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The influence of the staphylococcal Extracellular Adherence Protein on T cells functions *in vitro* in the context of the psoriasiform skin disease

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ABBREVIATIONS

Ab	antibody
APC	antigen presenting cell
BalB/C	inbread mouse strain
BCR	B cell receptor
°C	degree Celsius
CD	cluster of differentiation
$CD18^{hypo}$	$CD18^{hypomorphic}$ mutation in (tgb1<tm1Bay>) PL/J mice
$CD18^{wt}$	$CD18^{wildtype}$ PL/J mice
C57BL/6J	indicated mouse strain
CFSE	5- (and 6-) carboxyfluorescein diacetate succinimidyl ester
c.m.	complete medium
CO_2	Carbon dioxide
DC	dendritic cell
DMSO	Dimethylsulfoxide
EAP	Extracellular Adherence Protein
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorting
Fig.	Figure
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box proteine 3
GM-CSF	Granulocyte-macrophage colony stimulating factor
ICAM-1	Intercellular adhesion molecule-1 (CD54)
ICN	Initial cell number
IFN-γ	Interferon gamma
IL	Interleukin
iNOS	inducible nitric oxide synthase
kDa	kiloDalton
LFA-1	Lymphocyte function-associated antigen-1
mAB	monoclonal Antibody
Map	MHC II analogous protein
MHC	Major histocompatibility complex
µl/ml/µg/mM	micro liter/milli liter/micro gram/milli molar

min	minute
NF-κB	Nuclear factor-kappa B
NK cells	natural killer cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PL/J	inbread mouse strain
PRR	pathogen recognition receptor
PSOR	psoriasis susceptibility locus
rpm	rotations per minute
RT	room temperature
SA	Staphylococcus aureus
sec	seconds
SD	standard deviation
TCR	T cell receptor
TGF-β	Tissue growth factor beta
T_h cell	helper T cell
TNF-α	Tumor necrosis factor alpha
T_{resp}	responder T cell
T_{regs}	regulatory T cells
U	units
UV	ultraviolet
VCAM	vascular cell adhesion molecule
w/o	without

1. INTRODUCTION

Psoriasis is a very common chronic, genetic and inflammatory disease with a prevalence of 2 to 3 percent worldwide (National Psoriasis Foundation; http://www.psoriasis.org/learn_statistics). Affecting the skin and the joints, this autoimmune disease is appropriate for research due to the easy access to samples of affected skin. Thus, in the past years concomitant clinical, experimental and genetic findings have majorly contributed to the better understanding of the complex pathophysiology of psoriasis, leading to the concept of a 'T cell mediated disease' (Kess et al. 2003; Lew et al. 2004; Nestle et al. 2009; Nickoloff and Wrone-Smith 1999; Nograles et al. 2011; Sugiyama et al. 2005; Wang et al. 2008). However, the intimate mechanisms underlying psoriasis pathogenesis are still to be deciphered and efficient therapeutic tools targeting pathogenic T cells need to be developed. This thesis addresses the question of whether the staphylococcal Extracellular Adherence Protein (EAP) would modulate the interaction between different cell-types of the immune system relevant for the psoriasis pathogenesis *in vitro*, as EAP was previously found to resolve the psoriasiform skin disease *in vivo*, in the $CD18^{hypo}$ PL/J mouse model (Wang et al. 2010). The following chapters will introduce relevant topics for our research such as the immunology of psoriasis and the biology of EAP.

1.1. Immunology

There are two different subtypes of immune defence mechanisms which are supposed to prevent from severe infections by killing pathogens: the innate immunity and the adaptive immunity.

The innate immune defence is rather unspecific and effected mainly by granulocytes and macrophages (phagocytes) which engulf and destroy pathogens. For this, mechanisms such as recognition of pathogen-associated molecular patterns (PAMPs), which are present on most microorganisms, or pathogen opsonisation via the unspecific activation of complement are employed (Janeway 2008a). Activated phagocytes release cytokines which induce vasodilatation and

increased blood flow allowing further recruitment of T lymphocytes to the inflammatory site, thereby initiating the more specific adaptive immune response. T cell recruitment is additionally enabled by the upregulation of adhesion molecules such as the intercellular adhesion molecule-1 (ICAM-1) or the vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells, which allows leukocyte binding to endothelial cells and mediates extravasation of leukocytes to affected inflammatory sites, as for example to the skin in psoriasis (Janeway 2008a).

The adaptive immune system is more antigen-specific than the innate immune system. Antigens are processed and presented to lymphocytes by antigen presenting cells, mainly dendritic cells and macrophages. Efficient presentation of foreign antigens requires the direct contact between lymphocytes and dendritic cells. This includes the formation of the ‘immune synapse’ consisting of the T-lymphocyte- (or B-lymphocyte-) receptor (TCR, BCR) on the one hand and the major histocompatibility complex (MHC) localized on the antigen presenting cell (APC) on the other hand. Another two co-stimulatory signals between CD40 and CD40L (CD154) and between CD28 and CD86 respectively, as well as additional binding of LFA-1 to ICAM-1 are required for proper lymphocyte activation (Fig. 1).

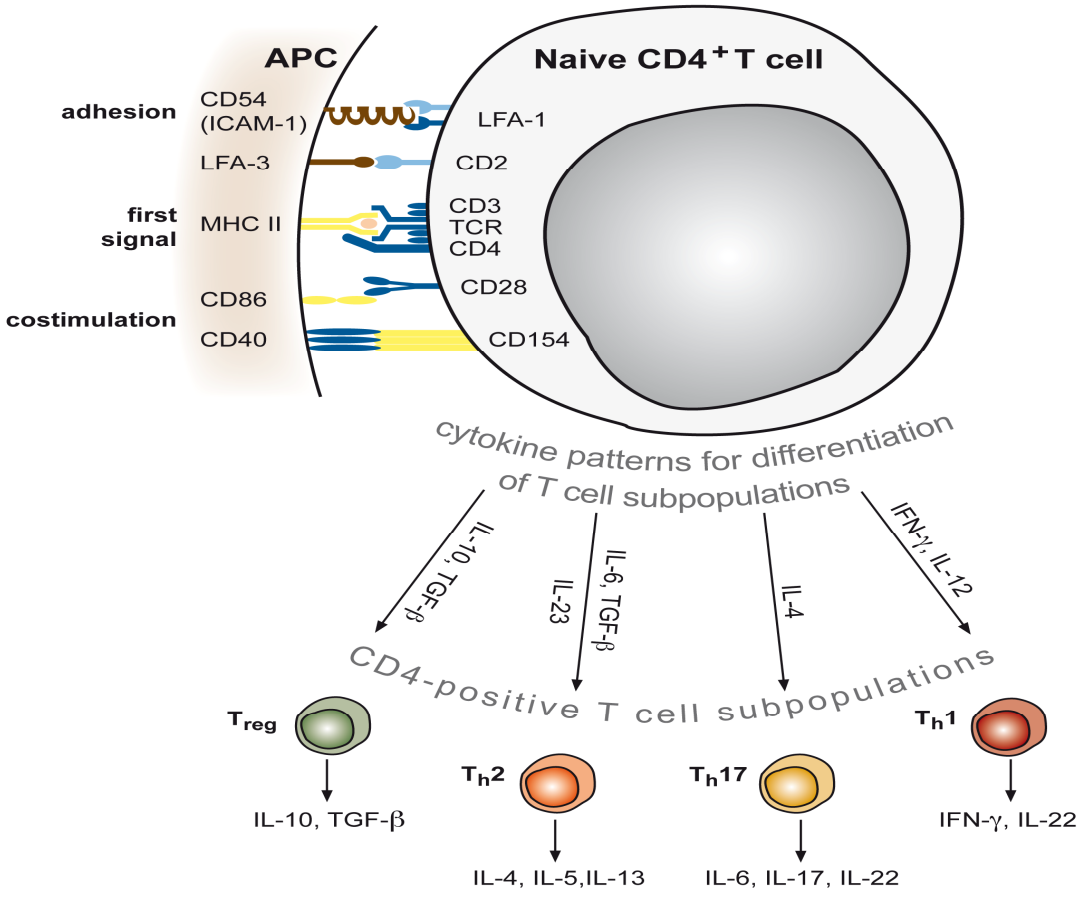

Fig. 1: **The activation and differentiation of T cells is induced by different signalling cascades**. *Binding of the T cell receptor complex (consisting of CD3, CD4 and TCR) to the specific peptide-MCH II molecule complex is the first obligatory signal. The two additionally required, co-stimulatory signals are provided by CD154-binding to CD40 and by CD28-binding to CD86. LFA-1-binding to ICAM-1 (CD54) additionally strengthens cell-cell contact. After activation, naive T cells differentiate into T cell subsets. Differentiation depends on APC-provided stimulatory cytokine patterns. Differentiated T cell subpopulations execute their special functions mainly by cytokines as well. (adapted from (Sabat et al. 2007))*

T and B lymphocytes acquire their antigen specificity by bearing unique sequences in their binding sites, differing from all other TCRs and BCRs. By somatic recombination, the adaptive immune system is able to produce a virtually unlimited number of T and B cell clones, specific to any antigen. T cells are 'educated' to recognize foreign antigens mainly in the thymus. The differentiation between self and foreign antigens is essential for mounting adequate immune responses and for the recognition of self-antigens as non-self leads to pathologic autoimmune reactions, as occurring in psoriasis (Janeway 2008b).

1.2. $CD4^+$ T helper cells

Psoriasis is regarded as an autoimmune disease with inflammation caused predominantly by autoreactive T_h1 cells. Contact to antigen presenting cells (APCs) and the influence of cytokines -released by APCs- are required to activate $CD4^+$ T cells and to give signals for further differentiation into different T cell subsets as T_h1, T_h2, T_h17 or into T_{reg} cells (Farrar et al. 2002; Janeway 2008a; Murphy and Reiner 2002).

The main features of different T cell subsets will be addressed in the following chapters.

1.2.1. T_h1 cells

T_h1 cell differentiation and proliferation requires the 'type 1' cytokine IL-12 which is mainly liberated by APCs, macrophages and NK cells, activated during immune responses against viruses and intracellular pathogens (Farrar et al. 2002; Murphy and Reiner 2002). Reciprocally, T_h1 cells are able to enhance macrophage

recruitment, maturation and activation by releasing IFN-γ, GM-CSF and TNF-α. IFN-γ is the signature cytokine of T_h1 cells which promotes the further T_h1 differentiation directly and by stimulating IL-12 production by macrophages (Boehm et al. 1997; Szabo et al. 2003). TNF-α released by macrophages and T_h1 cells induces the upregulation of adhesion molecules on endothelial cells such as VCAMs and ICAMs which bind to their counterpart integrins on leukocytes to promote their further recruitment to the inflammation site and enhanced bacterial killing (Choi et al. 2004; Pribila et al. 2004). To combat pathogens, activated macrophages and T_h1 cells release high amounts of reactive oxygen species (ROS), proteases and nitric oxide (NO) at the site of inflammation, resulting in a toxic environment for the intact tissue as well. Therefore, a well-regulated immune response to pathogens is absolutely required to avoid the damage of healthy tissue. This fine-regulation is imbalanced in autoimmunity where an exacerbated type 1 activation of leukocytes represents an important pathogenic cue in many autoimmune diseases, such as rheumatoid arthritis, autoimmune colitis and of course psoriasis (Sakaguchi et al. 1995).

1.2.2. T_h2 cells

Induction of T_h2 cell differentiation and proliferation requires 'type 2' cytokines such as IL-4. IL-4 is usually liberated by activated mast cells and differentiated T_h2 cells in response to helminths and allergens. T_h2 cells secrete IL-4 which acts on B cells to stimulate the production of IgE antibodies which bind to mast cells (Farrar et al. 2002; Murphy and Reiner 2002). The interaction with B cells requires the direct cell-cell contact which depends on ICAM-1 binding to LFA-1. The functions of these receptors will be detailed in chapter 1.4. Contact between T_{resp} cells and B cells additionally requires binding of the T cell receptor to the peptide which is presented by the MHC II molecule on B cells. Co-stimulation is also essential and is accomplished by the interaction between CD40 and the CD40-Ligand. This interaction together with the production of the T_h2 type cytokines IL-4 and IL-5 induce the transformation of B cells into antibody-secreting plasma cells which effect allergic responses and the defence against helminth and parasites (Janeway 2008b).

1.2.3. T_h17 cells

T_h17 cells are a subgroup of $CD4^+$ T cells which have been more recently discovered (Park et al. 2005) and have to be mentioned as they seem to play a distinguished role in inflammatory diseases (Hirota et al. 2011; Pene et al. 2008) and especially in psoriasis (Harper et al. 2009; Lowes et al. 2008). They were named by their most relevant secreted factor IL-17 that attracts and activates neutrophile leukocytes, especially in chronic inflammation (Janeway 2008b). Induction of T_h17 cells requires TGF-β and IL-6.

1.2.4. T_{reg} cells

Regulatory T cells (T_{regs}) were named according to their capacity of regulating T cell response at the site of inflammation. They are attracted and induced by APCs through TGF-β release in combination with antigen presentation. At the same time they suppress APCs via negative feedback. Their main suppressive function though is exerted on responder T cells in order to maintain peripheral self tolerance and to limit inflammation. T_{reg} activation was found to be primarily dependent on cell contact with APCs (Sakaguchi 2004), whereas the release of TGF-β and IL-10 cytokines was found to play an essential role for their suppressive functions (Toda and Piccirillo 2006). However, the various functions and the mechanisms of suppression still remain unclear. Reduction of IL-2 secretion, down regulation of MHC molecules and co-stimulatory signals may be part of these complex mechanisms (Thornton and Shevach 1998). The expansion and thereby the ability of T_{reg} cells to induce immunosuppression seems to require a higher affinity of T_{reg} cells to their specific peptide-MHC II molecule complex on the APC in comparison to T_{resp} cells (Wang et al. 2008; Wang et al. 2010).

T_{reg} cells characteristically express high levels of Foxp3, a transcription factor which is essential for T_{reg} cell differentiation, maintenance and function. Mutations in the Foxp3 gene as occurring in patients who suffer from the autoimmune-linked IPEX syndrome (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked), result in the development of various autoimmune diseases such as diabetes mellitus type 1, thyroiditis and autoimmune colitis due to decreased

numbers of T_{reg} cells in these patients (Bennett et al. 2001; Wildin et al. 2001). Ectopic expression of Foxp3 in $CD4^+CD25^+$ T cells conferred suppressive functions on T_{resp} cells *in vitro*, whereas reconstitution of neonatal Foxp3 deficient mice with wildtype $CD4^+CD25^+$ T_{reg} cells prevented from the development of autoimmune inflammation *in vivo* (Fontenot et al. 2003). These experimental findings highlight the importance of $Foxp3^+$ T_{reg} cells in suppression of uncontrolled inflammation during autoimmunity. However, it has to be mentioned that Foxp3 may also be expressed by activated $CD4^+$ T cells without regulatory/suppressive function on T_{resp} cells. Therefore, at least in pathologic inflammatory conditions, Foxp3 expression is not a reliable marker for the exclusive identification of T_{reg} cells (Allan et al. 2007). On the other hand, activated T_{resp} cells express Foxp3 only transiently in contrast to T_{reg} cells which constantly express Foxp3 at high levels (Wang et al. 2008). Another strategy to accurately distinguish between T_{regs} and T_{resp} cells is to characterize $CD4^+$ T cell populations according to CD127 expression. CD127, the α-chain of the IL-7 receptor, was highly expressed on T_{resp} cells (including $CD25^+$ T_{resp} cells) (Hartigan-O'Connor et al. 2007; Seddiki et al. 2006), thus negatively correlating with Foxp3 expression (Liu et al. 2006).

On average, about 9-12 % of T cells are T_{reg} cells. This ratio may vary, depending on the current challenges for the immune system. As previously described, autoimmunity is associated with a decreased number of T_{reg} cells, but a reduction of T_{reg} cells also occurs physiologically. For example, activation of Toll-like receptors by microbes on the DCs in the early phases of the adaptive immune defence requires a transitory suppression of T_{reg} cells in order to enable the successful initiation of pathogen-specific adaptive immune responses (Pasare and Medzhitov 2003) (Fig.2). Interestingly, the UV-A and UV-B light largely used in the therapy of psoriasis was shown to induce a decrease of T_{resp} cells but not of T_{reg} cells infiltrating the psoriasis plaques, which may mechanistically explain the high efficiency of phototherapy in psoriasis (Shehata and Elghandour 2007).

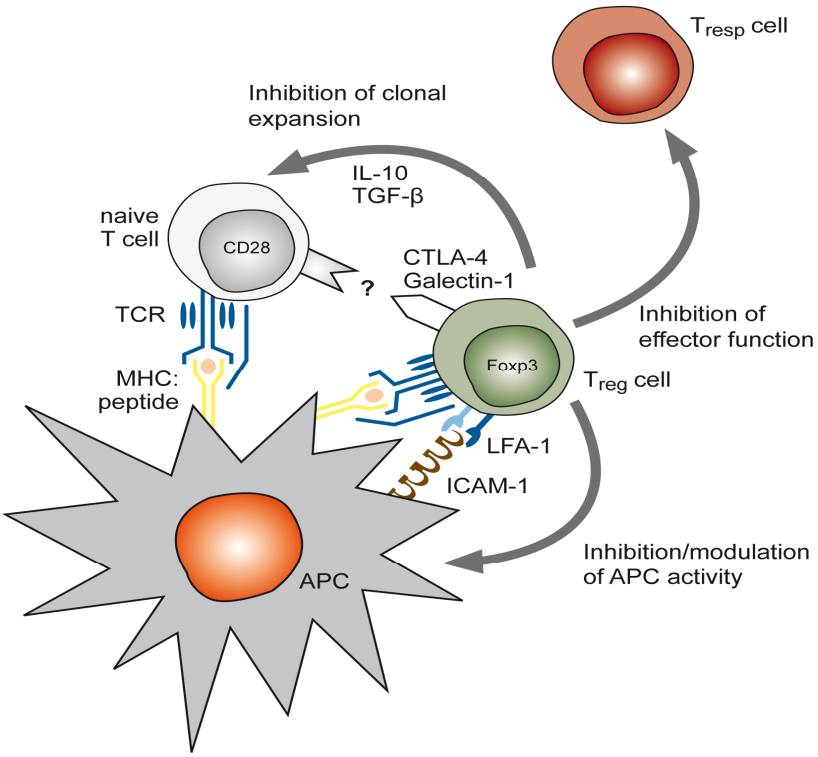

Fig. 2: **Interaction of T_{reg} cells with other cell types**. *APCs activate T_{reg} cells. T_{reg} cells suppress via cytokine release and cell-cell contact antigen presentation and the proliferation of T_{resp} cells, as well as the clonal expansion of naive T cells (adapted from (Mottet and Golshayan 2007)).*

Interestingly, latest research publications indicate that a subtype of Foxp3$^+$ T_{reg} cells from patients with severe psoriasis co-express the T_h17 transcription factor ROR-γ and easily differentiate *ex vivo* into IL-17-producing, inflammation-promoting T_h17 cells in presence of pro-inflammatory stimuli such as IL-23 (Bovenschen et al.; Soler and McCormick 2011). These findings suggest that T_{reg} cells maintain a fine balance between Foxp3 and ROR-γ expression and that the *in vivo* differentiation of T_{reg} cell subsets towards T_h17 cells possibly contributes to perpetuate the pathogenesis of psoriasis.

1.3. Psoriasis and its T cell mediated pathogenesis

Psoriasis vulgaris is regarded as a T cell mediated autoimmune disease with an ongoing inflammation promoted by activated T cells. The exact aetiology of psoriasis is still not elucidated, but there are several genetic determinants (psoriasis susceptibility locus 1 – *PSORS1* – encoding the *HLA-Cw0602* allele of

the MHC class I molecule (Gudjonsson et al. 2002; Russell et al. 1972) and other 9 additional segments – *PSORS2-10* – outside of the MHC, mutations in the *IL-23/T_h17* and in the *NF-κB* pathway genes (Perera et al. 2011)), and environmental cues (streptococcal infections (Valdimarsson et al. 2009), physical trauma (Raychaudhuri et al. 2008) or medications (Abel et al. 1986)) systematically associated with the onset and/or the exacerbation of the disease. With a prevalence of about 2-4 % in Caucasians it is a common disease (Christophers 2001). The typical skin manifestations are chronic stationary well demarcated erythematous plaques covered with white or silvery scales. However, psoriasis patients may exhibit with a variety of clinical phenotypes ranging from acute guttate forms to the generalised pustular psoriasis of von Zumbusch. Histological hallmarks which characterize lesional skin in psoriasis patients are acanthosis (thickening of the epidermis), papillomatosis (elongation of the rete ridges) and hypogranulosis (decrease or loss of the granular layer), all due to the increased turnover of the keratinocytes in the basal layer and clinically underlying the thickness of the psoriasis plaques. Parakeratosis (retention of nuclei in keratinocytes in the stratum corneum) due to the premature keratinocyte maturation in the upper epidermal layers with incomplete cornification is clinically manifested as silvery scales. Dilatation of dermal blood vessels due to inflammation associated with new blood vessel formation is clinically revealed by the pin-point bleeding, known as the Ausspitz sign (Fig. 3).

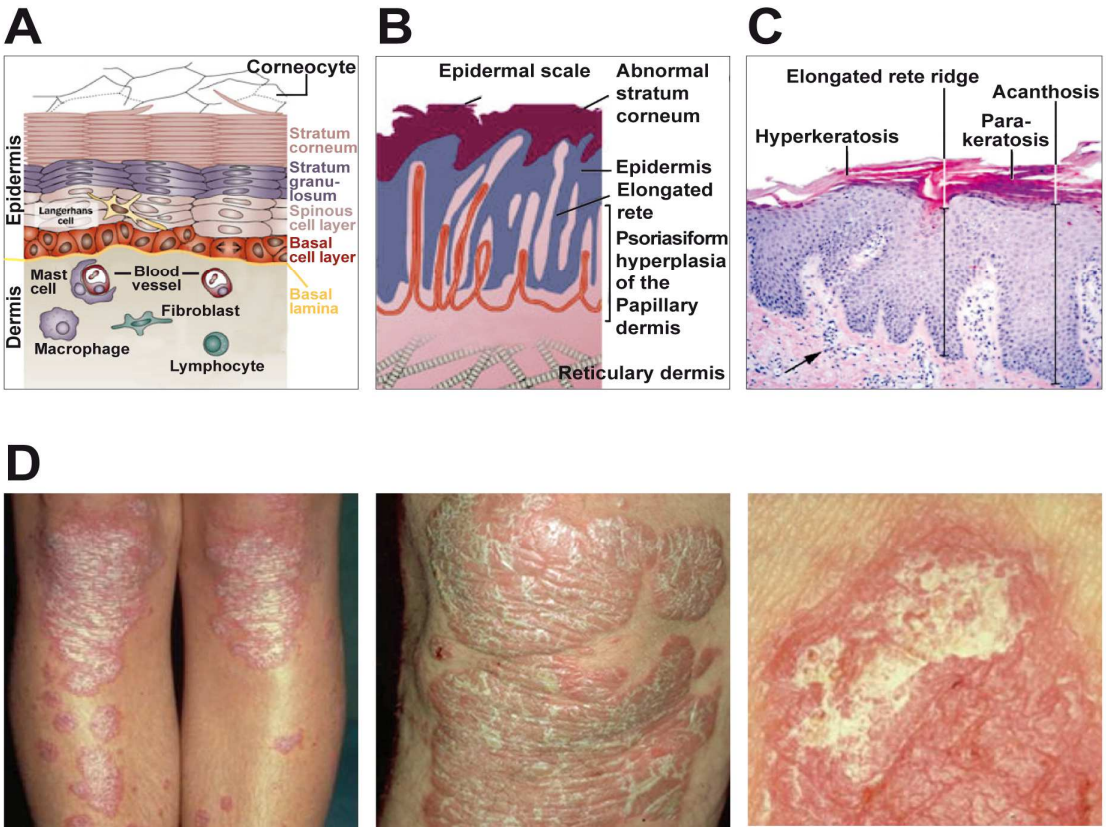

Fig. 3: Histological features of psoriasis plaques. *Schematic structure of the human skin (without subcutis) (**A**). The outermost layer is the stratum corneum which consists of corneocytes (dead keratinocytes) which is separated by the thin stratum granulosum from the stratum spinosum and the basal layer, consisting of dividing keratinocytes. (adapted from (Wagner et al. 2010)) (**B**) Schematic structure of psoriasis-affected skin with an enlarged stratum corneum due to a high proliferation turnover of keratinocytes, hyperproliferation of the stratum spinosum with elongated rete ridges and hyperplasia in the papillary dermis with angioneogenesis (adapted from (Lowes et al. 2007)), also shown histologically (**C**) with additionally parakeratosis (no loss of nuclei of keratinocytes in the stratum corneum (adapted from (Wagner et al. 2010)). (**A-C**) Histological changes in psoriasiform skin (**A-C**) correspond clinically to well demarcated erythematous plaques covered with 'silvery' scales ((**D**) University of Heidelberg and University of Erlangen, Germany; http://dermis.net/dermisroot/en/31346/diagnose.htm).*

In addition, the psoriasis plaque exhibits in the upper papillary dermis a dense immune cell infiltrate consisting of macrophages, DCs and T cells. Macrophages and DCs express high levels of pro-inflammatory cytokines (TNF-α) and inducible nitric oxide synthase (iNOS) which promote and perpetuate inflammation (Clark and Kupper 2006; Serbina et al. 2003; Wang et al. 2006b). DCs also produce the cytokines IL-23 and IL-20, which have the potential to activate T cells (Lee et al. 2004) and keratinocytes, respectively (Wang et al. 2006b). In this environment, T cells are polarized towards $CD4^+$ T_h1 and T_h17 cells and towards $CD8^+$ cytotoxic

cells which predominantly home within the epidermis, similar with the neutrophils which give rise to small foci in the stratum corneum – the microabscesses of Munro (Lowes et al. 2007). Many lymphocytes, macrophages, DCs and neutrophils are found to adhere to endothelial cells, suggesting active recruitment to the inflammatory plaque. Endothelial cells are activated in psoriatic lesions, as indicated by positive staining for ICAM-1, VCAM-1 and E-selectin (Krueger 2002). In affected psoriasis skin increased levels of T_h1 type pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-2, IL-6, IL-12, IL-17 and IL-23 were found, whereas T_h2 cytokines as IL-10 and IL-4 were hardly detected (Conrad et al. 2007; Nickoloff et al. 2007a), supporting the view of psoriasis as a T_h1 mediated disease. Besides, the cytokine profile described above explains the recruitment of macrophages in psoriatic skin, which are attracted and activated by T_h1 signalling (Wang et al. 2006b) (Fig. 4).

