant.

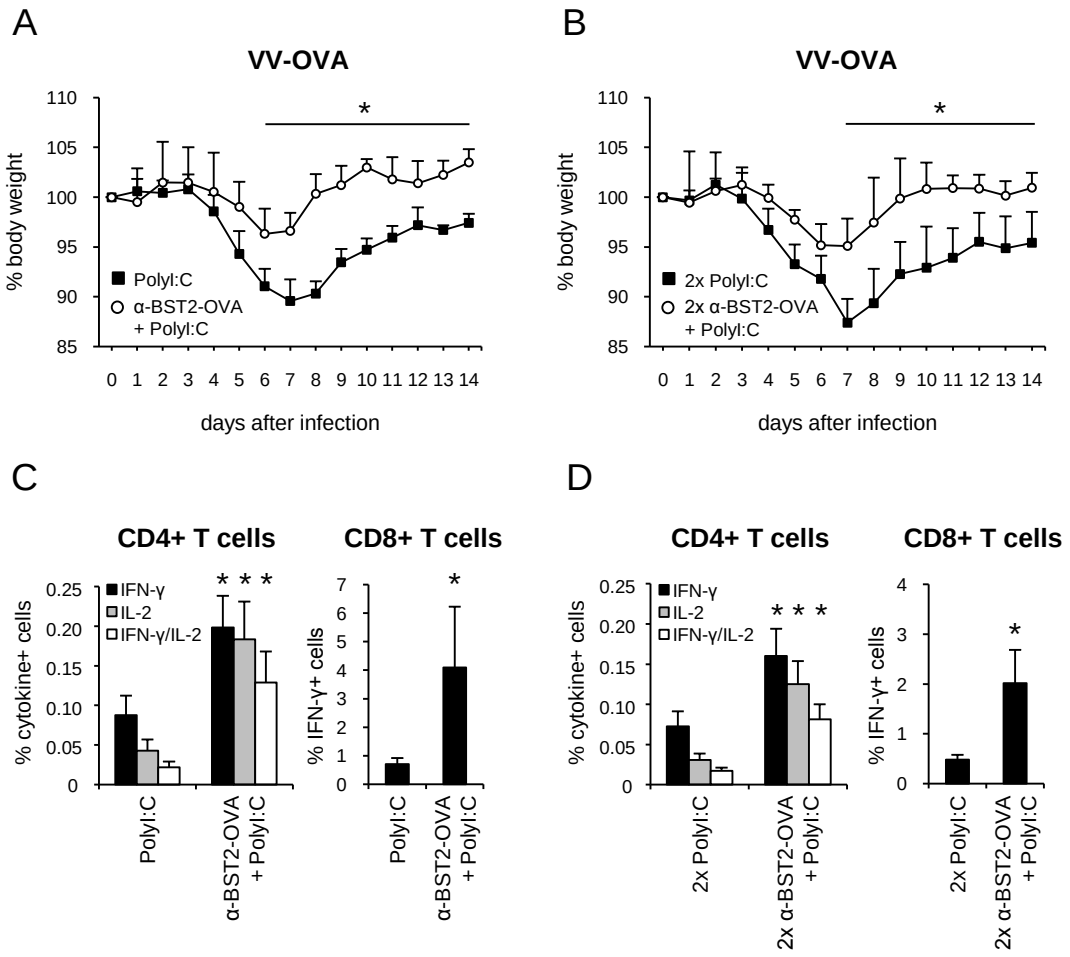

Fig. 45: Body weight loss and T cell responses after infection with VV-OVA.

C57BL/6 mice were immunized (i.p.) either once (A and, C) or twice (B and D) with 10 µg α-BST2-OVA and 50 µg PolyI:C or PolyI:C alone. Eight weeks after the last immunization mice were infected intranasally with $3*10^4$ PFU of VV-OVA. **(A)** and **(B)** display the loss of body weight compared to the weight before infection (mean ± SD, *n*=6, * p<0.05 Student's *t*-test). **(C)** and **(D)** show the frequency of OVA specific IFN-γ+, IL-2+ and IFN-γ+/IL-2+ CD4+ T cells and frequency of IFN-γ+ CD8+ T cells 14 days after viral challenge (mean ± SD, *n*=6, * p<0.05 Student's *t*-test).

4.9.8 Immunization with α-BST2-OVA together with PolyI:C delays the development of a rapidly growing tumor

To investigate if T_H1 and CTL responses induced by α-BST2 mediated delivery of antigen to pDCs can also confer anti-tumor immunity, a challenge with OVA expressing B16 melanoma cells (B16-OVA) was performed. Mice were immunized with α-BST2-OVA/PolyI:C and 14 days later B16-OVA cells were implanted subcutaneously. As control, a second group of mice received only PolyI:C but no antigen.

Mice that did not receive antigen developed rapidly growing tumors 10-12 days after implantation of tumor cells (Fig. 46 A). In contrast to that, α-BST2 mediated antigen delivery combined with PolyI:C as adjuvant provided protection against the rapidly growing tumor to a large extent (Fig. 46 A). The very slow progression of tumor growth correlated with an increased survival of immunized mice compared to mice that received PolyI:C but no antigen (Fig. 46 B).

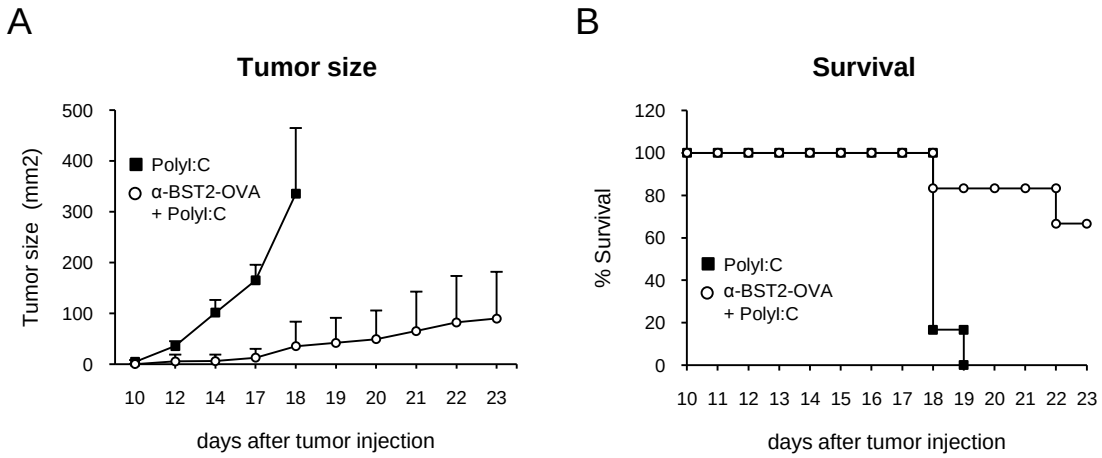

Fig. 46: Tumor growth and survival after implantation of OVA expressing tumor cells.
C57BL/6 mice were immunized (i.p.) with 10 µg α-BST2-OVA and 50 µg PolyI:C or PolyI:C alone. Two weeks later mice received $1*10^6$ B16-OVA melanoma cells (s.c.). **(A)** Tumor growth was monitored at the indicated time points (mean ± SD, *n*=6). **(B)** Survival after tumor implantation is shown (*n*=6).

