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In vivo expansion of regulatory T cells induces long-term suppression of contact hypersensitivity

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Asmaa El Beidaq

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ABSTRACT

Contact hypersensitivity (CHS) of murine skin serves as a model of allergic contact dermatitis. CHS is triggered by cutaneous exposure to small chemical structures called haptens. Skin dendritic cells (DCs) play an important role in the activation of hapten-specific CD8 T cells which represent the major effector cells driving this inflammatory reaction. In addition, neutrophils have been shown to play a critical role during sensitization and challenge phases. Over the past decade, several studies have demonstrated that forkhead-box-protein P3 $(Foxp3)^+$ regulatory T cells (Tregs) can control the magnitude of CHS-mediated inflammation. However, whether an experimental therapy that expands endogenous Tregs can down-regulate CHS response remains to be elucidated. In this thesis, this issue was addressed using injection of an interleukin (IL)-2/anti-IL-2 monoclonal antibody JES6-1 complex (IL-2/JES6-1) as a means of Treg induction in a 2,4,6 trinitrochlorobenzene (TNCB) induced CHS model. IL-2/JES6-1 injection before or after hapten sensitization led to a systemic expansion of both natural Tregs (nTregs) and peripheral Tregs (pTregs). IL-2/JES6-1 treatment resulted in suppressed CHS skin inflammation indicated by a considerable reduction of ear swelling. Elevated IL-10 and transforming growth factor-β (TGF-β) production by CD4 T cells, and lower numbers of hapten-specific interferon (IFN)-γ producing CD8 T effector cells in lymph nodes (LNs) were observed in treated mice. In inflamed ear tissue, neutrophil and CD8 T cell infiltration was reduced while cytotoxic T-lymphocyte-associated protein 4 $(CTLA\text{-}4)^+$ $Foxp3^+$ Treg frequencies were augmented. As shown by depletion studies, Tregs were required for the IL-2/JES6-1 mediated suppression of skin inflammation and impaired effector cells functions. IL-2/JES6-1 treatment during sensitization resulted in down-regulation of CD86 on hapten-bearing DCs and could inhibit effector cell priming in LNs indicating a suppression of T effector cell priming. Adoptive transfer of LN cells from sensitized mice into recipients treated with IL-2/JES6-1 showed impaired CHS, suggesting that IL-2/JES6-1 also affected the challenge phase

independent of the priming. Furthermore, IL-2/JES6-1 treatment showed long-lasting effects, i.e. it attenuated the inflammatory reaction during repeated challenges weeks after the last injection. These results show that IL-2/JES6-1 expanded Tregs use multiple mechanisms to mediate efficient and prolonged suppression of CHS mediated inflammation.

ZUSAMMENFASSUNG

Als Modell für die allergische Kontaktdermatitis wird die Kontakt-Hypersensibilität (CHS, *contact hypersensitivity*) der murinen Haut verwendet, welche durch den kutanen Kontakt mit niedermolekularen Chemikalien, den sog. Haptenen, induziert wird. Bei der Aktivierung von Hapten-spezifischen CD8 T-Zellen, die als die Haupteffektorzellen der inflammatorischen Reaktion gelten, spielen dendritische Zellen (DC, *dendritic cells*) in der Haut eine wichtige Rolle. Darüber hinaus sind auch Neutrophile involviert, sowohl bei der Allergen-Sensibilisierung, als auch und der akuten Entzündungsreaktion nach wiederholtem Allergen-Kontakt (*Challenge*-Phase). In den letzten Jahrzehnten haben verschiedene Untersuchungen die wichtige Rolle von *forkhead-box-protein P3* $(Foxp3)^+$ regulatorischen T-Zellen (Tregs) bei der Kontrolle der CHS-vermittelten Entzündung aufgezeigt. Ob jedoch eine therapeutische Expansion von endogenen Tregs die Symptome der CHS weiter verbessern zu vermag, war bislang noch unklar. In der vorliegenden Arbeit wurden daher Tregs mittels Injektion eines Interleukin (IL)-2/anti-IL-2 Antikörper Komplexes (IL-2/JES6-1) in einem 2,4,6-Trinitrochlorbenzol (TCNB)-induzierten CHS-Modell induziert. Die Injektion von IL-2/JES6-1 vor und/oder nach der Hapten-Sensibilisierung führte zu einer systemischen Expansion von sowohl natürlichen Tregs (nTregs) als auch peripheren Tregs (pTregs). Die IL-2/JES6-1-Behandlung äußerte sich in einer deutlichen Reduktion der CHS Hautentzündung, was durch eine verminderte Schwellung des Ohres gezeigt werden konnte. Außerdem zeigten sich eine erhöhte IL-10 und *transforming growth factor-β* (TGF-β) Produktion durch CD4 T-Zellen und eine geringere Anzahl von Hapten-spezifischen Interferon (IFN)-γ-produzierenden CD8 T-Effektorzellen in den Lymphknoten von behandelten Mäusen. Im entzündeten Gewebe des Ohres war die Infiltration von Neutrophilen und CD8 T-Zellen reduziert, wohingegen die *cytotoxic T-lymphocyte-associated protein 4* $(CTLA\text{-}4)^+$ $Foxp3^+$ Treg-Frequenz erhöht war. Wie durch Depletionsstudien gezeigt werden konnte, wurden Tregs für die IL-2/JES6-1-

vermittelte Suppression der Hautentzündung sowie die beobachteten Effekte auf die Effektorzellen benötigt. Die IL-2/JES6-1-Behandlung während der Sensibilisierung resultierte in einer Herunterregulation von CD86 auf Allergen-tragenden DC und einer Inhibition des *Primings* von CD8 Effektor T-zellen in den Lymphknoten. Der adoptive Transfer von Zellen der Lymphknoten aus sensibilisierten Mäusen zeigte eine beeinträchtigte CHS in Rezipienten, die zuvor mit IL-2/JES6-1 behandelt wurden. Diese Beobachtungen legen nahe, dass IL-2/JES6-1 auch die Allergen-*Challenge*-Phase unabhängig vom *Priming* beeinflusst. Die IL-2/JES6-1-Behandlung zeigte langanhaltende Effekte, d.h. sie führte zu einer Abschwächung der inflammatorischen Reaktion während wiederholter Allergen-*Challenges* auch noch Wochen nach der letzten IL-2/JES6-1-Injektion. Zusammenfassend zeigen die Ergebnisse, dass IL-2/JES6-1 Behandlung zu einer Expansion von Treg Populationen führt; und hierdurch über mehrere Mechanismen eine effiziente und anhaltende Suppression der CHS-vermittelten Inflammation bewirkt.

1. INTRODUCTION

1.1 Anatomy and functions of the skin

Skin is the largest organ of the body and most exposed to the environment. Long gone are the days when the skin was considered to resemble a passive barrier. Being constantly exposed to potential harms, the skin operates several functions in order to maintain homeostasis (1). The skin represents a physical and chemical barrier against microbial pathogens. In addition, it regulates the body temperature via sweat and hair. The skin acts also as a reservoir of hormones and vitamins. Moreover, the skin is a sensory-receptive area containing an extensive network of nerve cells sensing all environmental changes. The ability of the skin to perform various functions is tightly related to its sophisticated structure. The skin consists of an outer layer called epidermis, and an inner layer called dermis which provides the structural support of the skin, below which exists a loose connective tissue layer called hypodermis, an important depot of fat serving as an energy reserve. Hair follicles are complex structures implemented in the dermis and continued in the epidermis, from which hairs emerge from the skin (2).

1.2 The skin as an immune organ

Rich in immune cells, the skin forms a complex immune network consisting of both innate and adaptive immune cells (3). Keratinocytes are the predominant cells in the epidermis. These cells are responsible for keratin synthesis. Keratinocytes express many pattern recognition receptors, including Toll-like receptors (TLRs) that recognize a wide variety of pathogen components and self-components, which makes keratinocytes play a key role in innate immune responses and detection of pathogens (4). In response to pathogen invasions, keratinocytes produce many cytokines and chemokines including tumor necrosis factor (TNF), interleukin (IL)-1α, IL-1β, IL-6, IL-10, IL-18, IL-33, CXCL9, CXCL10, CXCL11 and CCL20 (5, 6). Cytokines are

important to direct the immune response to a specific effector mechanism (7), chemokines are a subclass of cytokines that are important to direct cellular migration, e.g. for T cell and innate effector cell recruitment to the skin (8). The epidermis also contains other immune cells ensuring different functions such as Langerhans cells, αβ T cells, and γδ T cells (Figure 1.1). The dermis consists of a different set of structural and immune cells. The dermal layer is mainly composed of collagen and elastin fiber, in addition to blood vessels and nerve cells which are distributed throughout the dermis. Dermal dendritic cells, mast cells, macrophages and T cells are the main immune cells residing in the dermis (Figure 1.1). Changes in lipid composition and epidermal differentiation can disturb the skin barrier, which plays a relevant role in the initiation of several immune-mediated skin pathologies, such as atopic dermatitis (9).

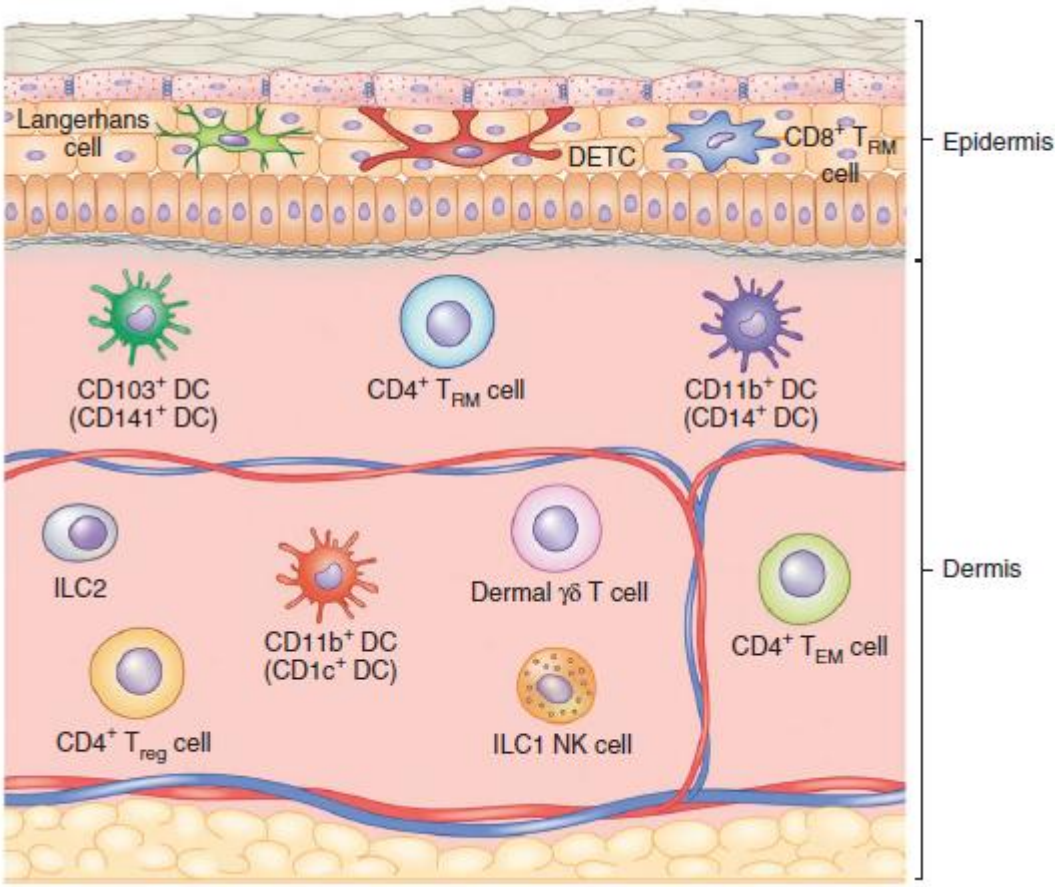

Figure 1.1: Immune cells residing in the skin. The figure describes the different innate and adaptive immune cells present in the epidermis and the dermis of murine skin. Modified from (3).

1.3 Irritant contact dermatitis

Contact dermatitis is a one of the most common skin inflammations. It is characterized by redness, itching, burning, and rash of the skin. These symptoms affect the epidermis and the outer layers of the dermis, and occur after direct contact with xenobiotic compounds that trigger the innate immune system present in the skin. There are two types of contact dermatitis, depending of the nature of the substance that comes in contact with the skin: Irritant contact dermatitis and allergic contact dermatitis.

1.3.1 Definition of irritant contact dermatitis

Irritant contact dermatitis is an antigen-nonspecific reaction of the skin to an irritant. It results from activated innate immunity to direct injury of the skin without prior sensitization (10). Irritant contact dermatitis is usually multifactorial. Different factors can influence the susceptibility to a cascade of pathophysiological events. These include skin barrier disruption, cellular damage, genetic predetermined strengths of pro-inflammatory mediator release, age, and sex. In addition to these endogenous factors, exogenous factors such as the nature of the irritant substance, the concentration of the chemical, the exposure volume, duration and exposure frequency do also modulate irritant contact dermatitis. Depending on these previously listed as well currently unknown factors, skin irritation can be presented as ten different clinical subtypes (10).

1.3.2 Mechanism of irritant contact dermatitis

Disruption of the skin barrier leads to the release of IL-1α by keratinocytes, which represents the initial step in the inflammatory cascade of skin irritation (11, 12). Activation of IL-1α induces further release of pro-inflammatory cytokines and chemokines such as IL-1β, TNF-α, IL-6, and IL-8 by epidermal and dermal cells (13). IL-1β plays a role in dendritic cell (DC) and T cell activation, it also induces the upregulation of intercellular adhesion molecule 1 (ICAM-1) by endothelial cells (11, 14). TNF-α secretion results in increased expression of major of histocompatibility complex (MHC) class II molecules and ICAM-1 on keratinocytes (15). The role of IL-6 in irritant contact dermatitis pathogenesis is not clear, and depends on the chemical nature of the irritant. It is thought that IL-6 contributes to the maintenance of skin inflammation by inducing the infiltration of mononuclear cells, whereas a recent study has reported an anti-inflammatory role of IL-6 during skin irritation (16). In humans, IL-8 is known for being responsible for neutrophil activation and attraction to the affected site of the skin. In mice, IL-8 homologues exist called KC, MIP-2, and LIX which exert similar functions (17). Since irritant

contact dermatitis is established independently of the adaptive immune system, the role of T cells in this inflammatory process is not well defined. It has been shown that CD4 and CD8 T cells infiltrate the skin following irritant exposure (18). Although the involvement of chemokines and cytokines has been highlighted, the precise mechanism of irritant contact dermatitis remains to be elucidated.

1.4 Allergic contact dermatitis

Allergic contact dermatitis is one of the most common skin diseases, affecting 15-20% of the global population (19). In contrast to irritant contact dermatitis, allergic contact dermatitis requires a prior sensitization to an allergen, which may resemble a xenobiotic chemical or hapten. Contact allergens are small organic molecules that only become immunogenic after binding to skin proteins, resulting in the generation of neo-antigens via a process called haptenization. This chemical reaction occurs between the electrophilic component of the hapten and the nucleophilic side chains of the target skin-protein. Haptenated self-proteins activate innate immune cells present in the skin, and this leads to a T cell mediated skin inflammation. The main difference between irritant contact dermatitis and allergic contact dermatitis is the involvement of hapten-specific T cells that amplifies skin inflammation, and generates allergen specific memory that results in increased inflammation following subsequent allergenic contacts. Many features are shared between these two diseases in the early innate response, i.e activation of innate immune cells by IL-1β and TNF-α. However, the upregulation of CXCR4 and CCR7 expression on Langerhans cells occurs only in allergic contact dermatitis. These two chemokines are required for Langerhans cell migration toward skin draining lymph nodes (LNs) (20). Moreover, studies focusing on gene expression analysis have also demonstrated that allergens but not irritants may lead to the upregulation of certain genes such as CCL23, CCL4, CYP27A1, HML2, NOTCH3, S100A4, and SLAM in DCs (21). In humans, the sensitization phase usually takes several weeks to months of repeated contact with the sensitizing allergen,

the patient becomes then sensitized and progressively develops allergy following further exposures to the same allergen, and this phase is called challenge. Contact hypersensitivity (CHS) is the experimental model used in mice to study the mechanisms and potential treatments of allergic contact dermatitis. In CHS models, skin allergy is induced using potent lipophilic compounds such as TNCB which are dissolved in an organic solvent and applied directly to the skin. In the CHS model used in this study, murine ear skin is challenged by a second application of the allergen five days after sensitization, and the magnitude of ear swelling during the following days reflects the intensity of the effector response induced (22).

1.5 Contact hypersensitivity: The sensitization phase

1.5.1 Hapten recognition and initial immune activation

Haptens often derive from unstable chemicals in an inactive form named pro-haptens. Once in contact with skin-proteins, pro-haptens are metabolized in the epidermis by endogenous enzymes resulting in an electrophilic hapten owning antigenic properties (23). Depending on their structural nature, haptens can trigger DC and other innate immune cell activation directly via TLRs recognition. TLRs are a family of receptors recognizing pathogen-associated molecular patterns (PAMPs), including lipids, lipoproteins, proteins, and nucleic acids (24). TLRs can also recognize damage-associated molecular patterns (DAMPs), which are self-molecules that can be released during necrotic cell death. Most TLRs, except for TLR3 and TLR4, signal exclusively via the adaptor MYD88, which activates the transcription factor nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) to induce the expression of pro-inflammatory cytokines, such as IL-1β (24). Recent studies have shown that mice deficient in TLR2 and TLR4 failed to establish sensitization to TNCB due to defective DC-T cell interactions (25). Haptens can also activate TLRs indirectly by generation of hyaluronic acid (HA) fragments which then trigger TLR activation and subsequent pro-inflammatory cytokine production. HA fragments are non-sulfated, linear polysaccharides

consisting of repeated units of (β,1–4)-d-glucuronic acid-(β,1–3)-N-acetyl-D-glucosamine expressed in the extracellular matrix and on the cell surface. It has been shown that HA fragments undergo rapid degradation at sites of inflammation, which provides maturation signals to DCs leading to their migration to LNs and priming of hapten-specific T cells (26, 27). In fact, the degradation of HA fragments results from the rapid generation of reactive oxygen species (ROS) that occurs during the interaction between contact allergens and skin proteins in an incompletely understood mechanism. ROS production is accompanied by adenosine triphosphate (ATP) and DAMPs release (28).

In addition to TLRs, innate immunity can be activated via NOD-like receptors (NLRs). Activation of NLRs by PAMPs or DAMPs involves the formation of an intracellular complex of proteins termed inflammasomes (29). Several studies have shown that keratinocyte activation and subsequent IL-1β production occurs in an NLR-dependent manner (30–32). In TNCB-induced CHS, ATP and purinergic receptor P2X7-dependent pathways have been reported as being crucial for NLRP3 (member of NLR family) inflammasome activation (33). Additionally, lack of NLRP12 resulted in impaired migration of skin DCs to LNs and neutrophil infiltration into the skin, and thus the impairment of the CHS response (34).

1.5.2 Keratinocyte activation

Keratinocytes have an active role in the induction of CHS. Their production of IL-1β and TNF-α after hapten exposure plays a critical role in Langerhans cell maturation and migration from the epidermis to LNs (35). Most CHS reactions involve IFN-γ producing Th1 cells. In response to IFN-γ stimulation by Th1 cells, keratinocytes upregulate the expression of ICAM-1 which promotes the infiltration of ICAM-1 ligand/CD11a-positive T cells into the epidermis (36). Keratinocytes are also able to influence T cell polarization after hapten exposure through conditioning skin-resident DCs. In contrast to most murine models, CHS to fluorescein isothiocyanate (FITC) involves Th2 cells. Sensitization to FITC in mice requires dibutyl

phthalate (DBP) which induces the expression of thymic stromal lymphopoietin (TLSP) by keratinocytes (37). TLSP stimulates DCs to induce the differentiation of naïve T cells into Th2 type cells (38).

1.5.3 Role of dendritic cells

Antigen presentation by DCs in the LNs and T cell priming is the key event during the sensitization phase. Various DC subtypes are present in the skin: Langerhans cells are dominating the epidermis while dermal DCs are present in the dermis, and can be either langerin positive or negative. Both populations efficiently acquire antigens and migrate to skin draining LNs (39). Due to their migration from the epidermis to draining LNs, Langerhans cell density decreases by approximately 50% at 24 hours after cutaneous penetration of the sensitizing hapten (40). Upon activation, DCs undergo change in morphology and exhibit increased expression of CD83 which is a maturation marker, and costimulatory molecules, including CD40, CD80, and CD86. These molecules are crucial in T cell priming, as they provide the second stimulatory signal during antigen presentation allowing an efficient T cell activation and proliferation (41–43). Different studies have shown that Langerhans cells and dermal DCs initiate the sensitization phase in a compensatory manner (44). Depletion of Langerhans cells did not affect CHS establishment, suggesting that its role can be taken over by other DC subsets (45). It has been reported that dermal DCs migrate faster to the LNs, while epidermal Langerhans cells take more time to migrate and need 48 hours to reach the LNs after skin sensitization (46). In Th1 driven CHS models, DCs secrete a combination of cytokines in the LNs that polarize the T cell response towards Tc1/Th1 and Tc17/Th17 effector/memory T cells. The inflammatory milieu is needed to overcome constitutive immune regulation. Following priming by hapten-bearing DCs, hapten-specific T cells undergo clonal expansion and then circulate throughout the blood until they are recruited back to the skin upon re-exposure of the same hapten.