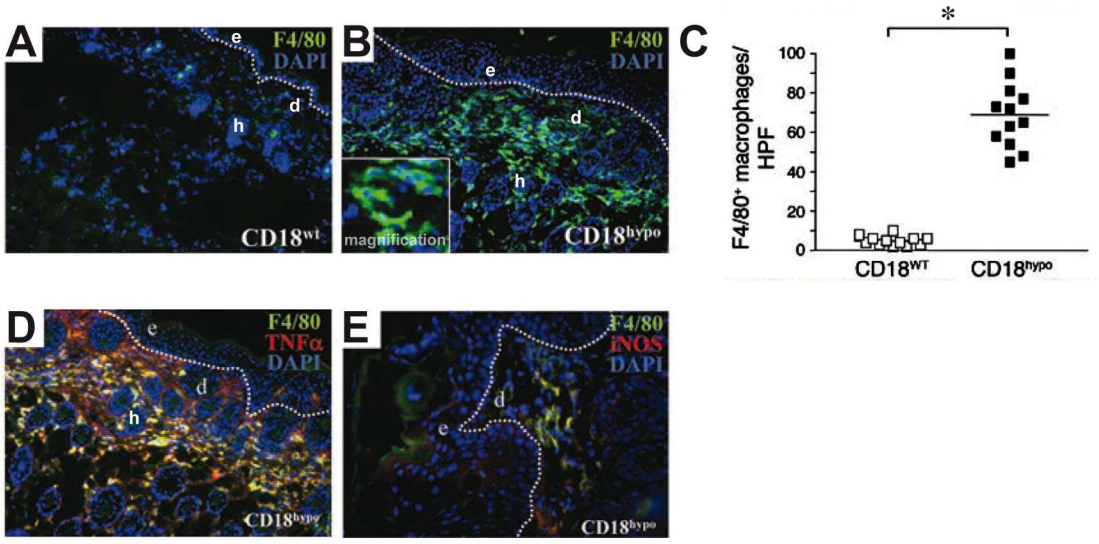

Fig. 4: Increase of macrophage infiltration in psoriasiform lesional skin in affected CD18^{hypo} mice *(Wang et al. 2006b). (**A** and **B**) Skin cryosections from CD18^{wt} (**A**) and affected CD18^{hypo} mice (**B**) were stained with F4/80–Alexa 488 which shows macrophages (green) abundantly infiltrating the skin in affected CD18^{hypo} mice (**B**) when compared with wildtype mice (**A**), as clearly demonstrated by the quantification of 12 HPF per mouse (n=4) (**C**). (**D** and **E**) Activated macrophages are an important source of TNF-α and NO in the lesional skin of affected CD18^{hypo} mice. Double immunostaining with anti-mouse TNF-α (**D**) or iNOS (**E**) and F4/80-Ab performed on cryosections derived from the lesional skin of affected CD18^{hypo} mice.*
Overlay (yellow) represents macrophages bearing the specific activation markers TNF-α and iNOS in the psoriasiform skin inflammation. Cell nuclei (blue) are counterstained with DAPI.
(e: epidermis; d: dermis; h: hair follicle. Dotted lines indicate the border between epidermis and dermis. Original magnification ×40; inset magnification ×100).

The finding of a limited T cell receptor repertoire in the psoriatic lesions suggests a rather clonal expansion of the skin-infiltrating T cells, most probably triggered by a so far unidentified peptide antigen by molecular mimicry, for example with streptococcal antigens (Conrad et al. 2007; Prinz et al. 1994). Similarly, K17 expressed by kerationocytes and human type-I-keratins were suggested to likely cross-react with streptococcal structures (Sabat et al. 2007). Furthermore these T cells show a memory cell phenotype which supports the hypothesis of an antigen-specific T cell-response. This may occur via perpetuation of self-antigen presentation to memory T cells.

Since it is known for a while that also affection of joints as psoriasis-arthritis occurs and requires intensive systemic therapy for prevention of i.e. mutilation, latest research results regard psoriasis more and more as a systemic disease with overall inflammation, especially affecting the skin, the joints and most likely also the cardiovascular system (Boehncke et al. 2009; Singh and Aneja 2011). Typical skin lesions in psoriasis patients are not seldom associated with a high psychological burden. The psychological pressure of psoriasis patients was found to be comparable to that of patients who suffered from an acute myocardial syndrome and just depression was found in a list of diseases to burden patients more than psoriasis does. This fact and the awareness of psoriasis as a systemic inflammatory disease point out even more the need of further investigation of psoriasis and the development of specific and effective therapeutic strategies.

1.4. The role of the Lymphocyte function-associated antigen-1 (LFA-1) for immune responses

LFA-1 is an important adhesion molecule mainly expressed by T and B lymphocytes which mediates leukocyte binding and signalling to different cell types, especially endothelial cells and APCs. LFA-1 is a member of the β_2 integrin family of leukocyte adhesion molecules consisting of two different subunits, the β_2-chain CD18 (beta-subunit) and the αL-chain CD11a (alpha-subunit) (CD11a/CD18). All members of the β_2 integrins family of adhesion membrane molecules are exclusively expressed on leukocytes and share the common β_2

chain which forms heterodimers with four different α-chains. For example, monocytes, neutrophils and macrophages express the β_2 integrin Mac-1 composed of the common β_2-chain CD18, and the CD11b αL-chain (CD11b/CD18), while antigen presenting DCs express gp150/95, consisting of CD18 and the αL-subunit CD11c (CD11c/CD18) (Hynes 1987) (Fig. 5A).

β_2 integrins mediate the binding of leukocytes to more than 20 different ligands, the more important one being the ICAM-1 receptor (Fig. 5B). In particular, LFA-1 expressed on the surface of activated lymphocytes mediates their binding to ICAM-1 on endothelial cells, thus promoting T cell migration through the endothelium and recruitment to the site of infection and inflammation. Accordingly, in CD18-deficient mice the extravasation of primed T cells in the skin upon specific challenging was severely decreased, resulting in a significantly suppressed inflammatory response in experimental models of allergic contact dermatitis and delayed-type hypersensitivity (Grabbe et al. 2002).

Additionally, LFA-1 binding to ICAM-1 molecules plays a major role in the cell-cell contact between different T cell subtypes and between T cell subsets and APCs in inflammation. Thus, LFA-1 was found to be at least co-responsible for preventing autoimmunity, as blocking LFA-1 with anti-CD11a-Ab in neonatal C57Br mice abrogated the thymic negative selection of self-reactive T cells which are normally deleted in this strain (Quddus et al. 1994). However, accumulating evidence suggests a key role of LFA-1 – ICAM-1 binding for breaking immune tolerance and uncontrolled T cell priming, activation and function during autoimmune diseases such as encephalitis (Gordon et al. 1995), diabetes (Moriyama et al. 1996) and psoriasis (Lebwohl et al. 2003). LFA-1 is expressed at high levels on T cells in psoriasis and inhibition of LFA-1 signalling by binding CD11a using the humanized antibody Efalizumab (Raptiva®) in humans resulted in strongly reduced numbers of T cells in psoriatic lesions and in a reduction of proinflammatory ‘type 1’ gene activation on mRNA levels (Gordon et al. 2003; Krueger and Bowcock 2005) (Fig. 5C). However, the exact effect of targeting LFA-1 – ICAM-1 interaction between T cell subsets or T cells and other cell types on molecular and functional level is largely unexplored so far.

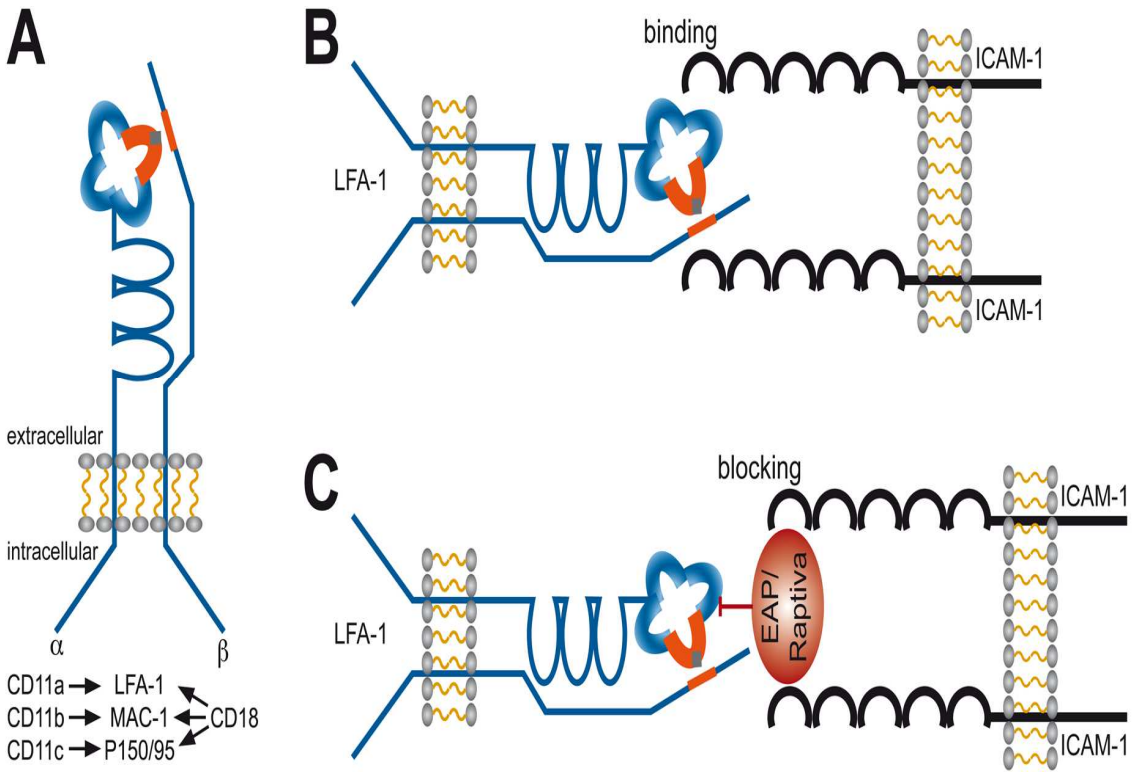

Fig. 5: **EAP and Efalizumab (Raptiva®) are able to block the binding of LFA-1 to ICAM-1.** *(**A**) The β_2 integrin LFA-1 is a heterodimere consisting of the common β chain CD18 and the variable α-chain CD11a and is exclusively expressed on lymphocytes. MAC-1 as a β_2 integrin that is expressed on macrophages and associates the common β-chain CD18 with the CD11b α-chain, whereas DCs express P150/95 consisting of CD18 and CD11c. (**B**) The leukocyte β_2 integrin LFA-1 binds to ICAM-1 on endothelial cells or DCs. (**C**) Immunmodulatory substances such as EAP and the humanized CD11a-Ab Raptiva® (Efalizumab) block the binding of LFA-1 to ICAM-1. (adapted from (Harris et al. 2000))*

1.5. The CD18^{hypo} PL/J mouse model for a T cell mediated psoriasiform skin disease

Leukocyte adhesion deficiency-1 patients presenting with reduced lymphocyte expression of LFA-1 frequently develop psoriasiform skin lesions, pointing out a possible relevance for CD18 in the pathogenesis of psoriasis (van de Kerkhof and Weemaes 1990). This hypothesis is strongly supported by the finding that PL/J mice bearing a mutation in the CD18 gene resulting in a rest-expression for CD18 of 2 – 16 % (depending on the leukocyte activation state) of the wild type level mount a psoriasis-like inflammatory skin disease (Bullard et al. 1996).

This so-called hypomorphic mutation was generated by introducing an insertion mutation resulting in the duplication of the exons 2 and 3 of the CD18 gene (Wilson et al. 1993). Remarkably, when the $CD18^{hypo}$ mutation was backcrossed on other mouse strains (C57BL/6J, Sv/129), no skin disease was observed. Therefore, the psoriasiform-like skin disease in $CD18^{hypo}$ PL/J mice shares a common polygenic base with the human psoriasis (Kess et al. 2006).

In addition, introduction of a CD18 null mutation in the PL/J strain failed as well to induce skin inflammation, suggesting that the impairment, and not the complete absence of signalling via β_2 integrins may contribute to psoriatic inflammation (Bullard et al. 1996).

In previous work we could show that the reduced CD18 gene expression resulted in the altered activation of β2-integrins on leukocytes and eventually in impaired T cell function in the $CD18^{hypo}$ PL/J mice. We found a high amount of $CD4^+$ T_{resp} cells and $CD8^+$ cytotoxic T cells accumulating in the inflamed skin of $CD18^{hypo}$ mice and producing high levels of 'type 1' pro-inflammatory cytokines IFN-γ and IL-2 (Kess et al. 2003). Of these, $CD4^+$ T_{resp} cells causally contributed to the development of the psoriasiform skin inflammation with typical clinical and histological features such as itching, erythema, parakeratosis and acanthosis, as the depletion of $CD4^+$ T cells by injection of anti-CD4-Abs completely resolved the psoriasiform skin phenotype, while the removal of $CD8^+$ cells had no effect (Kess et al. 2003). Furthermore, the numbers of T_{reg} cells were markedly reduced in the lesional skin, lymph nodes and the spleen of diseased $CD18^{hypo}$ PL/J mice when compared with wild type mice and the suppressive capacity of the $CD18^{hypo}$ T_{regs} on T_{resp} cell proliferation was severely reduced when compared to wild type T_{regs} (Fig. 6) (Wang et al. 2008).

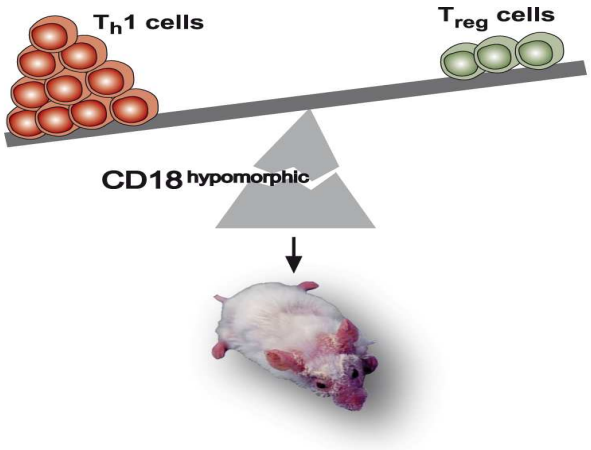

Fig. 6: **T cell mediated pathogenesis in the $CD18^{hypo}$ PL/J mouse.** *Hypomorphic mutation in the CD18-gene results in imbalance between uncontrolled proliferation of T_h1 type T_{resp} cells and impaired numbers and function of T_{regs} and eventually in autoimmunity. (adapted from (Wang et al. 2006b))*

Taken together, the hypomorphic mutation of CD18 induced dysfunctional T_{regs} which enabled the uncontrolled proliferation of T_h1 type T_{resp} cells and the development of clinically overt psoriasiform skin inflammation, suggesting that T_{regs} and T_{resp} cells require different levels of β_2 integrins expression for physiological activation and function (Clark and Kupper 2006; Wang et al. 2008).

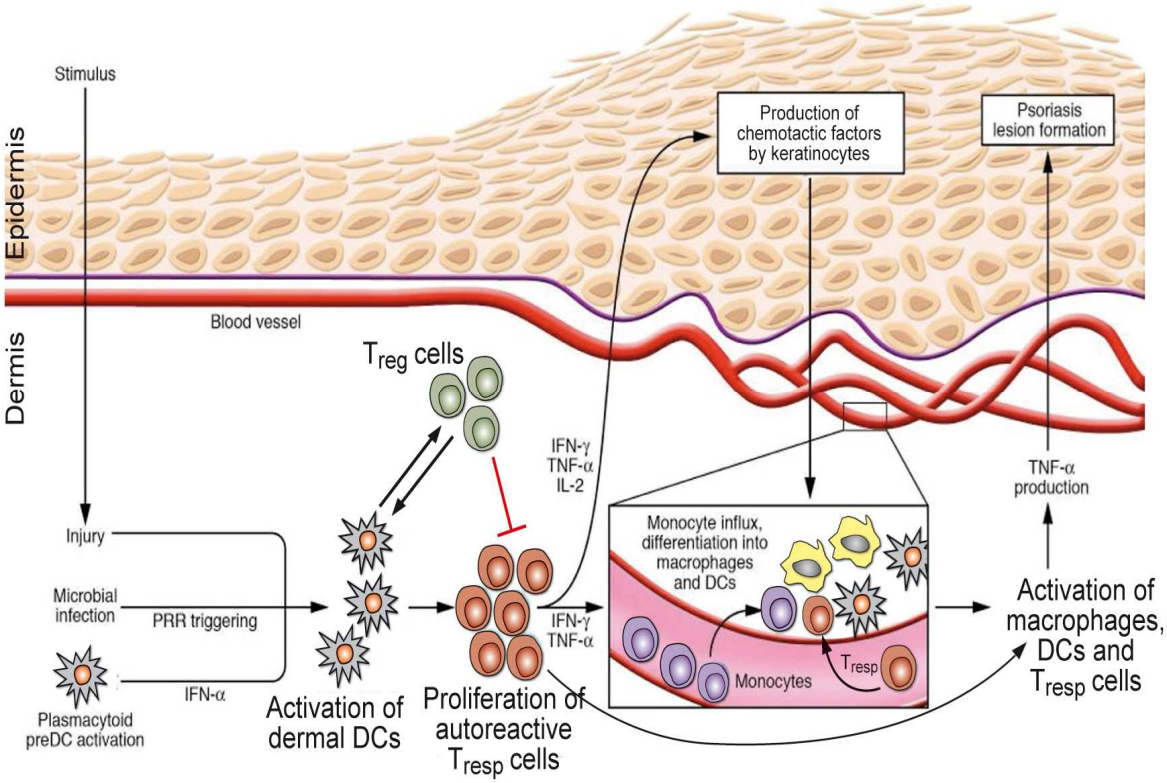

Fig. 7: Molecular and cellular triggers in the development of psoriatic skin lesions. *Many trigger factors can lead to the activation of dermal dendritic cells, a key initiating step in the development of psoriasis in predisposed individuals. Activated dendritic cells induce the proliferation of autoreactive T cells within the dermis, inducing production of IFN-γ and TNF-α, which in turn induces the production of chemotactic cytokines by epidermal cells. These chemotactic agents induce influx of monocytes from the blood, which undergo differentiation into macrophages and myeloid dendritic cells, as well as influx of T_{resp} cells. These dermal macrophages, once activated by T cell or dendritic cell cytokines, then produce large amounts of TNF-α, contributing to the development of skin lesions observed in psoriasis. T_{reg} cells are 'educated' by dendritic cells and have suppressive capacity to regulate the activity of T_{resp} cells, a control mechanism also in autoimmunity. (PRR, pathogen-recognition receptor) (adapted from (Clark and Kupper 2006))*

1.6. The staphylococcal Extracellular Adherence Protein (EAP)

Staphylococcus aureus (SA) is a bacterium capable of causing severe skin and systemic infections in humans once it had passed the skin protection barrier. SA is equipped with multiple virulence and survival mechanisms, as for example by secreting substances which are capable of suppressing the host immune response or facilitating immune defence evasion. One of these substances was previously found to be the Extracellular Adherence Protein (EAP), a cationic protein family with a diversity of structures and functions (Harraghy et al. 2003). EAP is a protein from the family of adhesins which is produced and secreted by several subgroups of SA. Its size, defined by the numbers of amino acids, differs between different strains of SA. Thus several analogues of EAP of 65 and 72 kDa have been identified, consisting of five to six tandem repeats of 93 to 110 amino acids, respectively (Lee et al. 2002). Further EAP analogues of 70 kDa consisting of six tandem repeat domains have been designated as p70 and MHC class II analog protein (Map), respectively (Jahreis et al. 1995). This structural complexity confers EAP a broad binding spectrum to extracellular matrix proteins (collagen), plasma proteins (pro-thrombin) and adhesion molecules such as ICAM-1, required for SA adhesion, invasion and inhibition of the host immune defence (Chavakis et al. 2002; Hussain et al. 2008; McGavin et al. 1993). Thus, EAP is a critical molecule for SA virulence and development of infection.

Of major importance for our research are the anti-inflammatory properties of EAP which are effected via different mechanisms. Thus, in some members of the EAP family, such as Map, each of the six repeated domains contains a 30 amino acid subdomain which shows a striking similarity with a sequence at the peptide-binding site of the MHC class II molecule of various mammals, suggesting that Map may affect antigen presentation and T cell function (Jonsson et al. 1995). Although the direct interaction between MHC class II molecules and TCR could not be blocked by EAP in a cell-free system (Massey et al. 2007), EAP was able to modulate immune responses by shifting T cell responses towards a T_h2 phenotype *in vitro* (Jonsson et al. 1995) and to suppress experimental autoimmune encephalitis (Xie et al. 2006) and arthritis (Lee et al. 2002) *in vivo*. This anti-inflammatory effect was long-lasting, persisting even after cessation of EAP

administration (Xie et al. 2006). The second important immunomodulatory property of EAP relies on its ability to bind to ICAM-1 on endothelial cells, thus preventing CD11b/CD18 (Mac-1)-dependent neutrophil adhesion to endothelial cells *in vitro* (Haggar et al. 2004) and β2 integrin-dependent leukocyte adhesion on endothelial cells and extravasation to the peritoneum in an experimental model of bacterial peritonitis (Chavakis et al. 2002). In a mouse model of wound healing, the inhibition of leukocyte-endothelial cell interaction by EAP-producing SA strains was found to inhibit leukocyte recruitment to the wound margin as well as the activation of the pro-inflammatory NF-κB pathway in leukocytes, which thus were less efficient in bacterial phagocytosis and killing. In addition, EAP revealed antiangiogenic properties by blocking alpha v-integrin mediated endothelial cell migration and capillary tube formation *in vivo* (Athanasopoulos et al. 2006), most likely by blocking the activation of RAS/RAF/MEK/ERK signalling pathway in endothelial cells (Sobke et al. 2006). Together, these mechanisms resulted in a severely delayed closure of EAP-treated wounds, supporting the clinical observation of disturbed wound healing in SA-infected wounds in humans. On the other hand, the potent antiangiogenic effect and the capacity of blocking the binding of alpha v beta3 integrin on breast tumour cells to extracellular matrix proteins such as osteopontin prevented tumour growth and metastasis to the bone in a murine model of mammary carcinoma (Schneider et al. 2007), qualifying EAP as an attractive therapeutic tool to prevent bone metastasis in breast cancer.