Inhibiting tumor growth in an antigen specific manner requires both efficient CD4+ and CD8+ T cell responses (Bonifaz *et al*., 2004). Thus, antigen specific T cells that are induced after immunization with antigen fused to α-BST2 in combination with adjuvant can not only mediate protection in an antigen specific viral infection model, but are also able to confer resistance against a rapidly growing tumor.

5 DISCUSSION

Antibody-antigen fusion proteins have been used in many studies to investigate the role of distinct DC populations for the initiation of antigen specific adaptive immune responses (see Table 2). Using recombinant antibodies for antigen delivery has several advantages. The unspecific binding of the antibody and therefore also the unspecific delivery of the antigen that is fused to the antibody *via* Fc receptors can be prevented by mutating the Fc part of the antibody. Furthermore, fusing the antigen to the C-terminus of the heavy chain allows the precise calculation of the amount of antigen that is delivered with the antibody. Chemical coupling of antigen to monoclonal antibodies has also been used in the past, however, this method does not provide the same accuracy as the fusion of antigen to an antibody does. Although the ratio of antibody and antigen can be calculated prior to the coupling reaction, the amount of antigen that is effectively connected to each antibody molecule completely depends on the efficiency of the chemical reaction and may vary within different preparations. In contrast to that, recombinant antibodies that are directly fused to antigen always carry the same amount of antigen (2 molecules antigen per 1 antibody molecule). Antibody mediated antigen delivery to DC subpopulations *in vivo* is an attractive approach to either induce or inhibit antigen specific immune responses avoiding cumbersome *ex vivo* manipulation and adoptive transfer of these rare cell types. Although pDCs constitute a well defined DC subpopulation, it has so far not been investigated whether antigen delivery to pDCs can be used to influence adaptive immune responses. The aim of this study was therefore to generate recombinant antibodies that facilitate the delivery of antigen specifically to murine pDCs *in vivo*.

5.1 Generation and use of recombinant antibodies for antigen delivery to murine pDCs *in vivo*

Siglec-H and BST2 were chosen as targets for antibody mediated antigen delivery, because of their unique expression on the surface of murine pDCs in the steady state and the availability of hybridoma cell lines producing monoclonal α-Siglec-H and α-BST2 antibodies (Asselin-Paturel *et al*., 2003; Blasius *et al*., 2006a; Blasius *et al*., 2006b; Zhang *et al*., 2006). Zhang *et al*. described intracellular expression of Siglec-H in a subset of marginal zone macrophages in the spleen and in medullary

macrophages in lymph nodes. In agreement with the published data, no surface expression of Siglec-H was detected by flow cytometry on other cells than pDCs in spleen, lymph nodes or bone marrow in the present study. Potential intracellular expression of Siglec-H in non pDCs does therefore not interfere with α-Siglec-H mediated antigen delivery.

PDC specific recombinant antibodies fused to OVA were generated as previously described for α-DEC205-OVA (Hawiger *et al*., 2001). The present study describes for the first time the use of recombinant pDC specific α-Siglec-H and α-BST2 antibodies fused to antigen. Zhang *et al*. have coupled OVA covalently to polyclonal sheep α-Siglec-H, however, the described influence on OVA specific CD8+ T cells after administration of sheep α-Siglec-H-OVA in combination with TLR9 ligand CpG 2216 has to be interpreted with caution (Zhang *et al*., 2006). As mentioned before, the use of polyclonal or monoclonal antibodies might result in unspecific antigen delivery after binding of the antibodies to Fc receptors. Moreover, the use of antibodies that originate from a different species for antigen delivery, especially in the presence of adjuvant, is not recommendable. Repeated injections of, for example, polyclonal sheep α-Siglec-H antibody might result in a strong anti sheep IgG antibody response that could block binding of polyclonal sheep α-Siglec-H to pDCs and could significantly affect the result. These potential difficulties were avoided by using recombinant α-Siglec-H and α-BST2 antibodies that consist of a mouse IgG1 scaffold that possesses distinct mutations in its Fc part to prevent binding to Fc receptors. Although the variable regions that were fused to the IgG1 scaffold originate from rat antibodies, the rat part of the recombinant antibody-antigen fusion proteins was restricted to a minimum. It is therefore very unlikely that α-rat IgG antibodies directed against the variable regions of recombinant α-Siglec-H or α-BST2 are generated after administration of α-Siglec-H-OVA or α-BST2-OVA.

Fusing pHEL to the C-terminus of α-Siglec-H and α-BST2 allowed to directly trace presentation of antigen on MHC II. Presentation of antigen on MHC II was restricted to pDCs at any time. This was also confirmed after co-administration of antibody-pHEL fusion proteins and TLR9 ligand CpG 1668 or TLR3/MDA5 ligand PolyI:C. This was remarkable, especially for α-BST2 mediated antigen delivery, as BST2 is known to be upregulated on other cells than pDCs after type I IFN signaling. However, it was shown that binding of α-BST2 to pDCs precedes the upregulation of BST2 on other

cells. Therefore both antibodies - α-Siglec-H and α-BST2 - mediate the delivery of antigen specifically to pDCs and are qualified tools to study the role of antigen presenting pDCs *in vivo*.