1.5.4 Contribution of mast cells, B1 cells, iNKT cells, and neutrophils to allergen sensitization

- **Mast cells:** The role of mast cells during CHS is quite controversial. Using different models, earlier studies have shown that CHS was impaired in mast-cell deficient mice (47, 48). However, transgenic mice used in these studies were not solely deficient for mast cells, but exhibited other immunological deficiencies, i.e these mice lacked neutrophils. More recently, conditional depletion of mast cells using diphtheria toxin (DT) resulted in reduced FITC and oxazolone induced CHS (49). In these studies, skin DC migration and T cell priming in the sensitization phase were impaired in mast cell-depleted mice.

- **B1 cells:** Considered as innate lymphocytes, B1 cells are T cell-independent, do form memory to a much lower extent than B2 cells (50), may not form germinal centers, and are the major source of IgM antibodies found in serum (51). B1 cells are activated during CHS sensitization, proliferate within 24 hours and produce hapten-specific IgM. CHS was found to be suppressed in B1 cell-deficient mice, and hapten-specific IgM restored skin inflammation (52). These antibodies activate complement which triggers inflammation through binding to complement receptors on mast cells (53), leading to the recruitment of effector T cells during elicitation.

- **iNKT cells:** They present a unique T cell lineage by bearing an invariant TCR which recognizes a small variety of glycolipid antigens in the context of CD1d. Cutaneous application of the hapten rapidly induces iNKT cell proliferation in the liver but not in lymphoid organs. When activated, iNKT cells produce IL-4 leading to the rapid activation of B1 cells (54).

- **Neutrophils:** Neutrophils represent the first line of defense against invading bacterial and fungal pathogens (55). In CHS, neutrophils are recruited by mast cells and endothelial cells via the upregulation of their chemoattractants CXCL1 and CXCL2, and their functions include ROS production and IL-1β secretion leading to the induction of DC migration to the LNs (56).

Infiltration of neutrophils into sensitized skin has been observed few hours following sensitization (56, 57). Depletion of neutrophils resulted in defective DC migration and hapten-specific T cell priming in the LNs (56). Hence, suggesting that neutrophils play an important role during sensitization.

In summary, sensitization to haptens is established through a series of activation processes during the CHS response. First, haptens bind to skin proteins and modify their structure into haptenated peptides which are further recognized by keratinocytes and DCs. Neutrophils, mast cells, and keratinocytes mediate DC activation, which leads to DC migration to skin draining LNs and activation/proliferation of allergen/hapten-specific T cells.

1.6 Mechanisms of the challenge phase in CHS

1.6.1 Innate immune response

The challenge phase is triggered by a second exposure to the sensitizing allergen/hapten. Stimulated by the hapten, keratinocytes produce IL-1β and TNF-α in a NLR-dependent manner (58), leading to the upregulation of ICAM-1 and P/E-selectins. Expression of these adhesion molecules is important to facilitate T cell infiltration to the skin. Additionally, mast cells produce histamine, which increases vascular permeability providing additional aid to effector T cell infiltration. The expression levels of CXCL1 and CXCL2 (chemoattractants for neutrophils) on keratinocytes and mast cells increases 3 hours after challenge, followed by elevated CXCL9 and CXCL10 expression (chemoattractants for Tc1 cells) 6-9 hours after challenge (59). These increases correlate with an early recruitment of neutrophils followed by effector T cells.

1.6.2 Hapten-specific T cell activation

Recruited by activated innate immune cells, T cells activated in LNs infiltrate the skin where they are locally further activated by hapten-bearing DCs. It has been shown that a stable interaction between cutaneous DCs and T cells takes places in skin (60), and CD86 inhibition

by siRNA resulted in reduced skin inflammation (61). The contribution of CD4 and CD8 T cells during CHS-mediated inflammation has been controversial. However, selective depletion of these T cell populations has revealed that CD8 T cells are the major effector cells. CHS was reduced after CD8 T cell depletion using monoclonal antibodies, whereas CHS was exaggerated following CD4 T cells depletion. However, simultaneous depletion of both CD4 and CD8 T cells only at the challenge phase further reduced the CHS response (62–64), suggesting that CD4 T cells contain both effector and suppressor populations. These results were confirmed using MHCI and MHCII deficient mice (63), indicating that CD8 T cells are pro-inflammatory effector cells while CD4 T cells contain both effector and regulatory T cells. Interestingly, CD8 T cells have been shown to be able to prevent hapten-specific CD4 T cell priming through the induction of Fas-mediated apoptosis in CD4 T cells, which results in CD8 T cell dominance in the effector phase (65). Neutrophils play an important role in directing hapten-specific CD8 T cells into the challenged skin via expression of Fas ligand (FasL) and perforin (66, 67). Kish *et al.* have shown that hapten-induced neutrophils specifically express perforin and FasL, which results in the induction of many chemokines that are chemoattractants for T cells (66). Infiltrated T cells then stimulate the neighboring cells to produce more inflammatory cytokines and chemokines, which amplifies T cell infiltration and chemokine production. Depending of their properties, different haptens can induce different types of cytokines, i.e TNCB induces IFN- γ production by CD8 T cells while FITC triggers Th2 cytokine production, such as IL-4 (68). Previous studies have demonstrated the detection of high levels of IFN-γ in challenged skin. IFN-γ is classically a Th1 cytokine, however, CD8 T cells are mainly responsible for its production in CHS-induced inflammation (64). It has been proposed that IFN-γ producing T cells trigger Fas-induced apoptosis of keratinocytes (69). This potent cytokine also induces mononuclear cell infiltration, triggers the upregulation of ICAM-1, and induces increased expression of MHCI on keratinocytes (70). Cytotoxicity is another important property CD8 T cells use in their effector functions during CHS. Mice double deficient in perforin and FasL

were able to prime hapten-specific CD8 T cells in LNs, but failed to develop CHS due to the lack of hapten-specific cytotoxic T lymphocytes. This hapten-specific toxicity was exclusively mediated by CD8 T cells through perforin or the Fas/FasL pathways (71). This inflammatory cascade leads to dermal edema and epidermal spongiosis, which peaks 24–48 hours after hapten challenge.

1.7 Control of CHS-induced inflammation

1.7.1 Regulatory T cells: The first line of control

Early studies have shown that MHCII deficient mice exhibit enhanced CHS (63), suggesting that CD4 T cells play a regulatory role in CHS. More recently, these cells were shown to constitutively express CD25 (IL-2 alpha receptor), as well as Foxp3 which is a master regulator of the regulatory pathway in the development and function of Tregs (72, 73). The characterization of Tregs has permitted a better understanding of their nature, origins, and functions during inflammation. Two distinct Treg subpopulations exist: nTregs that are generated in the thymus and are present without exposure to foreign antigens, and pTregs which are formed after antigen contact in the periphery. Previous studies have shown that Helios and Neuropilin-1 (Nrp-1) represent useful markers to distinguish nTregs from pTregs (74–76). Helios, the first identified nTreg marker, belongs to the Ikaros transcription factor family. 100% of thymic $Foxp3^+$ Tregs and about 70% of Tregs in the periphery express Helios which corresponds to nTregs frequencies found in thymus and spleen, respectively. *In vitro* TGF-β induced Tregs fail to express Helios, same observations were made for antigen-specific inducible Tregs (77). Furthermore, epigenetic Treg-specific demethylated region (TSDR) modifications have been used to distinguish nTregs from pTregs. $Foxp3^+$ $Helios^+$ Tregs were shown to have a fully methylated TSDR, while $Foxp3^+$ $Helios^-$ Tregs only showed a 45% methylated TSDR (78), demonstrating that $Helios^+$ Tregs are originated from the thymus. Nrp-1 is a receptor for the vascular endothelial growth factor family, and plays a role in Treg

suppressive functions (77, 79–81). It has been shown that Nrp-1^+ $Foxp3^+$ Tregs are important for effector T cell suppression (82). Tregs adopt various mechanisms to inhibit DC functions (Figure 1.2). Tregs can prevent the upregulation of costimulatory molecules CD80 and CD86 via the expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) which binds to these molecules (83). Lymphocyte activation gene 3 (LAG-3) is another molecule expressed on Treg cell surface that can inhibit DC maturation and stimulatory capacity by binding to MHCII molecules expressed by immature DCs (84). Additionally, Tregs can use extracellular ATP inactivation by CD39 which is an ectoenzyme that hydrolyzes ATP or ADP to AMP, preventing its usage by DCs (85). Another strategy Tregs can use for suppression of T effector cell activation is to promote long interactions between Treg cells and immature DCs via Nrp-1 (86), which prevents antigen presentation by DCs to effector cells.

Tregs can also suppress effector cells directly, using different strategies (Figure 1.3) (87). Production of anti-inflammatory cytokines such as IL-10, TGF-β, and IL-35 were among the first functions shown in Tregs that directly affect T effector cells (88). Moreover, Tregs have the capacity to compete with T effector cells for IL-2 due to their high expression levels of the IL-2 receptor CD25, resulting in IL-2 deprivation mediated apoptosis of T effector cells (89). Tregs can also kill T effector cells by granzymes (90), which is similar to the mechanism cytotoxic CD8 T cells use for their cytolytic activity. Furthermore, Tregs and T effector cells can bind to the same DC, which facilitate effector cells inhibition by Tregs in a CTLA-4-mediated fashion (Figure 1.4).

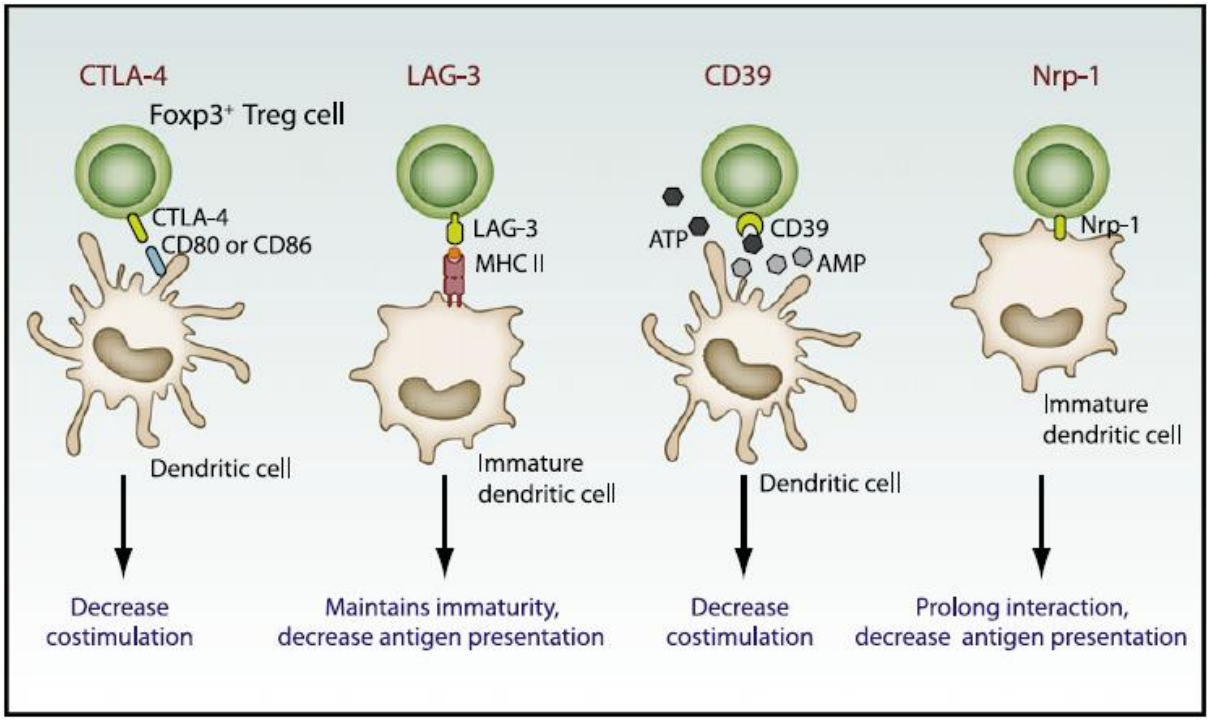

Figure 1.2: Possible mechanisms involved in DC inhibition by Tregs. This figure illustrates the major cell contact mediated mechanisms Tregs use to prevent DC maturation and subsequent co-stimulation of T effector cells. Modified from (86).

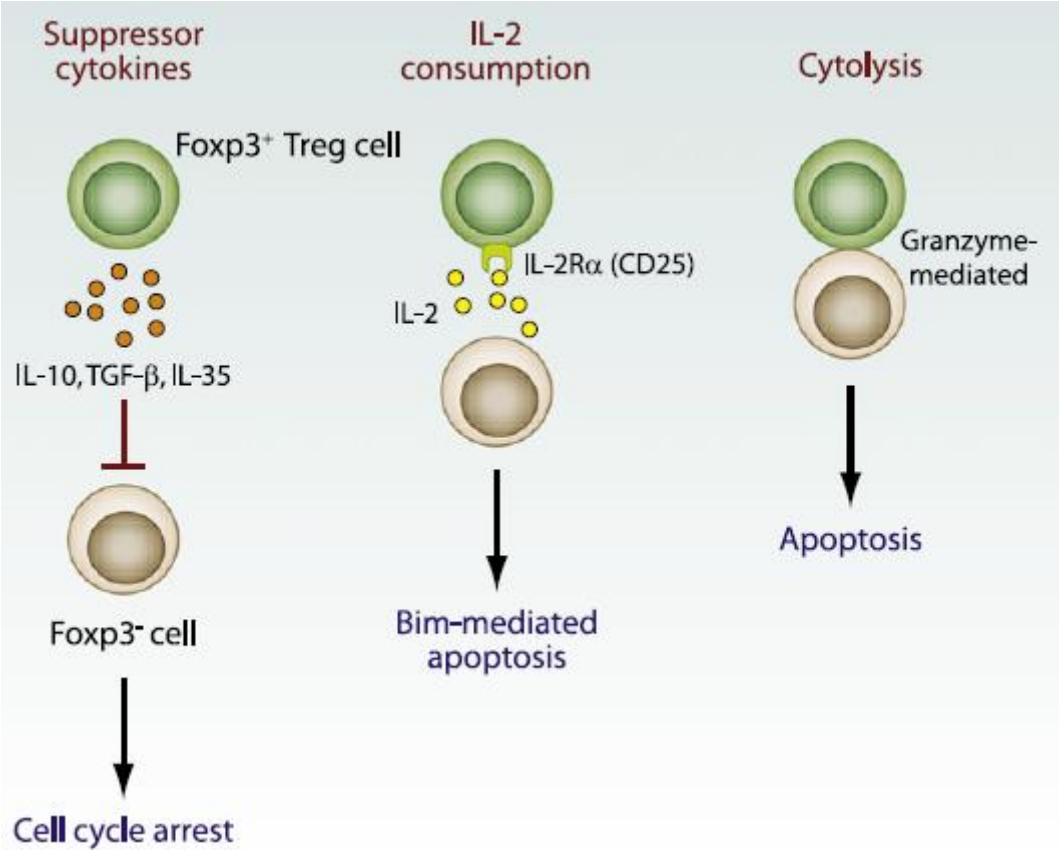

Figure 1.3: Mechanisms of direct T effector cell suppression by Tregs. The figure shows the different ways Tregs control effector cells, including anti-inflammatory cytokine production, IL-2 consumption, and granzyme-mediated apoptosis. Modified from (86).

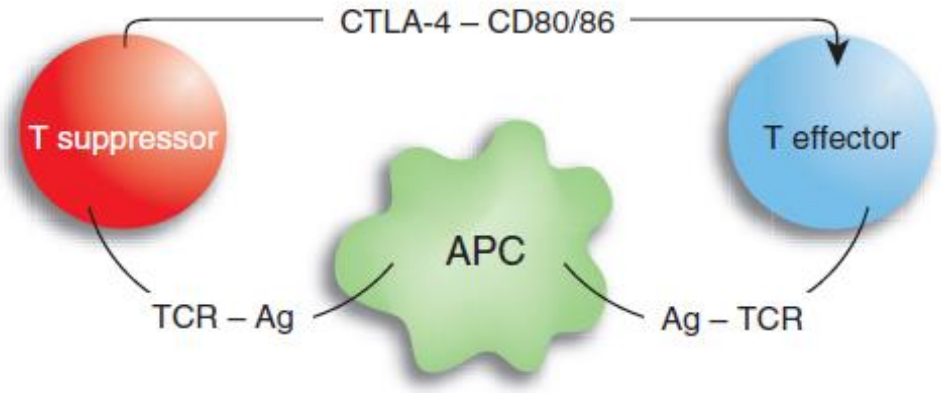

Figure 1.4: CTLA-4 mediated T effector suppression by Tregs via DC contacts. Effector T cells and Tregs (T suppressor) could meet at an antigen presenting cell (APC) during antigen (Ag) presentation, facilitating the binding of CTLA-4 on suppressors to CD80 and/or CD86 on activated effector cells. Modified from (87).

1.7.2 CHS regulation by Tregs

Treg depletion either before sensitization or before challenge resulted in enhanced and prolonged CHS response (91), while transfer of Tregs led to CHS suppression (92). During the sensitization phase, Tregs control the priming and expansion of hapten-specific T cells. It has been shown that hapten-specific CD8 T cells produce IL-2 in skin draining LNs during sensitization which is required for Tregs (93). Ring *et al.* have demonstrated that Tregs co-localized with DCs during sensitization in the LNs resulting in reduced expression of CD80 and CD86 by DCs, and thus impairment of hapten-specific CD8 T cell activation. This inhibition was mediated via gap junction formation (94). During the challenge phase, Tregs migrate to the affected skin and express high levels of CTLA-4 and IL-10, and exhibit more potent suppressive activities compared to Tregs residing in the LNs (95). Tregs have been shown to be able to block effector cell infiltration to the skin by downregulating E/P-selectin expression in blood endothelial cells (92). This blockade was mediated by IL-10 production and also by adenosine generation via ATP degradation (96). A possible induction of hapten-specific Tregs has been suggested in a model of photoimmunosuppression to DNFB (97). Previous studies have shown

that Tregs found in the challenged skin home back to the LNs in order to orchestrate the resolution phase, and keep the ability to migrate back to the skin (98). All these cited studies used either Treg depletion or Treg transfer to demonstrate the importance of Tregs in controlling the magnitude of the CHS response. However, so far, it was not known whether the expansion of endogenous Tregs will suppress CHS establishment. The present study addressed this question.

1.7.3 The contribution of regulatory B cells in CHS suppression

In addition to Tregs, regulatory B cells (Bregs) have been proposed as regulator cells during CHS (99). Bregs are identified as $CD19^{+}$ $CD5^{+}$ $CD1d^{hi}$ cells that produce anti-inflammatory IL-10. CD19-deficient mice exhibited augmented CHS responses, and the transfer of Bregs into CD19-deficient mice normalized the severity of CHS-mediated inflammation, indicating their involvement in the control of CHS response.

1.8 The IL-2/JES6-1 concept

IL-2 plays an important role in T cell activation and proliferation. IL-2 is thought to be mainly produced by CD4 T cells, and to a lesser extent, by CD8 T cells, natural killer (NK) cells, B cells, and natural killer T cells (100). During steady-state conditions, IL-2 production is low but constant and serves primarily for the development and homeostatic survival of Treg cells (101–103). These low levels of IL-2 are not sufficient to stimulate the proliferation of memory CD8 T cells *in vivo* (104). Induction of IL-2 production occurs following antigen activation of CD8 and CD4 T cells. This phenomenon is tightly regulated by silencing the IL-2 gene via the transcription factor B lymphocyte-induced maturation protein 1 (BLIMP1), which otherwise indicated by its name is also expressed in T cells.

The IL-2 receptor (IL-2R) consists of three subunits termed IL-2Rα (CD25), IL-2Rβ (CD122), and the common γ chain (CD132) (105–107). CD122 and CD132 subunits are critical for signal transduction, whereas CD25 doesn´t contribute to the signaling but confers high-affinity

binding of IL-2 to its receptor, therefore increasing ligand-receptor affinities (107–109). High-affinity αβγ IL-2Rs are constitutively found on Tregs, and transiently on recently-activated T cells (100, 110), while low-affinity βγ IL-2Rs are highly expressed on memory CD8 T cells and NK cells, and are present at a low level on naïve CD8 T cells (100, 110). Signal transduction of IL-2R activation occurs via several intracellular pathways, including the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, the phosphoinositide 3-kinase (PI3K)–AKT pathway, and the mitogen-activated protein kinase (MAPK) pathway (100, 111, 112).

Administration of low-dose IL-2 has been used as a therapy for several diseases (113, 114). However, its rapid renal elimination represents a major limitation for this treatment strategy. Usage of higher doses of IL-2 causes severe toxic side effects and therefore cannot be a solution to this problem. In order to extend the IL-2 half-life *in vivo* and strengthen its biological activity, previous studies have suggested IL-2 coupling to larger proteins such as IL-2- Immunoglobulin (Ig)G fusion proteins. These attempts however, had limited success (115, 116). More recently, injection of exogenous IL-2 complexed with anti-IL-2 monoclonal antibodies (mAbs) was shown to efficiently enhance the biological activity of IL-2 *in vivo* in mice (117).