Of notice, almost all described effects of EAP were tightly dependent on the dose in which EAP was administered. Thus, low concentrations of EAP seem to rather inhibit leukocyte activation, recruitment and proliferation, while too high concentrations were associated with cytotoxic effects (Athanasopoulos et al. 2006; Chavakis et al. 2002; Haggar et al. 2005; Xie et al. 2006).

Internalization of SA, blocking of leukocyte extravasation and recruitment to inflammation sites, inhibition of cell contact by receptor-blocking and down-regulation of pro-inflammatory cytokines are all mechanisms which make EAP capable of modulating the immune system and may be of benefit for re-balancing autoimmunity in psoriasis. Additionally, angiogenesis seems to be inhibited by EAP (Sobke et al. 2006), which is important in psoriasis where an increased angiogenesis may facilitate the increased infiltration of leukocytes. Interestingly, EAP was found to block the fifth binding site of ICAM-1, which is known to

specifically bind LFA-1 on lymphocytes (Chavakis et al. 2002; Scriba et al. 2008). This may result in an impaired binding capacity of ICAM-1 on endothelial cells or other leukocytes to LFA-1-bearing T cells, possibly leading to reduced migration and to impaired cell-cell contact of T cells to antigen presenting DCs. Preliminary *in vivo* studies performed by our group previous to the *in vitro* work presented in this thesis showed that ICAM-1 was up-regulated, particularly on $CD31^{+}$ endothelial cells, in lesional skin of $CD18^{hypo}$ mice and that injection of EAP in $CD18^{hypo}$ diseased mice resolved the psoriasiform skin inflammation most likely by inhibiting T cell-transmigration to the inflamed skin (Wang et al. 2010).

Therefore, we here studied whether the EAP-induced blockade of the contact between LFA-1 on T cells and ICAM-1 on APCs, would be able to modify T cell responses *in vitro*. In particular, we used *in vitro* proliferation assays to investigate whether EAP would modulate the proliferation of T_{resp} cells in different standardized stimulation settings, we asked whether the 'education' of T_{regs} by antigen presenting DCs to suppress T_{resp} cell proliferation would be influenced in presence of EAP and if the direct suppressive function of T_{regs} on T_{resp} cells would be affected by EAP *in vitro*. In a next set of experiments we tried to characterize mechanistically the EAP effects in these experiments by reproducing the results by blocking LFA-1 and ICAM-1 using specific neutralizing antibodies for the two molecules. Finally, we investigated the therapeutic potential of EAP for psoriasis by comparing the effects observed with EAP with the effects induced by the anti-LFA-1/CD11a-Ab Efalizumab, which has been already successfully employed in the treatment of human psoriasis, in the same settings.

1.7. Aim of the work

Psoriasis is a common inflammatory skin disease which has been associated with uncontrolled proliferation of type 1 cytokine-producing T_h1 autoreactive T cells due to defective function of regulatory T cells. The staphylococcal extracellular adhesion protein EAP which blocks cellular binding to a number of ligands including ICAM-1 resolved the psoriasiform skin inflammation in the $CD18^{hypo}$ mouse, an established murine model sharing its immune pathogenesis with the human psoriasis.

The aim of this study was to analyse the *in vitro* effects of EAP on T cell activation, proliferation and functions, which are relevant for the pathogenesis of psoriasis.
In particular, we asked whether:

- EAP would impact the proliferation of T cell subsets *in vitro*
- The education of regulatory T cells by antigen-presenting dendritic cells would be affected by EAP *in vitro* and if so, on which molecular level and
- Whether the physical contact between DCs and different T cell subsets would be influenced by EAP *in vitro.*

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Mice strains

CD18 hypomorphic (tgb1<tm1Bay>) PL/J mice and CD18 wild type PL/J mice	Deptartment of Dermatology and Allergology, University of Ulm, Germany
C57BL/6 mice and Balb/C mice	Charles River, Germany

2.1.2. Antibodies, cytokines and kits

CD3	BD Biosciences, Pharmingen
CD4	BD Biosciences, Pharmingen
CD8	BD Biosciences, Pharmingen
CD11a	BD Biosciences, Pharmingen
CD11c	BD Biosciences, Pharmingen
CD18	BD Biosciences, Pharmingen
CD25	BD Biosciences, Pharmingen
CD28	Merck KGaA, Darmstadt
CD54 (ICAM-1)	BD Biosciences, Pharmingen
CD127	NatuTec, Frankfurt am Main
EAP	Prof. Preissner, University of Giessen
Gr-1	Miltenyi Biotec, Bergisch Gladbach
Recombinant Murine IL-2	PeproTech Inc., USA
Recombinant Murine IL-4	Cell Concepts, Hamburg
Raptiva® (Efalizumab)	Serono Europe Ltd.
Rat IgG $_{2a, k}$ isotype	BD Biosciences, Pharmingen

Kits

CD4+ CD25+ Regulatory T cell isolation Kit (mouse)	Miltenyi Biotec, Bergisch Gladbach
FITC Annexin V Apoptosis Detection Kit I	BD Biosciences, Pharmingen
Foxp3 Kit	eBioscience, Frankfurt

2.1.3. Materials for T cell isolation and FACS analysis

ACK lysis buffer	Dept. of Dermatology and Allergology, University of Ulm
BD Falcon – cell strainer 40µm nylon	BD Biosciences, Pharmingen
BD Microlance 3 (0,45 mm)	BD Biosciences, Pharmingen
Centrifuge	Eppendorf, Hamburg
CFSE	Molecular Probes by Invitrogen, Karlsruhe
Dissection tool	Aesculap, Tuttlingen
Dulbecco's phosphate buffered saline (PBS) (1x) (w/o Ca^{2+}, Mg^{2+})	Biochrom AG, Berlin
Filter System PES 1000ml 0,45µm	VWR Int. GmbH, Germany
Fluorescence activated cell sorter (FACSCalibur)	Becton Dickinson, NL
HistoDenz™	Sigma-Aldrich, USA
Ice maker	Scotsman
LD-column	Miltenyi Biotec, Bergisch Gladbach
MACS buffer	Miltenyi Biotec, Bergisch Gladbach
Microscope (Diaphot-TMD)	Nikon, Duesseldorf
Microscope (Zeiss Axiophot)	Zeiss, Germany
Minifuge RF	Heraeus Sepatech GmbH
MiniMACS Separator	Miltenyi Biotec, Bergisch Gladbach

MS-columns	Miltenyi Biotec, Bergisch Gladbach
Neubauer chamber	Roth, Neu-Ulm
NUNC 12 wells plates	NUNC, Langenselbold
NUNC 24 wells plates	NUNC, Langenselbold
NUNC 96 wells plates	NUNC, Langenselbold
Omnifix-F Syringe (1ml, 5ml and 20ml)	Braun, Tuttlingen
Pipetts	Roth, Neu-Ulm
QuadroMACS Separation Unit	Miltenyi Biotec, Bergisch Gladbach
QuadroMACS 15ml Tube Rack	Miltenyi Biotec, Bergisch Gladbach
Refridgerator (4°C)	Liebherr, Ochsenhausen
RPMI complete medium	Gibco, Karlsruhe
Sterile bench Faster BHA 48	Faster, Italy
Vortex Genie	Scientific Industries, INC, USA

2.1.4. Ingredients of buffers and solutions

ACK lysis buffer (pH 7.2)	0,15 M NH_4Cl; 0,1 mM EDTA; 10 mM $KHCO_3$; H_20
CFSE (5 mM stock)	25 mg 5-carboxyfluorescein diacetate; 4,5 ml DMSO
MACS buffer (pH 7.2)	PBS; 0,5 % bovine serum albumin; 0,4 % EDTA
PBS	137 mM NaCl; 2,7 mM KCl; 8,4 mM; Na2HPO4; 1,4 mM KH2PO4; pH 7,4

RPMI 1640	100U/ml Penicilline; 100µg/ml Streptomycin; 1 % L-Glutamine; 10 % fetal calf serum (heat inactivated)

2.2. Mice

$CD18^{hypo}$ PL/J mice were generated as described in the introduction (Wilson et al. 1993). To minimize the risk of previous T cell activation mice were kept in specific pathogen free conditions/environment. In healthy mice T_{reg} cells are distinguished from T_{resp} cells mainly by their high expression of CD25, which is commonly used for T_{reg} cell isolation. However, in $CD18^{hypo}$ PL/J diseased mice a high expression of CD25 is more likely indicative for T cell activation than it characterizes T_{reg} cells (Wang et al. 2006b), and the previous activation of T cells may lead to isolation of $CD25^{+}$ activated T cells along with the T_{reg} cells. Therefore, T_{reg} cells were isolated exclusively from young $CD18^{hypo}$ PL/J mice at the age of 5-6 weeks in order to minimize the period of time which might lead to events causing activation of the immune system. Additionally, high Foxp3 expression and low CD127-expression were used as additional marker to increase the purity of 'real' T_{reg} cells. Experiments were accomplished in consideration with the German Law for Welfare of Laboratory Animals and were approved by the Institutional Review Board of the University of Ulm.

2.3. Isolation of $CD4^+$ T cell populations from $CD18^{wt}$ PL/J mice

2.3.1. Isolation of splenocytes

Isolation steps took place at a sterile bench, cells and solutions were kept cool on ice or for longer time periods stored in the refrigerator and work was performed quickly in order to reach a maximum outcome of viable cells.

Directly after sacrificing the mice by CO_2 inhalation, spleens were dissected and grinded through a 40µm cell strainer into a 50ml tube. 10ml PBS were used to solve and flush the grinded spleen through the cell strainer to allow a maximal cell yield.

Centrifugation of the PBS-diluted splenocytes was performed at a speed of 1200 rpm at 4°C for 10min. These centrifugation parameters were also used in the following steps of centrifugation.

Supernatant was wasted and the cell pellet resuspended and mixed with 4ml of ACK lysis buffer for 3min to eliminate red blood cells. 20ml of ice-cold PBS interrupted the lysing process by dilution of ACK lysis buffer to prevent overall cell cytotoxicity. Another step of centrifugation followed. After washing the cells again by resuspending the cell pellet with 10ml of PBS and one more centrifugation procedure, cells were diluted again and a sample of cells, diluted in a defined volume of PBS, was counted using a microscope at a magnification of 400x in a Neubauer cell counting chamber to determine the initial number of viable cells. Dead cells were excluded by Trypan blue staining. The splenocytes isolated at the end of this step contained a mixture of $CD4^+$ T lymphocytes and $CD4^-$ cells consisting of erythrocytes, monocytes, granulocytes, B lymphocytes and $CD8^+$ T cells.

2.3.2. Depletion of $CD4^-$ cells

In a next step, all $CD4^-$ cells were removed by magnetic cell depletion which was performed as recommended in the user´s manual for '$CD4^+CD25^+$ Regulatory T Cell Isolation Kit, mouse'. For this, the cell pellet was carefully mixed with 40µl of MACS buffer and 10µl of Biotin antibody-cocktail per 10^7 initial cell number (ICN);

this cocktail contains antibodies against $CD8^+$ cytotoxic T cells (anti-CD8a), granulocytes (anti-CD11b), B lymphocytes (anti-CD45R), NK cells (anti-CD49b) and erythrocytes (anti-Ter-119). After incubation for 10min at 4°C, cells were added with 30µl of MACS buffer and 20µl of CD25-PE antibody per 10^7 ICN which will be required for the positive selection of $CD4^+CD25^+$ T_{reg} cells in a further step, following $CD4^-$ cell depletion. After further incubation at 4°C for 15min the cell suspension was filled up with 2ml of MACS buffer per 10^7 ICN and washed by centrifugation. Supernatant was wasted and cells were solved in 500µl of MACS buffer. The separation was performed by running the cells through a LD-column, which was placed in the magnetic field of a 'QuadroMACS Separation Unit' and was previously rinsed with 2ml ice-cold MACS buffer. During this procedure all $CD4^-$ cells labelled with magnetic beads bound to the column, while $CD4^+$ cells passing in the flow through. After running the cell solution, the column was washed two times with 1ml of MACS buffer and the 2,5ml of $CD4^+$ cell solution were washed again by centrifugation. This $CD4^+$ fraction contained a mixture of $CD4^+CD25^-$ T_{resp} cells and $CD4^+CD25^+$ T_{reg} cells.

2.3.3. Positive selection of $CD25^+$ cells

To further enrich the $CD4^+CD25^+$ T_{reg} cell population, the $CD4^+$ cell pellet was resolved in 90µl of MACS buffer and 10µl anti-PE MicroBeads (volume referring always to 10^7 cells) were added to bind to the PE-conjugated anti-CD25 antibodies which were added previously during the depletion of $CD4^-$ cells. The cells were incubated at 4°C for 15min, washed by centrifugation and resuspended in 500µl of MACS buffer. A MS-column was fixed in a MiniMACS Separator unit and rinsed by MACS buffer, before the cell solution was pipetted onto the column. After running through, the MS-column was washed three times with 500µl MACS buffer. As the PE-conjugated anti-CD25-Abs were magnetically labelled with anti-PE MicroBeads, the $CD25^-$ cells of the $CD4^+$ cell fraction (T_{resp} cells) passed through the column and the $CD25^+$ cell fraction (T_{reg} cells) was magnetically bound to the column in the MiniMACS Separator – induced magnetic field. To recover the T_{reg} cell fraction separated on the column, the column was removed from the magnetic

field and was flushed with 1ml of MACS buffer with a plunger into a new 15ml tube.

As a high purity, as controlled by subsequent FACS analysis, of the T_{reg} cell fraction was required for our experiments, the $CD25^+$ positive selection was repeated by running the $CD4^+CD25^+$ cells through a second MS-column, as described before. T_{reg} cells and T_{resp} cells were washed and resuspended in 1ml of PBS per $1x10^6$ cells.

2.4. Isolation of $CD4^+$ T cell populations from $CD18^{hypo}$ PL/J mice

The isolation of splenocytes and the depletion of $CD4^-$ T cells were performed according to the protocols described for $CD18^{wt}$ PL/J mice (2.3.1 and 2.3.2), except for the timepoint of the addition of CD25-PE Ab. Due to the fact that $CD18^{hypo}$ PL/J mice have significantly lower numbers of T_{reg} cells compared to their wild type littermates, resulting in genetically determined immunodeficiency and defective suppression of autoimmunity (Wang et al. 2008), and a considerable number of the $CD4^+CD25^+$ T cells are rather activated T cells than T_{reg} cells (Wang et al. 2008), we adjusted and improved the protocol for T_{reg} cell isolation from $CD18^{hypo}$ PL/J mice in order to get a high purity of viable T_{reg} cells. To eliminate as many activated $CD4^+CD25^+$ T cells as possible and enrich 'real' T_{reg} cells, we used the low/absent expression of the IL-7 receptor (CD127) in T_{reg} cells as a further marker to discriminate between $CD4^+CD25^+CD127^{low}$ T_{reg} cells and $CD4^+CD25^+$ $CD127^{high}$ T_{resp} cells. For this, we additionally treated the previously isolated $CD4^+$ T cells with 5µl of CD127-PE antibody per 10^7 cells, aiming at eliminating the $CD127^+$ fraction of potentially activated T_{resp} cells. The subsequent incubation, centrifugation and MACS isolation procedure were performed as described above (see 2.3.2). Cells were centrifuged, supernatant was wasted and cells were resolved in 85µl of MACS buffer per 10^7 cells and treated with 15µl of anti-PE-MicroBeads per 10^7 cells, which bound to the PE-conjugated antibodies. We incubated the cells at 4°C for 15min, washed them and let the resuspended cells run through a second LD-column. After washing and counting, cells were treated with 90µl of MACS buffer and 10µl of CD25-PE-antibody per 10^7 cells. After 15min of incubation at 4°C, washing and resuspending cells in buffer, the same steps

were done with anti-PE-MicroBeads, before cells were flushed through the MS-columns as described in protocol 2.3.3. The result was a pure fraction of $CD4^+CD25^+CD127^{low}$ T_{reg} cells from $CD18^{hypo}$ PL/J mice. The expression of additional markers characteristic for T_{reg} cells, such as Foxp3 and TGF-β was further analysed by FACS (as detailed in Chapter 2.6).

2.5. Detection of apoptosis by Annexin V staining

Apoptosis is a physiologic process of programmed cell death which is important for maintenance of homeostasis in tissues. Apoptosis can be induced by a large variety of signals or stimuli. As opposed to apoptosis, the cell death by necrosis occurs rather in pathologic conditions, often being induced by toxicity. As EAP was previously reported to induce cellular apoptosis at high concentrations, for the herein described experiments it was necessary to investigate the potential pro-apoptotic effect of EAP on T_{resp} cells an T_{reg} cells at given concentration. This method would allow to rule out that variations in T cell proliferation was only due to an increased cell death, and not to active immune mechanisms induced by EAP. To assess apoptosis we measured the expression of Annexin V bound to the fluorochrome FITC on T cells in different experimental settings by FACS. Annexin V binds to phosphatidylserine, a phosphlipid of the inner plasma membrane of the cells, which is translocated to the outer side of the membrane when the cells undergo apoptosis. This process occurs very early in apoptosis, therefore early apoptotic cells, as well as late apoptotic and necrotic cells, can be reliably detected by Annexin V - FITC expression by FACS analysis (van Engeland et al. 1996). We compared Annexin V expression of T_{reg} cells or T_{resp} cells which were previously cultured in presence or absence of EAP for the intensity of FITC in FACS, which allowed us to assess the cellular toxicity of EAP.

We used samples of $1x10^5$ T_{reg} cells or T_{resp} cells which were previously cultured with DCs in presence or absence of EAP. Cells were washed twice with cold PBS and resuspended in diluted binding buffer according to the manufacturer's instruction in the manual of the 'FITC Annexin V Apoptosis Detection Kit I'. 5µl of FITC Annexin V were added per $1x10^5$ T_{reg} cells or T_{resp} cells, cells were mixed

gently and incubated for 15 min at RT. 400µl of diluted binding buffer were added and cells were immediately analysed by FACS for Annexin-FITC expression.

2.6. FACS analysis of the isolated T cell subsets

The fluorescence activated cell sorting (FACS) is a common method in cell biology with a large range of application and of great importance. Depending on plotting forward scatter (FSC, measurement of cell size) versus side scatter (SSC, a measure of cell granularity), different cell subsets can be characterized by their unique morphology and separated from cell populations with different characteristics from the selected ones. We employed this analysis method to study the purity of the isolated T cell subsets and dendritic cells, as well as to measure T cell proliferation by CFSE dilution.

All stainings for FACS analysis were performed in recipients protected from light.

After washing, each sample of 10^6 T cells was diluted in 100µl of PBS and subsequently incubated with 3µl of anti-CD4-APC Ab, 1µl of anti-CD8-PerCP Ab and 1µl of anti-CD25-PE Ab or 1µl of anti-Foxp3-PE Ab for 20min at 4°C in a 1,5ml Eppendorf tube. One sample of T cells was incubated with 100µl rat-IgG-Ab, serving as unspecific isotype control. Cells were washed by addition of 500µl PBS and a short spin for 12sec at $14x10^3$ rpm. Supernatant was wasted and the cell pellet was resuspended in 300µl PBS and transferred into a FACS tube. Cells were fixed with 30µl of 4% paraformaldehyde and immediately analysed or stored at 4°C in the dark if FACS analysis could not be performed immediately after staining. This procedure was used to determine and quantify the purity of the different T cell fractions by FACS analysis.

2.6.1. CFSE-labelling of isolated T cells

Carboxyfluorescein diacetate succinimidyl ester (CFSE) can be used to measure cell proliferation by FACS at the same wavelength as FITC (Fluorescein isothiocyanate) fluorochromes. CFSE is a dye which accumulates intracellular and equally dilutes in the daughter cells after cell division. As CFSE-intensity is sensitive to light, it is required to work in a shaded room. Due to the cytotoxic

effects of DMSO, the vehicle of CFSE, staining has to be done fast, strictly according to the protocol.

One ml of a 20µM CFSE solution obtained by diluting 20µl of a 5mM stock solution with 5ml PBS was added to each $1x10^6$ cells and mixed carefully. After incubating the cells at RT for 6min in the dark, the reaction was stopped with a five times volume of RPMI complete medium. Cells were washed thoroughly by centrifugation two times to completely remove the DMSO, thus preventing further cytotoxicity. The cells were diluted with 100µl RPMI c.m. per 10^6 cells and stored at 4°C and protected from light. The labelling efficiency was analysed by FACS.

2.6.2. FACS analysis of CFSE-labelled cells

To investigate CFSE intensity of different subsets of T lymphocytes, we set gates for the appropriate cell population in order to filter the lymphocyte populations. We measured the intensity of CFSE-FITC of each cell within the gates. The data is shown as a histogram of cell counts per CFSE intensity (proliferation histogram). The intensity of CFSE directly after isolation and staining of the cells was considered 100% (CFSE-labelling efficiency). After each cell division, the fluorescence intensity of the progenitor cells is equally divided to the daughter cells, resulting in CFSE dilution as a measure of proliferation. For each analysis a limit of $25x10^3$ counted events was set.

2.7. Isolation of bone marrow-derived dendritic cells

Allogeneic dendritic cells required for *in vitro* suppression assays were isolated from C57BL/6 mice as previously described (Wang et al. 2006a). For this, mice were sacrificed by CO_2 inhalation, both femurs were removed and dissected from muscles and tendons using sterile instruments. The epiphyses were cut and the bone marrow was flushed out with 5 ml ice-cold PBS through a 21G needle into a 50ml tube. The tube was filled up to 30ml with PBS and cells were washed three times by centrifugation at 1200rpm and 4°C for 10min. Cells were counted and diluted at $1x10^6$ cells/ml with pre-warmed RPMI c.m. + IL-4 and GM-CSF (50ml RPMI c.m. supplemented with 100µl of a 10ng/ml IL-4 solution and 150µl of a

40ng/ml GM-CSF solution). Cells were carefully transferred into 12 well NUNC plates ($2x10^6$ cells per well) and incubated in a humidified 5% CO_2 atmosphere at 37°C. 72h later we refreshed the RPMI c.m. + IL-4 and GM-CSF by carefully removing the supernatant from each well with the help of a pipette and refilling the wells slowly with prewarmed fresh medium. Mature DCs were harvested after 6 days of culture. For this, the cells were detached from the bottom by repetitive vigorous washing of each well and the pooled cell suspensions (10ml with approximately $3x10^5$ DCs per mouse) were washed by centrifugation. Supernatant was wasted and DCs were resuspended in 10ml supplemented RPMI c.m. in a 15ml tube. Thereafter 2ml of HistoDenz™ gradient solution were very carefully and slowly pipetted on the bottom of the DC-containing 15ml tube, creating a lower HistoDenz™-phase and an upper cell-medium-phase. Without shaking or mixing the liquids, the gradient tubes were centrifuged for 20min at 600g at RT w/o break. Centrifugation resulted in the separation of 3 different phases, corresponding to different densities of the medium and the dendritic cells-containing interphase. The DCs fraction was harvested by aspiration of 4ml interphase solution, which was apparent at the 2ml calibration mark of the tube and washed twice with RPMI c.m. by centrifugation. Cells were counted using a Neubauer chamber under the microscope and resuspended in 1ml of RPMI c.m. To exclude ongoing proliferative responses, DCs were irradiated for 20min at 40Gy. After irradiation DCs were washed by centrifugation and resuspended at $1x10^6$ cells per 1ml PBS for further experiments.