It has been proposed that CCR9 expression distinguishes functionally different subpopulations of pDCs. Hadeiba *et al.* have identified a population of CCR9- pDCs with higher expression of co-stimulatory molecules (Hadeiba *et al*., 2008). It has also been shown that CCR9- pDCs have the ability to develop under the influence of certain proinflammatory environmental conditions into a cDC like cell type with higher antigen presentation capacity (Schlitzer *et al*., 2011). Antigen delivery to CCR9- pDCs could be responsible for the observed immune responses or could result in the presentation of antigen on cDC like cells under certain circumstances. However, after antigen delivery *via* Siglec-H and BST2 peptide/MHC II complexes were detected only on CCR9+ but not CCR9- pDCs or cDCs. This clearly excludes a role for CCR9- pDCs in the observed antigen specific immune responses. CCR9- pDCs probably fail to present antigen delivered *via* Siglec-H and BST2 because they express only low levels of MHC II indicating that this cell subset is not designated to present antigen (Schlitzer *et al*., 2011). When pDCs were identified not only by the expression of CD11c and B220 but with pDC specific markers (Siglec-H, BST2), higher expression levels of co-stimulatory molecules on CCR9- pDCs as reported by Hadeiba *et al.* could not be confirmed (Schlitzer *et al*., 2011). Therefore, it can be concluded that CCR9- pDCs, irrespective of their role *in vivo*, did not contribute to the immune responses that were elicited by targeting antigen to Siglec-H or BST2.

As the ability of pDCs to cross-present exogenous antigen is still a matter of controversial debate, this question was also addressed. In the present study, CD8+ T cell responses were analyzed to investigate the potential of antigen fused to α-BST2 to induce protective adaptive immune responses. The ability of pDCs to cross-present exogenous antigen was therefore only investigated after antigen delivery *via* BST2. A commercially available antibody that detects the OVA specific CD8+ T cell epitope SIINFEKL presented on MHC I (Porgador *et al*., 1997) failed to detect SIINFEKL/MHC I complexes after *in vivo* delivery of OVA to pDCs *via* BST2 or to CD8α+ DCs *via* DEC205 and also after administration of high doses of soluble OVA. The ability of pDCs to cross-present antigen was therefore analyzed in an indirect approach by co-cultivating pDCs and OVA specific CD8+ OT-I T cells in the absence

or presence of α-BST2-OVA. In contrast to Sapoznikov *et al.,* who claimed that pDCs fail to cross-present exogenous antigen, CD8+ T cell proliferation was observed after co-cultivation of pDCs and CD8+ OT-I T cells in the presence of α-BST2-OVA. This provides direct evidence that murine pDCs are able to cross-present exogenous antigens on MHC I after antigen delivery with α-BST2. Sapoznikov *et al*. used transgenic CD11c-DTR mice that permit ablation of cDCs but not pDCs by diphtheria toxin (DTx) injection. They claimed that after injection of DTx all observed T cell responses to systemically administered antigen are mediated by pDCs. In this experimental setting no CD8+ T cell activation was observed. However, there have been growing doubts that injection of DTx really only depletes cDCs in the described CD11c-DTR mouse (personal communication). It is possible that pDCs were also depleted to some extent and therefore no CD8+ T cell responses were observed. Moreover, Sapoznikov *et al*. used CpG as adjuvant in their study. Administration of CpG leads to less IFN I production compared to stimulation with PolyI:C. A systemic IFN I response is however critical for the induction of CD8+ T cell responses (Le Bon *et al*., 2006; Le Bon *et al*., 2003). An alternative explanation for the disparate results would be that interactions of pDCs and cDCs in secondary lymphoid organs are required to enable pDCs to fulfill their entire activity. These interactions were prevented in cDC depleted mice.

5.2 Influence of antigen presenting pDCs on antigen specific immune responses

Administration of pDC specific antibody-antigen fusion proteins resulted in a dose dependent proliferation of antigen specific T cells *in vivo*. Proliferation was enhanced after antigen was delivered to pDCs in the presence of CpG 1668. PDCs in the steady state only express low levels of co-stimulatory molecules. The increase in T cell proliferation after pDC maturation reflects the common model that after upregulation of co-stimulatory molecules (e.g. CD86) stronger T cell responses can be induced. These results confirm that antigen presenting pDCs are able to prime naïve antigen specific T cells *in vivo* (Bjorck *et al*., 2008; Villadangos and Young, 2008). Although it was shown that pDCs can prime antigen specific naïve T cells after antibody mediated antigen delivery *in vivo*, cDCs are clearly more potent in inducing T cell responses to soluble antigen (Young *et al*., 2008). The main role of pDCs might therefore rather be to influence antigen specific T cell responses and

direct them into a certain direction instead of initiating them (Krug *et al*., 2003). To investigate how antigen presenting pDCs can influence an antigen specific T cell response that was initiated by other APCs, soluble OVA was administered simultaneously with α-Siglec-H-OVA and α-BST2-OVA, respectively. Co-administration of soluble antigen and pDC specific antibody-antigen fusion proteins revealed that administration of α-Siglec-H-OVA dampened proliferation of OVA specific T cells in response to soluble OVA whereas α-BST2-OVA further increased the proliferative response. Fig. 47 illustrates the diverse influence of antigen presenting pDCs, dependent on whether the antigen was delivered with α-Siglec-H or α-BST2. On the basis of these results, the ability of α-Siglec-H mediated antigen delivery to suppress effector T cell responses as well as the potential usefulness of antigen fused to α-BST2 to induce effector T cell responses was further investigated.

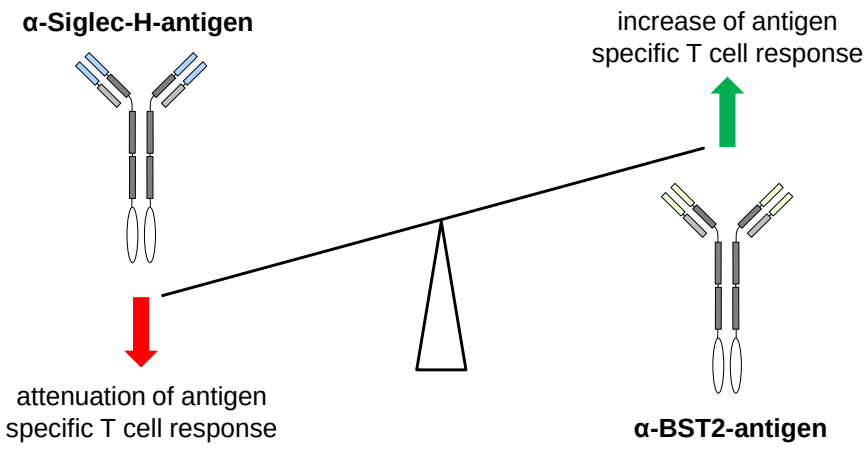

Fig. 47: Contrary T cell responses after antigen delivery to pDCs *via* Siglec-H and BST2.
Administration of α-Siglec-H-OVA is able to suppress OVA specific T cell responses, whereas antigen delivery to pDCs *via* BST2 increases antigen specific T cell responses to soluble OVA.