Two functionally distinct murine IL-2/mAb complexes are distinguished (117–119). Complexing IL-2 with anti-mouse IL-2 mAb clone S4B6 leads to IL-2/S4B6 complexes that preferentially stimulate cells expressing high levels of βγ IL-2Rs, notably memory CD8 T cells and NK cells (119). Conversely, the usage of anti-mouse IL-2 mAb clone JES6-1A12 generates IL-2/JES6-1 complexes which interact almost exclusively with cells expressing high levels of αβγ IL-2Rs. Hence resulting in induction of selective expansion of Treg populations (117, 119). The difference of the functional properties of IL-2/S4B6 and IL-2/JES6-1 complexes is explained by the binding of the two mAbs to different IL-2 epitopes (119, 120). S4B6 recognizes and binds to the CD25 binding site, and thereby covers the IL-2's epitope binding to

CD25, thus biasing IL-2/S4B6 complexes to preferentially stimulate CD122^{hi} cells (memory CD8 T cells and NK cells) (Figure 1.5). Vice versa, JES6-1A12 is attached to IL-2's epitopes binding to CD122 and CD132, which makes IL-2/JES6-1 complexes selectively stimulate CD25^{hi} cells, such as Treg cell (118).

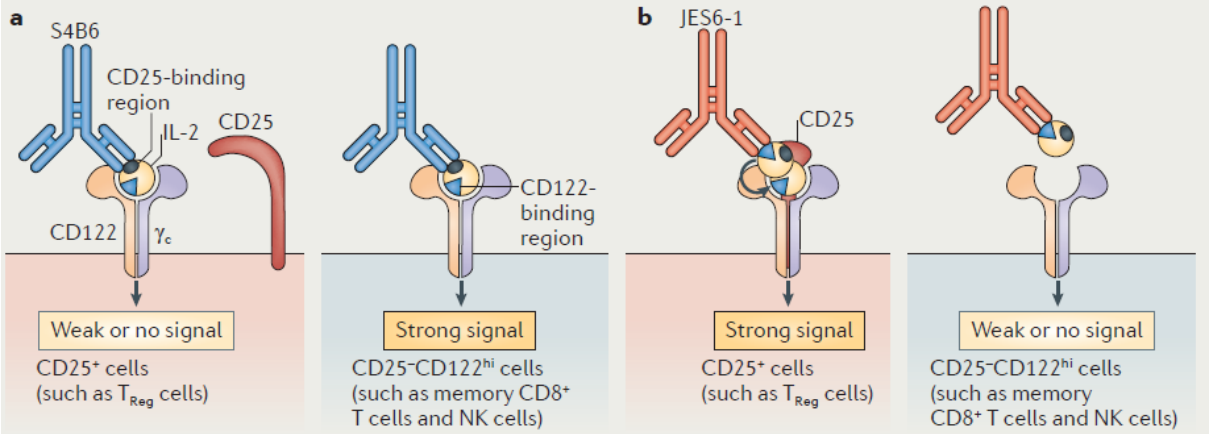

Figure 1.5: Mechanism of action of IL-2/mAbs complexes. IL-2/mAbs complexes can selectively target a specific cell subset depending on the epitope recognized by the monoclonal antibody used in the complex. (a) The antibody S4B6 binds to the region of IL-2 that interacts with CD25 and therefore stimulates CD122^{hi} cells, notably memory CD8 T cells and NK cells. (b) JES6-1 antibody interacts with the region of IL-2 that makes contact with CD122 which leads to Treg induction. Modified from (119).

2 MATERIALS AND METHODS

2.1 Materials

Table 2.1.1: Buffers and medias

Name	Composition	Source	Storage
PBS (1X)	8 g/l NaCl 0.2 g/l KCl 1.44 g/l Na2HPO4	Calbiochem Calbiochem Sigma-Aldrich	RT
PBS-BSA	PBS with 0.5% BSA	Sigma-Aldrich For BSA	4°C
RPMI medium 1640 with Glutamine	medium + 10% FCS 200 µg/ml Penicillin 200 U/ml Streptomycin 5 x 10-5 M Beta 2-mercaptoethanol	Gibco Gibco Invitrogen Invitrogen Sigma-Aldrich	4°C
Red blood cell lysis buffer (10X)	8.02 g NH4Cl 0.84 g NaHCO3 0.37 g C10H16N2O8 100 ml H2O	Sigma-Aldrich Geyer Th. GmbH Geyer Th. GmbH	4°C

Table 2.1.2: Kits

Name	Method	Composition	Source	Storage
Foxp3 Fix/Perm Buffer set	Intracellular Foxp3 staining	Foxp3 Fix/Perm Foxp3 Perm buffer	Biolegend	4°C
Fixation/Permeabilization Solution Kit	Intracellular cytokine staining	Fixation buffer Perm/Wash buffer	Biolegend	4°C

Table 2.1.3: Antibody list

Anti-mouse antibodies	Clone	Conjugation	Source
Anti-CD11b	M1/70.15.11	A700	In house production
Anti-Ly6G	1A8	APC	Biolegend, Germany
Anti-IFN-γ	XMG1.2	APC	Biolegend, Germany
Anti-IL-10	JES-16E3	FITC	Biolegend, Germany
Anti-CD19	1D3	APC-Cy7	Biolegend, Germany
Anti-CD138	281-2	PE	Biolegend, Germany
Anti-Foxp3	FJK-16s	PE	eBiosciences, Germany
Anti-B220	RA3.B2	A700	In house production
Anti-CD5	53-7.3	A405	eBiosciences, Germany
Anti-Helios	22F6	FITC	Biolegend, Germany
Anti-Nrp-1	3E12	PE-Cy7	Biolegend, Germany
Anti-LAP-1	TW7-20B9	PerCP-eFluor 710	Biolegend, Germany
Anti-CTLA-4	UC10-4B9	APC	Biolegend, Germany
Anti-CD4	GK1.5	eF450	eBiosciences, Germany
Anti-CD8	53-6.7	A700	In house production
*Anti-IL-2	JES6-1	Purified	In house production
Anti-CD1d	K253	PE	Biolegend, Germany

*Anti-IL-2 was used for injection of IL-2/JES6-1 complexes
All listed antibodies were stored at 4 °C.
Rat IgG1, IgG2a, IgG2b (eBioscience) with the matching fluorochromes were used as isotype controls.

Table 2.1.4: List of reagents used in CHS induction

Name	Usage	Source	Storage
TNCB	Hapten sensitization and challenge	Sigma-Aldrich	RT
Acetone	Dissolvent	J.T.Baker®	RT
FITC	Hapten sensitization	Sigma-Aldrich	4 °C
DBP	Dissolvent	Sigma-Aldrich	RT
Croton oil	Skin irritation	Sigma-Aldrich	RT
Oxazolone	Hapten sensitization and challenge	Sigma-Aldrich	RT
Diphtheria toxin	Treg depletion	Sigma-Aldrich	-80 °C

Table 2.1.5: Other reagents

Name	Usage	Source	Storage
PMA/Ionomycin	Polyconal stimulation of cells for intracellular staining	Sigma-Aldrich	-20 °C
Brefeldin-A	Inhibition of protein transport within cells	eBioscience, Germany	4 °C
Ketamin	Mice anesthesia	Sigma-Aldrich	4 °C
Dispase	Tissue dissociation	Roche	-20 °C
Collagenase A	Collagen digestion	Roche	-20 °C
DNAse	DNA degradation	Sigma-Aldrich	4 °C
Hematoxylin	H&E staining	Sigma-Aldrich	RT
Eosin	H&E staining	Sigma-Aldrich	RT
TNBS	Antigen restimulation of cells	Sigma-Aldrich	4 °C
O.C.T compound medium	Tissue embedding	Sakura-Finetek, Netherlands	RT
Mitomycin C	Antigen restimulation of cells	Sigma-Aldrich	4 °C

Table 2.1.6: List of laboratory equipment

Name	Manufacturer
Centrifuge (Microfuge 22R)	Beckman Coulter, Germany
Flow cytometer LSR II	BD Biosciences, Germany
Cell Sorter FACS Aria III	BD Biosciences, Germany
Centrifuge 5810 R	Eppendorf, Germany
Laminar Hood (LabGard Class II Biological Safety Cabinet NU-437)	NuAire, USA
Cryostat CM1850	Leica Microsystems, Germany
Direct Heat CO2 Incubator (NU5510/E)	NuAire, USA
Light Microscope Leica DMi1	Leica Microsystems, Germany
Micrometer	Mitutoyo, Leonberg, Germany

Table 2.1.7: List of consumables

Name	Usage	Source
5 ml FACS tube	Flow cytometry	Sarstedt AG & Co.
1,5 ml Eppendorf tube	Flow cytometry	BD Bioscience, Germany
EDTA containing blood collection tube	Flow cytometry	BD Vacutainer, Germany
70 µm cell strainer	Flow cytometry	BD Falcan, Germany
48 well plate	Cell stimulation	Geyer Th. GmbH
Super frost glass slide	Tissue section preparation	Geyer Th. GmbH

Table 2.1.8: Software list used for data analysis

Name	Developer
FACS Diva	BD Biosciences, Germany
Flow jo	FlowJo LLC, USA
GraphPad prism	GraphPad Software, Inc, USA

2.2 Mice

8-12 week old, Female C57BL/6 mice were purchased from Charles River Laboratory (Sulzfeld, Germany) and maintained under pathogen-free conditions. Severe combined immunodeficiency (SCID) beige mice (121), and depletion of regulatory T cell (DEREG) mice (122) were provided by Prof. Ralf Ludwig, Department of Dermatology, University of Lübeck, Germany. Experiments were performed at the animal facility of University of Lübeck. All animal studies were performed with approval from the respective authorities (Ministry of Energy, Agriculture, the Environment and Rural Areas) and performed by certified personnel.

2.3 Induction of CHS

2.3.1 TNCB-induced CHS model

Sensitization of mice: At day 0, the abdomen of mice was shaved manually using a scalpel. The purpose of shaving the abdomen is to facilitate skin contact with the hapten, and ameliorate its penetration into the skin layers. Then, 100µl of 3% TNCB dissolved in acetone was applied on the shaved abdomen using a pipette. The role of acetone is to extract lipids from the outer layer of the epidermis called stratum coreneum, this process will cause the disruption of the epidermal barrier and ensure an efficient antigen administration.

Challenge of mice: At day 5, the right ear was challenged by applying a total volume of 20µl of 1% TNCB dissolved in acetone onto both sides of the ear. As a control, the left ear was painted with acetone alone. Ear swelling reaction was measured 24 h later or at the indicated time points after challenge. In the second challenge experiments, right or left ear was painted with 20 µl of 1% TNCB/acetone 12 days after first challenge, and ear swelling was tracked for the following 5 days.

2.3.2 Model of double CHS induction with TNCB and oxazolone

In this model, a first sensitization was performed by applying 150 µl of 3% oxazolone dissolved in ethanol on the shaved back of mice. Fifteen days later, a second sensitization was applied by painting the shaved abdomen with 100 µl of 3% TNCB dissolved in acetone. For the challenges, a first challenge was performed by applying a total volume of 20µl of 1% TNCB dissolved in acetone onto both sides of the right ear, and 20µl of 1% oxazolone dissolved in ethanol onto both sides of the left ear. Second challenges were performed respecting the same order 12 days after the first challenge.

2.3.3 Ear swelling measurement

Before each challenge, ear thickness of both ears was measured 3 times using a micrometer, the mean of values obtained is the baseline used for calculating the ear swelling. Twenty four hours after challenge or at indicated time points, ear thickness was measured again 3 times and mean value was calculated. Ear swelling was determined by subtracting the mean value of the ear thickness after challenge from the mean value of the ear thickness measured before challenge.

2.4 Skin irritation

Irritant contact dermatitis is induced following skin irritation by a chemical. The response to this irritation occurs rapidly after irritant exposure to the skin because it´s mediated by innate immune cells and established independently of the adaptive immune system. To induce antigen-nonspecific skin inflammation, 1% of croton oil dissolved in 70% acetone, and 10 µl of the mixture was applied onto each side of the right ear of SCID beige mice. The left ear was painted with acetone only and used as control. Twenty four hours later, ear thickness was measured and ear swelling was calculated as previously described in paragraph 2.3.3.

2.5 IL-2/JES6-1 complex treatment

To induce Treg expansion, three injections of IL-2/JES6-1 complexes are needed (123). For each injection, 1 µg of recombinant mouse IL-2 was complexed with 5 µg of anti-IL-2 monoclonal antibody (clone JES6-1A12). The mixture was diluted in 150 µl sterile PBS and incubated for 30 min at 37°C. The injections were performed intraperitoneally (i.p.) on 3 consecutive days (days 0, 1 and 2). PBS injections were used for controls. The i.p. injection was performed using a 27 G needle in the lower quadrant of the abdomen but close to the midline. Injections in the nipples or any harm to the tissue was avoided in order to have a successful i.p. injection.

2.6 Diphtheria toxin treatment

DEREG transgenic mice express a diphtheria toxin receptor-enhanced green fluorescent protein (DTR-eGFP) fusion protein under control of the endogenous forkhead box P3 promoter/enhancer regions on the bacterial artificial chromosome (BAC) transgene (124). In these mice, fully functional $Foxp3^+$ $CD4^+$ regulatory T cells express DTR-eGFP allowing fluorescent detection and diphtheria toxin-induced depletion of $Foxp3^+$ Treg cells (125). For Treg depletion, DEREG mice were injected i.p. with 1 µg of Diphtheria toxin dissolved in 100 µl sterile PBS one day before sensitization. DT injection into WT mice was used a control.

2.7 Adoptive transfer

LN cell transfer from sensitized donors into naïve recipients is a model used to passively induced CHS in mice. Effector cells are transferred after priming in donor mice, which enables recipients to respond to a challenge after cell transfer within 24 hours. In the adoptive transfer experiment, donor mice were treated with either IL-2/JES6-1 or PBS on days -5, -4 and -3, and sensitized to TNCB on day 0. Five days later, cell suspensions were prepared from draining LNs and injected intravenously in the tail vein into recipient mice (2×10^7 cells/mouse). Recipient mice were treated with either IL-2/JES6-1 complexes or PBS on days 0, 1, 2. The adoptive transfer was performed at day 5. One hour after the adoptive transfer, mice were challenged in the right ear and ear swelling was measured one day later.

2.8 *In vivo* DC migration assay

In order to assess DC accumulation in the skin draining LNs after hapten sensitization, mice were sensitized by applying 20 µl of 0.5% FITC dissolved in acetone/ DBP at a ratio of 1:1 on both sides of the ear. FITC is a skin sensitizing fluorochrome permitting its tracking when transported by DCs from the skin to the draining LNs. DBP is used as an adjuvant that augments the ability of topically applied FITC to stimulate the proliferative responses in LNs. Auricular

LNs were removed 24 hours and 48 hours after FITC sensitization, and FITC-bearing DCs cells were assessed using flow cytometry.

2.9 Cell isolation from ear tissue

The inflamed ear was split into dorsal and ventral halves using forceps, chopped into small pieces and incubated in 1.2 mg/ml Dispase for one hour at 37°C in a total volume of 2 ml RPMI medium. This step serves at an initial enzymatic digestion resulting in collagen and fibronectin cleavage by proteases and tissue dissociation. Ear skin pieces were then digested using 1 mg/ml collagenase A and 60 µg/ml DNAse I for one hour at 37°C. This step permits to further digest the tissue and liberate the cells into the medium. Tissues were passed through a 70 µm cell strainer using a 5 ml syringe and washed in 0.5% PBS-BSA. Isolated cells were surface stained for flow cytometry.

2.10 Blood preparation

Blood was collected from the heart of anesthetized mice by cardiac puncture using a 27 G needle connected to a syringe. Blood was collected in EDTA (anticoagulant) coated tubes. Up to 1 ml of blood can be collected using the cardiac puncture. For red blood cells lysis, blood was transferred into tubes containing 14 ml of 1X RBC lysis buffer (pre-diluted in water) and incubated in the dark at 4 °C for 10 min with gentle agitation every two minutes. Blood was then centrifuged at 300 g for 10 min. The supernatant was removed and cells were washed with 0.5% PBS-BSA buffer. Cells were then ready for flow cytometry.

2.11 H&E staining

Hematoxylin and Eosin (H&E) staining is a standard diagnosis method used in histology. Hematoxylin alone is not technically a dye, it is therefore coupled with aluminum salts. The complex acts as a basic dye positively charged and can react with negatively charged, basophilic

cell components, such as nucleic acids in the nucleus resulting in a blue stain. The nuclear staining is followed by counterstaining with an aqueous Eosin solution. Eosin is anionic, acts as an acidic dye and therefore reacts with positively charged, acidophilic components in the tissue coloring them in red/pink.

To perform H&E staining of ear tissue, 5 µm sections of frozen tissue were generated using Cryostat CM1850, and sections were collected in super frost glass slides. Sections were dried overnight at RT and then fixed in ice cold acetone/ethanol 1:1 for 10 min. Tissue sections were washed with 1X PBS three times for 5 min, then stained with hematoxylin in a jar for 10 min. Sections were washed with 1X PBS twice for 5 min and with water for 3 min. Eosin staining was performed by dipping the slides into a jar full of 1% eosin for 1-2 min. Slides were quickly washed in water for 30 seconds and then dipped in 50% ethanol, 70% ethanol, and 96% ethanol for 30 seconds each. Tissue sections were then incubated in xylene for 5 min, then slides were cleaned and mounted with entellan and coverslip.

2.12 Hapten-specific CD8 T cell detection

Auricular and axillary LNs were isolated at indicated time points and single cell suspension was prepared, washed, and resuspended in RPMI media supplemented with 10% FCS (Fetal Calf Serum), 1% Penicillin-Streptomycin and 0,1% β-Mercaptoethanol. To prepare hapten-loaded APCs, a single cell suspension was prepared from spleen of naïve mice and were used as source for APCs. Splenic cells were then treated with mitomycin C for 30 min at 37 °C to inhibit their mitotic activation, and washed with warm media. In order to load APCs with the allergen, 3 mM of 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was added and cells were incubated for 7 min at 37°C. TNBS is a water soluble analog of TNCB and therefore is used for *in vitro* stimulation of cells. Splenic cells were washed and resuspended in the same media. For LN cells restimulation in vitro, 10^7 LN cells were mixed with 10^7 TNBS-modified splenic cells in a 48 well-plate (total volume of 1 ml), and incubated at 37°C for a total of 5 hours.

Brefeldin A was added for the last 4 hours. Cells were harvested and stained intracellularly for IFN-γ.

2.13 Flow cytometry

2.13.1 Surface staining

Single cell suspension was prepared from spleen or LNs in PBS-BSA, and approximately 1 million cells were used for staining. Cells were first centrifuged at 300 g for 10 min. In order to block non-specific binding of staining antibodies to Fc receptors, 50 µl of anti-CD16/CD32 (FcγR block) antibody with a concentration of 10 µg/ml was added to the cells and incubated for 10 min on ice. Fluorochrome-conjugated antibodies in a concentration of 2-5 µg/ml were prepared in 50 µl PBS-BSA and added to the cells. The cocktail was kept in the dark on ice for 10-15 min, and the staining was stopped by adding 1 ml PBS-BSA. Cells were then centrifuged at 300 g for 10 min (4 °C), resuspended in 300 µl PBS-BSA, and acquired in the flow cytometer LSR II.

2.13.2 Intracellular staining

Intracellular staining was used in this thesis to detect cytokines in T and B cells. For this purpose, freshly isolated cells were first stimulated for five hours and intracellular staining was performed. First, single cell suspensions were prepared from spleen or LNs. Cells were then centrifuges at 300 g for 10 min, and resuspended in RPMI media containing 10% FCS, 1% Penicillin-Streptomycin and 0,1% β-Mercaptoethanol. To prepare the stimulation master mix, phorbol 12-myristate 13-acetate (PMA) (a specific activator of kinase C), ionomycin (an ionophore that triggers calcium release) were added to RPMI media at a final concentration of 500 ng/ml. For B cell stimulation, lipopolysaccharide (LPS) was also added (10 µg/ml, final concentration). To prepare the cell culture, 10^7 cells suspended in 500 µl of media were mixed with 500 µl of the stimulation mix in a 48 well plate and incubated at 37 °C for 5 hours. Brefeldin A (inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus,

2 mM final concentration) was added for the last 3 hours of culture. Unstimulated cells were used as control. After the 5 hour stimulation, cells were harvested from each well into Eppendorf tubes and centrifuged at 300 g for 10 min. Surface staining was performed as described above (paragraph 2.13.1). After surface staining, cells were fixed with 100 µl of Biolegend fixation buffer for 20 min at RT. 100 µl of Biolegend intracellular staining permeabilization wash buffer was added to the cells for 20 min at RT. Cells were centrifuged at 350 g for 10 min and the supernatant was discarded. After this permeabilization step, cells were stained intracellularly with the fluorescently labeled monoclonal antibodies against the desired cytokines for 15 min at 4 °C. Cells were washed again with Perm/Wash buffer as before and resuspended in 300 µl PBS-BSA. Cells were acquired in a flow cytometer.

2.13.3 Treg staining

Foxp3 is a master regulator of the development and function of Tregs. It is considered as a key marker used to identify Tregs by flow cytometry. Being a transcription regulator, Foxp3 is present in the nucleus and therefore its detection requires the permeabilization of the nuclear membrane. First, single cell suspension was prepared (10^7 cells/ sample) and surface staining was performed as described in paragraph 2.13.1. For Treg staining, cells were fixed with 200 µl of 1X Fix/Perm solution (pre-diluted in PBS) for 20 min in the dark at RT. Cells were first washed with PBS-BSA and centrifuged at 300 g for 10 min, a second washing step was performed using 200 µl of 1X Foxp3 Perm buffer (pre-diluted in PBS). Cells were resuspended in the Foxp3 Perm buffer and incubated in the dark for 15 min at RT. After permeabilization, cells were centrifuged at 300 g for 10 min and resuspended in 100 µl Foxp3 Perm buffer containing anti-mouse Foxp3, anti-mouse CTLA-4, and anti-mouse Helios antibodies in a concentration of 2-5 µg/ml. After 45 min incubation in the dark at 4 °C, cells were washed with PBS-BSA and resuspended in 300 µl PBS-BSA. Cells were then analyzed by flow cytometry.