2.7.1. FACS analysis of dendritic cells

The purity of the bone marrow-derived dendritic cells was analysed according to the expression of the specific marker CD11c by flow-cytometry. For this, the staining was performed as previously described for T cells (chapter 2.6). $200x10^3$ DCs were equally divided and filled into two 1,5ml Eppendorf tubes. One aliquot of the DCs ($100x10^3$ cells) was stained with 100µl rat-IgG-Ab for the unspecific isotype control, the other aliquot ($100x10^3$ cells) was stained with 1µl anti-CD11c-PE Ab. We incubated both samples for 20min at 4°C, added afterwards 500µl PBS and washed the cells for 12sec at $14x10^3$ rpm. Supernatant was wasted and the

cell pellet was resolved in 300µl PBS and transferred into a FACS-tube, where cells were fixed with 30µl of 4% paraformaldehyde and either immediately analysed or stored at 4°C for later FACS analysis.

2.8. Experimental settings

2.8.1. Proliferation assays of T cells stimulated with plate-bound anti-CD3-Ab in presence or absence of EAP

To investigate the effect of EAP on the proliferation capacity of different T cell subsets, we first set up different proliferation assays for T_{resp} cells and T_{regs}. Briefly, T_{resp} cells were stimulated by TCR cross-linking with plate-bound anti-CD3-Ab alone, as this is known to be sufficient for inducing robust T_{resp} cell proliferation (Jenkins et al. 1990). For T_{reg} cell proliferation we established a stimulation protocol with soluble anti-CD3-Ab and co-stimulation with anti-CD28-Ab and IL-2 (Thornton and Shevach 1998).

To induce T_{resp} cell proliferation we coated 30µl of 2µg/ml anti-CD3-Ab to each bottom of a 24 wells flat-bottom microtiter plate. The plate was incubated at 37°C for 2 hours, before the solution was removed from the wells. For T_{reg} cell proliferation we added 0,2µg/ml soluble anti-CD28-Ab and 500U/ml of IL-2 -along with $1x10^6$ isolated CFSE-labelled $CD18^{wt}$ or $CD18^{hypo}$ T_{resp} cells respectively T_{reg} cells which were diluted in 100µl of PBS. In defined wells we added different concentrations of EAP to the cells. Four different concentrations of EAP (0; 5; 10 or 20µg/ml) were tested in quadruplicate for each cell type. After treatment the cells were incubated for 7 days in a humidified 5% CO_2 atmosphere at 37°C. Afterwards FACS analysis of the CFSE-labelled cells was performed to identify possible effects of increasing EAP-concentrations on cell proliferation (Fig. 8).

To exclude that changes in the proliferation of the differently treated T cell fractions were due to cytotoxic effects of EAP on T lymphocytes, we assessed cellular apoptosis by measuring Annexin V expression.

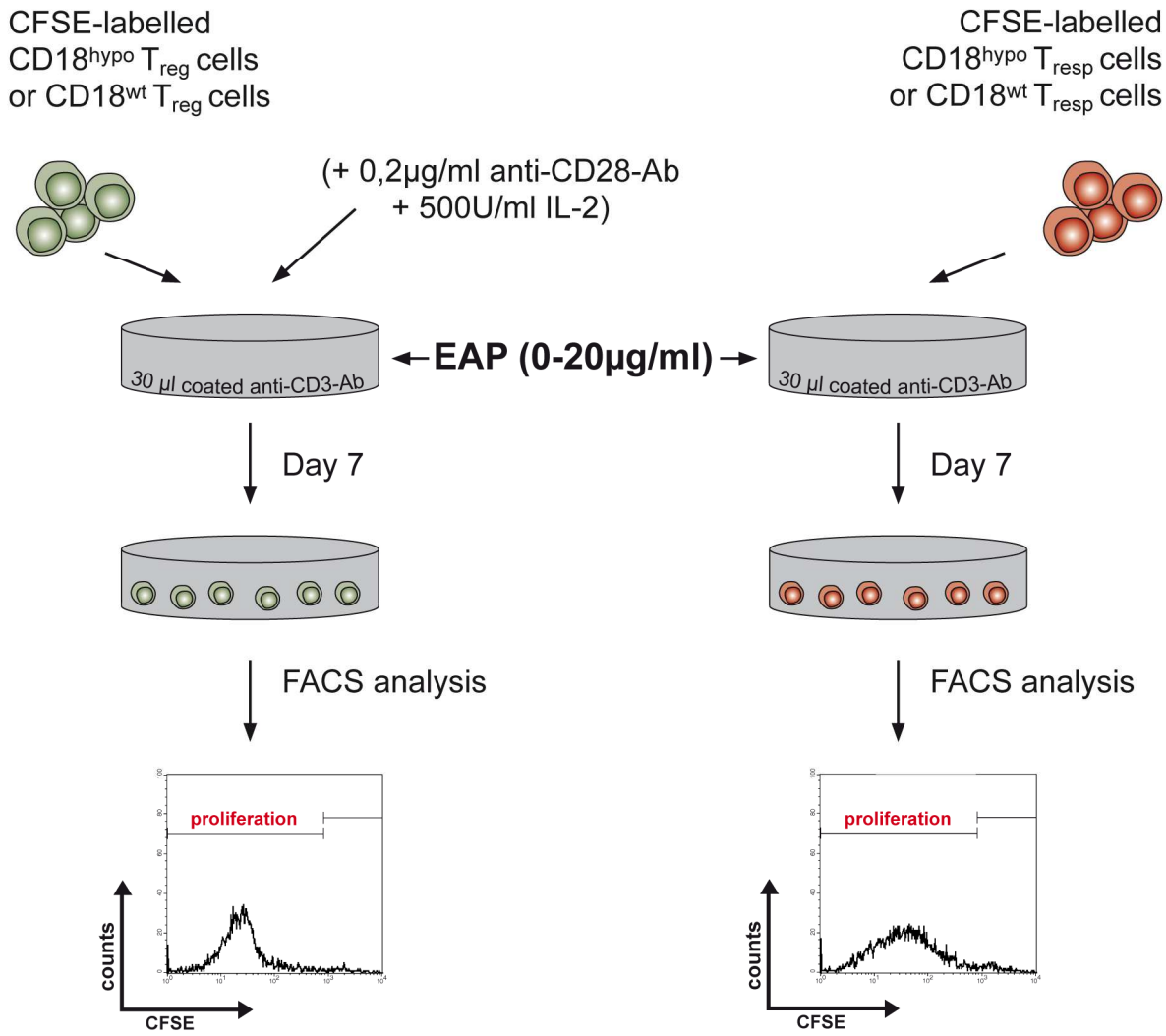

Fig. 8: Proliferation assay of T cell subsets stimulated with coated anti-CD3-Ab. *Anti-CD3-Ab was bound to the wells bottoms of a 24 well NUNC plate. Different subsets of CFSE-labelled T cells from both CD18^{wt} and CD18^{hypo} mice were plated in the wells (just one T cell subset per well) and treated with different concentrations of EAP (0; 5; 10 or 20µg/ml). Cells were cultured for 7 days in 100µl PBS, with 0,2µg/ml anti-CD28-Ab and 500U/ml IL-2 additionally supplemented in the experimental settings with T_{regs}. After 7 days proliferation of CFSE-labelled T cells was analysed by FACS.*

2.8.2. Proliferation assays of T cells stimulated with soluble anti-CD3-Ab in presence or absence of EAP

In parallel with T cell stimulation by plate-bound anti-CD3-Ab, we established alternative stimulation methods with soluble anti-CD3-Ab which require co-stimulation with anti-CD28-Ab and IL-2 for all T cell populations. As described in 2.8.1, we cultured 1x10^6 T cells in 24 well plates and added this time 500U/ml of IL-2 and 0,2µg/ml of anti-CD28-Ab for all T cell subtypes. Instead of anti-CD3-Ab

which was in the previous experiment bound to the wells bottom, we used 2µg/ml of soluble anti-CD3-Ab as stimuli. Again the proliferation assays were performed in absence or presence of different concentrations of EAP in the wells (0; 5; 10µg or 20µg/ml) to study the potential effect of EAP on T cell proliferation in these experimental settings.

After stimulation we stored the plates for 7 days in the cell culture incubator at 37°C. Afterwards a FACS analysis of the CFSE-labelled cells was performed to identify possible differences in cell proliferation induced by the increasing concentrations of EAP.

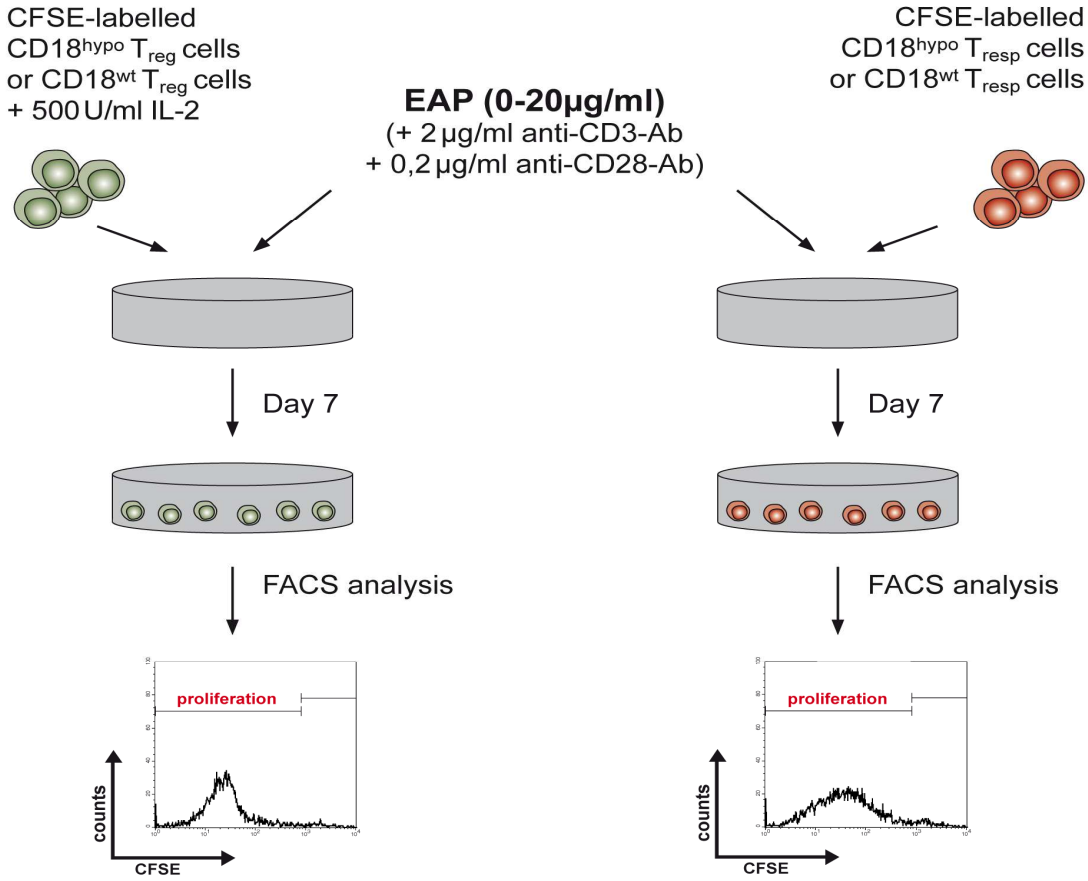

Fig. 9: Proliferation assay of T cell subsets stimulated with soluble anti-CD3-Ab. *Different subsets of CFSE-labelled T cells from both CD18^{wt} and CD18^{hypo} mice were cultured with 2µg/ml soluble anti-CD3-Ab in a 24 well NUNC plate (just one T cell subset per well) and for each subset different concentrations of EAP (0; 5; 10 or 20µg/ml) were supplemented. Cells were cultured for 7 days in 100µl PBS with 0,2µg/ml anti-CD28-Ab and 500U/ml IL-2. After 7 days proliferation of CFSE-labelled T cells was analysed by FACS.*

2.8.3. Expansion of T_{reg} cells by allogeneic dendritic cells in presence or absence of EAP

With this set of experiments we wanted to simulate a more physiologic proliferation of T_{reg} cells, which occurs *in vivo* by expansion of T_{reg} cells by antigen presenting DCs in the draining lymph nodes. We used in our experiments allogeneic DCs from C57/Bl6 mice (major histocompatibility complex H-2^b) which are known to have a much stronger effect on T cell expansion than autologous DCs. This was especially important for immunodeficient $CD18^{hypo}$ PL/J T cells in order to get the best possible stimulus for proliferation. We incubated $5x10^5$ T_{reg} cells from $CD18^{wt}$ and $CD18^{hypo}$ PL/J mice (MHC H-2^u) together with the same amount of allogeneic DCs (MHC H-2^b), 500U/ml of IL-2 and different EAP-concentrations (0; 5; 10 or 20µg/ml) in a 96 well flat-bottom microtiter plate at 37°C for 7 days. In another control experiment, we aimed at disrupting the immune synapse between DCs and T_{reg} cells by blocking LFA-1 using different concentrations of neutralizing anti-CD11a-Ab or anti-CD18-Ab or its counterpart ligand ICAM-1 using anti-ICAM-1-Ab respectively. Also Efalizumab as a humanized monoclonal anti-CD11a-Ab was used in this experimental setting.

If the mechanism of action of EAP is by impairing the proper formation of this synapse, we would expect to measure similar effects of EAP treatment and of LFA-1 or ICAM-1 inhibition on the expansion of T_{reg} cells by DCs. After 7 days we separated the two cell types by MACS. For this, we labelled the cells with 1µg/ml of anti-CD11c-MicroBeads per 10^6 DCs and incubated them for 15min at 4°C. Afterwards we washed the cells and placed a MS-column in the magnetic field of a MiniMACS separator, rinsed the column with 500µl of PBS, put the cell solution onto the column and let the unlabelled T cells run through. The column was washed 3 times with 500µl of PBS and the T_{reg} cells were collected, washed and resuspended in 2ml RPMI c.m. per 10^6 T_{reg} cells to be further investigated for suppression.

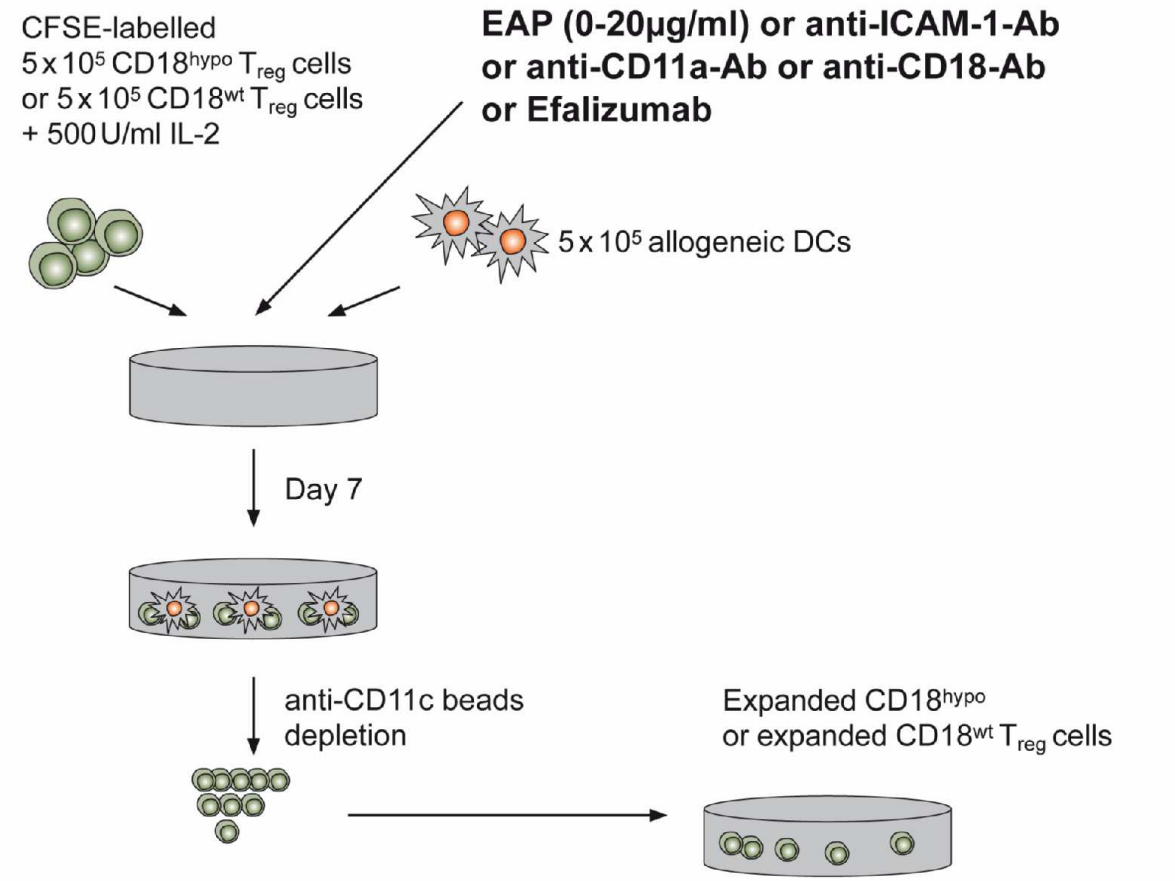

Fig. 10: Expansion of T_{reg} cells with allogeneic dendritic cells in presence or absence of EAP. *$5x10^5$ T_{reg} cells from both $CD18^{wt}$ respectively $CD18^{hypo}$ mice were cultured with the same amount of allogeneic DCs in a 96 well NUNC plate and for each subset different concentrations of EAP (0; 5; 10 or 20µg/ml) respectively anti-ICAM 1-Ab, anti-CD11a-Ab, anti-CD18-Ab or Efalizumab in different concentrations were added. Cells were cultured for 7 days in 100µl PBS with 500U/ml IL-2. After 7 days of expansion, DCs were removed by MACS sorting after labelling with anti-CD11c-MicroBeads. Expanded T_{reg} cells from both $CD18^{wt}$ and $CD18^{hypo}$ mice were supplemented with 2ml RPMI c.m. for use in further suppression assays.*

2.8.4. Suppression assays in presence or absence of EAP

We used the $CD18^{wt}$ and $CD18^{hypo}$ T_{reg} cells which were previously expanded by allogeneic DCs in absence or presence of EAP in co-culture with freshly isolated T_{resp} cells to investigate the suppressive function of the T_{reg} cells on the proliferation of T_{resp} cells in different genetic settings. Both cell types were cultured in 24 wells plates. We incubated $1x10^6$ CFSE-labelled T_{resp} cells with $2{,}5x10^5$ T_{reg} cells in 1ml RPMI c.m. (enriched with 500U/ml IL-2) for 7 days at 37˚C. After 7 days, the proliferation of T_{resp} cells was analysed by CFSE-dilution in FACS. As

controls, freshly isolated T_{reg} cells from both genotypes were used in co-cultures to determine if the suppressive capacity on T_{resp} cell proliferation was influenced by EAP for T_{reg} cells which were not previously expanded by DCs.

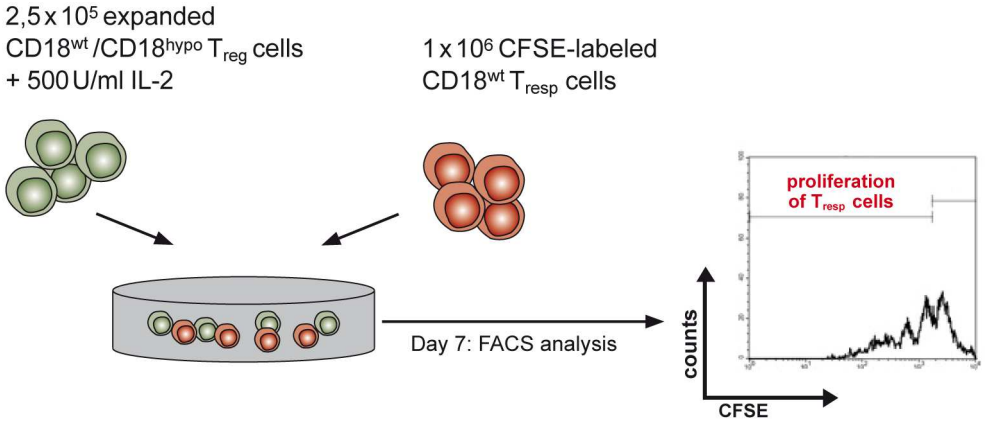

Fig. 11: Suppression assay in presence or absence of EAP. *2,5x10^5 expanded T_{reg} cells from CD18^{wt} respectively CD18^{hypo} mice were co-cultured with 1x10^6 CFSE-labelled CD18^{wt} T_{resp} cells in a 24 well NUNC plate. T_{reg} cells were previously expanded by allogenic DCs in presence or absence of different concentrations of EAP. Cells were cultured for 7 days in 1ml RPMI c.m. with 500U/ml IL-2. After 7 days proliferation of CFSE-labelled T_{resp} cells was analysed by FACS.*

2.8.5. Aggregation assays in presence or absence of EAP

Clustering of T cells around antigen presenting DCs is a prerequisite for primary stimulation of proliferative responses *in vivo* and *in vitro*. Our next approach was set up to visualize possible differences in cell-cell contacts and on the aggregation pattern induced by EAP. We studied the aggregation of CD18^{wt} T_{resp} cells respectively T_{reg} cells, co-cultured with allogeneic DCs in presence or absence of different concentrations of EAP in Petri dishes for 7 days. After 7 days the dishes were photographed at a 20x original magnification using a Zeiss Axiophot microscope and corresponding software (Zeiss). The photomicrographs were divided into defined fields by overlaying a grid and the clusters of DCs and T cells were counted in at least 100 high-power fields per experimental sample. The numbers of counted aggregates for both CD18^{wt} T_{reg} cells and CD18^{wt} T_{resp} cells in absence or presence of different concentrations of EAP were finally compared.

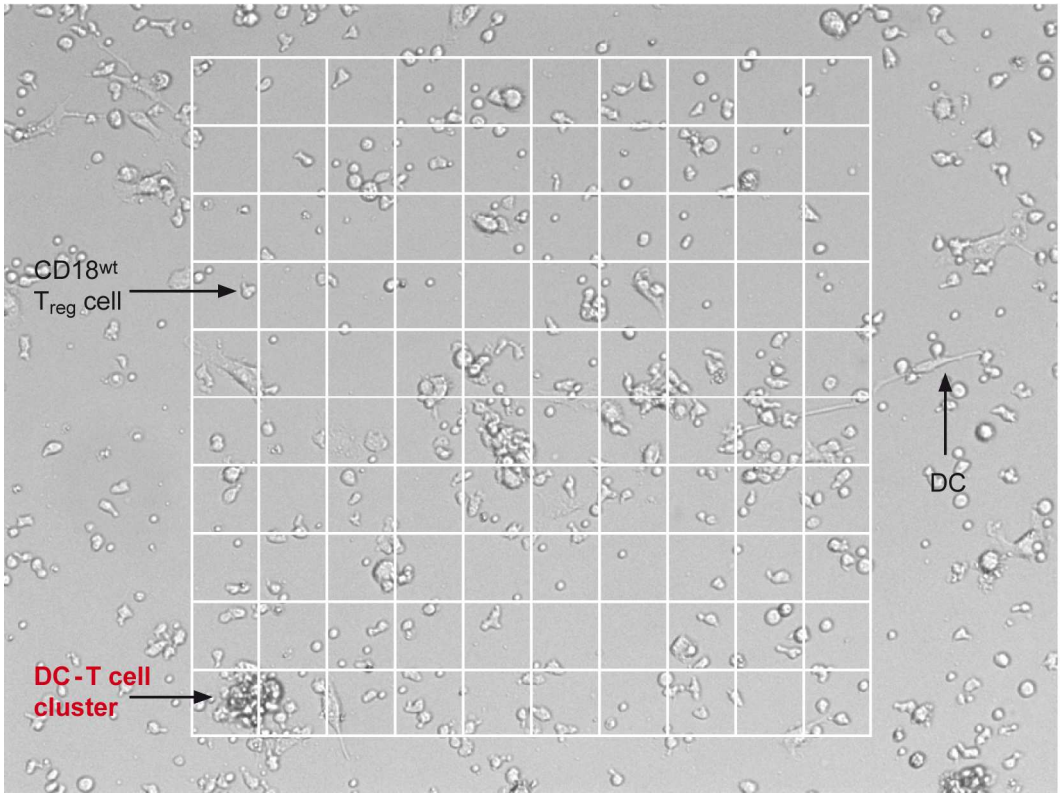

Fig. 12: Aggregation assay in presence or absence of EAP. *T_{reg} cells respectively T_{resp} cells from $CD18^{wt}$ respectively $CD18^{hypo}$ mice were co-cultured with DCs in Petri dishes in presence or absence of different concentrations (0; 5; 10 or 20µg/ml) of EAP. Cells were cultured for 7 days, thereafter the dishes were photographed at a 20x original magnification using a Zeiss Axiophot microscope. A randomly placed grid with 100 high-power fields was used to count clusters of DCs and T cells, indicating a close cell-cell contact which is supposed to require a tight contact between both cell types in the immune synapse. The picture indicates few cell-cell contact in presence of 20µg/ml EAP. Arrows point at a dendritic cell (DC), a $CD18^{wt}$ T_{reg} cell and at a cluster of T_{reg} cells surrounding a DC.*

2.9. Statistics

Quantitative data of cluster formation is presented as mean values of quadruplicates ± SD. Statistical significance was determined by the unpaired two-tailed Student's *t* test using GraphPad Prism® software. P values less than 0.05 were considered statistically significant; * indicates p values <0.05, ** indicate p<0.01.