5.2.1 Attenuation of effector T cell responses by antigen targeting to pDCs *in vivo*

It has been shown previously in different studies that antigen presenting pDCs can have tolerogenic effects *in vivo* in several murine disease models including asthma, allogeneic transplant rejection and autoimmune diseases such as type I diabetes, systemic lupus erythematosus, rheumatoid arthritis and experimental autoimmune encephalomyelitis (de Heer *et al*., 2004; Hadeiba *et al*., 2008; Irla *et al*., 2010; Kang *et al*., 2007; Nikolic *et al*., 2009; Ochando *et a*l., 2006). In addition, pDCs in the liver

and mesenteric lymph nodes are required for oral tolerance induction (Goubier *et al*., 2008). It has however not been investigated so far, if targeting antigen specifically to pDCs is effective in inducing antigen specific tolerance, either by inducing the generation of adaptive Tregs (active tolerance) or by inducing T cell anergy, deletion or hyporesponsiveness (passive tolerance).

An active mechanism for the induction of adaptive tolerance mediated by pDCs can be excluded, as antigen delivery to pDCs failed to promote the expression of the key transcription factor Foxp3 in naïve T cells and the differentiation of antigen specific Tregs was not observed. The generation of IL-10 producing Foxp3- T cells with regulatory function (Tr1 cells) was not detected either. This failure in inducing active antigen specific tolerance might be due to the fact that pDCs are not constitutively producing TGF-β (Moseman *et al*., 2004). In contrast, CD8α+ DCs that are known to promote Foxp3 expression in the steady state were shown to secrete TGF-β (Yamazaki *et al*., 2008). Inefficient activation of the mTOR pathway in the presence of specific environmental cues and in the absence of TGF-β leads to induction of hyporesponsiveness or anergy in T cells while similar signals in the presence of TGF-β lead to the induction of Foxp3+ Tregs (Chappert and Schwartz, 2010). Studies that described elevated levels of Foxp3+ T cells after transfer of pDCs (Ochando *et al*., 2006) did not differentiate between *de novo* induction and expansion of natural Tregs. The present study clearly showed that antigen delivery to pDCs does not lead to *de novo* expression of Foxp3 in naïve T cells. Therefore, the elevated levels of Foxp3+ Tregs that were reported after pDC transfer are most likely the result of Treg expansion and not *de novo* generation.

Although antigen presenting pDCs did not promote the differentiation of Foxp3+ Tregs, antigen delivery to pDCs *via* Siglec-H was able to dampen antigen specific CD4+ T cell responses to soluble antigen even in the presence of TLR9 ligand CpG 1668. Analysis of the density and the stability of peptide/MHC II complexes revealed that only low levels of peptide/MHC II were present on the surface of pDCs after antigen had been delivered with α-Siglec-H. However, these complexes were stably presented for up to 8 days. Thus, long lasting low level antigen presentation by pDCs after antigen delivery to Siglec-H correlated with attenuated CD4+ T cell expansion, even in the presence of a proinflammatory stimulus.

It has been shown in several reports that continuous or repeated administration of low doses of peptides without adjuvant induces immune tolerance (Apostolou and von Boehmer, 2004; Kang *et al*., 2007; Verginis *et al*., 2008). It has also been reported that low level antigen presentation by DCs in the absence of co-stimulation can lead to hyporesponsiveness of antigen specific CD4+ T cells after initial proliferation (Kawahata *et al*., 2002). Moreover, a transgenic model that allows inducible expression and presentation of antigen in DCs revealed that persistent antigen presentation rapidly induces a dysfunctional state in CD4+ T cells (Han *et al*., 2010). Antigen persistence is necessary in the expansion phase of CD4+ T cells, however, a switch occurs later that turns the stimulatory TCR signal into an inhibitory one (Han *et al*., 2010). This impairment of CD4+ T cell responses is partially reversed after the persistent antigen presentation disappears (Han *et al*., 2010).

These observations are similar to the results that were obtained after antigen delivery to pDCs *via* Siglec-H. Adoptively transferred antigen specific T cells initially proliferated after antigen had been delivered to pDCs *via* Siglec-H. However, T cells were impaired to respond to an additional systemic antigen pulse when pDCs continuously presented low levels of antigen on MHC II during this stimulation. This was also the case in the presence of TLR9 ligand CpG 1668 which directly activates pDCs and induces a systemic immune activation. Thus, antigen delivery to pDCs *via* Siglec-H can be harnessed to attenuate antigen specific CD4+ T cell responses even in an activated environment that is usually more prone to mediate effector T cell responses than to promote tolerance.

The attenuation of antigen specific immune responses after antigen delivery to pDC *via* Siglec-H was also confirmed in naïve mice without transfer of antigen specific CD4+ T cells. Immunization with soluble OVA in combination with the TLR3/MDA5 ligand PolyI:C induced a strong OVA specific antibody response with different isotypes including isotype switched IgG2b and IgG2c. This indicated that a strong T helper cell response that is required for isotype switching was initiated (Boscardin *et al*., 2006; Stavnezer, 2000). The OVA specific antibody response was almost completely abrogated when mice were immunized with OVA/PolyI:C and simultaneously received α-Siglec-H-OVA. This result suggested that antigen delivery to pDCs *via* Siglec-H and the subsequent presentation of antigen on pDCs inhibited the generation of OVA specific effector T cells that were induced by immunization

with OVA/PolyI:C and are required for a strong antibody response (Boscardin *et al*., 2006). Antigen fused to α-DEC205 can be used to induce the differentiation of Foxp3+ Tregs in naïve mice and can be harnessed to mediate antigen specific tolerance (Yamazaki *et al*., 2008). However, in combination with an adjuvant such as PolyI:C, antigen fused to α-DEC205 does not mediate tolerance but instead promotes the differentiation of effector T cells and induces the production of high levels of antigen specific antibodies (Boscardin *et al*., 2006; Longhi *et al*., 2009). Thus, in contrast to α-DEC205, α-Siglec-H can also be used to suppress or inhibit endogenous immune responses in the presence of strong innate stimuli such as TLR ligands.