2.14 Data analysis

2.14.1 Flow cytometry data analysis

During sample measurement, cell acquirement and event recording was done using FACSDiva software. FCS files (3.0 version) were exported from the FACSDiva software and analyzed using flow jo software. All gates were set according to isotype controls. Compensation was used to reduce signal spillover between the emission spectra of two fluorochromes.

2.14.2 Statistical analysis

Statistical analysis of data was performed using GraphPad Prism. Unpaired two-sided Student's t test was used. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. In case of bar plots, data are expressed as mean ± SD.

3 RESULTS

3.1 IL-2/JES6-1 treatment expands endogenous Tregs *in vivo*

Depletion of Tregs results in enhanced and sustained ear swelling following CHS induction (126). In contrast, adoptive transfer of $CD4^+$ $CD25^+$ Tregs either before sensitization or before challenge strongly suppresses CHS-mediated ear swelling by blocking effector cell influx into inflamed skin (92), suggesting that these cells exhibit the capacity to control CHS-mediated inflammation. However, it is not clear whether *in vivo* induction of Treg populations will suppress the response to an antigen-specific skin allergy. In order to address this issue, mice were treated either with IL-2/JES6-1 complexes or PBS (as controls). Subsequently, CHS was induced by local application of TNCB.

3.1.1 IL-2/JES6-1 injection before sensitization efficiently suppresses CHS

In order to reach maximal Treg induction at the time of sensitization, mice were treated with injections of IL-2/JES6-1 on three consecutive days starting on day -5, then mice were sensitized on day 0, challenged on day 5, and analyzed 24 hours after challenge. The scheme of this treatment regimen is shown below (Figure 3.1). Later in this thesis, this particular treatment regimen will be called IL-2/JES6-1 BS. As expected, $Foxp3^+$ Treg cell frequencies and numbers were increased in spleen and LNs on day 0 in IL-2/JES6-1 treated mice compared with PBS treated controls (Figure 3.2). This expansion was no longer observed on day 6, which is in accordance with previous findings showing that IL-2/JES6-1 treatment induces a rapid and systemic, but only transient Treg expansion that vanishes within few days (127). Notably, IL-2/JES6-1 treatment strongly reduced ear swelling after challenge (Figure 3.3).

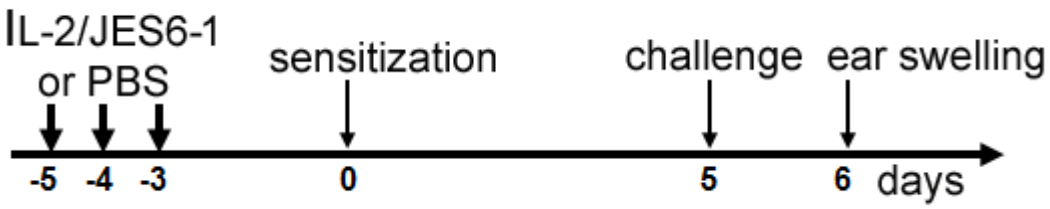

Figure 3.1: Scheme of CHS induction and treatment regimen before sensitization. Mice were injected with either IL-2/JES6-1 or PBS on days -5, -4 and -3, and sensitized with TNCB on day 0 as indicated. On day 5, the right ear was challenged with TNCB while the left ear was treated with vehicle. Ear swelling was measured on day 6. Analysis of Treg frequencies and cell counts were analyzed on days 0 and 6.

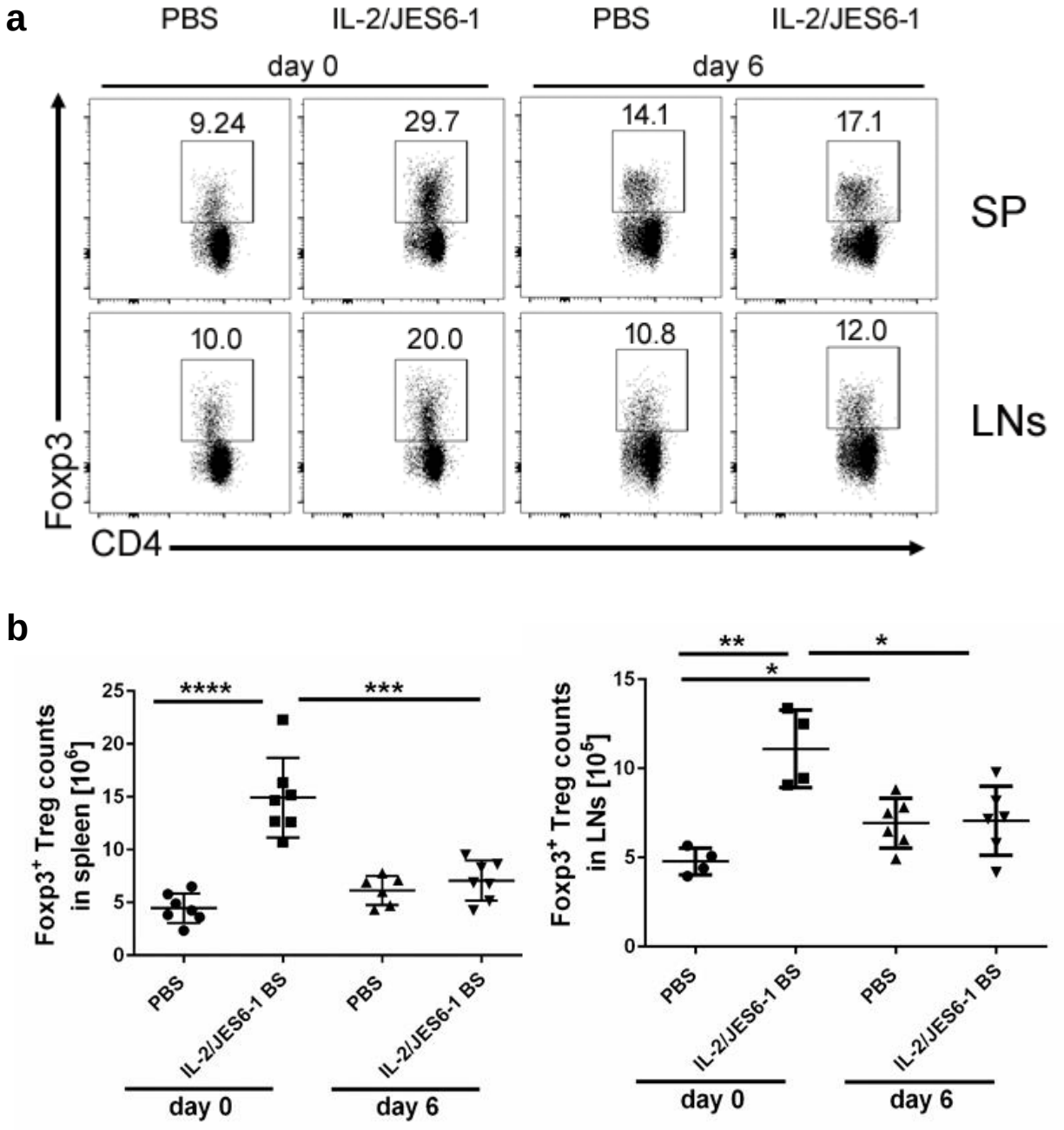

Figure 3.2: Rapid and transient expansion of Tregs upon IL-2/JES6-1 treatment. (a) FACS analysis of Foxp3$^+$ Treg cells on days 0 and 6 after sensitization (IL-2/JES6-1 BS = IL-2/JES6-1 complexes treated before sensitization; PBS = control group) in spleen (SP) and pooled (axillary and auricular) LNs. Cells were gated on CD4$^+$ T cells. (b) Treg cell numbers in spleen (right data plot) and lymph nodes (left data plot). Data are pooled from at least two independent experiments (n= 4-6), *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 (two-tailed unpaired Student's t-test). Data are expressed as mean ± SD.

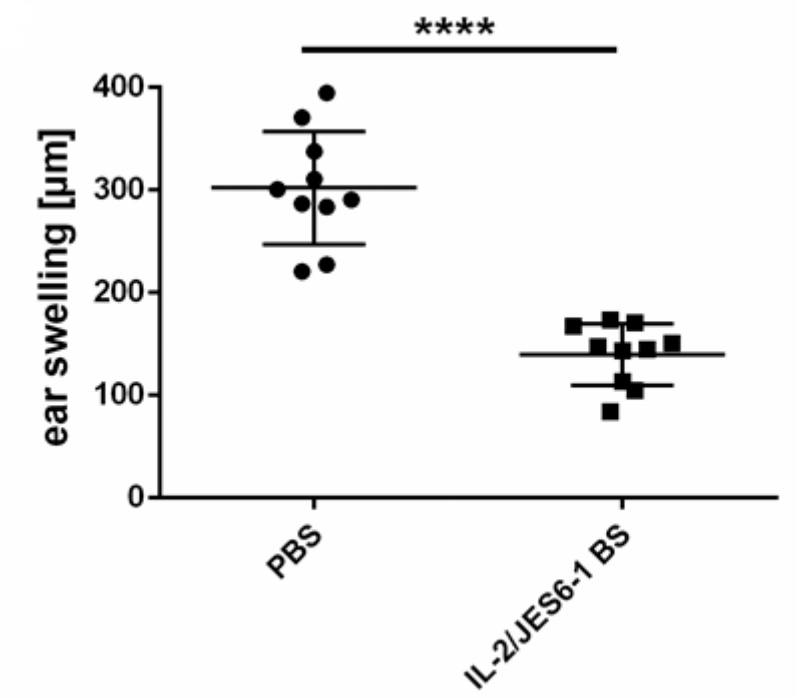

Figure 3.3: IL-2/JES6-1 injection before sensitization results in CHS suppression. Ear thickness was measured before challenge (used as baseline) and 24 hours post-challenge, and mean of ear swelling was calculated. Data show the increase in ear thickness compared to the value before challenge. Data are pooled from at least two independent experiments (n= 4-6), ****P < 0.0001 (two-tailed unpaired Student's t-test). Data are expressed as mean ± SD.

3.1.2 IL-2/JES6-1 treatment expands both nTregs and pTregs

Several studies have shown that IL-2/JES6-1 leads to *in vivo* Treg expansion (117, 127, 128). However, it had remained unclear if this treatment selectively affects nTregs or pTregs, or both. To address this issue, Treg subsets were distinguished by Helios and Nrp-1 expression. Interestingly, Both Nrp-1^+ Foxp3^+ T cells and Nrp-1^- Foxp3^+ T cells were expanded in spleen and LNs upon IL-2/JES6-1 treatment (Figure 3.4), Suggesting that both nTreg and pTreg populations were systemically affected by this treatment. To ensure that Nrp-1^+ Foxp3^+ T cells represent nTregs, their Helios expression was analyzed by flow cytometry. Nrp-1 expression by Foxp3^+ Treg cells strictly correlated with Helios expression (Figure 3.5), which is in accordance with the previous findings reported in literature (80).

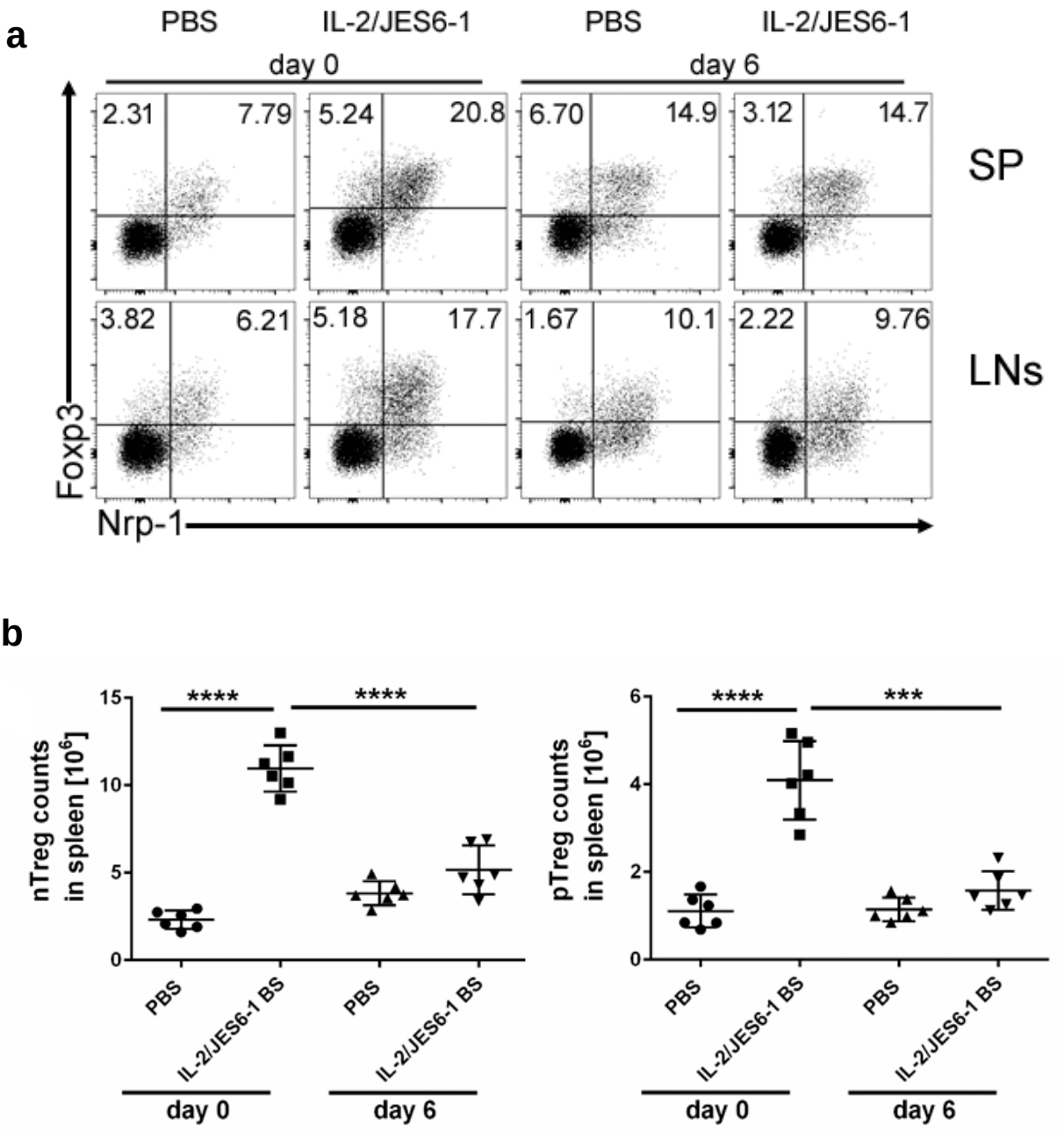

Figure 3.4: Induction of nTregs and pTregs upon IL-2/JES6-1 treatment. Mice were treated as shown in figure 3.1 and Treg subsets were analyzed. (a) FACS analysis of nTregs and pTregs using Nrp-1 on days 0 and 6 after sensitization (IL-2/JES6-1 BS = IL-2/JES6-1 complexes treated before sensitization; PBS = control group) in spleen (SP) and pooled (axillary and auricular) lymph nodes (LNs). Cells were gated on $CD4^+$ T cells. (b) nTreg and pTreg cell numbers in spleen. n= 6, ***P < 0.001 and ****P < 0.0001 (two-tailed unpaired Student's t-test). Data are shown as mean ± SD.

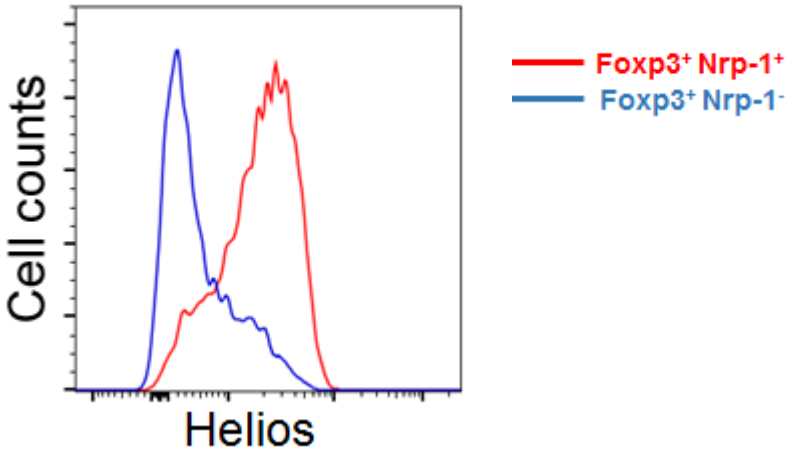

Figure 3.5: Foxp3$^+$ Nrp-1$^+$ Tregs co-express Helios. Mice were treated as shown in figure 3.1, and Treg subsets were analyzed at day 0. Histogram plot shows Helios expression of Foxp3$^+$ Nrp-1$^+$ cells.

3.1.3 Characterization of the suppressive capacity of IL-2/JES6-1 expanded Tregs

Tregs expanded following IL-2/JES6-1 injections were further analyzed for the anti-inflammatory cytokine TGF-β and activation marker CTLA-4. The first form synthesized of TGF-β is called pro-TGF-β which will be cleaved resulting in latency-associate peptide (LAP). TGF-β production by Tregs was measured by analyzing LAP-1 expression which reflects the latency-associate peptide of the isoform TGF-β 1. Before sensitization (day 0), TGF-β expressing Tregs are present in small numbers in spleen and LNs. Upon IL-2/JES6-1 treatment, Treg populations were approximately 10-fold expanded. Interestingly, about 2-fold increased numbers of TGF-β$^+$ Foxp3$^+$ Tregs were observed on day 6 in the absence of IL-2/JES6-1 treatment after hapten challenge in the PBS treated controls, indicating that hapten exposure itself stimulated Treg expansion (Figure 3.6). Similarly, CTLA-4$^+$ Treg cell populations were also affected by IL-2/JES6-1 treatment and were approximately 10-fold expanded on day 0. Again, those cells were already increased in numbers in PBS treated mice on day 6 after challenge (Figure 3.7), but to a much lower extent compared with the IL-2/JES6-1 treated

group. Expression levels of CTLA-4 in individual Foxp3$^+$ Treg cells was upregulated in IL-2/JES6-1 treated mice (Figure 3.8). These data indicate that TGF-β and CTLA-4 might be involved in controlling CHS-mediated inflammation via IL-2/JES6-1.

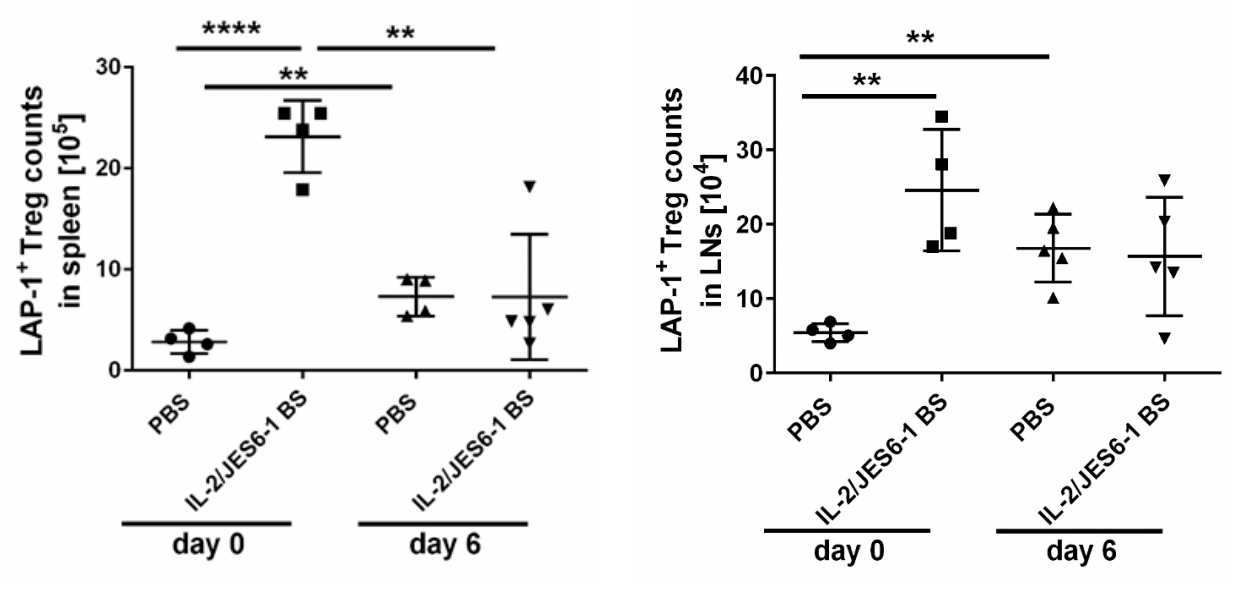

Figure 3.6: Increased numbers of LAP-1$^+$ Tregs in IL-2/JES6-1 treated mice. Mice were sensitized and challenged by TNCB on days 0 and 5, respectively. Before sensitization, mice were treated with IL-2/JES6-1 complexes. Cell numbers were calculated following flow cytometric analysis of LAP-1$^+$ Foxp3$^+$ Tregs in spleen (left panel) and lymph nodes (right panel). Data are representative of at least two independent experiments (n= 4-6), **P < 0.01 and ****P < 0.0001 (two-tailed unpaired Student's t-test). Data are expressed as mean ± SD.