3. RESULTS

3.1. Analysis of purity of isolated cell fractions

To investigate interactions of cells in presence of neutralizing antibodies and EAP, we first isolated cells from mice tissue as described before. To minimize the risk of undesired interaction of the target cells with additional cell types, we had to reach a high purity of our isolated cell fractions. For this purpose we controlled the grade of purity of the isolated cells by FACS analysis.

When T_{resp} cells were isolated, samples of the isolated cell fractions were stained with fluorochrome-labelled anti-CD4-Ab and anti-CD25-Ab. Anti-CD8-Ab staining detected and excluded $CD8^+$ T lymphocytes. T_{regs} were additionally stained with Abs against CD127 and Foxp3. Previous to analysing specific marker expression, the T lymphocytes were morphologically identified and gated according to their localization in FSC-SSC setting as shown in Fig. 13A. Next, the cells in the lymphocyte gate were plotted according to CD4, CD25 and CD127 expression to measure the purity of T_{regs} and T_{resp} cell populations from $CD18^{wt}$ and $CD18^{hypo}$ mice respectively. A control staining with isotype-matched Rat-IgG (Fig. 13F) allowed to distinguish specific from unspecific signals. Fig. 13 depicts an example of purity analysis for $CD4^+CD25^+$ T_{regs} isolated from a $CD18^{wt}$ mouse (Fig. 13B), compared with $CD4^+CD25^+$ T_{regs} (Fig. 13C) isolated from a $CD18^{hypo}$ mouse. Figure 13B demonstrates the isolation strategy for T_{regs} from $CD18^{wt}$ mice. Here, more than 75% of the $CD4^+CD25^+$ T cells (Fig. 13B, upper panel) expressed the CD127 marker at very low levels (Fig. 13B, lower panel), qualifying them as 'real' T_{reg} cells. By contrast, only 15% of the $CD4^+CD25^+$ gated $CD18^{hypo}$ T cells (Fig. 13C, upper panel) showed a low expression of CD127 (Fig. 13C, lower panel), clearly demonstrating the necessity for additional purification of $CD4^+CD25^+$ T cells according to CD127 expression in $CD18^{hypo}$ diseased mice. The quantified results from our analysed probes are presented at subtopics 3.1.1 - 3.1.3.

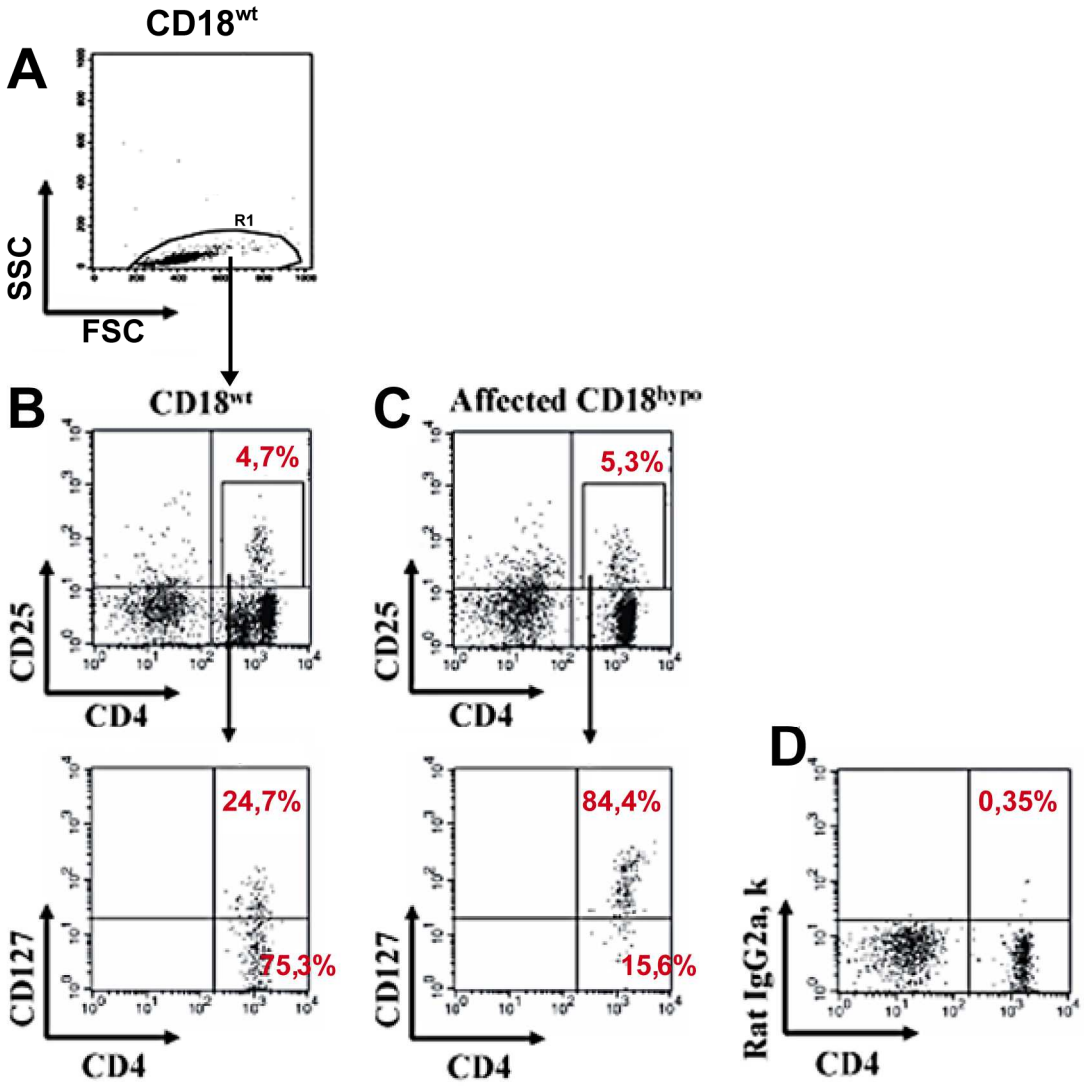

Fig. 13: Example for detection of T cell populations by FACS analysis. (**A**) *Isolated cells plotted on forward and side scatter; live lymphocytes gated (R1). Forward scatter is on the x-axis (FSC) and side scatter is on the y-axis (SSC). Gated $CD18^{wt}$ lymphocytes (**B**, upper panel) and $CD18^{hypo}$ lymphocytes (**C**, upper panel) as dot plot diagrams. CD4 is on the x-axis and CD25 on the y-axis. Cells in the upper right quadrant are $CD4^+CD25^+$ T_{reg} cells. Isolation of $CD4^+CD25^+$ $CD18^{wt}$ T cells (**B**, upper panel) yielded high percentages of $CD127^-$ T_{regs} (**B**, lower panel). The $CD4^+CD25^+$ fraction of $CD18^{hypo}$ T cells (**C**, upper panel) was strongly contaminated with $CD127^+$ activated T_{resp} cells (**C**, lower panel), indicating a reduced fraction of $CD4^+CD25^+CD127^-$ T_{reg} cells in $CD18^{hypo}$ mice. (**D**) Staining with isotype IgG excluded unspecific staining. Representative results for at least 3 independent experiments performed in triplicates (n=3). (adapted from (Wang et al. 2008))*

3.1.1. Purity of $CD4^+CD25^-$ T_{resp} cells

After isolation of splenocytes and depletion of $CD4^-$ T cells, $CD4^+CD25^-$ T_{resp} cells were purified by MACS sorting. To control the purity of this isolated cell fraction, i.e. the percentage of cells expressing the marker pattern characteristic for T_{resp} cells, an aliquot from the fraction was stained with fluorochrome-labelled Abs against CD4 and CD25 to make the expression of CD4 and CD25 visible in FACS, as shown in Fig. 12. Almost all $CD4^+$ gated T_{resp} cells were $CD25^-$. For $CD4^+CD25^-$ T_{resp} cells isolated from wild type animals we obtained a purity of 89-96%, for T_{resp} cells isolated from $CD18^{hypo}$ mice of up to 95%.

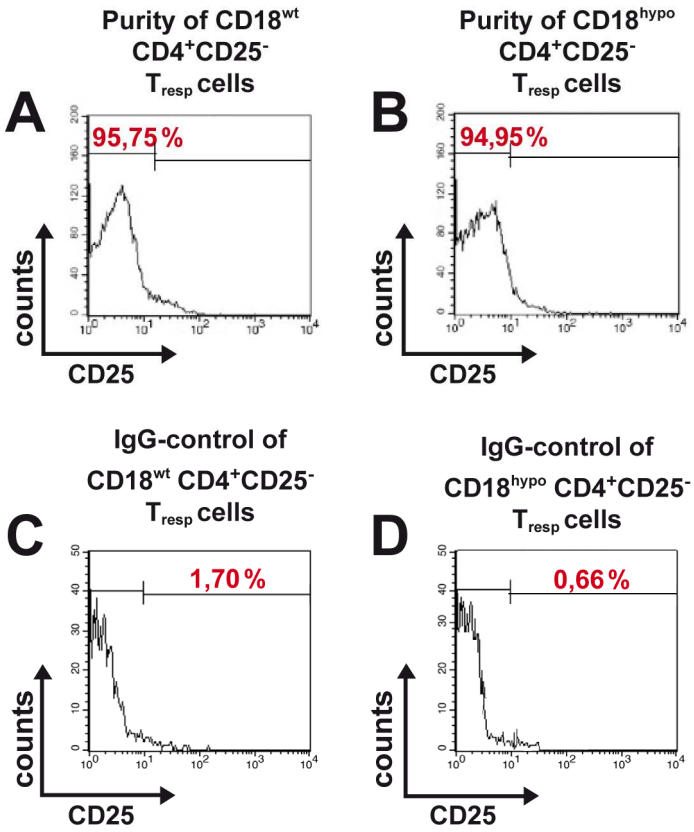

Fig. 14: Purity of T_{resp} cells. *Purity of T_{resp} cells of $CD18^{wt}$ (**A**) and of $CD18^{hypo}$ mice (**B**) and corresponding IgG-controls (lower panels **C** and **D**). Purity of $CD4^+CD25^-$ T_{resp} cell isolation yielded regularly in ranges of 89% – 96%. Representative results for at least 3 independent experiments performed in triplicates (n=3).*

3.1.2. Purity of $CD4^+CD25^+$ T_{reg} cells

For the evaluation of the purity of the $CD4^+$ $CD25^+$ T_{reg} cell fraction cells were stained with antibodies against CD4 and CD25. This time, gates were set for cells with $CD4^+$ and $CD25^+$ expression (as shown in Fig. 13 B and C). For T_{reg} cells from wild type animals we obtained a purity of up to 95%, for T_{reg} cells from $CD18^{hypo}$ mice a purity of even 98% (Fig. 15, upper panels). T_{reg} cells from $CD18^{hypo}$ mice were isolated from young (5-6 weeks old) and healthy animals to reduce the number of -in parallel to real T_{reg} cells isolated- activated $CD25^+$ T_{resp} cells. Staining with isotype IgG excluded unspecific staining (Fig. 15, lower panels).

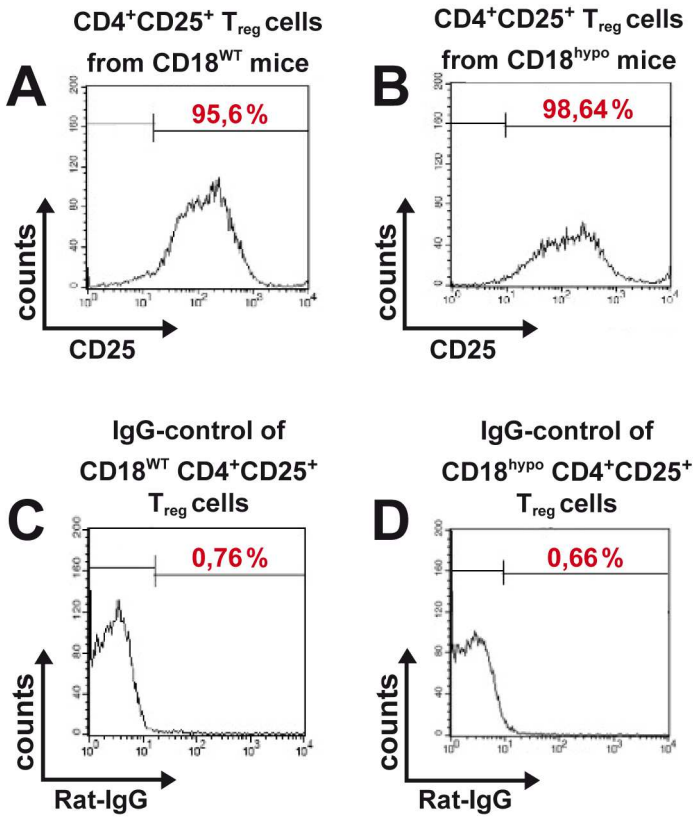

Fig. 15: Purity of T_{reg} cells. *Purity of T_{reg} cells of $CD18^{wt}$ (**A**) and of $CD18^{hypo}$ mice (**B**) and corresponding IgG-controls (lower panels, **C** and **D**). Purity of $CD4^+CD25^+$ T_{reg} cell isolation yielded regularly in ranges of 93% – 98%. Representative results for at least 3 independent experiments performed in triplicates (n=3).*

3.1.3. Purity of dendritic cells

DCs with high purity were required for the expansion of T_{reg} cells. DCs were isolated from C57BL/6 mice and stained with anti-CD11c-PE Ab to subsequently assess the purity of this cell fraction by FACS. Setting of gates for the evaluation of DCs purity was performed after exclusion of unspecific staining given by the isotype control again. The purity for DCs isolated from C57BL/6 mice was within limits from 88-94% (Fig. 16).

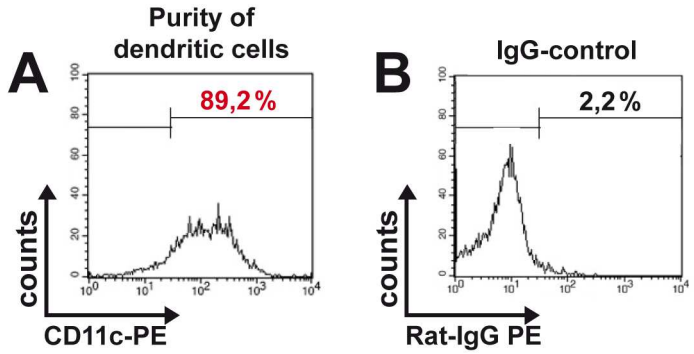

Fig. 16: Purity of dendritic cells. *Purity of CD11c-positive dendritic cells isolated and matured from C57Bl/6 mice (left panel) and IgG-control (right panel). Representative results for at least 3 independent experiments performed in triplicates (n=3).*

3.2. EAP inhibits the proliferation of T_{resp} cells in the in vitro proliferation assay

EAP is considered to block the LFA-1 (CD11a/CD18)-dependent adhesion of T cells to the ICAM-1 ligand (Xie et al. 2006). Therefore we first investigated whether EAP would inhibit the homophilic interaction required for the proliferation of T_{resp} cells upon cross-linking the TCR with plate-bound or respectively soluble anti-CD3-Ab in absence or presence of different concentrations of EAP. A higher percentage of MACS-purified $CD4^+CD25^-$ T_{resp} cells proliferated upon activation with the combination of soluble anti-CD3-Ab, co-stimulatory anti-CD28-Ab and IL-2 (37% of the T_{resp} cells; Fig. 17A) when compared with the amount of T_{resp} cells which had proliferated after stimulation with plate-bound anti-CD3-Ab alone (13-14% of the T_{resp} cells, Fig. 17A), which is known as a less effective proliferation

stimulus. Interestingly, addition of EAP to T_{resp} cells stimulated with soluble anti-CD3-Ab did not suppress T cell proliferation, even when given at the highest concentration of 20µg/ml of EAP, most likely indicating that such potent proliferative stimuli are able to overcome the reduction in the tight T cell – T cell contact induced by EAP (Fig. 17C). However, when anti-CD3-Ab was bound to the well bottom, the proliferating T_{resp} cell fraction decreased to less than 50% of the control cells when 20µg/ml of EAP were added (Fig. 17E). These results indicate that EAP may inhibit T_{resp} cell proliferation in presence of less potent, more physiologic stimuli, when obviously a tighter cell-cell contact is required, in this case accomplished by adhesion to coated anti-CD3-Ab. Furthermore, the finding that the proliferation of T_{resp} cells stimulated with soluble anti-CD3-Ab was not suppressed by EAP in any concentration up to 20µg/ml strongly indicates that EAP has no relevant cytotoxic effect on T_{resp} cells.

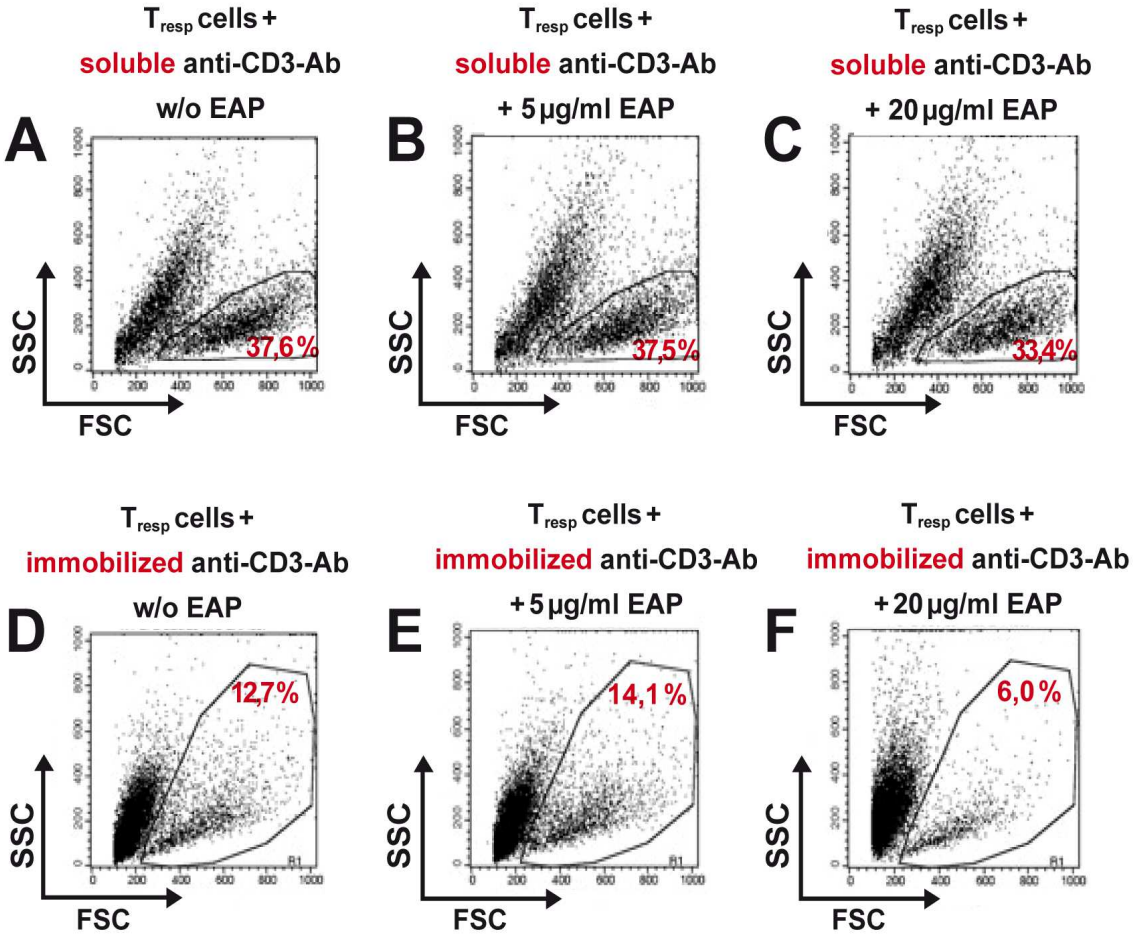

Fig. 17: EAP inhibits proliferation of T_{resp} cells induced by plate-bound anti-CD3-Ab, but not by soluble anti-CD3-Ab. *Dot-plot diagram showing the gated proliferative fraction of T_{resp} cells that were either stimulated with 2µg/ml soluble anti-CD3-Ab + 500U/ml IL-2 + 0,2µg/ml anti-CD28-Ab (**A**) or with immobilized anti-CD3-Ab (**D**). The results indicate soluble anti-CD3-Ab to be a stronger*

*proliferation stimuli (gated cell fraction) than plate-bound anti-CD3-Abs. Furthermore, a reduction of T_{resp} cell proliferation induced by EAP could only be observed when the T_{resp} cells were stimulated with immobilized anti-CD3-Ab (**E**+**F**). In contrast, addition of EAP to T_{resp} cells stimulated with soluble anti-CD3-Ab did not affect the proliferation of T_{resp} cells (**B** and **C**). Representative results for at least 3 independent experiments performed in triplicates (n=3).*

However, cytotoxicity of EAP in higher doses (100µg/ml up to 400µg/ml) has been reported in the literature (Lee et al. 2002). Therefore we analysed the expression of Annexin V, a classical apoptosis marker, to assess potential cytotoxic effects of EAP in our experiments. The numbers of Annexin V-positive cells were not enhanced by treatment with the highest used dose of 20µg/ml EAP when compared to untreated control cells, clearly indicating that EAP has no toxic effects on the cells up to this concentration (Fig. 18). Accordingly, non-cytotoxic doses of up to 20µg/ml of EAP were used in all experiments.