In accordance with these findings, it was shown that antigen delivery to pDCs with α-Siglec-H efficiently inhibited the expansion and differentiation of endogenous CD4+ helper T cells that were induced by peptide immunization with CFA and PTx. Reduced frequencies of CD4+ helper T cells producing IL-2, IL-17 and IFN-γ were observed when OVA was delivered to pDCs with α-Siglec-H the day before mice were immunized with pOVA/CFA/PTx compared to mice that were not pretreated with α-Siglec-H-OVA. A shift from T_H1/T_H17 T cell differentiation to IL-4 producing T_H2 or IL-10 producing Tr1 cells was not observed. This supports the hypothesis that the observed attenuated effector T cell responses were not the result of an active suppression but were the consequence of impaired helper T cell differentiation.

This impairment to generate antigen specific effector T cell responses correlated with a less severe progress of the CD4+ T cell mediated antigen specific autoimmune disease EAE after pMOG had been delivered to pDCs *via* Siglec-H. In the pMOG specific EAE model, administration of α-Siglec-H-pMOG the day before mice were immunized with pMOG/CFA/PTx successfully attenuated T cell mediated autoimmunity. Injection of α-Siglec-H-pMOG one week before mice were immunized with pMOG/CFA/PTx resulted in a delayed onset of the disease, but did not as efficiently reduce the incidence or severity of the disease as did administration of α-Siglec-H-pMOG 1 day before the immunization. Antigen delivery to pDCs *via* Siglec-H results in presentation of the antigen on MHC II and peptide/MHC II complexes were detectable on the surface of pDCs for up to 8 days. In mice that received pMOG fused to α-Siglec-H 7 days before they were immunized with pMOG/CFA/PTx, it is very likely that the antigen was not presented on the surface of pDCs for a long

enough time during T cell priming to successfully inhibit the development of pathogenic CD4+ T cells. Administration of α-Siglec-H-pMOG the day before the induction of EAE was however effective in inhibiting EAE, probably due to continuous presentation of antigen on pDCs during T cell priming which leads to T cell hyporesponsiveness. This again supports the hypothesis that pDCs actually have to present the antigen to suppress T cell responses and that the tolerogenic effects are not mediated by induced Tregs.

Irla *et al.* have shown that mice lacking expression of MHC II in pDCs develop a significantly more severe pMOG induced EAE than wildtype mice. They therefore postulated that pDCs present the autoantigen during EAE induction and this prevents pathogenic T cell responses. Although this result nicely correlates with the findings of this study, the conclusions that were made by Irla *et al*. are not fully substantiated. They could show without doubt that the lack of MHC II expression in pDCs resulted in a more severe disease after pMOG induced EAE. However, they miss to provide direct evidence that only the specific presentation of antigen by pDCs and not innate immune functions of pDCs mediated the observed effect. PDCs lacking MHC II expression were unable to efficiently interact with pMOG specific CD4+ T cells. Due to these less frequent pDC/T cell contacts, pDCs may not be able to transmit their innate immune signals to the CD4+ T cells and may fail to influence T cell priming in an antigen unspecific way. Therefore, the observed effect in mice lacking expression of MHC II in pDCs could be mediated, at least in parts, by an antigen unspecific contribution of pDCs. Targeting antigen to pDCs with α-Siglec-H is not accompanied by such limitations. The phenotype of pDCs is not changed and pDC/T cell interactions are not affected by the binding of α-Siglec-H to pDCs, whereas they are innately precluded in a system where pDCs do not express MHC II. Therefore, the delivery of antigen to pDCs with α-Siglec-H allows investigating the influence of antigen presenting pDCs in an experimental setting without any manipulations of the immune system such as depletion of distinct cell types or impaired expression of essential surface molecules such as MHC II.

The present study shows for the first time that antigen delivery to pDCs *via* Siglec-H is able to inhibit the generation of CD4+ effector T cells and can be used to dampen an antigen specific CD4+ T cell mediated autoimmune disease. This study provides clear evidence that antigen presenting pDCs fail to induce the *de novo* generation of

regulatory T cells. More likely, the continuous presentation of low levels of antigen on pDCs leads to hyporesponsiveness of antigen specific CD4+ T cells preventing further expansion and differentiation.

5.2.2 Induction of effector T cell responses by antigen targeting to pDCs *in vivo*

PDCs differ substantially from cDCs with regard to their antigen presentation function. PDCs even when activated by TLR ligands have a high turnover of peptide/MHC II complexes, whereas cDCs shut down the formation of new peptide/MHC II complexes upon activation and stabilize high levels of peptide/MHC II complexes on the cell surface, thus allowing stable contacts with naïve T cells (Young *et al*., 2008). Therefore, cDCs can effectively present antigens which are present for only a short period of time, whereas pDCs are specialized to present antigens on MHC II which are continuously internalized or generated intracellularly (such as during viral infections). Thus, pDCs that have long been believed to play a rather subordinate role might actually play a more prominent role as APC, especially during viral infections. PDCs constitute a major and well characterized subpopulation of DCs also in the human immune system, which are able to directly respond to TLR7 and 9 agonists. Therefore, targeting antigen to pDCs is an attractive and promising strategy for vaccinations to induce antigen specific immune responses also in humans. However, it has been shown in many studies that antigen presenting pDCs mediate tolerance instead of inducing protective immune responses. This has also been observed in this study after antigen had been delivered to pDCs with α-Siglec-H. Co-administration of soluble OVA and α-BST2-OVA was however followed by an increased T cell response compared to soluble antigen alone. This indicates that pDCs *per se* are able to induce effector T cell responses and do not necessarily mediate tolerance. It was therefore investigated, whether antigen fused to α-BST2 can be used to induce strong and protective adaptive immune responses.

Many previous studies that used α-DEC205 to deliver antigen to CD8α+ DCs used a stimulatory α-CD40 antibody to activate DCs (Boscardin *et al*., 2006; Idoyaga *et al*., 2011). Although α-CD40 is certainly a suitable tool to activate DCs and enhance effector T cell responses in mice *in vivo*, this antibody was not used as adjuvant in the present study. Potential devastating side effects of α-CD40 might prohibit the use

of α-CD40 in humans. The use of adjuvants was therefore restricted to more applicable substances such as TLR9 ligand CpG 1668 and TLR3/MDA5 ligand PolyI:C. These adjuvants are well characterized and were already tested for application in humans or in studies with non-human primates (Flynn *et al*., 2011; Klinman, 2006). Experiments using OVA specific transgenic CD4+ and CD8+ T cells revealed that both - CpG 1668 and PolyI:C - are qualified to boost antigen specific T cell responses after administration of α-BST2-OVA.