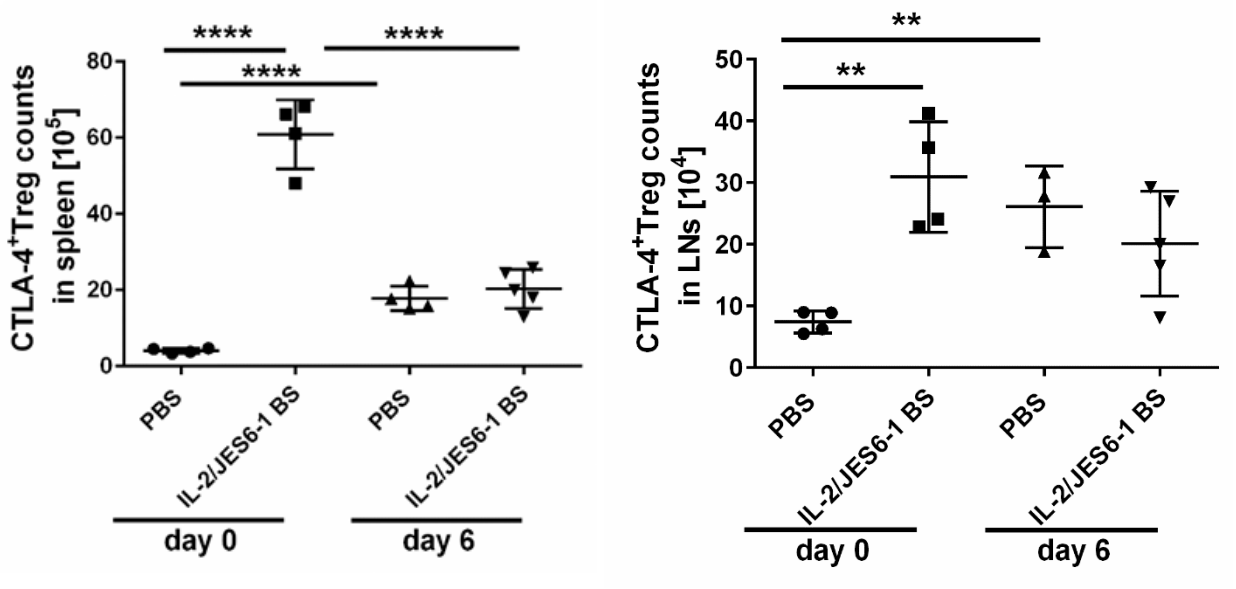

Figure 3.7: Expansion of CTLA-4$^+$ Tregs in IL-2/JES6-1 treated mice. Mice were sensitized and challenged by TNCB on days 0 and 5, respectively. Before sensitization, mice were treated with IL-2/JES6-1 complexes. Cell numbers were calculated following flow cytometric analysis of CTLA-4$^+$ Foxp3$^+$ Tregs in spleen (left panel) and LNs (right panel). Data are representative of at least two independent experiments (n= 4-6), **P < 0.01 and ****P < 0.0001 (two-tailed unpaired Student's t-test). Data are presented as mean ± SD.

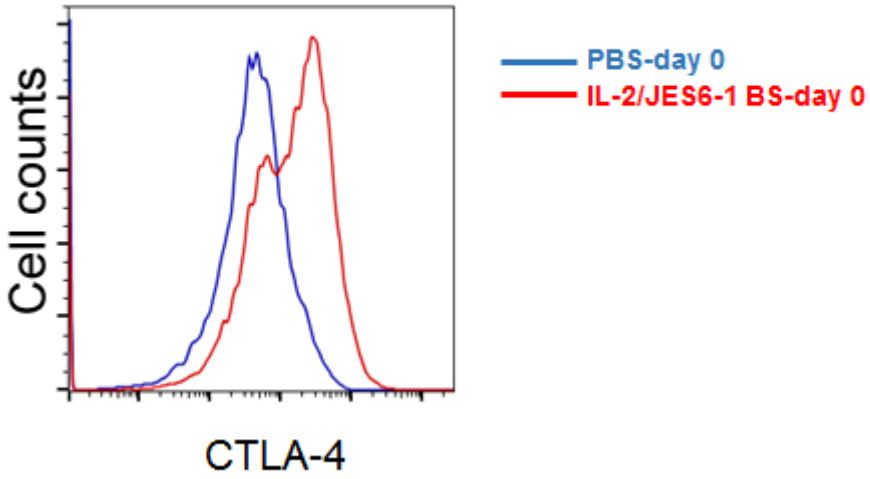

Figure 3.8: Up-regulation of CTLA-4+ by Tregs after IL-2/JES6-1 treatment. CTLA-4 expression by Foxp3^{+} Tregs in spleen after IL-2/JES6-1 injection before sensitization (day 0). Data shown are gated on Foxp3^{+} Tregs and are representative of two independent experiments (n= 4-6).

3.1.4 Increased IL-10 expression by CD4 T cells in IL-2/JES6-1 treated mice

Previous studies have demonstrated that CD8 T cells are the effector cells in murine contact sensitivity while CD4 T cells play a regulatory role mainly by IL-10 secretion (62–64). The effect of IL-2/JES6-1 treatment before sensitization on CD4 T cells was investigated. Flow cytometry analysis show that IL-10 expression by CD4 T cells was elevated following IL-2/JES6-1 injection in spleen and LNs. It is of note that the IL-10 increase was more pronounced in spleen. A slight increase of IL-10 expressing CD4 T cell numbers was also observed in PBS treated mice after challenge (Figure 3.9), confirming the involvement of CD4 T cells in CHS regulation by IL-10 production.

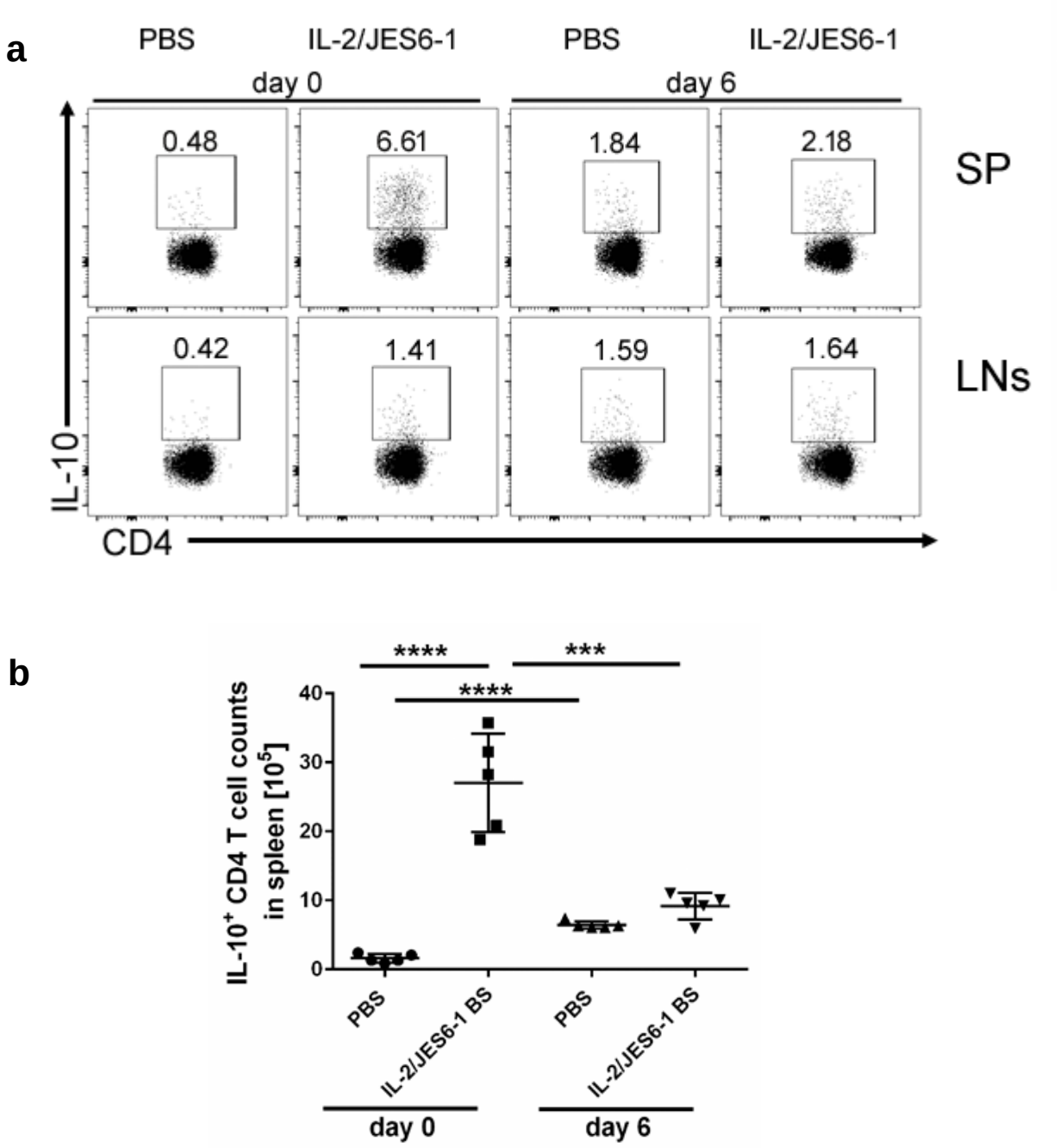

Figure 3.9: Induction of IL-10 expression by CD4 T cells upon IL-2/JES6-1 treatment. Mice were treated as shown in figure 3.1 and IL-10 expression by CD4 T cells was analyzed. (a) Flow cytometry analysis of IL-10 expressing CD4 T cells on days 0 and 6 after sensitization (BS = IL-2/JES6-1 treated before sensitization; PBS = control group) in spleen (SP) and pooled (axillary and auricular) lymph nodes (LNs). Cells were gated on CD4+ T cells. (b) IL-10+ CD4 T cells counts in spleen. n= 6, ***P < 0.001 and ****P < 0.0001 (two-tailed unpaired Student's t-test). Data are shown as mean ± SD.

3.2 Treg expansion before the challenge phase results in CHS suppression

During challenge, previously primed T cells are activated in the skin and the draining LNs, which makes their recruitment to the site of hapten exposure as fast as 24 hours after hapten challenge (129, 130). To examine the impact of the IL-2/JES6-1 treatment on Tregs and CHS when injected after sensitization but before challenge (IL-2/JES6-1 BC), mice were sensitized on day 0 and IL-2/JES6-1 or PBS injections were given on days 0, 1, and 2. On day 5, challenge was performed on the right ear, while the left ear was treated with vehicle (Acetone alone). Ear swelling measurement was performed 24 hours after challenge (Figure 3.10). Again, a reduction in ear swelling was observed in IL-2/JES6-1 treated mice before challenge. Numbers of nTregs and pTregs were still increased on day 6 upon IL-2/JES6-1 treatment, suggesting that hapten-sensitization does not disturb Treg expansion upon IL-2/JES6-1 injections. Likewise, IL-10^+ CD4 T cell numbers were increased after IL-2/JES6-1 treatment before the challenge phase (Figure 3.11). There was no significant difference in TGF-β^+ Treg counts between IL-2/JES6-1 BC and PBS treated groups, Tregs expressing TGF-β mainly resemble nTregs (Figure 3.12). CTLA-4 expression was also upregulated on Tregs in IL-2/JES6-1 treated mice. Interestingly, CTLA-4^+ Tregs observed following IL-2/JES6-1 treatment were mostly pTregs (Figure 3.13).

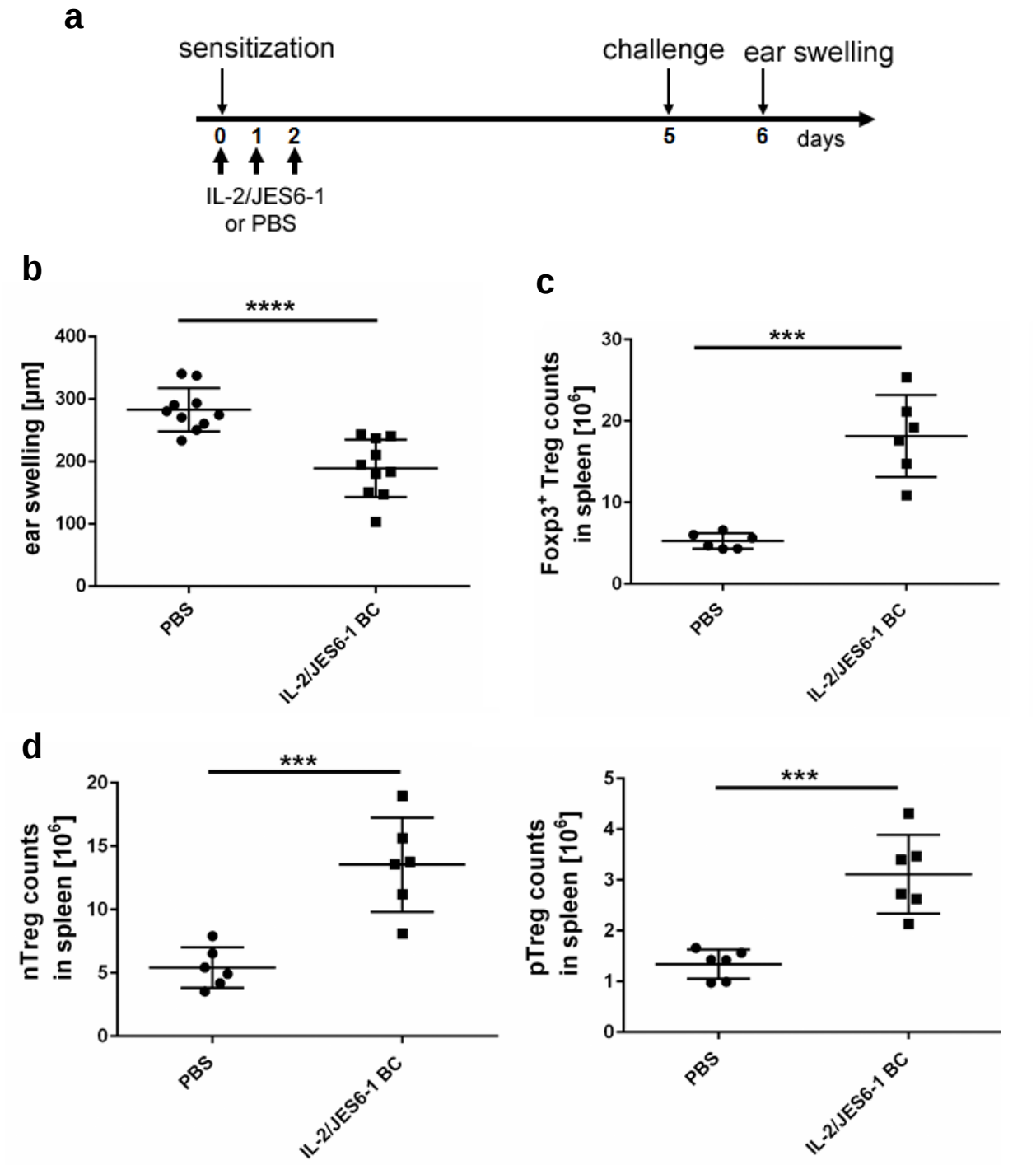

Figure 3.10: IL-2/JES6-1 treatment before challenge efficiently suppresses CHS. (a) Scheme of treatment schedule used for Treg induction before challenge. (b) Mean of ear swelling 24 hours post challenge. (c) Total Foxp3$^+$ Treg cell numbers in spleen on day 6. (d) Cell counts of nTregs and pTregs in spleen on day 6. Data are compiled from at least two independent experiments n= 5-6, ***P < 0.001 (two-tailed unpaired Student's t-test). Data are shown as mean ± SD.

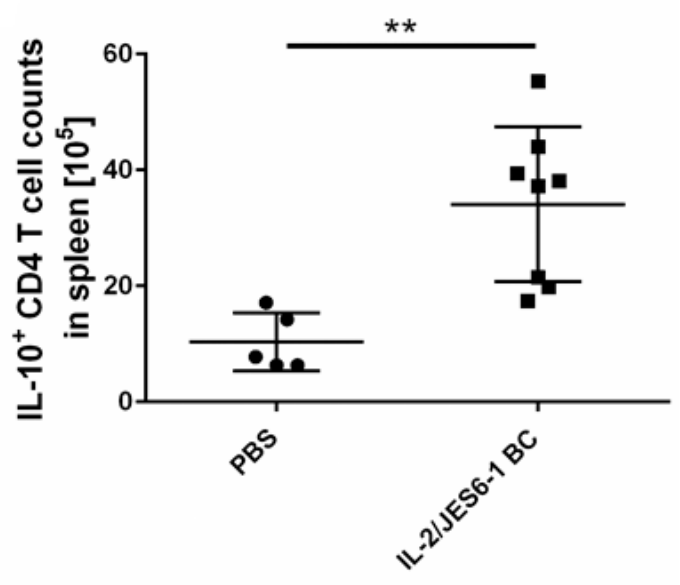

Figure 3.11: Augmented IL-10 expression by CD4 T cells in IL-2/JES6-1 treated mice before challenge. Mice were treated as shown in figure 3.10 and IL-10^+ CD4 T cell numbers were analyzed on day 6. **P < 0.01 (two-tailed unpaired Student's t-test). Data are presented as mean ± SD.

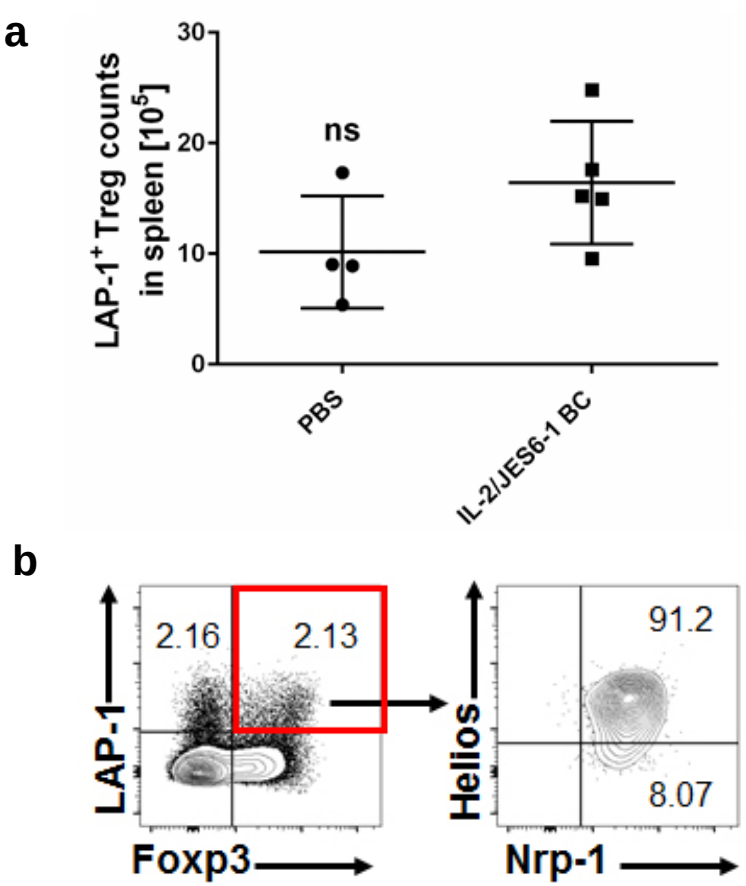

Figure 3.12: TGF-β expressing Tregs remain unaffected by IL-2/JES6-1 treatment before challenge. Mice were treated as shown in figure 3.10 and TGF-β^+ Foxp3$^+$ Tregs were analyzed on day 6. (a) TGF-β^+ Treg numbers in spleen. (b) Gating strategy for analysis of Nrp-1 and Helios expression in LAP-1$^+$ Tregs (quadrants were set according to isotype control). Dot plot of the left image was gated on CD4 T cells. Data are presented as mean ± SD, n= 4-5.