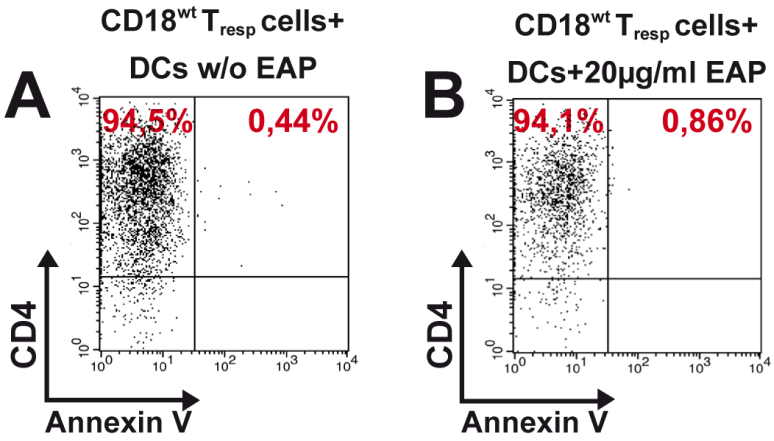

Fig. 18: EAP does not induce apoptosis in T_{resp} cells. *Dot- diagram of proliferating T_{resp} cells which were stimulated with 2µg/ml soluble anti-CD3-Ab + 500U/ml IL-2 + 0,2µg/ml anti-CD28-Ab in presence of DCs without EAP (**A**) and in presence of 20µg/ml of EAP (**B**). After 7 days of co-culture a sample of the T_{resp} cells was stained with Annexin V. The results show a comparable low expression of the apoptosis marker Annexin V for both settings, indicating that addition of EAP does not induce apoptosis. Representative results for at least 3 independent experiments performed in quadruplicates (n=3).*

3.3. EAP inhibits the suppressive function of DCs-expanded T_{regs} in vitro, similar with the antibody-mediated disruption of the LFA-1 – ICAM-1- mediated DC - T_{reg} contact

T_{reg} cells play a crucial role in suppressing uncontrolled inflammation in general (Sakaguchi 2000). Improper expansion, activation and function of T_{regs}, as also occurring in the $CD18^{hypo}$ PL/J mouse model (Wang et al. 2010), results in autoimmune diseases such as psoriasis, rheumatoid arthritis, encephalitis or colitis (Fontenot et al. 2003). *In vivo*, the adequate expansion of T_{regs} is accomplished by activated DCs, by direct antigen presentation and release of TGF-β, and this process is impaired in psoriasis patients and in the $CD18^{hypo}$ mouse model (Wang et al. 2008). However, the mechanism of how β_2-integrins control DC – T cell contacts and T_{reg} expansion, has not been elucidated so far. We hypothesised that proper T_{reg} expansion depends on the ideal strength of the immune synapse between these cells, which may be controlled by β_2-integrins. To study this hypothesis, we employed EAP as an established inhibitor of the LFA-1 – ICAM-1-dependent cell-cell interactions in co-cultures of T_{reg} cells with allogeneic DCs during T_{reg} cell expansion. The effect of targeting the immune synapse between DCs and T_{regs} with EAP was subsequently assessed by measuring the capacity of T_{reg} cells (expanded in absence or presence of EAP) to inhibit T_{resp} cells proliferation in *in vitro* suppression assays.

3.3.1. Expansion of T_{reg} cells by dendritic cells increases their suppressive function towards T_{resp} cell proliferation *in vitro*

First we investigated if the suppressive capacity of T_{reg} cells towards T_{resp} cells was improved if T_{reg} cells were expanded with dendritic cells- previous to co-culture with T_{resp} cells. Therefore we co-incubated $1x10^5$ $CD4^+CD25^+$ T_{regs} isolated from $CD18^{wt}$ mice with the same amount of allogeneic DCs isolated from C57Bl/6 mice in 96 wells microtiter plates and stimulated them with 500U/ml IL-2. After 7 days of co-culture, DCs were separated from $T_{regs.}$ by MACS sorting. 10^5 of expanded

purified T_{regs} were thereafter co-cultured for 7 days with $4x10^5$ CFSE-labelled T_{resp} cells freshly isolated from $CD18^{wt}$ mice which were stimulated with 500U/ml IL-2, 2µg/ml soluble anti-CD3-Ab and 0,2µg/ml anti-CD28-Ab and the proliferation was assessed by CFSE-dilution in FACS. In parallel, freshly isolated T_{regs} were directly co-cultured with T_{resp} cells under the same stimulation conditions. The results indicated a strong capacity of freshly isolated $CD4^+CD25^+$ T_{regs} to suppress T_{resp} cell proliferation at ratios of T_{reg}: T_{resp} cells 1:4 (Fig. 19B) when compared to the proliferation of stimulated T_{resp} cells alone (Figure 19A). However, the suppressive effect of T_{reg} cells on T_{resp} cells proliferation was much stronger when T_{reg} cells were previously 'licensed' by dendritic cells (Figure 19C). Because of the sufficient suppressive capacity of 'educated' T_{reg} cells already at a 1:4 ratio of T_{reg} cells to T_{resp} cells, the following suppression assays were all performed with T_{reg}: T_{resp} cells ratios of 1:4.

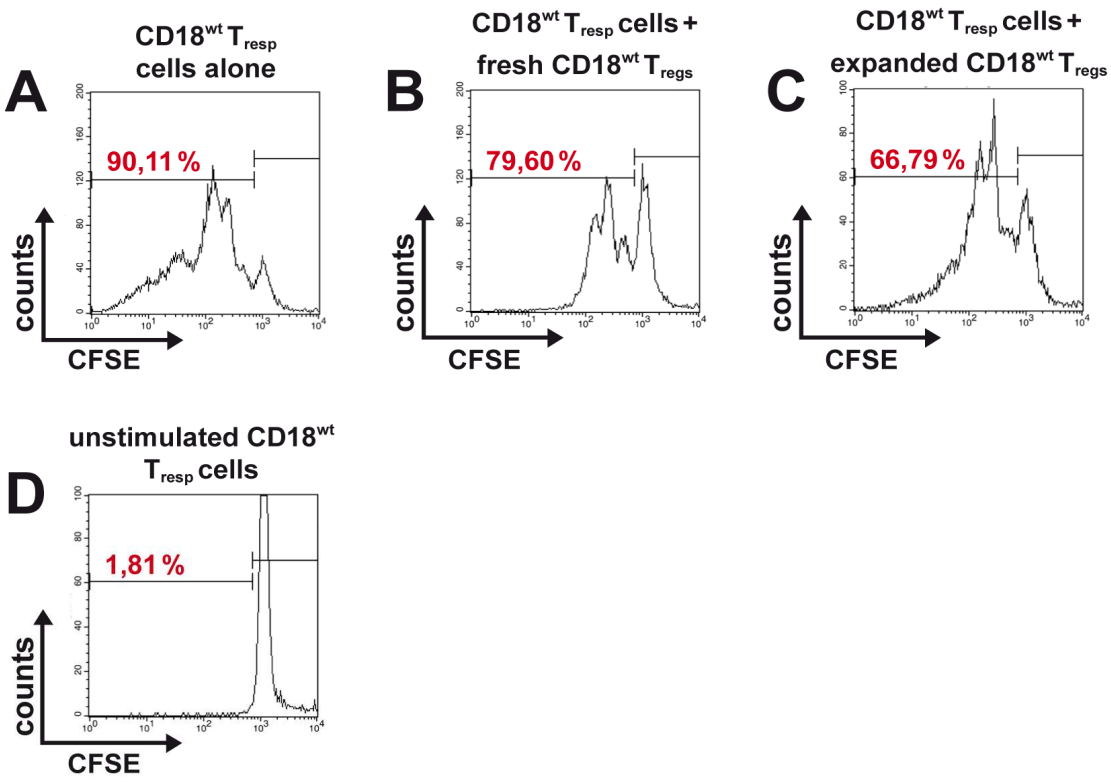

Fig. 19: **Dendritic cells enhance the suppressive effect of T_{regs} towards T_{resp} cells.** *(**A**) Histogram indicating the proliferation of T_{resp} cells after stimulation with soluble anti-CD3-Ab, anti-CD28-Ab and IL-2, (**B**) unexpanded fresh T_{reg} cells suppress the proliferation of T_{resp} cells and (**C**) even stronger suppressive effect of T_{reg} cells which were previously expanded with DCs on T_{resp} cell proliferation. (**D**) Unstimulated T_{resp} control cells do not proliferate. Representative results for at least 3 independent experiments performed in triplicates (n=3).*

3.3.2. EAP inhibits the suppressive function of expanded T_{reg} cells towards the proliferation of T_{resp} cells *in vitro*

Finally we investigated whether the interruption of the immune co-stimulatory synapse between LFA-1 on T_{regs} and ICAM-1 on dendritic cells by EAP would influence the suppressive function of T_{reg} cells on T_{resp} cells. Therefore we performed the 'education' of T_{reg} cells by dendritic cells in absence or presence of different concentrations of EAP. We co-incubated $1x10^5$ $CD4^+CD25^+$ T_{regs} isolated from $CD18^{wt}$ mice and stimulated with 500U/ml IL-2 with the same amount of allogeneic DCs in 96 wells microtiter plates in presence of different concentrations of EAP (0; 5; 10 or 20µg/ml). After 7 days of co-culture, DCs were separated from T_{regs} by MACS sorting. The expanded T_{regs} were now co-cultured for 7 days with $4x10^5$ freshly isolated CFSE-labelled T_{resp} cells from $CD18^{wt}$ mice which were activated with 500U/ml IL-2, 2µg/ml soluble anti-CD3-Ab and 0,2µg/ml anti-CD28-Ab and the proliferation was assessed by CFSE dilution in FACS. Remarkably, addition of EAP resulted in a dose-dependent reduction of the suppressive capacity of $CD18^{wt}$ T_{regs} towards T_{resp} cell proliferation (Fig. 20C,D), with the maximal dose of 20µg/ml completely abolishing the suppressive T_{reg} function (Fig. 20E), strongly suggesting that EAP may impair the suppressive function of T_{regs} by compromising the adhesion, and thus the 'education' of T_{regs} by DCs during their expansion.

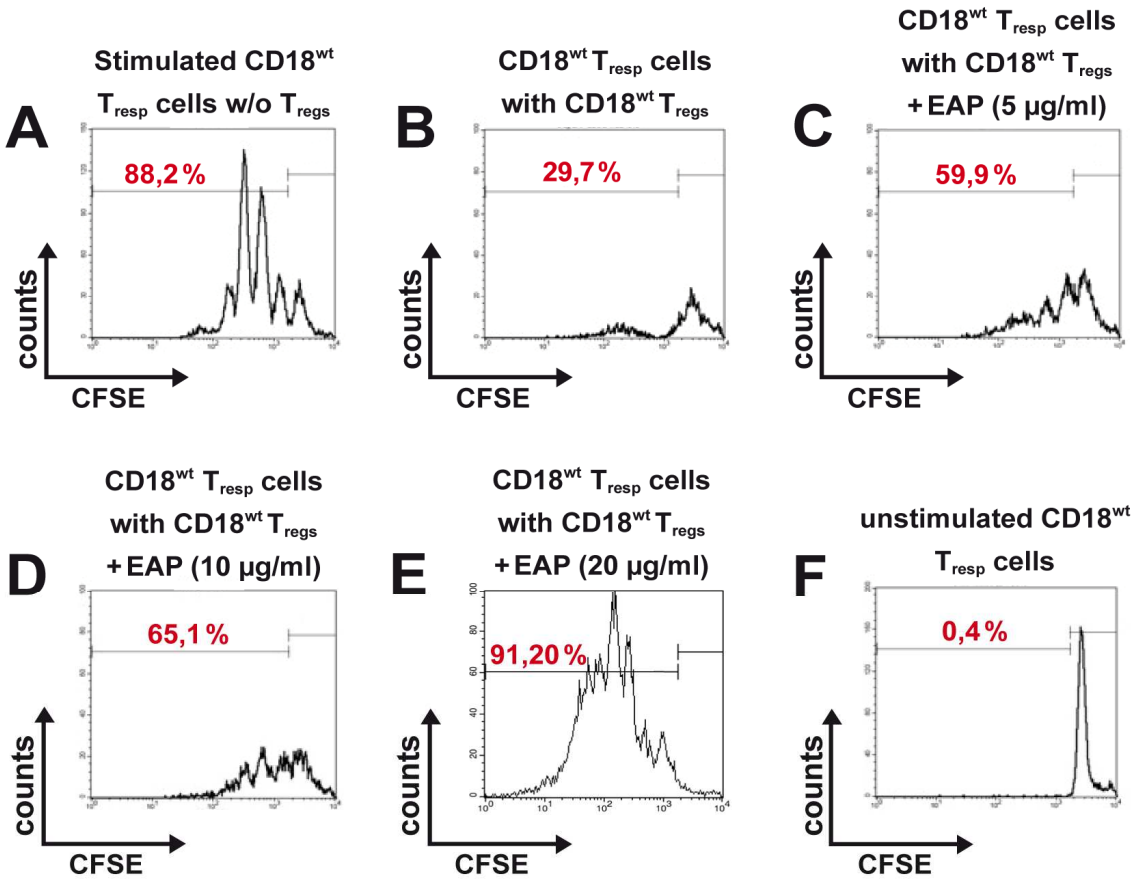

Fig. 20: Inhibition of LFA-1 signalling by EAP inhibits the suppressive function of CD18^{wt} T_{reg} cells towards CD18^{wt} T_{resp} cell proliferation. *(**A**) Proliferation of T_{resp} cells after stimulation with 2µg/ml soluble anti-CD3-Ab and 0,2µg/ml anti-CD28-Ab (**B**) Strong suppression of T_{resp} cell proliferation by co-culture with T_{reg} cells expanded in absence of EAP. Dose-dependent increase in the proliferation of T_{resp} cells co-cultured with T_{reg} cells previously expanded with DCs in presence of EAP at concentrations of 5µg/ml (**C**), 10µg/ml (**D**) and 20µg/ml (**E**). (**F**) Unstimulated T_{resp} control cells do not proliferate. Representative results for at least 3 independent experiments performed in quadruplicates (n=4).*

On the other hand, addition of EAP during expansion of CD18^{hypo} T_{regs} with DCs did not result in a change of the suppressive capacity of CD18^{hypo} T_{regs} towards CD18^{wt} T_{resp} cell proliferation (Fig. 21). In fact, no suppressive effect on CD18^{wt} T_{resp} cell proliferation could be observed even when no EAP was used previously, during T_{reg} cell expansion. This effect was most likely due to the impaired CD18, and consequently the affected LFA-1 expression on the surface of the T_{reg} cell membrane, which resulted in impaired cell-cell contact of T_{reg} cells towards both DCs during expansion, and towards T_{resp} cells in the suppression assay.

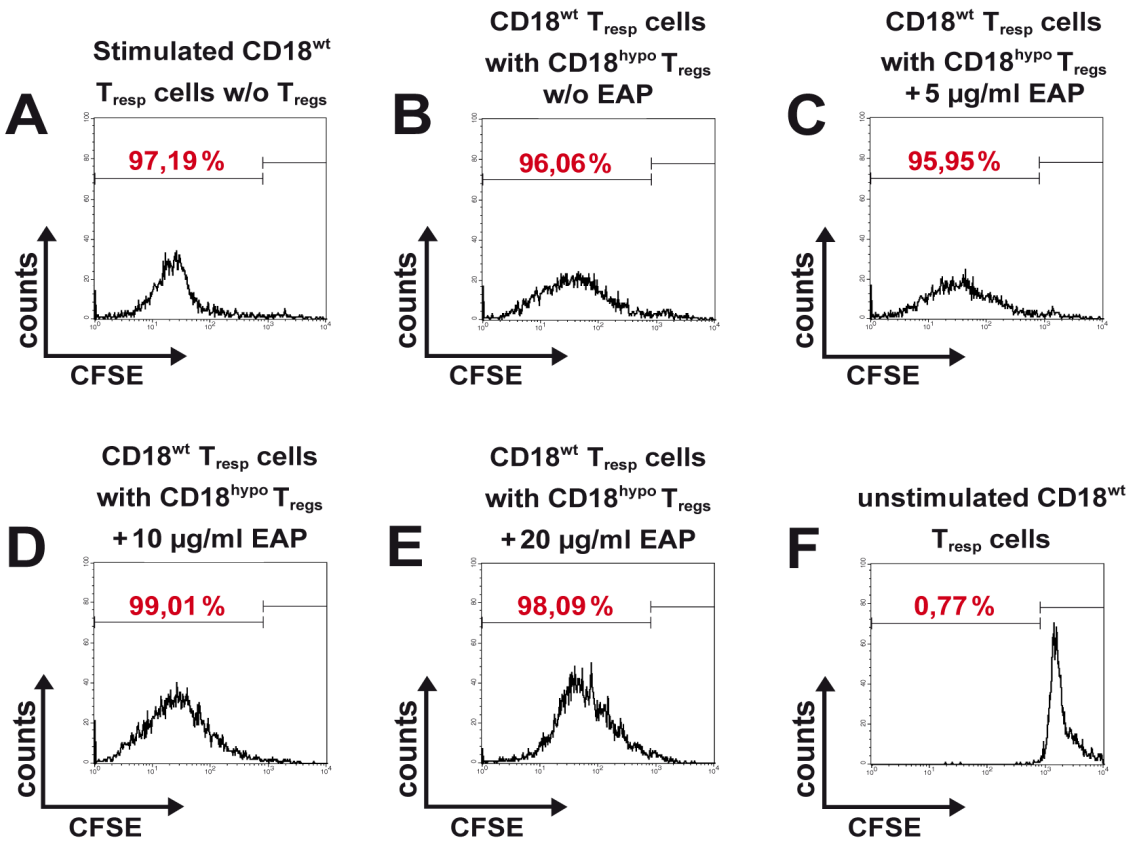

Fig. 21: Suppressive function of CD18^{hypo} T_{reg} cells towards CD18^{wt} T_{resp} cell proliferation is strongly impaired and not further affected by EAP. *(**A**) Proliferation of T_{resp} cells after stimulation with 2µg/ml soluble anti-CD3-Ab and 0,2µg/ml anti-CD28-Ab. (**B**) In vitro suppression of T_{resp} cell proliferation by co-culture with expanded T_{reg} cells in absence of EAP. No suppression of T_{resp} cell proliferation in presence of CD18^{hypo} T_{reg} cells is observed, even in absence of EAP. No dose-dependent increased proliferation of T_{resp} cells co-cultured with CD18^{hypo} T_{reg} cells previously expanded with DCs in presence of EAP at concentrations of 5µg/ml (**C**), 10µg/ml (**D**) and 20µg/ml (**E**). (**F**) Unstimulated T_{resp} control cells do not proliferate. Representative results for at least 3 independent experiments performed in quadruplicates (n=4).*

3.3.3. Disruption of LFA-1 – ICAM-1 dependent contact between DCs and T_{regs} inhibits the suppressive function of T_{regs} towards T_{resp} cell proliferation *in vitro*

We further wanted to investigate whether an interruption of the immune synapse by specific blocking the co-stimulatory signals between LFA-1 on T_{reg} cells and ICAM-1 on dendritic cells would result in the similar inhibition of the suppressive function of T_{reg} cells on T_{resp} cells as observed with EAP. For this we added

different concentrations of neutralizing anti-ICAM-1-Ab, respectively anti-CD18-Ab or anti-CD11a-Ab to the co-cultures of $1x10^5$ $CD4^+CD25^+$ T_{regs} stimulated with 500U/ml IL-2 (isolated from $CD18^{wt}$ mice) with the same amount of allogeneic DCs. T_{reg} cells expanded with DCs in presence or absence of neutralizing anti-CD11a, anti-CD18 or anti-ICAM-1 antibodies were subsequently co-cultured for 7 days with $4x10^5$ freshly isolated CFSE-labelled T_{resp} cells from $CD18^{wt}$ mice stimulated with 500U/ml IL-2, 2µg/ml soluble anti-CD3-Ab and 0,2µg/ml anti-CD28-Ab and the proliferation was assessed by CFSE-dilution in FACS. Addition of neutralizing anti-ICAM1-Ab (Fig. 22), of anti-CD18-Ab (Fig. 23) and of anti-CD11a-Ab (Fig. 24) dose-dependently reduced the capacity of T_{regs} to suppress T_{resp} cell proliferation, clearly demonstrating that a proper signalling via the co-stimulatory molecules ICAM-1 and LFA-1 enables the formation of a tight immune synapse between DCs and T_{regs} and is required to instruct and expand T_{regs} with proper suppressive functions.

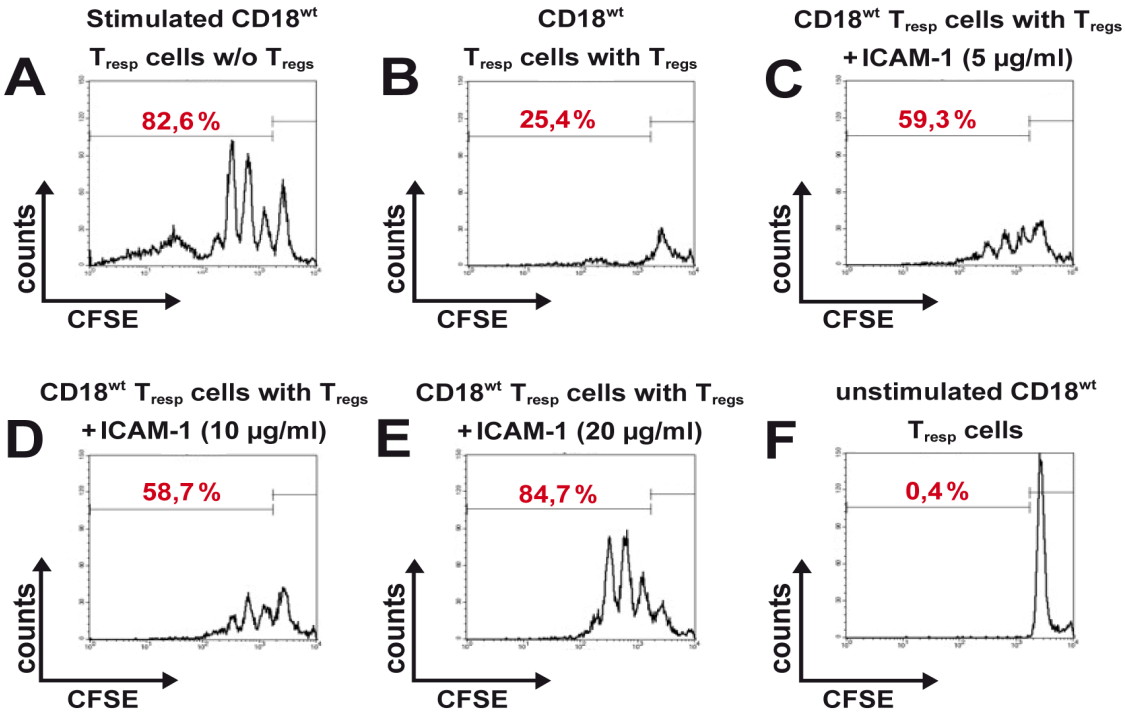

Fig. 22: Inhibition of ICAM-1 signalling with blocking anti-ICAM-1-Ab inhibits the suppressive function of T_{reg} cells towards T_{resp} cell proliferation. *(**A**) Proliferation of T_{resp} cells after stimulation with 2µg/ml soluble anti-CD3-Ab and 0,2µg/ml anti-CD28-Ab. (**B**) Suppression of T_{resp} cell proliferation by co-culture with T_{reg} cells expanded in absence of anti-ICAM-1-Ab. Dose-dependent increase in proliferation of T_{resp} cells co-cultured with T_{reg} cells previously expanded with DCs in presence of anti-ICAM-1-Ab at concentrations of 5µg/ml (**C**), 10µg/ml (**D**) and 20µg/ml (**E**). (**F**) Unstimulated T_{resp} control cells do not proliferate. Representative results for at least 3 independent experiments performed in triplicates (n=3).*