Immunizing mice with α-Siglec-H-OVA without adjuvant or in combination with CpG 1668 or PolyI:C failed to induce endogenous adaptive immune responses. This was in accordance with the finding that antigen delivery *via* Siglec-H dampens antigen specific immune responses. Administration of α-BST2-OVA only induced antigen specific immune responses in naïve mice when the antibody-antigen fusion protein was injected in combination with adjuvant. This was expected as naïve pDCs express lower levels of MHC class II and co-stimulatory molecules than cDCs (Young *et al*., 2008). Therefore, pDCs need to be stimulated either directly (e.g. TLR9 ligand CpG 1668) or indirectly (e.g. PolyI:C) to efficiently upregulate MHC II and co-stimulatory molecules.

Immunizing mice with α-BST2-OVA in combination with adjuvant using a prime boost protocol successfully induced antigen specific effector CD4+ T cells producing IFN-γ. This T_H1 commitment was expected as it has been described that a systemic type I interferon response is required for and followed by a strong T_H1 immune response (Longhi *et al*., 2009; Sano *et al*., 2003). PolyI:C and to a lower extent also CpG oligonucleotides provide this systemic IFN I response. The strong T_H1 responses were also reflected in the production of high titers of antigen specific antibodies with different isotypes, especially IgG2b and IgG2c which are known to be associated with T_H1 directed immune responses (Boscardin *et al*., 2006; Stavnezer, 2000). Administration of α-BST2-OVA in the presence of adjuvant also successfully induced the generation of activated CD8+ T cells that acquired cytolytic function and efficiently killed antigen pulsed target cells *in vivo*. The finding that only immunization with α-BST2-OVA/PolyI:C but not α-BST2/CpG efficiently induced CTL responses is in accordance with reports showing that a strong type I interferon response is required to induce strong CD8+ T cell responses (Le Bon *et al*., 2006; Le Bon *et al*.,

2003). Administration of PolyI:C is known to induce high levels of IFN I and is therefore a qualified adjuvant to promote CD8+ T cell activation (Kato *et al*., 2006).

The evidence that murine pDCs can cross-present antigen that was delivered *via* BST2 *in vivo* and moreover are able to activate antigen specific CD8+ T cells was adduced for the first time in this study. As mentioned before, Sapoznikov *et al*. claimed that pDCs cannot prime CD8+ T cells. However, the requirement of pDCs for the induction of CD8+ T cell proliferation after antigen delivery *via* BST2 was clearly demonstrated in the present study. CD11c+ DCs isolated from a mouse that was immunized with α-BST2-OVA/PolyI:C mediated OT-I T cell proliferation. In contrast to that, CD11c+ DCs isolated from a mouse that was immunized with α-BST2-OVA/PolyI:C and was treated with α-Gr-1 to deplete pDCs *in vivo* failed to induce OT-I T cell proliferation.

The described adaptive immune responses triggered by pDCs were able to mediate protection from severe loss of body weight after infection with a vaccinia virus strain encoding OVA. This model has been used previously to test the quality of adaptive immune responses that were elicited by targeting antigen to CD8α+ DCs with α-DEC205 (Bonifaz *et al*., 2004). The observation that mice that were immunized with α-BST2-OVA/PolyI:C lost significantly less body weight than mice that only received PolyI:C correlated with significantly higher levels of antigen specific CD4+ and CD8+ T cells producing effector cytokines (IL-2, IFN-γ). A definite conclusion about whether the protection was mediated mainly by OVA specific CD4+ or CD8+ T cells cannot be drawn. It is most likely that both T cell subsets contributed significantly to the protection as it has been described for wildtype vaccinia virus (Xu *et al*., 2004).

The strength of the adaptive immune response that was elicited by antigen delivery to pDCs *via* BST2 was also tested in an antigen specific tumor model. OVA expressing melanoma cells were implanted into mice that had been immunized with α-BST2-OVA/PolyI:C or PolyI:C alone. Mice that received only the adjuvant developed much faster growing tumors than mice immunized with α-BST2-OVA/PolyI:C and died or had to be sacrificed much earlier. These results show that the CD4+ and CD8+ T cell responses that were induced after immunization with α-BST2/PolyI:C are capable to provide protection against a rapidly growing tumor.

Targeting antigen to pDCs in combination with adjuvant is an attractive tool to develop new vaccination strategies that aim to induce strong and protective T cell responses. The capability of antigen specific antibodies that were produced after antigen delivery with α-BST2 to mediate protection was not investigated in this study, because appropriate pathogens expressing OVA on their surface were not available. Fusion of proteins to the C-terminus of α-BST2 that are present on the surface of vaccinia virions or *Chlamydia trachomatis* failed, because expression of those recombinant α-BST2-antigen fusion proteins was not possible most likely due to steric hindrance and subsequent disruption of the tertiary structure of the protein. However, antibody responses could be exploited to mediate protection against viral infections. Fusion of virus specific antigens (e.g. B5R from vaccinia virus, hemagglutinin from influenza virus) to pDC specific antibodies might therefore be an attractive strategy to mediate full protection, because blocking antibodies could additionally prevent binding of virus particles to target cells. It is clearly beneficial for the success of a vaccine to induce both - cellular and humoral immunity. The only obstacle that has to be overcome is to find a protein sequence that contains relevant T and B cell epitopes and can be fused to antibodies without interfering with proper protein folding.

5.3 Differences of α-Siglec-H and α-BST2 mediated antigen delivery

The conflicting results regarding the influence of pDCs on adaptive immune responses that were obtained after antigen delivery *via* Siglec-H and BST2 were surprising, as both antibodies were shown to deliver the antigen specifically to pDCs and a role for other APCs could be excluded. However, despite the extensive use of different antibodies to deliver antigen to distinct cell types, few studies have actually compared antibodies that deliver antigen to the same cell type but use different receptors. One recent study compared antigen delivery to CD8α+ DCs *via* DEC205, Clec9A and Langerin (Idoyaga *et al*., 2011). In this study, comparable T_H1 and CD8+ T cell responses were observed after HIV gag p24 had been targeted to CD8α+ DCs using antibodies for DEC205, Clec9A and Langerin. It was however not investigated, whether the density of peptide/MHC complexes or the duration of antigen presentation differed after the antigen had been delivered to CD8α+ DCs with the described antibodies. Similar amounts of antigen presented and a comparable

stability of the antigen/MHC complexes could account for the similar immune responses.