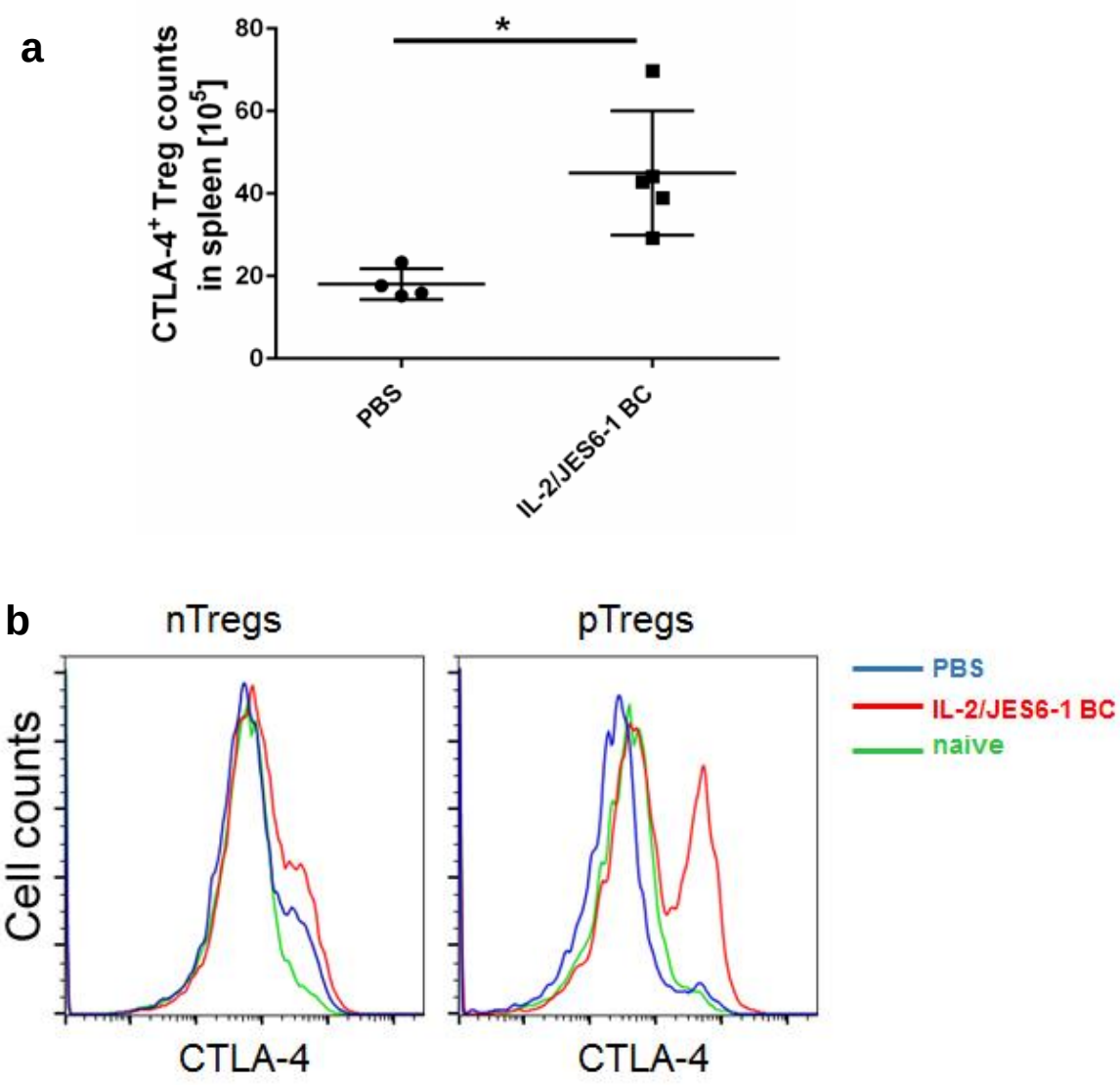

Figure 3.13: Characterization of CTLA-4+ Tregs following IL-2/JES6-1 treatment. (a) Numbers of CTLA-4 expressing Tregs. (b) CTLA-4 expression by nTregs and pTregs on day 6. Data shown are gated on Nrp-1^{+} Foxp3^{+} and Nrp-1^{-} Foxp3^{+} Tregs for nTregs and pTregs, respectively. *P < 0.05, n= 4-5 (two-tailed unpaired Student's t-test). Data are expressed as mean ± SD.

3.3 Effects of IL-2/JES6-1 treatment on leukocyte infiltration into inflamed skin

Neutrophils start accumulating in the skin 6 hours after challenge, followed by lymphocytes within 12 hours after challenge, which leads to an extensive neutrophil and T cell infiltration 24 hours post-challenge (59). To investigate the effect of IL-2/JES6-1 treatment on inflamed skin, histology of the ears was analyzed, and effector cell infiltration into the affected skin was inspected.

3.3.1 IL-2/JES6-1 treatment results in reduced neutrophil and CD8 T cell infiltration into challenged skin

Histological analysis of ear sections showed a general decrease in cell infiltration in groups treated with IL-2/JES6-1 either before sensitization or before challenge compared with the PBS controls (Figure 3.14). These results confirm the anti-inflammatory effects of the IL-2/JES6-1 treatment. In addition to CD8 T cells, neutrophils represent potent effector cells during both sensitization and challenge in CHS (56, 57). To test the effect of IL-2/JES6-1 treatment on neutrophil and CD8 T cell infiltration, these cells were quantified in ear tissue after challenge, and in blood. CHS induction as such did not alter neutrophil frequencies in blood 24 hours after sensitization. However, when mice were treated before sensitization with IL-2/JES6-1, neutrophil frequencies were reduced in this tissue 24 hours post-sensitization (Figure 3.15). In skin, neutrophil frequencies were decreased 24 hours after challenge following IL-2/JES6-1 treatment (Figure 3.15). Additionally, CD8 T cell frequencies were reduced in IL-2/JES6-1 treated groups before sensitization and before challenge (Figure 3.16). These results demonstrate that IL-2/JES6-1 treatment results in decreased infiltration of effector cells into the inflamed skin.

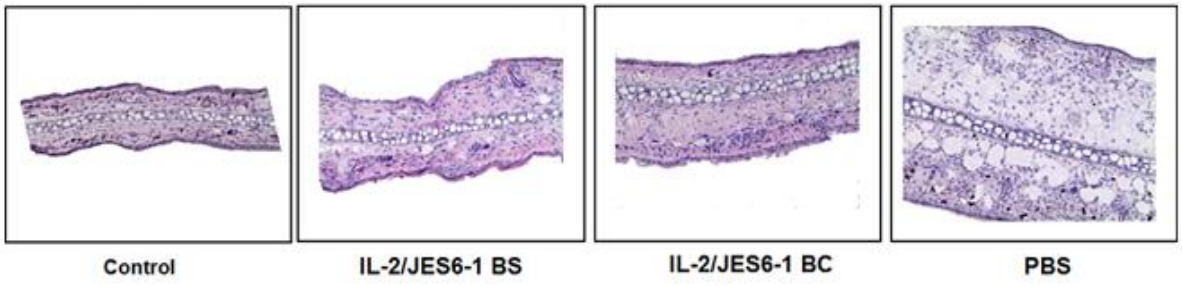

Figure 3.14: Reduced cellular influx in challenged skin following IL-2/JES6-1 treatment. Representative histological sections of 5 µm after hematoxylin and eosin (H&E) staining (200x magnification). Tissue sections were prepared from inflamed ears 24 hours after challenge.

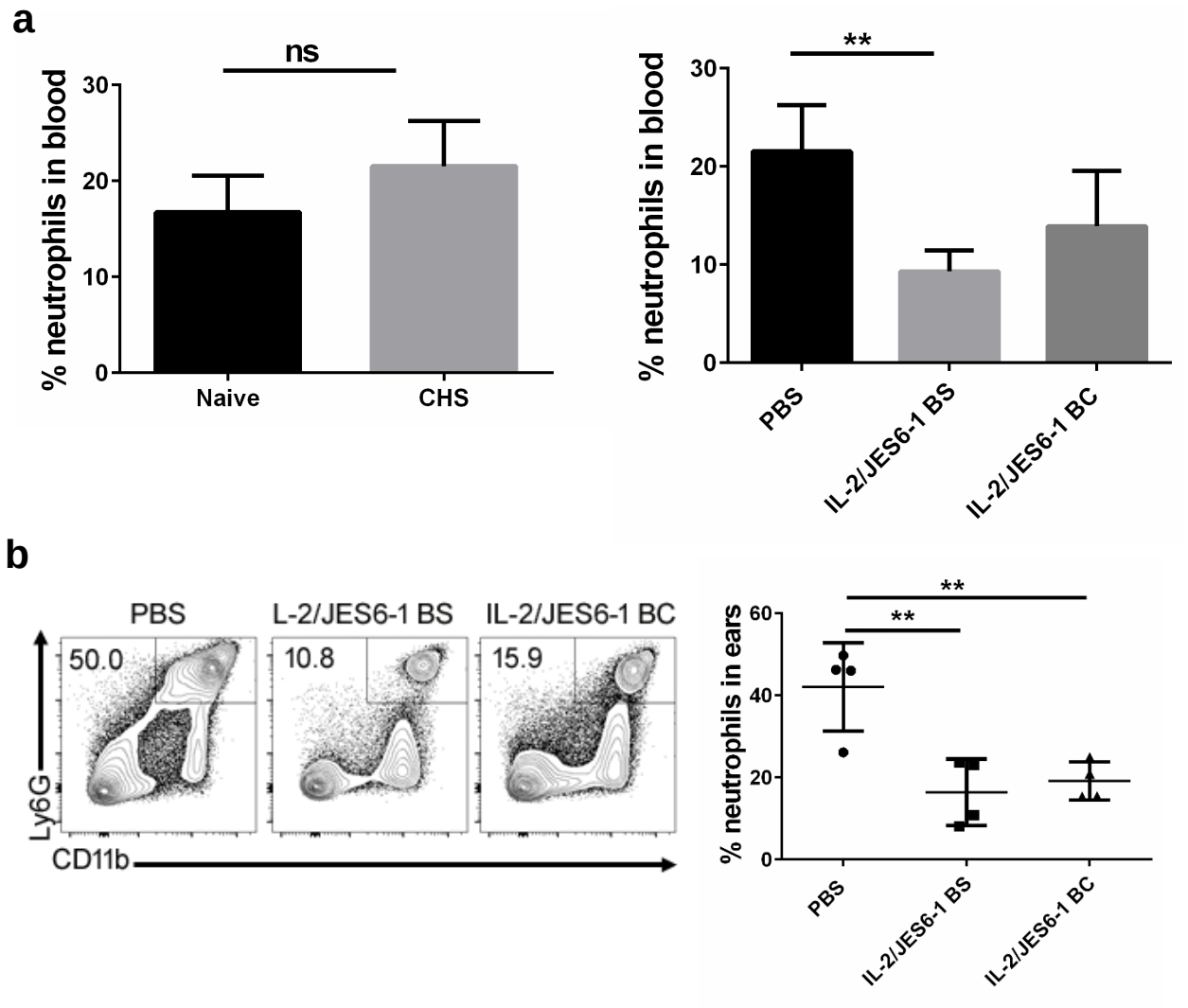

Figure 3.15: Effect of IL-2/JES6-1 on neutrophils. Mice were treated with IL-2/JES6-1 either before sensitization or before challenge and neutrophils were analyzed. (a) Neutrophil frequencies in blood in naïve or sensitized mice 24 hours after sensitization (left panel). Neutrophil frequencies in blood in IL-2/JES6-1 or PBS treated mice 24 hours after sensitization (right panel). (b) Flow cytometry analysis of neutrophils in ear tissue 24 hours after challenge (left panel), cells were pre-gated on singlets. Percentage of neutrophils in ear tissue (right panel). **P < 0.01 (two-tailed unpaired Student's t-test), n=4-6. Data are presented as mean ± SD.

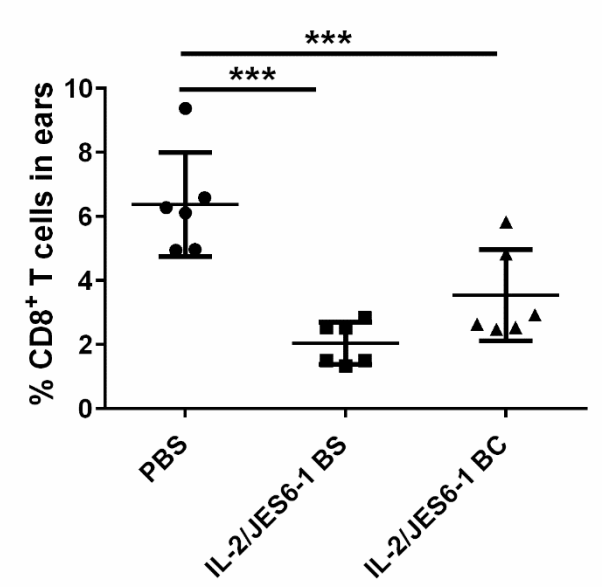

Figure 3.16: Reduced CD8 T cell accumulation in the skin upon IL-2/JES6-1 treatment. Mice were treated with IL-2/JES6-1 either before sensitization or before challenge and CD8 T cells were analyzed, PBS controls received PBS injections using the same schedule as IL-2/JES6-1 BS group. Frequencies of CD8 T cells in ear tissue 24 hours after challenge. n= 5-6, ***P < 0.001 (two-tailed unpaired Student's t-test). Data are shown as mean ± SD.

3.3.2 Increased Treg frequencies in inflamed skin following IL-2/JES6-1 treatment

Tregs are the most important suppressor cells during CHS-mediated inflammation. Tregs migrate to the inflamed tissue upon challenge, and migrate back to the skin draining LNs, these circulating Tregs have the capacity to migrate back to the skin (98). It has been shown that these Tregs express high levels of CTLA-4 and have potent suppressive activities (98). To examine the effect of IL-2/JES6-1 on Treg cells in the skin, Tregs were analyzed in ear tissue 24 hours after challenge. In ears, $Foxp3^+$ Treg cells were increased in both groups that received IL-2/JES6-1 treatment (Figure 3.17). Notably, 100% of $Foxp3^+$ Tregs found in the skin were $CTLA\text{-}4^+$ (Figure 3.17). This molecule has been shown to be very important for Treg mediated suppression of effector T cells (131). These data show that IL-2/JES6-1 treatment supports the presence of suppressor cells in inflamed tissue.

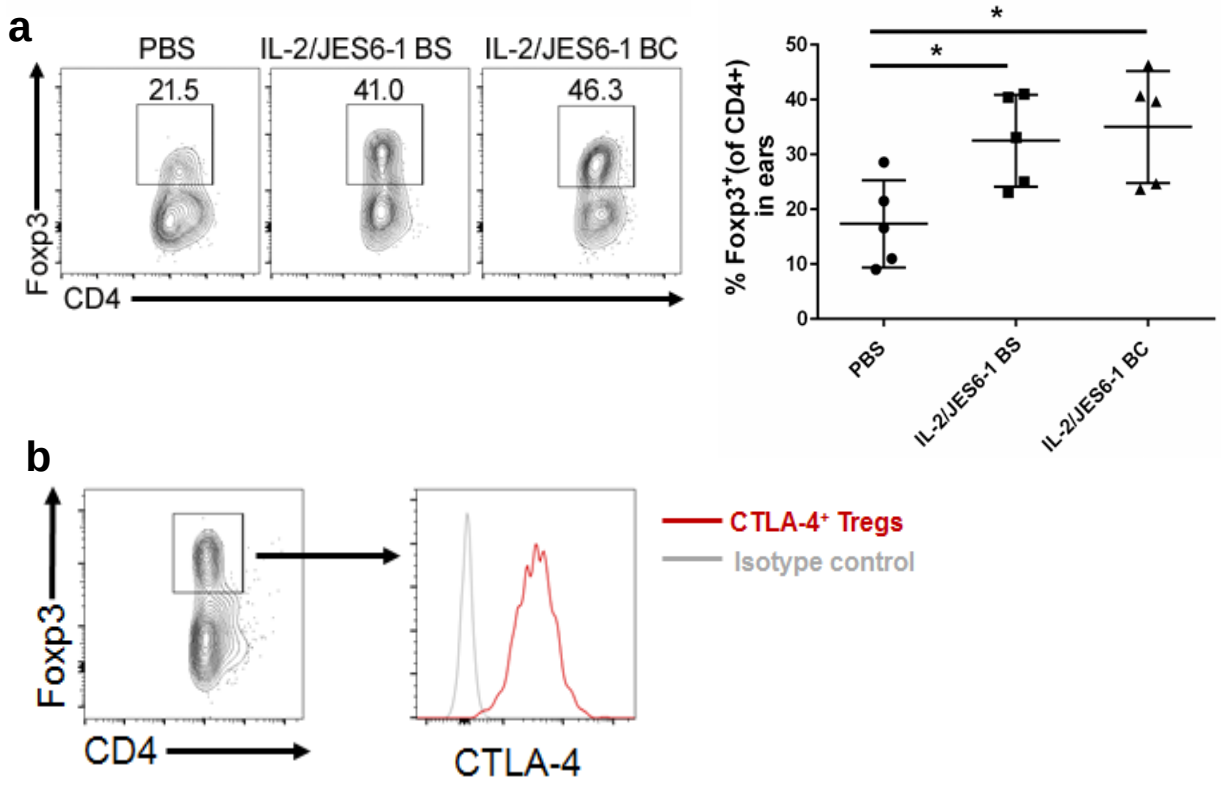

Figure 3.17: IL-2/JES6-1 treatment results in increased Treg frequencies in inflamed skin. Mice were treated with IL-2/JES6-1 either before sensitization or before challenge and CHS was induced. PBS controls received PBS injections using the same schedule as IL-2/JES6-1 BS group. Tregs were analyzed in skin 24 hours after challenge. (a) Flow cytometry analysis of Tregs in ears, cells were gated on CD4 T cells (left panel). Treg frequencies detected in ear tissue (right panel). (b) CTLA-4 expression by Tregs found in the skin. *P < 0.05, n= 5 (two-tailed unpaired Student's t-test). Data are expressed as mean ± SD.

3.4 IL-2/JES6-1 effect on neutrophils is lymphocyte-dependent

In order to test whether IL-2/JES6-1 can affect neutrophil-mediated skin inflammation directly, or via T cells, SCID mice were used. SCID mice are characterized by the absence of T and B cells, which makes them a good tool for this purpose. However, induction of CD8 T cell-mediated CHS wouldn´t be possible in these mice. To resolve this issue, antigen-nonspecific skin irritation was induced using croton oil as a model of irritant contact dermatitis. This cutaneous irritation is characterized by the activation of the innate immune system and skin barrier disruption after contact to chemicals. Neutrophils and eosinophils are the main populations that infiltrate the affected skin after chemical exposure, while basophiles have a regulatory role in this process (132, 133). In the absence of lymphocytes, induction of non-specific skin irritation showed similar neutrophil infiltration in both IL-2/JES6-1 treated mice and PBS controls (Figure 3.18). Moreover, ear swelling was similar between these two groups (Figure 3.18), indicating that the inhibitory effect of IL-2/JES6-1 on neutrophil recruitment depends on lymphocytes. These data are in accordance with the idea that IL-2/JES6-1 complexes stimulate Tregs selectively, which leads to the hypothesis that the observed effect of IL-2/JES6-1 on neutrophils might be mediated by Tregs.

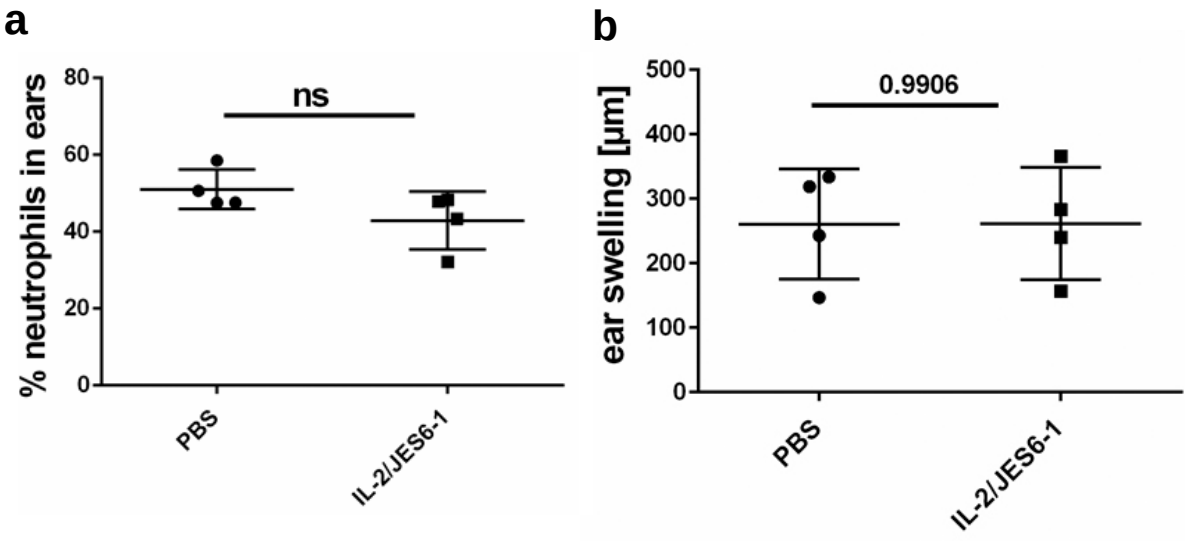

Figure 3.18: IL-2/JES6-1 does not affect neutrophils directly. SCID beige mice were treated on days -5, -4, and -3 with IL-2/JES6-1 or PBS, and skin irritation was induced using croton oil on day 0. (a) Neutrophils frequency in ears 24 hours after skin irritation (b) Ear swelling at 24 h after skin irritation.

3.5 Foxp3$^+$ regulatory T cells mediate IL-2/JES6-1 induced CHS suppression

Despite the numerous studies showing that IL-2/JES6-1 selectively expands Tregs and improves different inflammatory diseases, there was no clear evidence that these observed ameliorations are Treg-dependent. Recent data have shown that IL-2/JES6-1 can affect dermal innate lymphoid cells type 2, their activation leads to an increase in IL-5 production, which causes eosinophilia (134, 135). Taken together, IL-2/JES6-1 complexes might also have a direct effect on innate lymphoid cells, and thus indirectly affect eosinophils. Consequently, it was necessary to identify the cells that are responsible for CHS suppression after IL-2/JES6-1 treatment. To assess the role of Foxp3$^+$ Tregs in TNCB-induced CHS suppression after IL-2/JES6-1 treatment, DEREG mice were analyzed. These mice express a diphtheria toxin receptor under the control of the Foxp3 promoter, allowing effective and selective Treg depletion (122). DEREG mice were treated with IL-2/JES6-1 before sensitization, and Foxp3$^+$ Tregs were depleted one day before sensitization (Figure 3.19). 24 hours after DT injection, Tregs were depleted from blood (Figure 3.19). Treg frequencies were still reduced even 7 days later in LNs (Figure 3.19). Ear swelling on day 6 revealed that the CHS response was completely restored in Foxp3$^+$ Treg depleted mice. In addition, Neutrophil infiltration into the inflamed ear tissue was similar to PBS treated controls, and Treg frequencies were decreased (Figure 3.20). These observations demonstrate that Tregs are essential for IL-2/JES6-1 mediated suppression of CHS.