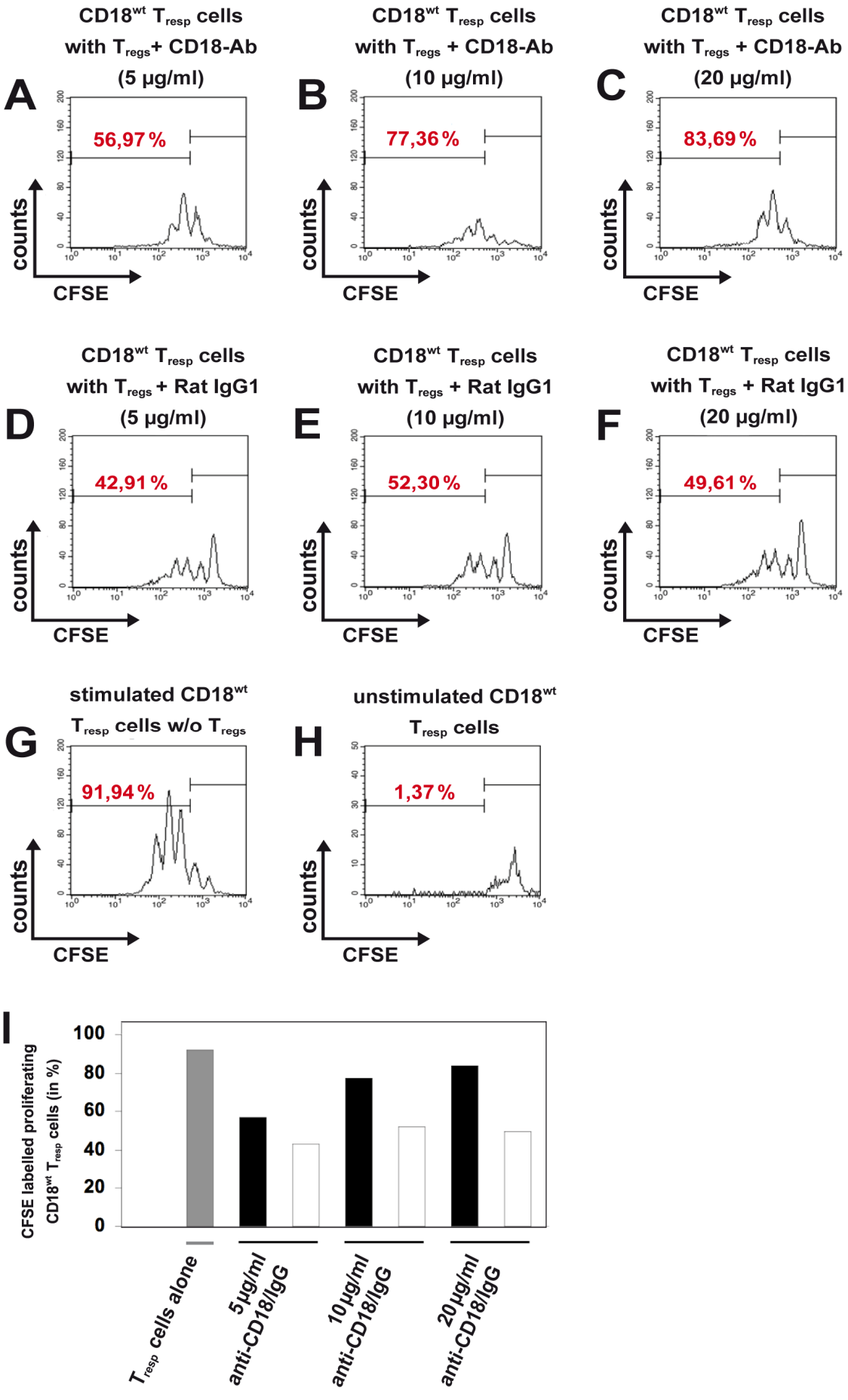
A
CD18wt Tresp cells
with Tregs+ CD18-Ab
(5 µg/ml)
56,97 %
counts
CFSE
B
CD18wt Tresp cells
with Tregs + CD18-Ab
(10 µg/ml)
77,36 %
counts
CFSE
C
CD18wt Tresp cells
with Tregs + CD18-Ab
(20 µg/ml)
83,69 %
counts
CFSE
D
CD18wt Tresp cells
with Tregs + Rat IgG1
(5 µg/ml)
42,91 %
counts
CFSE
E
CD18wt Tresp cells
with Tregs + Rat IgG1
(10 µg/ml)
52,30 %
counts
CFSE
F
CD18wt Tresp cells
with Tregs + Rat IgG1
(20 µg/ml)
49,61 %
counts
CFSE
G
stimulated CD18wt
Tresp cells w/o Tregs
91,94 %
counts
CFSE
H
unstimulated CD18wt
Tresp cells
1,37 %
counts
CFSE
I
CFSE labelled proliferating
CD18wt Tresp cells (in %)
100
80
60
40
20
0
Tresp cells alone
5µg/ml
anti-CD18/IgG
10µg/ml
anti-CD18/IgG
20µg/ml
anti-CD18/IgG

Fig. 23: Inhibition of LFA-1 signalling with blocking anti-CD18-Ab inhibits the suppressive function of T_{reg} cells towards T_{resp} cell proliferation. The *strongest inhibition of T_{resp} cell proliferation was observed when they were co-cultured with DC-expanded T_{reg} cells, as described in Fig. 22 (data not shown). Dose-dependent increase in the proliferation of T_{resp} cells co-cultured with T_{reg} cells previously expanded with DCs in presence of anti-CD18-Ab at concentrations of 5µg/ml (**A**), 10µg/ml (**B**) and 20µg/ml (**C**). Rat IgG isotype control with corresponding concentrations as in histograms (**A-C**) of 5µg/ml (**D**), 10µg/ml (**E**) and 20µg/ml (**F**) show no influence on T_{resp} cell proliferation. (**G**) Proliferation of T_{resp} cells w/o T_{reg} cells stimulated with 500U/ml IL-2, 2µg/ml soluble anti-CD3-Ab and 0,2µg/ml anti-CD28-Ab. (**H**) Unstimulated T_{resp} cells do not proliferate. (**I**) Representative column bar diagram with anti-CD18-Ab as an example for all of our experiments with blocking Abs and EAP showing the dose-dependent decrease of T_{resp} cell proliferation by T_{reg} cells. Black-coloured columns represent the influence of increasing concentrations of anti-CD18-Ab on T_{resp} cell proliferation, white-coloured columns represent the corresponding IgG isotype controls. Representative results for at least 3 independent experiments performed in triplicates (n=3).*

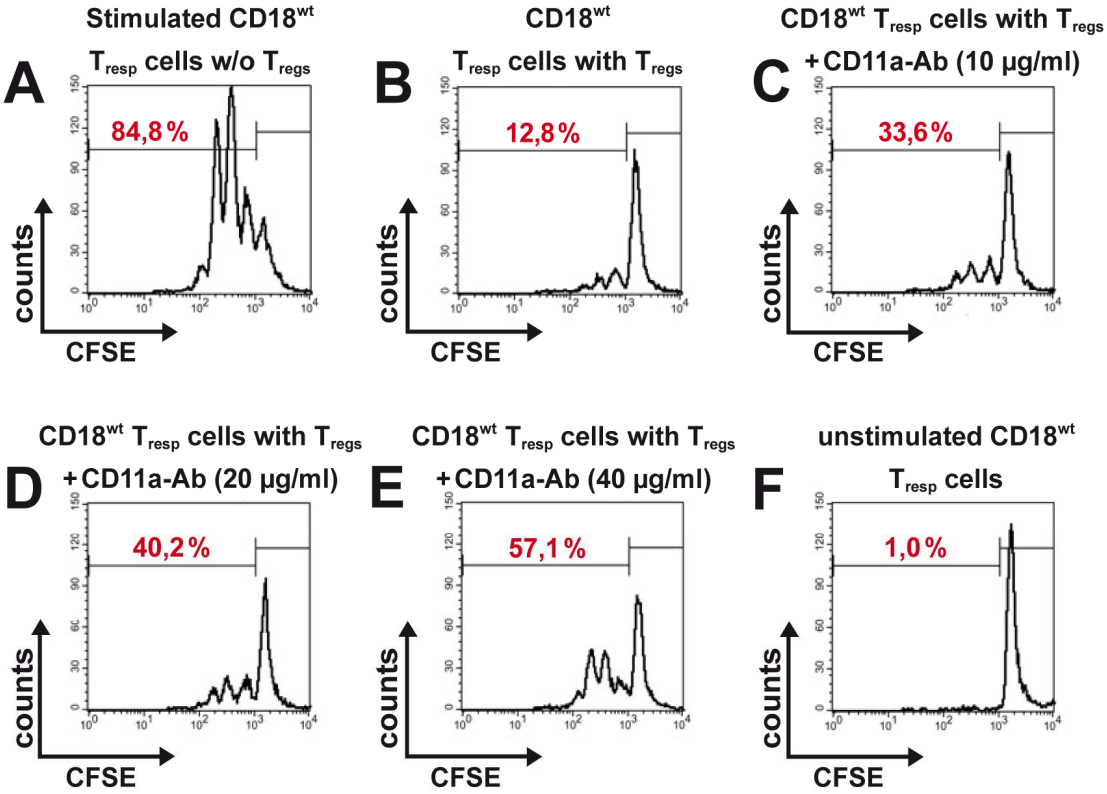

Fig. 24: Inhibition of LFA-1 signalling with blocking anti-CD11a-Ab inhibits the suppressive function of T_{reg} cells towards T_{resp} cell proliferation. *(**A**) Proliferation of T_{resp} cells after stimulation with 2µg/ml soluble anti-CD3-Ab and 0,2µg/ml anti-CD28-Ab. (**B**) Suppression of T_{resp} cell proliferation by co-culture with T_{reg} cells expanded in absence of anti-CD11a-Ab. Dose-dependent increase in the proliferation of T_{resp} cells co-cultured with T_{reg} cells which were previously expanded with DCs in presence of anti-CD11a-Ab at concentrations of 10µg/ml (**C**), 20µg/ml (**D**) and 40µg/ml (**E**). (**F**) Unstimulated T_{resp} cells do not proliferate. Representative results for at least 3 independent experiments performed in triplicates (n=3).*

3.3.4. The humanized anti-CD11a-Ab Efalizumab (Raptiva®) inhibits the suppressive capacity of T_{reg} cells towards T_{resp} cell proliferation *in vitro*

The humanized anti-CD11a-Ab Efalizumab has been previously shown to interfere with LFA-1–ICAM-1 dependent adhesion mechanisms and thus to attenuate a broad range of T cell mediated immune responses *in vitro* (Dustin and Springer 1988) and in animal models (Gordon et al. 1995; Nakakura et al. 1993). In humans, Efalizumab has been successfully employed for the treatment of moderate to severe plaque psoriasis, acting via inhibition of T cell trafficking and blockade of memory T cells entering the inflamed skin (Lebwohl et al. 2003). We further asked whether Efalizumab would also decrease the suppressive function of T_{regs} in our herein established *in vitro* proliferation assays, similarly with the results obtained with EAP or with the murine neutralizing anti-CD11a-Ab, anti-CD18-Ab or anti-ICAM-1-Ab. T_{reg} cells and dendritic cells were co-cultured as described in 3.3.2, this time in presence of Efalizumab instead of EAP or murine Abs against CD11a, CD18 or ICAM-1. As expected, addition of Efalizumab in the culture medium during the expansion of T_{regs} with DCs resulted in a dose-dependent inhibition of the suppressive function of T_{reg} cells on T_{resp} cells proliferation (Fig. 25). These results demonstrate that EAP shares a common mechanism with anti-CD11a-Abs with the specific inhibition of physiologic LFA-1 – ICAM-1-dependent cell-cell contacts, thus interrupting the antigen presentation to T_{reg} cells and eventually resulting in impaired suppression of pathogenic T_{resp} cells and autoimmunity.

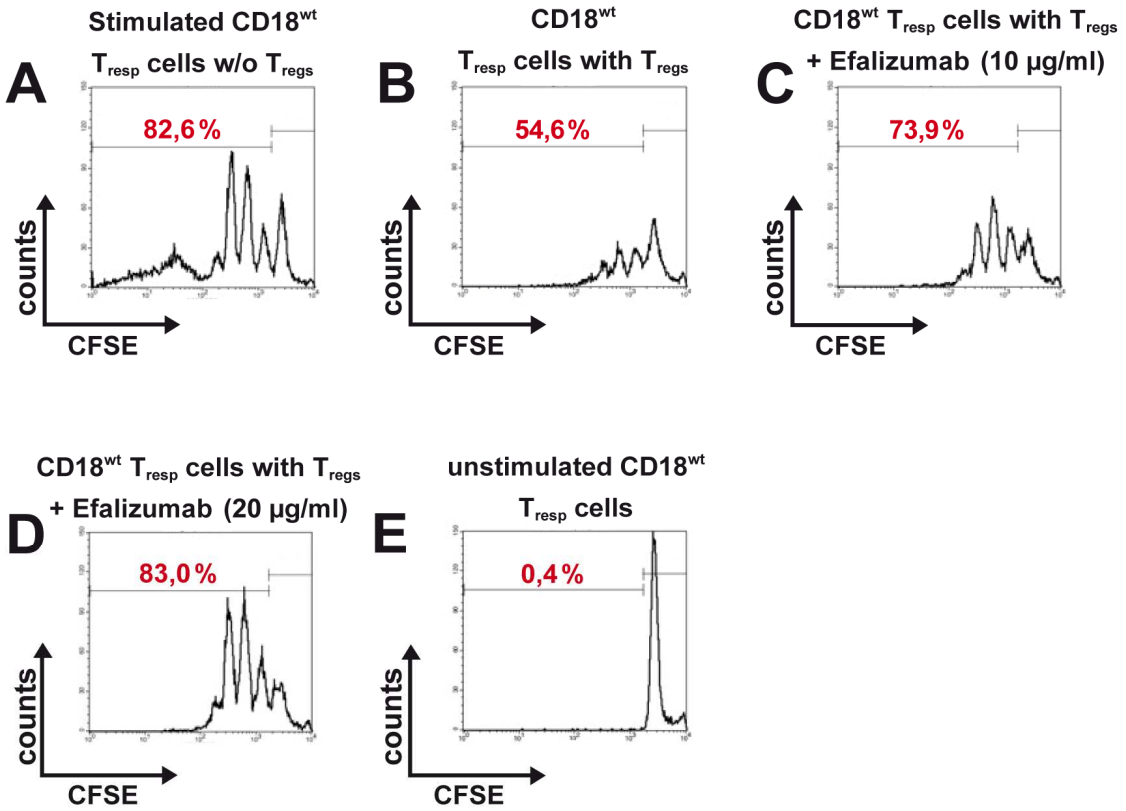

Fig. 25: Inhibition of LFA-1 signalling with Efalizumab inhibits the suppressive function of T_{reg} cells towards T_{resp} cell proliferation. *(**A**) Proliferation of T_{resp} cells after stimulation with 2µg/ml soluble anti-CD3-Ab and 0,2µg/ml anti-CD28-Ab. (**B**) Suppression of T_{resp} cell proliferation by co-culture with T_{reg} cells expanded in absence of Efalizumab. Dose-dependent increase in the proliferation of T_{resp} cells co-cultured with T_{reg} cells previously expanded with DCs in presence of Efalizumab at concentrations of 10µg/ml (**C**) and 20µg/ml (**D**). (**E**) Unstimulated T_{resp} cells do not proliferate. Representative results for at least 3 independent experiments performed in triplicates (n=3).*

3.4. *EAP inhibits cluster formation between T_{regs} and DCs in vitro*

The results of our suppression assays with addition of EAP suggested that EAP compromised the formation of the immune synapse between T_{reg} cells and dendritic cells. Similar results were obtained in similarly designed suppression assays with blocking antibodies against LFA-1 and ICAM-1. We therefore hypothesised that the deficient immune synapse formation may be due to the impaired direct physical contact between antigen presenting cells and T_{regs}. If this was true, T_{regs} may not even come in contact with DCs and thus would not cluster around DCs in presence of EAP. To verify this hypothesis, we co-cultured

$CD4^+CD25^+$ T_{reg} cells isolated from $CD18^{wt}$ C57BL/6J mice with dendritic cells isolated from BALB/C mice in absence or presence of EAP for 7 days in an *in vitro* aggregation assay. Cluster formation between T_{regs} and DCs was microscopically assessed by taking photomicrographs from 100 randomly selected high-power fields and counting cell aggregates as previously described (Wang et al. 2008). The results of the aggregation assays clearly show an inhibitory effect of EAP on the formation of cell contacts between dendritic cells and T_{reg} cells resulting in reduced numbers of cell clusters in presence of EAP (Fig. 26A). In analogy with the suppressive effect on T_{resp} cell proliferation, the decrease in cluster formation was dose-dependent, with less clusters at higher concentrations of EAP. A control aggregation assay of $CD18^{hypo}$ T_{reg} cells and DCs showed reduced numbers of clusters, comparable to aggregation assays of $CD18^{wt}$ T_{reg} cells and DCs in addition of EAP. Interestingly, an identical inhibitory effect on cluster formation was observed when DCs were co-cultured with T_{resp} cells in presence of EAP, suggesting that EAP does not exclusively affect LFA-1-dependent T_{reg} functions, but most likely all T lymphocyte populations. However, it is still not clear how the functions of different T cell subsets are influenced by EAP which remains to be studied.

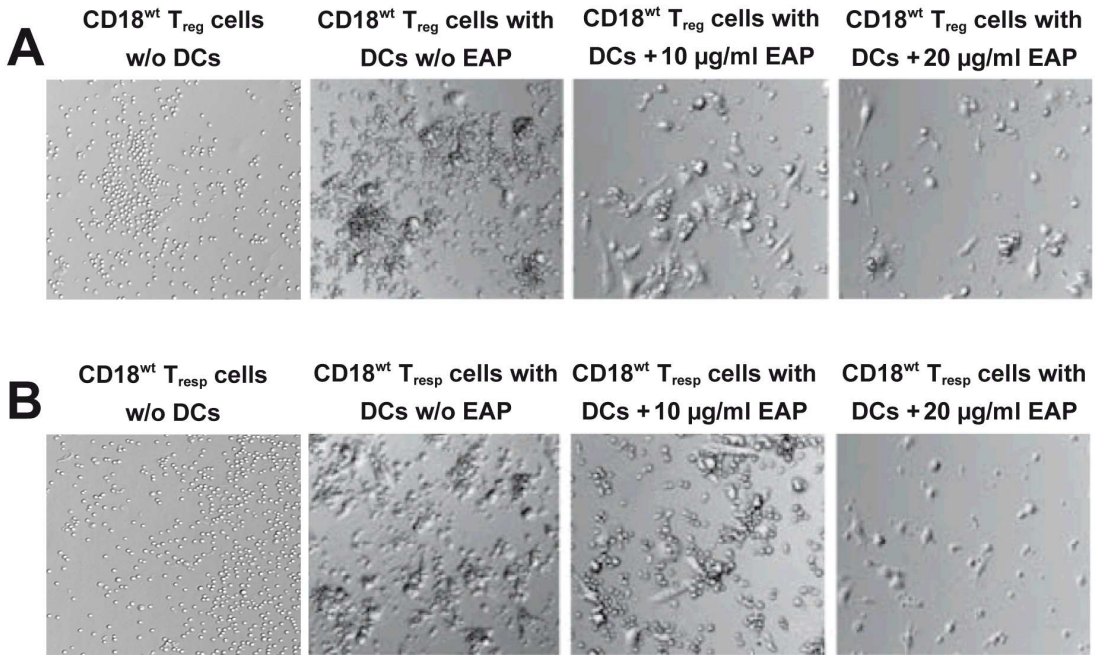

Fig. 26: **EAP inhibits cluster formation between DCs and T_{regs}, respectively T_{resp} cells.** *Representative photomicrographs showing cluster formation of T_{regs} (**A**) and T_{resp} cells (**B**) co-cultured with or w/o DCs in presence of different concentrations of EAP. The pictures clearly show a decrease of cluster formation in presence of increaing concentrations of EAP. (adapted from (Wang et al. 2010))*

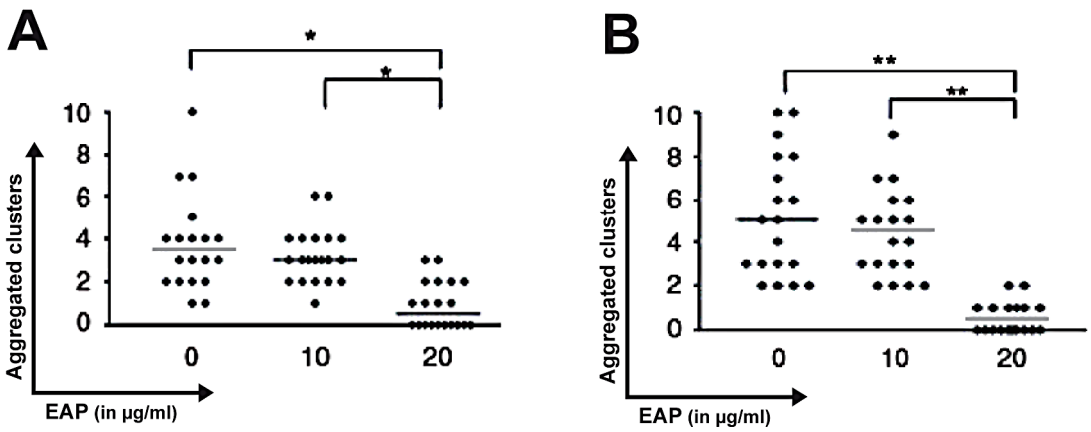

Fig. 27: Quantitative analysis of cluster formation between DCs and T_{regs}, respectively T_{resp} cells in presence or absence of EAP. *(**A**) Cluster quantification in aggregation assays of T_{reg} cells and DCs in addition of different concentrations of EAP. (**B**) Counted clusters of T_{resp} cells and DCs in presence of different concentrations of EAP. Results are given as mean ± SD in scatter columns representing the number of clusters of T_{reg} cells and DCs counted from 100 randomly chosen high-power fields ($^{*}P<0.05$, $^{**}P<0.01$ by Student's t-test). One representative experiment in triplicate out of three independent experiments is shown. (adapted from (Wang et al. 2010))*

4. DISCUSSION

Psoriasis is a common inflammatory disease of the skin with a complex and so far not completely resolved pathogenesis (Nestle et al. 2009). Clinical and histopathologic features, as well as the response to specific therapy all suggest psoriasis to be a T_h1 mediated autoimmune disease with a persistent activation of autoreactive pro-inflammatory 'type 1' T cells due to an impaired function of T_{reg} cells (Nestle et al. 2009; Nograles et al. 2011). T_h1 cells are actively recruited to psoriatic plaques at the level of high endothelial venules via integrin-dependent adhesion to highly up-regulated endothelial adhesion molecules E-selectins, ICAMs and VCAMs (Krueger 2002; Terajima et al. 1998; Yusuf-Makagiansar et al. 2002). Here they persistently release type 1 pro-inflammatory cytokines, especially TNF-α and IFN-γ, which then perpetuate the inflammatory cascade in psoriasis (Nestle et al. 2009; Nickoloff et al. 2007b). Therefore, therapeutic approaches targeting T cell recruitment and the release of inflammatory cytokines proved to be very efficient in resolving psoriasis inflammation in the past years (Krueger 2002; Marble et al. 2007).

The staphylococcal extracellular adherence protein is an immunomodulatory substance secreted by Staphylococcus aureus species which is known to bind to ICAM-1 (Harraghy et al. 2003), thus blocking β_2 integrin signalling. This results in an inhibition of leukocyte migration and extravasation, thus protecting SA from leukocyte killing (Chavakis et al. 2002; Hussain et al. 2008). Interestingly, in recent preliminary work of our group, daily injections of EAP resolved the psoriatic skin inflammation in the established $CD18^{hypo}$ PL/J murine model of psoriasiform skin disease (Wang et al. 2010), indicating a valuable therapeutic potential of EAP for psoriasis. However, which LFA-1 – ICAM-1-mediated immune processes were causally controlled by EAP in this model was not addressed so far.

In this thesis, we analysed *in vitro* the cellular mechanisms possibly influencing the control of the psoriasiform skin disease by EAP *in vivo*. We found that EAP inhibited the suppressive function of T_{regs} on T_{resp} cell proliferation. This was most likely due to an impaired direct interaction between T_{regs} and DCs during antigen presentation resulting in an impaired 'licensing' of the Treg cells. An identical

decrease of the suppressive T_{reg} function could be reproduced by targeting the co-stimulatory signal between LFA-1 (CD11a/CD18) on T_{regs} and ICAM-1 on DCs during T_{reg} 'education' by using neutralizing antibodies against CD18, CD11a and ICAM-1 respectively. Moreover, the addition of the humanized anti-CD11a-mAb Efalizumab in the same experimental settings resulted in a very similarly impaired suppressive function of the T_{regs}, suggesting EAP to share the mechanism of action with this antibody which had already been employed in human psoriasis with a comparable efficiency.