The amount of peptide/MHC II complexes on the surface of pDCs and also the stability of those complexes differed significantly dependent on whether the antigen was delivered with α-Siglec-H or α-BST2. Low levels of peptide/MHC II complexes were detected after antigen delivery *via* Siglec-H. Those complexes were however stable and present for up to 8 days after a single administration of antigen fused to α-Siglec-H. In contrast, antigen that was fused to α-BST2 was presented to a higher extent, but antigen/MHC II complexes were not very stable and disappeared about 48 h after the administration of α-BST2-antigen fusion proteins. It was not further explored, whether the different routes of antigen delivery (Siglec-H *vs*. α-BST2) were the only cause for the observed disparities in MHC II restricted antigen presentation. It is possible that binding of α-Siglec-H and α-BST2, although it did not affect the activation state of pDCs, changes the process of antigen trafficking, processing and presentation within the cell. Among those possible changes could for example be differences in the ubiquitination of MHC II molecules. It has been shown that differences in the ubiquitination of MHC II account for the different antigen presenting features of pDCs and cDCs (Young *et al*., 2008). However, more likely, the differences between antigen delivery *via* Siglec-H and BST2 become noticeable earlier. BST2 is expressed at higher levels compared to Siglec-H. This results in a faster uptake of the antigen, because more α-BST2 molecules can bind to a single cell compared to α-Siglec-H. This faster ingestion of antigen may result in the formation of more peptide/MHC II complexes and the shortened presence of those complexes on the cell surface, because a faster uptake is also followed by a faster usage of the limited amount of α-BST2-antigen fusion proteins.

Since pDCs have a high turnover of peptide/MHC II complexes, it might also be possible that Siglec-H mediated antigen delivery provides a long lasting intracellular source of antigen for the continuous formation of new peptide/MHC II complexes to maintain a constant level of peptide/MHC II on the cell surface. Possibly, antigens are delivered to a less degradative compartment after they have been targeting to Siglec-H compared to BST2. Long lasting, low level antigen presentation by pDCs after antigen delivery to Siglec-H correlated with attenuated T cell responses, even in the presence of a proinflammatory stimulus, whereas a short but intense antigen

pulse that followed antigen delivery *via* BST2 was able to mediate protective immunity when the α-BST2-antigen fusion protein was administered with adjuvant. These data raised the hypothesis that differences in intracellular antigen trafficking, processing and loading onto MHC II result in a significant different presentation of antigen on MHC II that is fused to α-Siglec-H or α-BST2. This differential MHC II antigen presentation in turn accounts for the contrary T cell responses after antigen delivery to pDCs *via* Siglec-H and BST2.

5.4 The appeal of antigen delivery to pDCs for applications in humans

In mice, especially CD8α+ DCs have been used in many studies to induce strong and protective antigen specific adaptive immune responses. A human equivalent of these cells was recently described (Poulin *et al*., 2010). Although those cells might be an attractive target for antigen delivery in humans, using pDCs as target cells has several advantages. In humans, pDCs occur in much higher frequencies in the blood as well as in the spleen than the described BDCA3+ DCs (Mittag *et al*., 2011). Another advantage of antigen targeting to pDCs *versus* cDCs is the lower expression of co-stimulatory molecules and lack of peptide/MHC II stabilization in response to stimulation. This may allow tolerance induction even in the context of inflammation, when low level persistent antigen presentation is achieved by the targeting strategy. In contrast to that, antigen delivery to human pDCs leading to a high density of peptide/MHC II complexes in combination with a potent adjuvant could be used to induce protective adaptive immune responses.

In contrast to murine pDCs, Siglec-H is not expressed on human pDCs. As suitable alternative, the pDC specific surface molecule BDCA-2/CD303 could be used to deliver antigen specifically to pDCs, although it remains to be determined if the ensuing T cell response would be tolerogenic or immunogenic. In contrast to Siglec-H, BST2 is expressed also on human pDCs. However, its expression is not restricted to pDCs, but also other human immune cells express BST2. Nevertheless, it might be a useful target for antigen delivery to human DCs. Antibodies directed against human BST2 have been described to enhance type I IFN and proinflammatory cytokine responses of pDCs by interrupting interaction of BST2 with ILT7 (Cao *et al*., 2009). This might be beneficial for the use of α-BST2 in vaccination approaches.

5.5 Model for the role of pDCs for the induction of antigen specific immune responses

The quintessence of this study is summarized and illustrated in the model depicted in Fig. 48. The common understanding is that immature cDCs in the steady state can mediate tolerance by inducing the differentiation of Tregs. In the presence of danger signals, such as PAMPs, cDCs undergo several changes and become mature and professional APCs that are very potent in inducing effector T cell responses (CD4+ and CD8+). In the depicted model, pDCs are not able to induce the differentiation of Tregs. PDCs can however suppress effector T cell responses when antigen is presented at low levels for a long period of time, as it is the case after antigen delivery with α-Siglec-H. However, pDCs are also very efficient in mediating effector T cell responses when high levels of antigen are presented and the antigen is administered in combination with a strong adjuvant. α-BST2 mediated antigen delivery fulfills these requirements and antigen fused to α-BST2 can induce strong effector T cell responses (CD4+ and CD8+) when administered in combination with TLR3/MDA5 ligand PolyI:C. Thus, pDCs similar to cDC subpopulations can be harnessed to modulate antigen specific immune responses. This strategy might also be useful in humans where pDCs constitute a major subpopulation of DCs.