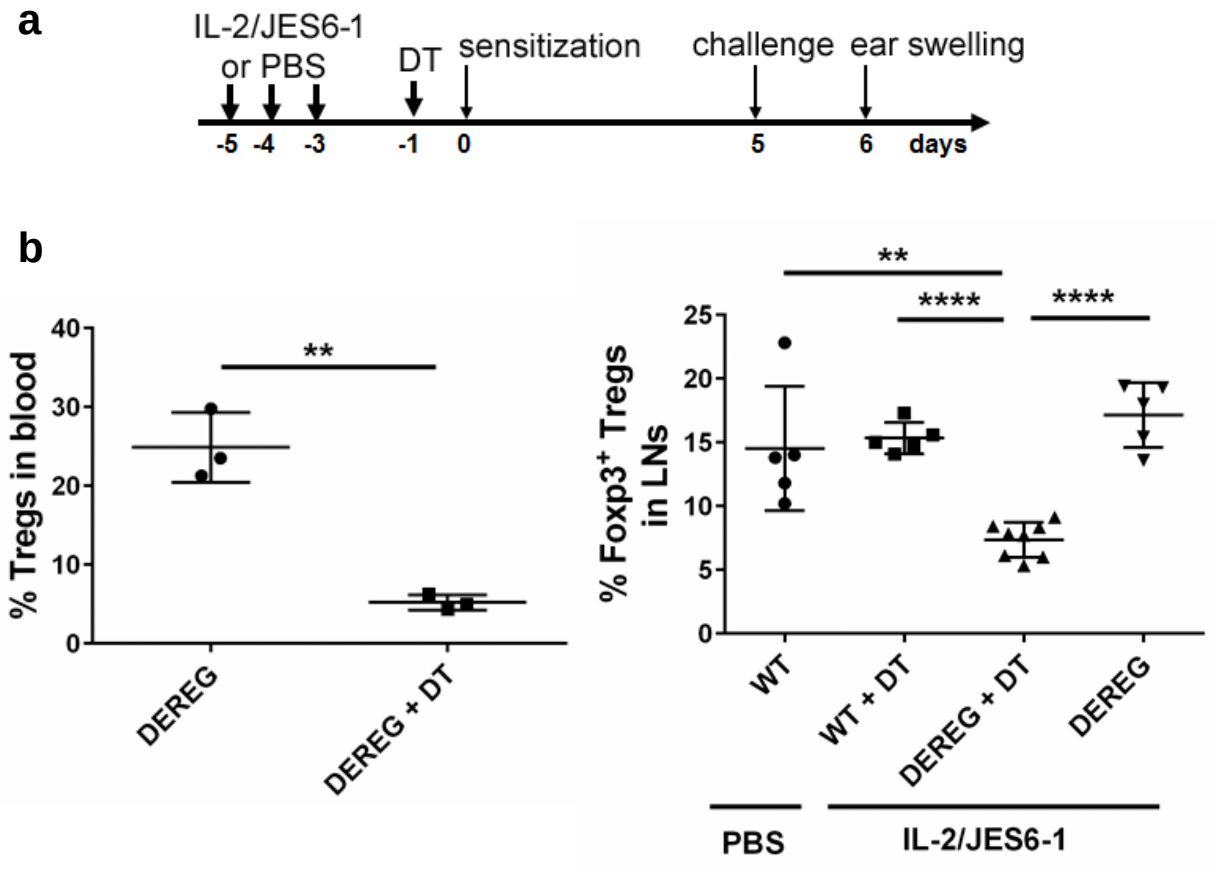

Figure 3.19: Depletion of Foxp3$^+$ Tregs following diphtheria toxin injection. DEREG or WT mice were treated with IL-2/JES6-1 or PBS before sensitization and Tregs were deleted by DT injection before CHS was induced. (a) Treatment regimen used in DEREG mice. (b) Percentage of Foxp3+ Tregs in blood on day 0 (left panel). Frequency of Foxp3$^+$ Tregs in LNs on day 6 (right panel). Data are depicted as mean ± SD (n= 3-8 mice per group) **P < 0.01 and ****P < 0.0001.

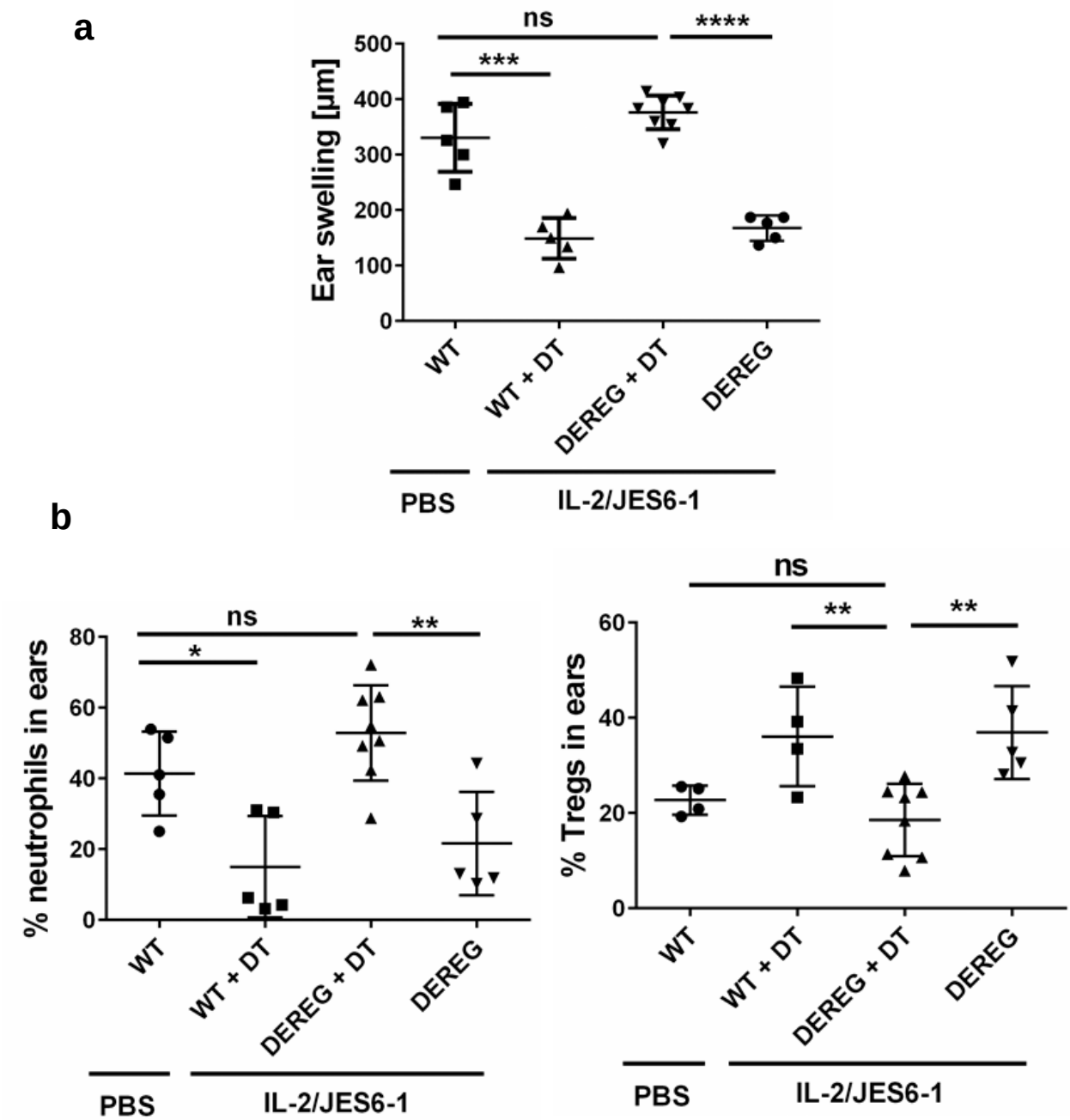

Figure 3.20: Treg depletion restores the CHS response in IL-2/JES6-1 treated mice. DEREG or WT mice were treated as described in Figure 3.19. (a) Mean of ear swelling 24 hours post-challenge. (b) Frequencies of neutrophils and Foxp3$^+$ Tregs in inflamed ear tissue on day 6. Data are depicted as mean ± SD (n= 5-8 mice per group) *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

3.6 IL-2/JES6-1 treatment affects CHS via multiple mechanisms

During the last decade, several studies have focused on the role of Tregs during CHS, as well as their localization in inflamed skin and LNs (91, 98, 136). However, the effect of Tregs on CD8 T effect cell priming remains ambiguous. To investigate if IL-2/JES6-1 can restrain the priming of effector cells in LNs during sensitization, and/or the effector functions after challenge, adoptive transfer of LN cells from sensitized mice was used as passive model for CHS. Donor mice were treated with IL-2/JES6-1 or PBS before sensitization to TNCB. Five days later, skin draining LN cells were isolated and subsequently injected intravenously into recipient mice. Before challenge, recipients were either treated with IL-2/JES6-1 or PBS. Ear swelling measurement revealed a reduction in the CHS response in mice that received LN cells from IL-2/JES6-1 treated donors. CHS was also suppressed in recipients treated with IL-2/JES6-1 before challenge (Figure 3.21). These results show that $Foxp3^+$ Treg expansion via IL-2/JES6-1 treatment can suppress CHS reactions through two mechanisms: Inhibition of effector cell priming during sensitization, and by suppressing the effector phase after challenge.

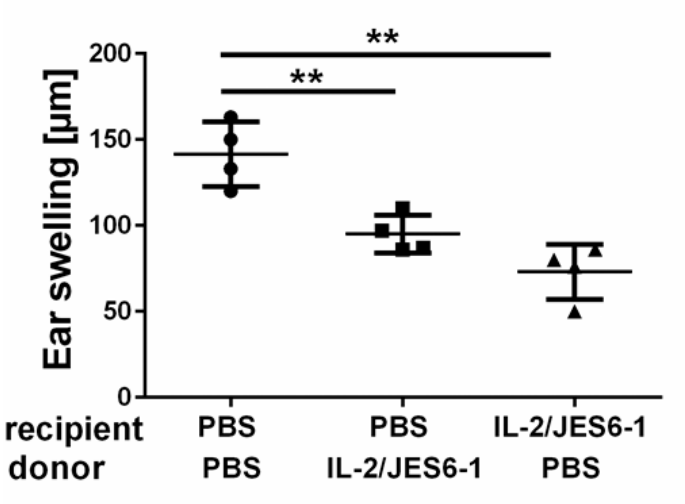

Figure 3.21: IL-2/JES6-1 treatment suppresses sensitization and challenge phases of CHS. Donor mice were treated with IL-2/JES6-1 or PBS and sensitized with TNCB. Five days later, inguinal and axillary LN cells were isolated, pooled and adoptively transferred into recipient mice which were challenged one hour after the adoptive transfer. The ear swelling 24 hours post challenge is relative to basal ear thickness before CHS induction and is presented as mean ± SD (n= 4 mice per group).

3.7 IL-2/JES6-1 expanded Tregs down-regulate CD86 expression by dendritic cells

DCs are responsible for antigen uptake, transport and subsequent presentation to T cells in LNs. In order to examine the effect of IL-2/JES6-1 on antigen transport by DCs during the sensitization phase, a different CHS model was induced using FITC as a contact sensitizer. Following skin sensitization, this fluorescent hapten allows its tracking in tissues and on individual antigen-presenting cells. Mice were treated with IL-2/JES6-1 or PBS on days -5, -4, and -3 and ear skin was sensitized with FITC on day 0. Auricular LNs were analyzed on days 1 and 2. Localization of hapten-bearing DCs in LNs was assessed by flow cytometry. Twenty four hours after sensitization, 8-10% of FITC-bearing DCs were observed in LNs. Cell frequencies of $FITC^+$ $CD11c^+$ DCs were not altered by IL-2/JES6-1 (Figure 3.22). Additionally, CD80 and CD86 expression by these cells was similar in IL-2/JES6-1 treated group and PBS controls (Figure 3.22). When analyzed at 48 hours post-sensitization, higher frequencies of $FITC^+$ $CD11c^+$ DCs were detected in LNs. Hence, suggesting that DC accumulation in LNs was not affected by the IL-2/JES6-1 treatment. However, CD86 expression by FITC-bearing DCs was reduced 48 hours post-sensitization in IL-2/JES6-1 treated mice compared to controls, while CD80 expression remained similar between the two groups (Figure 3.22). These data suggest that IL-2/JES6-1 treatment does not affect DC accumulation in draining LNs but modulates DC maturation which is important for T cell priming in LNs.

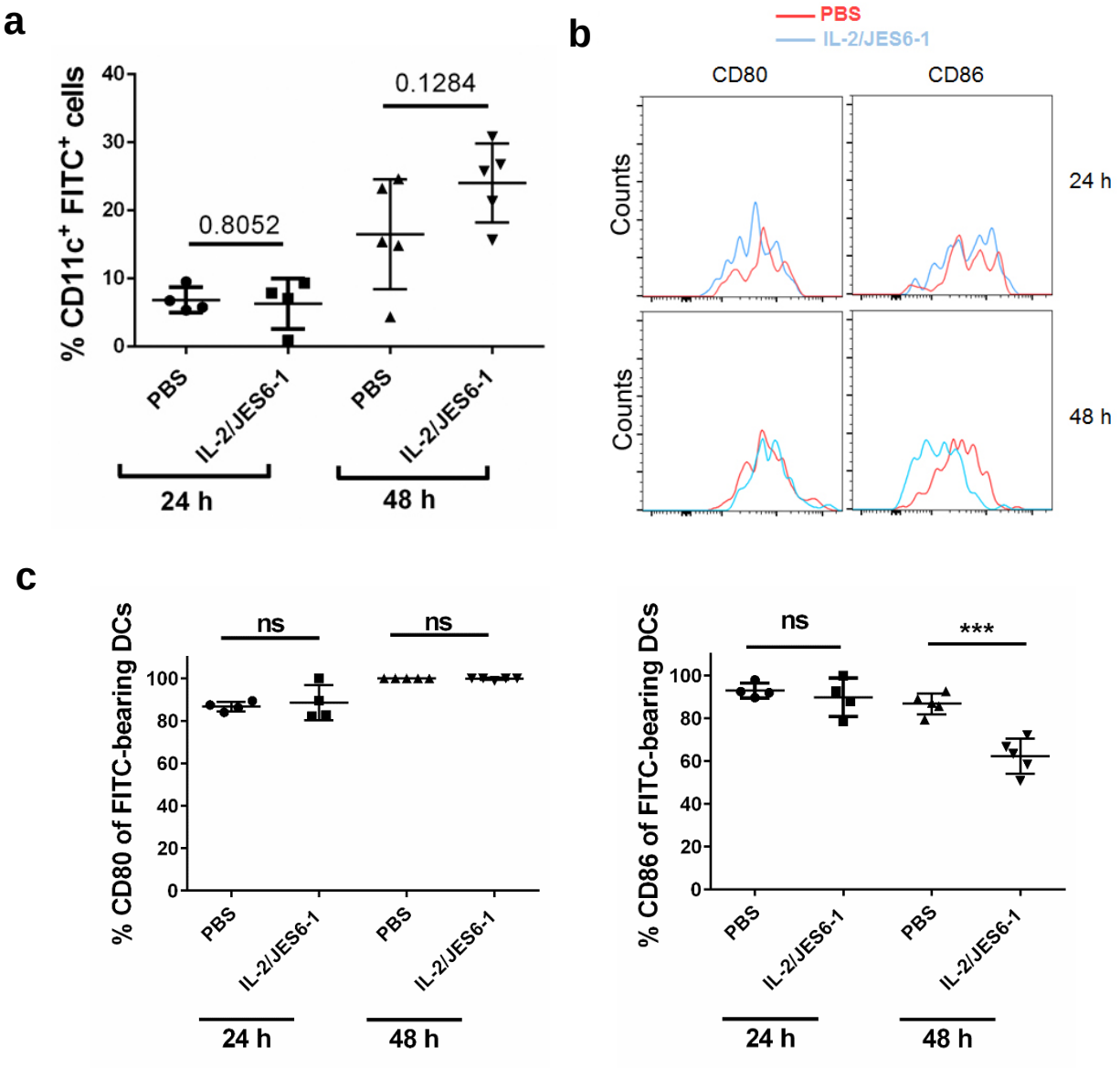

Figure 3.22: Down-regulation of CD86 by DCs in IL-2/JES6-1 treated mice. Mice were sensitized with FITC, and LNs were harvested 24 and 48 hours later. (a) Frequencies of FITC bearing DCs among MHCII^{hi} DCs in auricular LNs 24 and 48 hours after skin sensitization. Cells were gated on CD11c^{+} MHCII^{hi} DCs and FITC^{+} DC were analyzed. Data are presented as mean ± SD (b) Representative examples for CD80 and CD86 expression by FITC^{+} DCs at 24 and 48 hours post-sensitization. (c) Frequencies of CD80^{+} FITC-bearing DCs (left data plot) and CD86^{+} FITC-bearing DCs (right data plot) at 24 and 48 hours post-sensitization. Cells were gated on FITC^{+} CD11c^{+} MHCII^{hi} cells. Data are presented as mean ± SD.

3.8 CHS suppression by Foxp3$^+$ Treg expansion is long-lasting and not restricted to the originally inflamed tissue

To better characterize the Treg-mediated CHS suppression upon IL-2/JES6-1 treatment, different aspects were investigated: The duration of suppression upon repeated challenges, the limitation of this suppression to originally affected skin, and its antigen-specificity.

3.8.1 Long-term CHS suppression following IL-2/JES6-1 treatment

Allergic contact dermatitis is a skin disease that only occurs after repeated exposures to the same allergen. Using the IL-2/JES6-1 experimental therapy, it is necessary to test its effect on a second challenge applied long after the IL-2/JES6-1 injection. Therefore the duration of the immunosuppressive effect of IL-2/JES6-1 treatment was investigated. Mice were treated with IL-2/JES6-1 either before sensitization or before challenge, and CHS was induced. Control groups received 3 PBS injections on days -5, -4, and -3. When the ear swelling was resolved 12 days after the first challenge, the same ear received a second challenge (Figure 3.23). In control groups, the second challenge induced a more pronounced ear swelling than after the first challenge. Groups treated with IL-2/JES6-1 either before sensitization or before the first challenge showed impaired CHS compared with PBS treated controls for at least 5 days after the second challenge, i.e. 22 days after the last injection of IL-2/JES6-1 (Figure 3.23). Hence, the therapeutic effect of the IL 2/JES6-1 expanded Tregs is not limited to the acute treatment period, but lasts for weeks, at least.

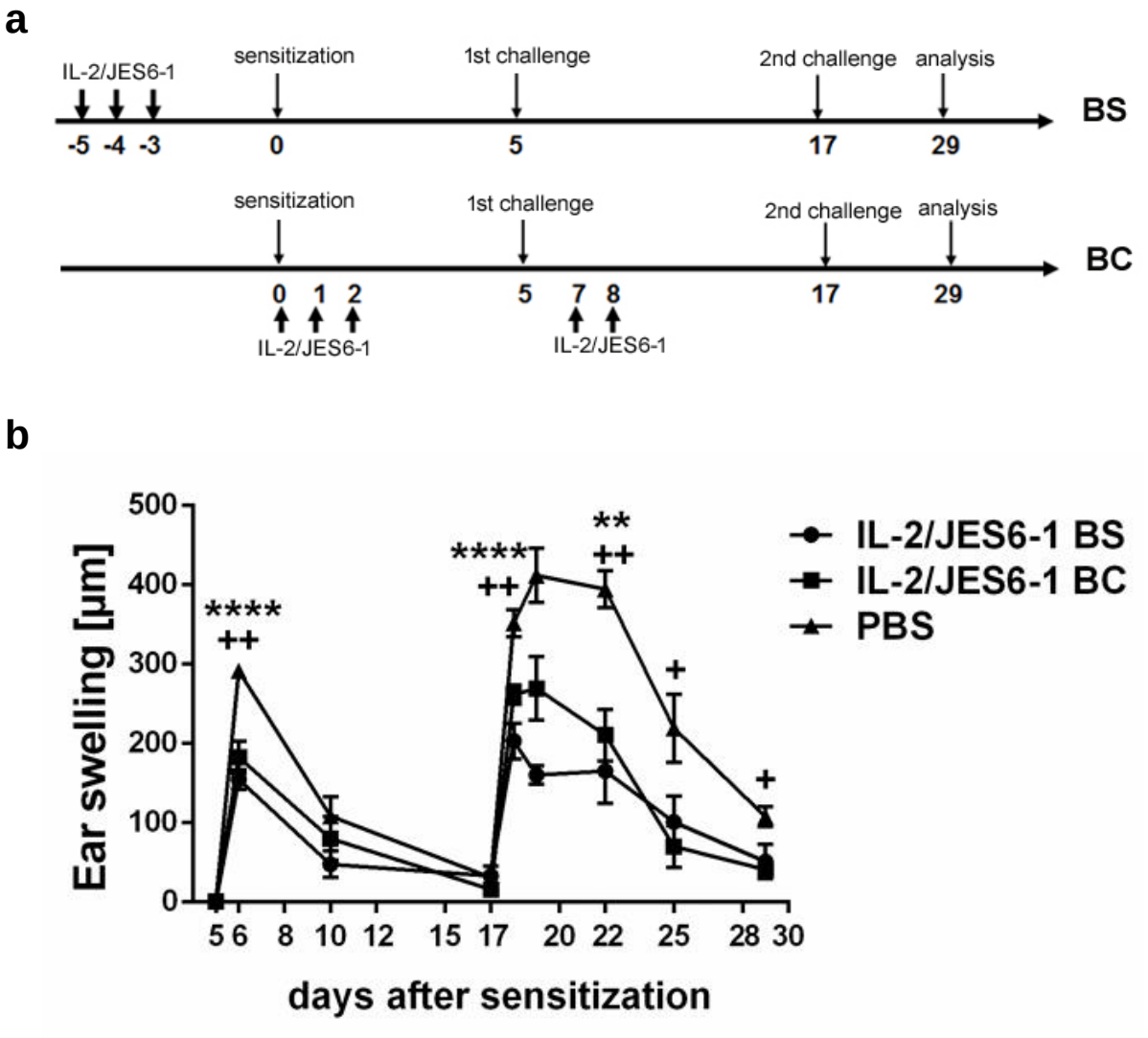

Figure 3.23: Treg expansion by IL-2/JES6-1 results in long-term CHS suppression. (a) Scheme of BS and BC treatment regiments for IL-2/JES6-1 injections and TNCB challenges. IL-2/JES6-1 was injected either before sensitization (BS) or before challenge (BC). PBS controls received three injection on days -5, -4, and -3. Both challenges were performed on the right ear. (b) Kinetics of ear swelling after first and second challenge of the same ear. Data are shown as mean ± SEM and representative of one out of two independent experiments (n=5). * reflects BS vs PBS and + reflects BC vs PBS. */+P < 0.05, **/++P < 0.01, and ****/++++ P < 0.0001.