Addition of EAP during the expansion and education of T_{regs} with DCs *in vitro* had two important consequences: first, EAP induced a severe impairment of the immune suppressive capacity of T_{regs} on T_{resp} cells. This effect was dose-dependent and, at the maximal concentration of 20µg/ml, the suppressive function of T_{regs} was virtually abolished (Fig. 20). This *in vitro* function of EAP is interesting and has never been described so far. However, the *in vivo* relevance of this mechanism is questionable, as administration of EAP in different mouse models of inflammation did not primary affect T_{reg} function, but rather directly inhibited T_{resp} cell recruitment and proliferation (Lee et al. 2002; Schneider et al. 2007; Xie et al. 2006). However, the specific effect of EAP on T_{regs} *in vivo* is still not elucidated and should be addressed in the future using appropriate genetic tools. By contrast to this, addition of EAP during expansion of T_{regs} isolated from $CD18^{hypo}$ mice did not further affect the suppressive capacity of $CD18^{hypo}$ T_{regs} on $CD18^{wt}$ T_{resp} cell proliferation, which was severely impaired even in the absence of EAP (Fig. 21), indicating that the impaired LFA-1 expression due to the low expression of CD18 causally drives the impaired contact between T_{regs} and DCs during antigen presentation and the compromised suppressive function of T_{reg} cells *in vitro* and in the $CD18^{hypo}$ PL/J psoriasis model. These data strongly suggest that the interaction between LFA-1 and ICAM-1 is involved in the antigen presentation and T_{reg} expansion *in vitro,* and that EAP disrupts this tight interaction, most likely via its MHC analogy (Jonsson et al. 1995; Lee et al. 2002). This may be of disadvantage in the context of psoriatic inflammation, where EAP may amplify the already impaired suppressive function of T_{regs}. On the other hand, a similar inhibitory effect was induced on T_{resp} cell proliferation by EAP (Fig. 17)(Wang et al. 2010), which may be of major importance for psoriasis and for other T_h1-mediated autoimmune reactions. However, *in vivo*, we did not detect any decrease in the

proliferation of adoptively transferred T_{reg} and T_{resp} cells in recipient $CD18^{wt}$ mice treated with EAP (Wang et al. 2010). This suggests that EAP most likely does not interfere with the education and activation of T_{reg} and T_{resp} cells by DCs *in vivo*, but may control other pathogenic mechanisms in psoriasis such as leukocyte recruitment to the psoriasis plaque.

Interestingly, the hypothesis of functionally impaired T_{regs} promoting unrestrained generation and proliferation of pathogenic T cells has been proposed for human psoriasis as well (Sugiyama et al. 2005), thus qualifying the $CD18^{hypo}$ mouse as a valuable model for studying T cell-mediated pathogenic pathways in human psoriasis and autoimmune diseases in general. One possible explanation may be the absolute requirement of physiological, high expression levels of CD18 for proper T_{regs} function. Accordingly, in the $CD18^{hypo}$ mouse model T_{regs} have to be impaired in their function of suppressing proliferation of T_{resp} cells in the inflamed skin. Furthermore, the reduction of $CD18^{hypo}$ T_{reg} function in the thymus might result in a reduced suppression of autoreactive thymocytes, thereby breaking central immune tolerance and contributing to development of autoimmunity. Recently, the transformation of anti-inflammatory T_{regs} into pro-inflammatory IL-17-producing T_h17 cells especially in severely affected psoriasis patients was proposed, indicating a possible ambivalent role of T_{regs} in this autoimmune disease (Soler and McCormick 2011). Here, EAP may also play a crucial role because of its anti-inflammatory potential which is in part mediated by the inhibition of IL-15 expression in a Ras/Raf/MEK/ERK-dependent inhibitory mechanism (Sobke et al. 2006). Interestingly, both IL-15 and IL-17 were found to be abundantly expressed in psoriatic plaques and to be regulated via Ras pathway signalling. By inhibiting this pathway, EAP may reduce the expression and thus decrease the pro-inflammatory effects of IL-15 and especially IL-17 in psoriasis (Li et al. 2006; McLInnes 2006). Besides these, some other mechanisms have been incriminated in the suppression of inflammation in several other disease models. In a model of experimental autoimmune encephalitis (EAE) EAP was found to significantly inhibit the recruitment of inflammatory cells to the central nervous system and thus to prevent the development of EAE. Similarly, administration of EAP resulted in reduced tissue edema and decreased influx of T cells to the inflammation site in a model of delayed-type hypersensitivity (Xie et al. 2006). These remarkable effects were corroborated with the finding that EAP reduced adhesion and transmigration

of T cells to TNF-α activated human endothelial cells and the binding of T cells to immobilized ICAM-1 *in vitro* (Xie et al. 2006). Other immunomodulatory properties for the EAP-analogue protein Map from SA were revealed using infection models. Infection of mice with Map-producing SA strains resulted in a significantly higher incidence of arthritis, osteomyelitis and abscess formation when compared with mice infected with Map-deficient SA. The full development of infection with Map^+ SA strains depended on T cell functions, as infection of T cell-deficient nude mice with Map^- SA strains did not result in a decreased severity of arthritis and osteomyelitis as compared with control mice infected with wildtype SA (Lee et al. 2002). Furthermore, Map was found to shift the T cell response from a T_h1 type, which was associated with efficient bacterial clearance, to a T_h2 polarization which may be responsible for SA survival and persistence (Lee et al. 2002). Interestingly, EAP was also shown to induce an upregulation of IL-4, a master T_h2 type cytokine, paralleled with a decrease of IFN-γ release from leukocytes isolated from a subgroup of patients with atopic dermatitis, suggesting EAP to induce a T_h1 towards T_h2 shift of the immune response and thus to contribute to the pathogenesis of allergic skin inflammation (Jahreis et al. 2000). This shift from a T_h1 immune response, as also occurring in psoriasis, to a T_h2 mediated immune response may have additionally contributed to the resolution of the psoriasiform skin disease after injection of EAP in the $CD18^{hypo}$ mouse model. In fact, established first-line therapies in psoriasis such as UV-light, corticosteroids, fumaric acid esters, vitamin D analogues and methotrexat are all known to induce high levels of IL-4 along with the direct suppressive effects on inflammatory cells (Ghoreschi et al. 2003; Singh et al. 2010).

In an experimental model of bacterial peritonitis EAP-binding to ICAM-1 and the extracellular matrix proteins fibrinogen and vitronectin significantly reduced inflammation by inhibiting leukocyte adhesion and β_2 integrin-dependent neutrophil recruitment to the site of infection (Chavakis et al. 2002). These observations are in line with the finding of reduced inflammation, including reduced numbers of neutrophilic abscesses of Munro, in our previous *in vivo* studies with EAP injections in the $CD18^{hypo}$ mouse model. Finally, in a murine model of wound healing the wound closure in presence of EAP-producing SA strains and EAP-deficient SA strains was compared. Again, administration of EAP-producing SA-strains resulted in delayed wound healing *in vivo* and the impairment of ICAM-1

dependent interactions of leukocytes with endothelial cells *in vitro* (Athanasopoulos et al. 2006).

A second remarkable effect of EAP was the disruption of the interaction between DCs and T cell subpopulations *in vitro*. EAP dose-dependently inhibited the contact between DCs and T_{regs} or T_{resp} cells respectively, resulting in a significantly decreased cluster formation between the two cell populations (Fig. 26 and Fig. 27). This mechanism most likely underlies the observed severe reduction in the proliferation capacity of T_{regs} and T_{resp} cells, as well as the impaired suppressive effect of T_{regs} induced by EAP. This finding is novel and so far not described in the literature. However, its relevance *in vivo* remains to be analyzed in the future. In our model, by contrast to the strong inhibitory effect of EAP on cluster formation between DCs and T_{resp} or T_{reg} cells *in vitro*, injection of EAP at concentrations of 50 or even 100µg/ml in affected $CD18^{hypo}$ mice did not inhibit the proliferation of either T_{resp} or T_{reg} cells, indicating that EAP does not affect T cell proliferation *in vivo* when injected in non-toxic concentrations (Wang et al. 2010).
EAP was found to specifically target LFA-1–ICAM-1-mediated interactions, but also to bind to various other extracellular matrix proteins such as fibronectin, fibrinogen, vitronectin or prothrombin, enabling SA to efficiently colonize different tissues (Hussain et al. 2002; McGavin et al. 1993). Thus, we further asked whether the specific targeting of the LFA-1 – ICAM-1 binding using blocking antibodies would reproduce the effects on T cell responses observed with EAP. Indeed, blocking of ICAM-1 by addition of neutralizing anti-ICAM-1-Ab during T_{reg} cell expansion by DCs resulted in an identical, dose-dependent inhibition of the suppressive T_{reg} function as produced by EAP (Fig. 22). These results are in line with previous reports showing a dose-dependent inhibitory effect of similar magnitude exerted by both EAP and antibodies against ICAM-1 on neutrophil binding and transmigration across the endothelium *in vitro* (Haggar et al. 2004). A very similar outcome was induced by blocking the β_2 integrin counterpart of ICAM-1 in the immune synapse with neutralizing anti-CD18-Ab (Fig. 23). These results strongly suggested that EAP specifically disrupted the β_2 integrin – ICAM-1 binding between T cells and DCs in the described experiments, and that the suppressive effects on T cell proliferation were not due to blocking other targets of EAP. However, considering the analogy of EAP with an epitope of the MHC molecule, in

the future it will be interesting to analyse a potential effect of EAP on the MHC – TCR binding in the immune synapse. So far it has been suggested that EAP may directly block MHC-TCR interactions, thus mediating anergy and immunosuppression (Foster 2005; Jonsson et al. 1995). Alternatively, EAP has been proposed to directly mediate the cross-linking of MHC molecules with TCR, thus inducing MHC-TCR signalling via superantigen-like mechanisms (Geisbrecht et al. 2005). However, a recent structural study using peptide-MHC tetramer technology demonstrated that EAP does not block MHC-TCR interactions and is not a superantigen, but has rather a non-specific cross-linking activity depending on the numbers of the 110 amino-acids repeats (Massey et al. 2007).

To finally demonstrate that, of the β_2 integrin subunits, LFA-1 was specifically targeted in its binding to ICAM-1 in our experiments, we employed the humanized anti-CD11a-mAB Efalizumab (Raptiva®) in the same experimental settings. Indeed, blocking LFA-1 with Efalizumab dose-dependently decreased the suppressive function of T_{regs} on T_{resp} cell proliferation *in vitro*, similarly with the results obtained with the neutralizing anti-CD11a-Ab, anti-CD18-Ab, anti-ICAM-1-Ab or EAP (Fig. 25). The humanized monoclonal LFA-1-antibody Efalizumab (Raptiva®) is part of the repertoire of 'biologicals' and has been used since 2004 to modulate the immune response in psoriasis by preventing extravasation of leukocytes and thus reducing the release of high levels of T_h1 cytokines in the inflammatory skin plaques (Krueger and Bowcock 2005). The selective binding of Efalizumab to the α-subunit of LFA-1 (CD11a) was supposed to specifically inhibit the adhesion of lymphocytes to ICAM-1 on endothelial cells which is required for the transmigration of lymphocytes from the blood vessels to the inflamed skin and to produce less global immune suppression compared with other T cell-depleting treatments (Wang et al. 2010). Unfortunately, these expectations on Efalizumab treatment of psoriasis failed to be met. In 2009 a drug safety mail from the European Medicines Agency (EMEA; http://www.emea.europa.eu/humandocs/PDFs/EPAR/raptiva/2085709en.pdf) and the Food and Drug Administration (FDA; http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/ucm133337.htm) suspended the marketing authorisation for Raptiva and recommended to carefully discontinue the use of this drug in patients with psoriasis due to severe adverse events. The highest safety concerns included the occurrence of three cases of progressive multifocal leukoencephalopathy (Korman

et al. 2009; Talamonti et al. 2011), associated with an impaired T cell response to Human Poliomavirus 2 (Schwab et al. 2012), most likely due to the systemic blockade of LFA-1. In addition, treatment with Raptiva® was shown to induce a prolonged state of T cells hyporesponsiveness which may increase the risk of severe, life-threatening infections and malignancies (Leonardi et al. 2006) and of severe generalized pustular psoriasis which could be observed in several patients upon treatment withdrawal (Gaylor and Duvic 2004). In this line, EAP may represent a valuable therapeutic alternative to Efalizumab, as we and others could show a clear dose dependency of EAP on T cell functions *in vitro* and *in vivo* (Wang et al. 2010), as well as on binding to potent ICAM-1 stimuli such as immobilized ICAM-1 or on ICAM-1 on TNF-α-stimulated endothelial cells (Xie et al. 2006). Besides this, recent data suggests that a failure to respond to EAP treatment may be reliably identified in initial phases of the treatment. Thus, anti-EAP-Abs could be identified in the serum of patients with SA-infection (Haggar et al. 2005), but not in infected mice (Joost et al. 2011). A similar induction of antibodies against therapeutic approaches such as TNF-α-inhibitors was also reported in patients suffering from rheumatoid arthritis (Bartelds et al. 2011) or psoriasis (Lecluse et al. 2010) with a poor response to therapy, suggesting that the titer of anti-EAP-Abs may be indicative for the therapeutic response. Alternative to inhibition of LFA-1 signalling, the identification of new key molecules in the pathogenesis of psoriasis, especially in its T_h1-mediated autoimmune response, offered targets for new therapeutical approaches. During the past years several such so-called 'biologicals' have been developed. Especially neutralizing antibodies against the master pro-inflammatory cytokine in psoriasis, TNF-α (Etanercept, Adalimumab and Infliximab), were successfully and so far safely used to cure and to prevent severe progression of psoriasis and especially psoriasis arthritis (Weinberg et al. 2005). Interestingly, recent studies demonstrate that the reduction in TNF-α levels resulted in a decrease in effector T cells numbers and an increase in T_{reg} cells in 10 out of 14 patients treated with Etanercept, Adalimumab or Infliximab, which have correlated with a positive therapeutic response to the treatment (Richetta et al. 2011). Recently a new therapeutic approach using antibodies directed against another T_h1 type cytokine, IL-23

(Ustekinumab), has been developed and proved very promising in clinical trials (Krulig and Gordon 2010).

Importantly, *in vivo* studies performed in our department in parallel with this *in vitro* work showed a significant improvement of the inflammatory skin lesions as measured by a decrease in erythema, hair loss and scaling in severely diseased $CD18^{hypo}$ mice after intraperitoneal injection of EAP. In a further step, adoptive transfer of CFSE-labelled $CD18^{hypo}$ or $CD18^{wt}$ T_{regs} respectively T_{resp} cells in $CD18^{hypo}$ mice was performed together with injection of EAP or vehicle control, in order to investigate the influence of EAP on T cell proliferation *in vivo*. Surprisingly, despite a substantial clinical improvement of the skin phenotype, no significant changes in T_{resp} or T_{reg} cell proliferation induced by EAP could be observed in the lymph nodes of the $CD18^{hypo}$ recipient mice, indicating that EAP does not reduce proliferation of T_{regs} or T_{resp} cells in lymphoid organs *in vivo*. Instead, analysis of skin samples of $CD18^{hypo}$ mice revealed a strong reduction in numbers of infiltrating $CD4^+$ T cells and in levels of the pro-inflammatory cytokine TNF-α occurring after treatment with EAP and correlating with the positive disease outcome (Wang et al. 2010). This observation is in line with our *in vitro* data showing EAP to interrupt the contact between LFA-1 on T cells and ICAM-1 (Fig. 20). In contrast with the finding that EAP does not influence T cell proliferation *in vivo* (Wang et al. 2010), our herein described experiments clearly demonstrated a decrease in proliferation of T cell fractions when cultured in presence of EAP *in vitro*.

A possible reason for the absence of T cell proliferation in the skin draining lymph nodes *in vivo* may consist in the primary blockade of ICAM-1 by EAP at the level of high endothelial venules, resulting in the impaired ability of T cells to transmigrate and subsequently proliferate into lymph nodes or inflamed skin. A similar mechanism has been previously suggested for the preventive effect of EAP in the development of experimental autoimmune encephalitis (Xie et al. 2006). Alternatively, it is possible that at least a fraction of the adoptively transferred T cells may be directly attracted to the inflammatory sites in the skin and thereby circumvents the lymphoid organs and the possible influence of EAP at this site.

Taken together, our *in vitro* data corroborated with the *in vivo* findings qualify EAP as a potential therapeutic alternative, especially to T cell targeting agents, for

human psoriasis. LFA-1 blockade was predicted to produce less global immune suppression compared with whole T cell depleting agents such as anti-CD4-Ab (Nickoloff et al. 1990). However, treatment with Efalizumab induced in psoriasis patients a characteristic state of severe T cell hyporesponsiveness (Guttman-Yassky et al. 2008), while its withdrawal induced a severe pustular relapse of the disease in some patients (Gaylor and Duvic 2004; Johnson-Huang et al. 2012), possibly due to the massive acute recruitment of T_{resp} cells in parallel with the inhibition of the suppressive function of T_{regs}. Although our *in vitro* data suggests that this may occur with EAP as well, there are studies indicating a long-lasting effect for EAP (Chavakis et al. 2002; Harraghy et al. 2003; Sobke et al. 2006) which recommends EAP as a safer approach than Efalizumab, which recently has been withdrawn from the market. By contrast to other biologicals, we found that EAP specifically targets ICAM-1, which is predominantly up-regulated on the inflamed endothelium in psoriasis plaques (Wang et al. 2010). Thus, EAP seems to primarily interfere with LFA-1–ICAM-1-mediated transendothelial recruitment of lymphocytes to the inflammation site. Although our *in vitro* data demonstrate a clear inhibitory effect of EAP on T_{reg} cell expansion and suppressive function, this effect was not translatable *in vivo*, where the T cell proliferation remained unchanged (Wang et al. 2010). In the future, it will be interesting to investigate the relevance of the anti-angiogenic function of EAP for psoriasis, as it was documented for wound healing (Athanasopoulos et al. 2006; Sobke et al. 2006) and breast cancer bone metastasis (Schneider et al. 2007). Finally, the immunomodulatory effect of EAP in shifting T_h1 towards T_h2 immune responses as previously postulated (Harraghy et al. 2003) will be of great interest especially for T_h1-mediated autoimmunity but also for T_h2-controlled allergic reactions.

A series of *in vitro* experiments described in this doctoral thesis highlighted some novel, so far undescribed immunomodulatory effects of the staphylococcal protein EAP. Especially the property of EAP to disrupt the contact between antigen presenting DCs and different T cell subsets may have important therapeutic implications. On one hand, the inhibition of tight contacts between DCs and T_{regs} resulted in the impaired T_{reg} cell 'education' and thus abrogated their suppressive function on T_{resp} cells. This effect should be critically considered in T_h1-mediated autoimmune diseases where EAP might amplify the already impaired T_{reg} function. On the other hand, disruption of the contact between DCs and T_{resp} cells would be

of benefit in such diseases by inhibiting uncontrolled T cell proliferation and interrupting the pro-inflammatory cascade. In the $CD18^{hypo}$ murine model of psoriasform skin disease there was no *in vivo* relevance detectable for these mechanisms (Wang et al. 2010). Here, rather the inhibition of the LFA-1 – ICAM-1-dependent lymphocyte recruitment to the inflammatory sites was primarily responsible for the improvement of the skin disease. Nevertheless, these and other so far unidentified effects of EAP may become relevant with different dosage and therapy regimens or in different models of T_h1-driven immune reactions and may be successfully exploited as alternative therapeutic tools to specifically modulate pathologic immune responses.

5. SUMMARY

Psoriasis is a common inflammatory disease of the skin with a complex and so far not completely resolved pathogenesis. Especially the pathologic shift of the immune response towards uncontrolled proliferation of type 1 cytokines-producing autoreactive responder T cells (T_{resp}), most likely due to an impaired suppressive function of regulatory T cells (T_{regs}), have been incriminated to drive inflammation in psoriasis. Therefore, immune modulators aimed to rebalance these pathologic immune responses are currently developed and successfully employed in the treatment of patients with severe disease.

The staphylococcal extracellular adherence protein (EAP) was shown to exert immunomodulatory, anti-inflammatory and anti-angiogenic effects in several inflammatory disease models by binding to a number of ligands, including the intercellular adhesion molecule ICAM-1. ICAM-1 is the main ligand of the β_2 integrin Lymphocyte function-associated antigen-1 (LFA-1) which is expressed on lymphocytes and is required for lymphocyte activation, proliferation and transmigration. Our group found EAP to be capable of resolving the psoriasiform skin disease in the $CD18^{hypo}$ mouse, a murine model for human psoriasis characterised by reduced expression of LFA-1 on lymphocytes with impaired function of T_{regs} resulting in enhanced proliferation of T_{resp} cells.

This MD thesis addresses several T cell-related mechanisms possibly underlying the favourable *in vivo* effects of EAP on the psoriasiform skin disease in $CD18^{hypo}$ mice. In detail, a series of *in vitro* experiments were performed in order to analyse the effect of EAP on T_{resp} cell proliferation, on T_{reg} 'licensing' by antigen presenting cells, on T_{reg} suppressive function on T_{resp} cells and on the contact between antigen presenting dendritic cells (DCs) and different T cell subsets.

We show that EAP significantly inhibits the proliferation of wildtype T_{resp} cells stimulated with physiological stimuli (plate-bound anti-CD3-antibody (Ab)) but not with strong stimuli as soluble anti-CD3-Ab, anti-CD28-Ab and Interleukin-2. Of notice, no inhibition of EAP on the proliferation of $CD18^{hypo}$ T_{resp} cells could be detected in the same *in vitro* experimental conditions, correlating with the unaffected proliferation of T_{resp} cells *in vivo* in $CD18^{hypo}$ mice treated with EAP. Next we asked whether EAP would affect the antigen presentation and thus the

licensing of T_{regs} to suppress T_{resp} cell proliferation. We clearly show that addition of EAP during the education of T_{regs} by DCs was sufficient to dose-dependently impair the suppressive function of T_{reg} cells towards T_{resp} cell proliferation.

On molecular level, EAP interrupted the cell-cell contact between antigen presenting DCs and T_{regs} on the level of ICAM-1 – LFA-1 binding. Thus, an identical dose-dependent decrease of the suppressive effect on T_{resp} cell proliferation was obtained when expanding T_{regs} with DCs in presence of neutralizing antibodies directed against ICAM-1 or the two chains of the LFA-1 β_2 integrin, CD18 and CD11a. Moreover, the addition of the humanized anti-LFA-1 antibody Efalizumab (Raptiva®) resulted as well in a dose-dependent decrease of the suppressive function of T_{regs} towards T_{resp} cells proliferation, indicating that EAP specifically targets the interaction between ICAM-1 and LFA-1. In fact, blocking of ICAM-1 on endothelial cells with decreased transmigration of T_{resp} cells at the inflammatory sites in the skin most probably contributed to the improvement of the psoriasiform skin inflammation *in vivo*.

Finally, using cluster formation assays between DCs and T_{regs} or T_{resp} cells respectively, we could prove that EAP disrupts the physical interaction between the two cell populations, as the numbers of DCs-T_{regs} and of DCs-T_{resp} cells clusters were significantly and again dose-dependently reduced in presence of EAP. Therefore, we concluded that EAP induces a specific and long-lasting inhibition of ICAM-1 – LFA-1 contacts with impact on T cell transmigration, proliferation and function which may be of benefit for the modulation of autoimmune responses as occurring in psoriasis.

In the discussion part we assess the relevance of our findings and place them in the context of the *in vivo* data reported by our group and of the most recent knowledge regarding psoriasis immunology and EAP biology. We examine in detail data which are in line, but also the controversial aspects between our *in vitro* findings and *in vivo* data from our group and others. We try to reconcile such issues by clearly defining our *in vitro* models and comparing them with other published models. Finally, we discuss interesting parallels between EAP effects on lymphocytes and on other leukocyte subsets and open perspective questions on putative EAP-related mechanisms which may be addressed in the future.

Our results are important, as they highlight novel mechanistic aspects of EAP as an immunomodulatory molecule.

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