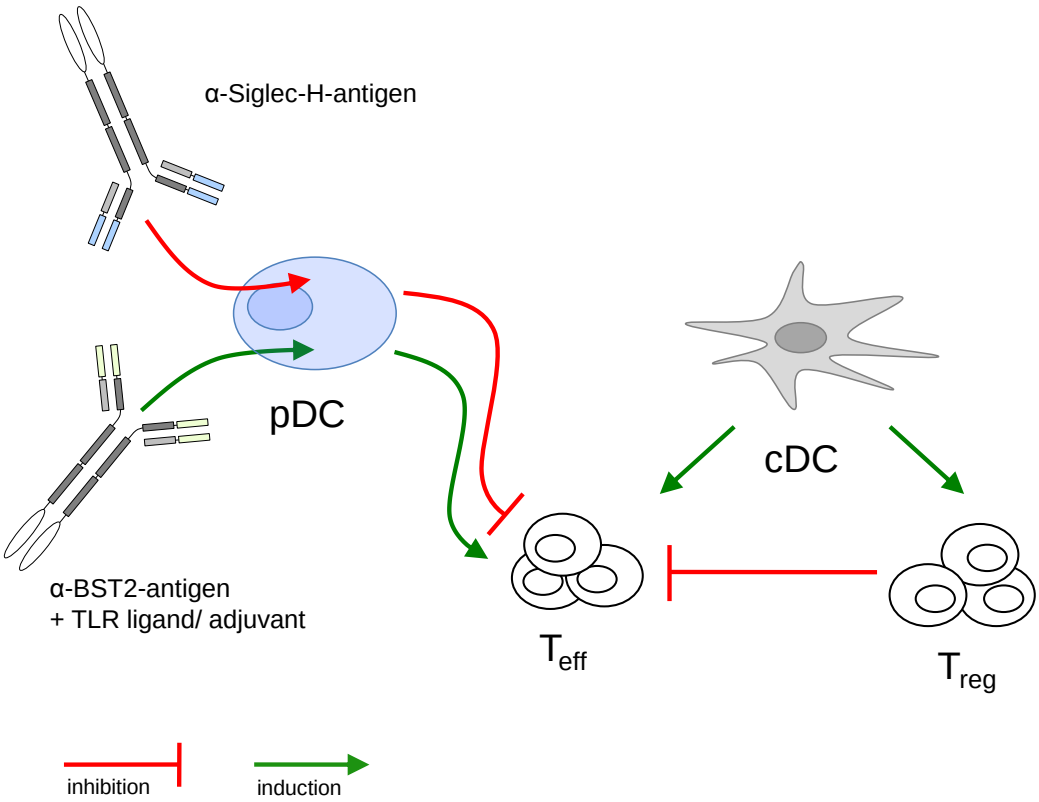

Fig. 48: Model describing the role of antigen presenting pDCs in influencing T cell responses.

CDCs are able to mediate tolerance by inducing the expression of Foxp3 and the differentiation of Tregs. Beyond that, activated and mature cDCs can induce the differentiation of effector T cells (CD4+ and CD8+). In contrast to cDCs, pDCs are unable to induce the differentiation of Tregs. However, pDCs that present antigen at low levels for a long period of time (e.g. antigen delivery *via* Siglec-H) can suppress effector T cell differentiation and mediate tolerance independently of Tregs. Antibody mediated antigen delivery *via* BST2 that results in a short but intense period of antigen presentation can induce strong and protective effector T cell responses (CD4+ and CD8+) when the antigen is injected in combination with adjuvant (e.g. CpG 1668 or PolyI:C).

6 SUMMARY

To investigate the influence of plasmacytoid dendritic cells (pDCs) on the initiation of adaptive immune responses, recombinant antibodies to Siglec-H (sialic acid binding Ig-like lectin H) and BST2 (bone marrow stromal cell antigen 2) were generated that enable the delivery of antigen to murine pDCs *in vivo*. Delivery of antigen to pDCs with α-Siglec-H and α-BST2 resulted in specific presentation of the antigen by pDCs.

Co-administration of soluble antigen and antigen fused to α-Siglec-H and α-BST2, respectively, revealed that delivery of antigen to pDCs *via* Siglec-H dampens antigen specific CD4+ T cell responses, whereas antigen delivery *via* BST2 further enhances these T cell responses. Therefore, it was investigated whether administration of antigen fused to α-Siglec-H can be used to prevent pathogenic CD4+ T cell responses and in addition whether antigen delivery to pDCs *via* BST2 can induce protective immunity.

Delivery of a peptide derived from myelin oligodendrocyte glycoprotein (MOG) to pDCs *via* Siglec-H significantly attenuated antigen specific effector T cell responses. This attenuation correlated with greatly reduced disease severity in a murine model of multiple sclerosis. This effect was not mediated by regulatory T cells but due to T cell hyporesponsiveness following a long lasting antigenic signal provided by pDCs after antigen delivery *via* Siglec-H. In contrast to that, antigen delivery to pDCs with α-BST2 resulted in a strong antigen signal for a shorter period of time on pDCs. This antigen pulse was able to successfully induce CD4+ and CD8+ effector T cell responses when α-BST2-antigen fusion proteins were co-administered with adjuvant (TLR9 agonist CpG 1668 or TLR3/MDA5 agonist PolyI:C). These effector T cell responses were able to mediate protection in a viral infection model as well as in an antigen specific tumor model.

Although pDCs constitute only a small cell population within all antigen presenting cells, the present study shows for the first time that antigen delivery to murine pDCs can be used to tune antigen specific T cell responses. Targeting antigen to pDCs can prevent autoreactive CD4+ T cells responses, but in the context of adjuvant stimulation can also be used to induce antigen specific protective immunity.

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CURRICULUM VITAE

Jakob Loschko

Personal data

Date of birth	7. November 1981
Place of birth	Gräfelfing, Germany

Education

Since 12/2007	PhD thesis in the laboratory of PD Dr. Anne Krug, II. Medical Department, Klinikum rechts der Isar, Technical University Munich, Germany
10/2002- 11/2007	Studies of biology at the University of Regensburg, Germany
01/2007- 11/2007	Diploma thesis in the laboratory of Prof. Dr. Susanne Modrow , Institute for Medical Microbiology and Hygiene, University of Regensburg, Germany
11/2006	Final examinations, Diploma (1.0)
08/2005- 06/2006	Independent studies in the laboratory of Dr. Jennifer Martin, Department of Molecular Cellular und Developmental Biology, University of Colorado at Boulder, USA
09/2004	Intermediate diploma (2.0)
08/2001-06/2002	Civilian service
1992- 2001	Secondary school (Abitur 2.2) Otto-von-Taube Gymnasium, Gauting, Germany

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Congress Abstracts

Keystone Symposia ”Dendritic cells and the initiation of adaptive immunity“, Santa Fe, USA (11.2.2011 - 17.2.2011)

Loschko Jakob, Heink Sylvia, Hackl Daniela, Dudziak Diana, Reindl Wolfgang, Krug Anne. Targeting antigen to plasmacytoid dendritic cells via Siglec-H prevents disease in a murine model of multiple sclerosis. (poster)

World Immune Regulation Meeting, Davos, Switzerland (29.03.10 - 01.04.10)

Loschko Jakob, Dudziak Diana, Reindl Wolfgang, Krug Anne. Antigen targeting to plasmacytoid dendritic cells and their influence on tolerance and immunity. (talk)

Toll2008 Meeting, Lisbon, Portugal (24.09.2008 - 27.09.2008)

Jakob Loschko, Emina Savarese, Christian Steinberg, Rahul D. Pawar, Wolfgang Reindl, Hans-Joachim Anders, Anne Krug. Role of TLR7 and IRAK1 for Pristane-induced Autoantibody Production and Development of Lupus Nephritis. (poster)