3.8.2 CHS suppression by IL-2/JES6-1 is not limited to originally affected skin

In order to address whether the long-lasting effect observed in IL-2/JES6-1 treated mice following a second challenge is limited to the site of original inflammation, mice received the same treatment regimen shown in figure 3.23. However, the first challenge was performed on the right ear while the second challenge was applied on the left ear, which was not challenged before. As indicated by the time course of the ear swelling, IL-2/JES6-1 also suppressed the inflammation on the ear that had not previously yielded an inflammatory response (Figure 3.24). These results suggest that the suppressive effect of IL-2/JES6-1 is not only localized in the site of original inflammation, and that expanded Tregs by IL-2/JES6-1 might induce a systemic suppression of CHS.

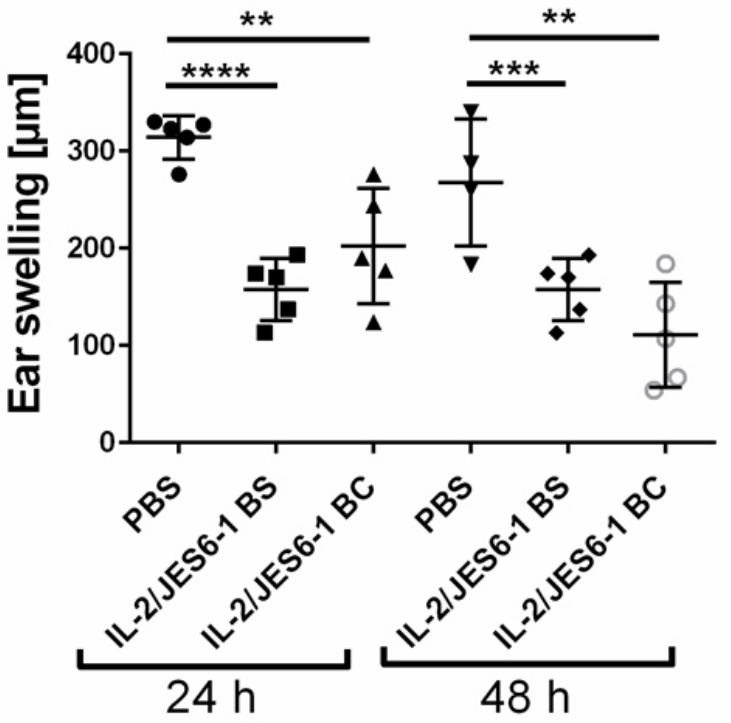

Figure 3.24: IL-2/JES6-1 mediates a systemic CHS suppression. Mice were treated using the treatment regimen shown in Figure 3.23, except that the second challenge was performed on the left ear. Data show the ear swelling at 24 and 48 hours after the second challenge. Data are shown as mean ± SD (n=5). ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

3.8.3 *In vivo* expanded Tregs control CHS in an antigen-specific manner

To determine if IL-2/JES6-1 treatment during sensitization to TNCB will also suppress CHS to a different allergen, on day -15 mice were first sensitized to oxazolone, and 15 days later to TNCB. On days -5, -4, and -3 i.e. when sensitization to oxazolone was already established, but before sensitization to TNCB, mice were treated with IL-2/JES6-1 (Figure 3.25). According to the results described above, this treatment regimen leads to maximum expansion of Treg populations at the time of the TNCB sensitization, which means that sensitization to oxazolone was established in the absence of expanded Tregs. Subsequently, the right ear was challenged with TNCB and the left with oxazolone. Once the ear swelling was resolved 12 days after these first challenges, each ear received a second challenge with the same antigen. Notably, CHS response to TNCB was suppressed after the second challenge, whereas there was no significant difference in the ear swelling in response to oxazolone between IL-2/JES6-1 treated mice and PBS controls (Figure 3.25). In conclusion, IL 2/JES6-1 induces a long-lasting effect that is not limited to previously inflamed tissues, but which is specific for allergens that induce an acute response at the time of the treatment.

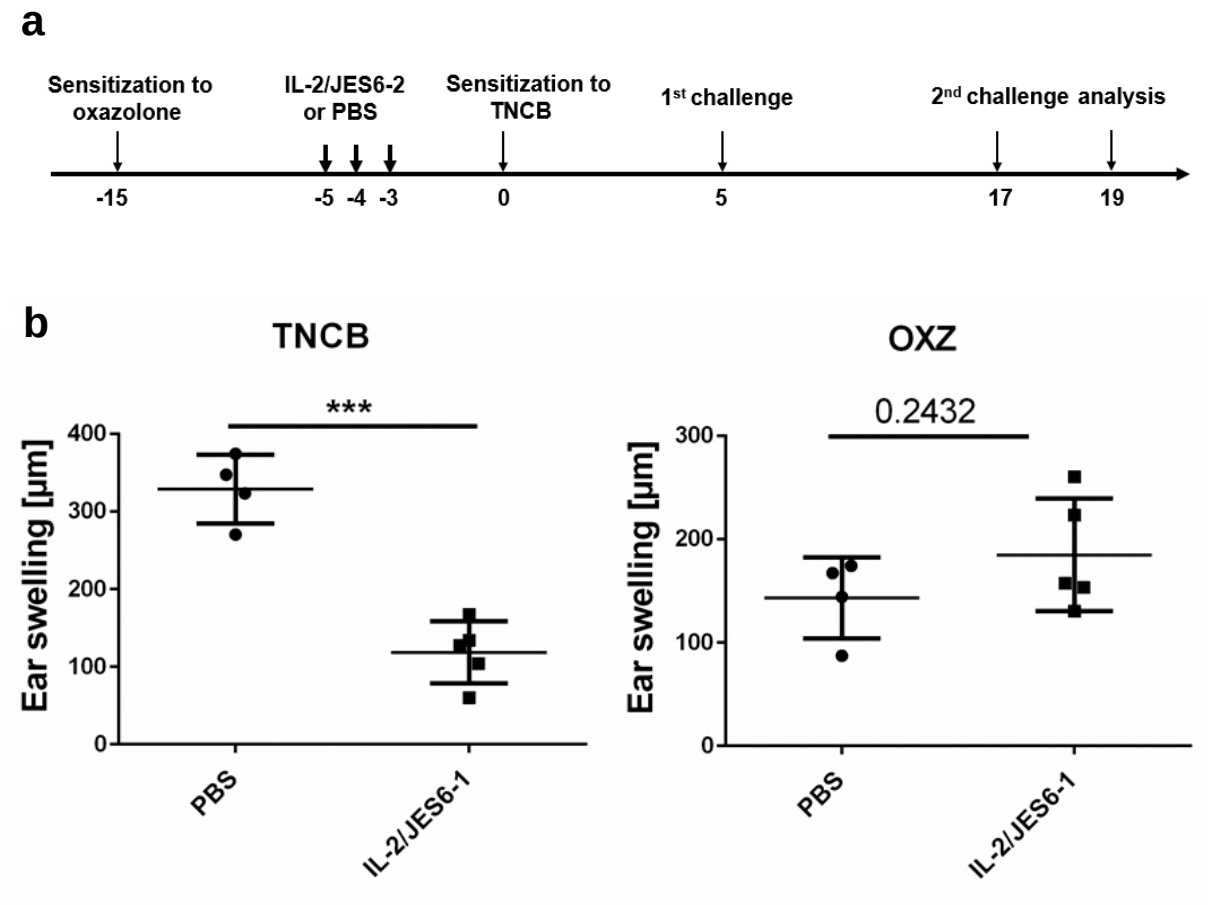

Figure 3.25: Treg expansion induces an antigen-specific suppression of CHS. Mice were sensitized to oxazolone, and IL-2/JES6-1 or PBS was injected 10, 11, and 12 days later. On day 0, mice were sensitized to TNCB. For the challenges on day 5, the right ear received TNCB and the left ear was painted with oxazolone. The same order was respected when applying the second challenge. (a) Scheme of treatments regimen used. (b) Ear swelling measured 48 hours after the second challenge. Data are displayed as mean ± SD, n=5 (two-tailed unpaired Student's t-test). *** $P < 0.001$.

3.9 *In vivo* Treg expansion results in prolonged reduction of hapten-specific IFN-γ$^+$ CD8 T cells in favor of increased populations of IL-10 and TGF-β producing T cells

To investigate the effects of IL-2/JES6-1 on effector cells driving CHS inflammation in skin draining LNs. Hapten-specific CD8 T cells and suppressor Tregs were assessed.

3.9.1 Decrease of hapten-specific CD8 T cells in IL-2/JES6-1 treated mice

IFN-γ producing CD8 T cells are considered to be the main effector cells in the CHS inflammatory response (64, 137), which makes the establishment of reliable and precise methods to detect hapten-reactive CD8 T cells very essential. A common method used for this purpose is the enzyme-linked immunospot (ELISPOT). This technique uses the functional properties of activated T cells to detect the presence and measure the frequency of antigen-specific T cells following *in vitro* restimulation with the antigen. The minimum duration of restimulation that is sufficient to allow detection of antigen-specific T cells is 24 hours. In the TNCB-induced CHS model, this short-term stimulation results in selective cytokine secretion by TNP-specific T cells which can be detected by ELISPOT. In order to allow further analysis of the hapten-specific T cells, this assay was modified by reducing the *in vitro* restimulation time to 5 hours and substitution of the ELISPOT assay by flow cytometric analysis of IFN-γ expression. Compared to controls, IL-2/JES6-1 treated groups showed a considerable reduction of hapten reactive IFN-γ$^+$ CD8 T cells (Figure 3.26). This reduction was still detectable on day 12 after the second challenge. In contrast, total IFN-γ$^+$ CD8 T cells were not altered in all groups, indicating that IL-2/JES6-1 treatment acts specifically on hapten-specific IFN-γ producing CD8 T cells without affecting total IFN-γ production in the system. As shown in DEREG mice, the IL-2/JES6-1 induced reduction of hapten-specific IFN-γ$^+$ CD8 T cells was dependent on Foxp3$^+$ Tregs (Figure 3.26). These data show that Treg expansion induced a persistent reduction in hapten-specific CD8 T cells.

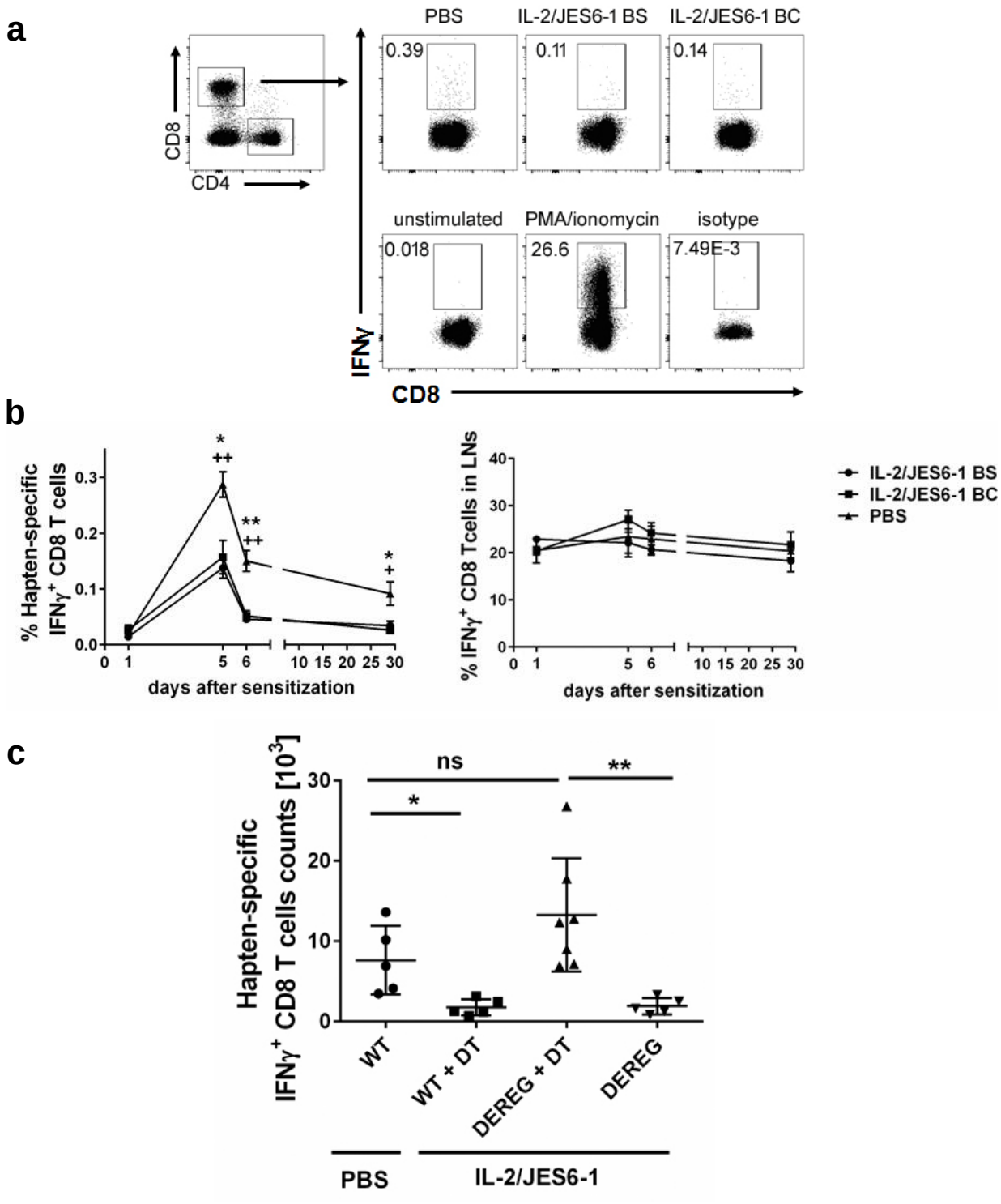

Figure 3.26: Foxp3$^+$ Tregs selectively reduce hapten-specific CD8 T cells. (a) Mice were treated as shown in figure 3.23, and IFN-γ expression was analyzed in hapten-specific CD8 T cells in pooled (auricular and axillary) LNs. Dot plots show the gating strategy used to identify hapten-specific IFN-γ^+ CD8 T cells after 5 hour re-stimulation with hapten-loaded APCs. Unstimulated controls consisted of LN cells from sensitized mice co-cultured with non-loaded splenic cells from

naïve mice. (b) Kinetics of the frequencies of hapten-specific IFN-γ^+ CD8 T cells (left panel) and total IFN-γ^+ CD8 T cells (right panel). (c) DEREG mice and their WT controls were treated with IL-2/JES6-1 or PBS, and CHS was induced as shown in figure 3.19. The graph shows cell numbers of hapten-specific IFN-γ+ CD8 T cells in LNs one day after challenge. Data are presented as mean ± SD n=5-8. * reflects BS vs PBS and + reflects BC vs PBS. */+P < 0.05 and **/++P < 0.01.

3.9.2 Increased Treg frequencies reappear in IL-2/JES6-1 treated mice long after treatment

IL-2/JES6-1 treatment induced a long-lasting suppression of CHS. It has been previously shown that IL-2/JES6-1 leads to a rapid but transient Treg expansion, which was also confirmed in the presented data. In order to test whether the long-term CHS suppression was associated with a second Treg increase, Treg populations were analyzed after repeated challenges late after the IL-2/JES6-1 treatment. Following a second allergic challenge but in the absence of new IL-2/JES6-1 injections, nTreg and pTreg frequencies were increased even 17 days after the first challenge, i.e. 32 days after the last IL-2/JES6-1 injection, and this increase was associated with higher frequencies of TGF-β^+ Foxp3$^+$ Tregs (Figure 3.27). Interestingly, this increase was only detected in skin draining LNs but not in spleen (Figure 3.27). In conclusion, while IL-2/JES6-1 treatment alone induces a transient increase in Treg populations, if combined with allergic challenges, it leads to a re-establishment of expanded Treg populations much later.

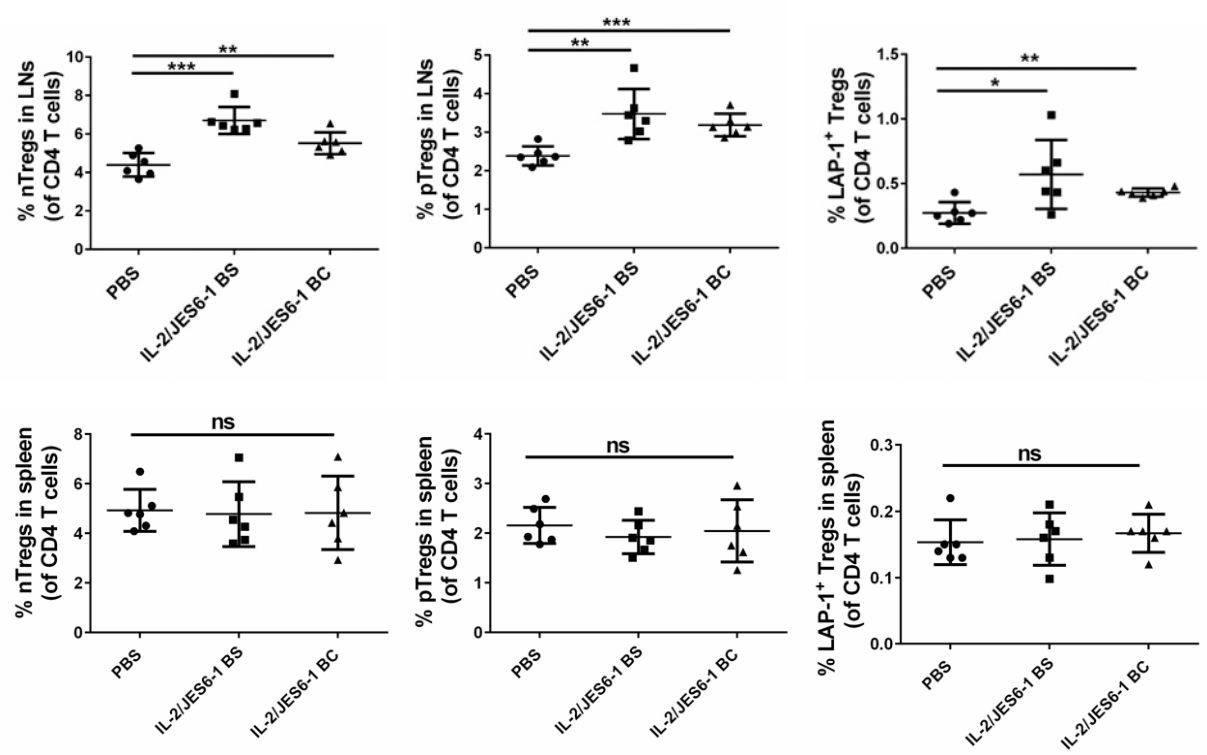

Figure 3.27: Increased Tregs were detected in IL-2/JES6-1 treated mice after a second challenge. Mice were treated as shown in figure 3.23, and Treg populations were analyzed 5 days after the second challenge. Graphs show frequencies of nTregs, pTregs and LAP-1$^+$ Tregs in LNs (upper row) and spleen (lower row). Results are shown as mean ± SD, n= 6 mice per group. (two-tailed unpaired Student's t-test).*P < 0.05, **P < 0.01, and ***P < 0.001.

3.10 Decrease in regulatory B cells in IL-2/JES6-1 treated mice in spleen

The role of B cells in CHS mechanism is controversial. CD19-deficient mice showed enhanced CHS response while human CD19 transgenic mice showed impaired CHS (99). In contrast, B cell knock out mice expressed lower levels of ear swelling compared with their WT controls (138). Additionally, previous studies have shown that Bregs can control the severity of CHS responses via IL-10 production (139). To determine whether IL-2/JES6-1 complexes have an effect on Breg cells, these cells were analyzed. Again, mice were treated with IL-2/JES6-1 or PBS either before sensitization or before challenge, and B cells were analyzed 24 hours after challenge. Numbers of total $CD19^+$ B cells were not altered in spleen upon IL-2/JES6-1 treatment. IL-10 expression by these cells was not significantly different from controls (Figure 3.28). Interestingly, IL-2/JES6-1 treatment seems to reduce Breg cell numbers and IL-10 expression by these cells, at least in spleen (Figure 3.28).

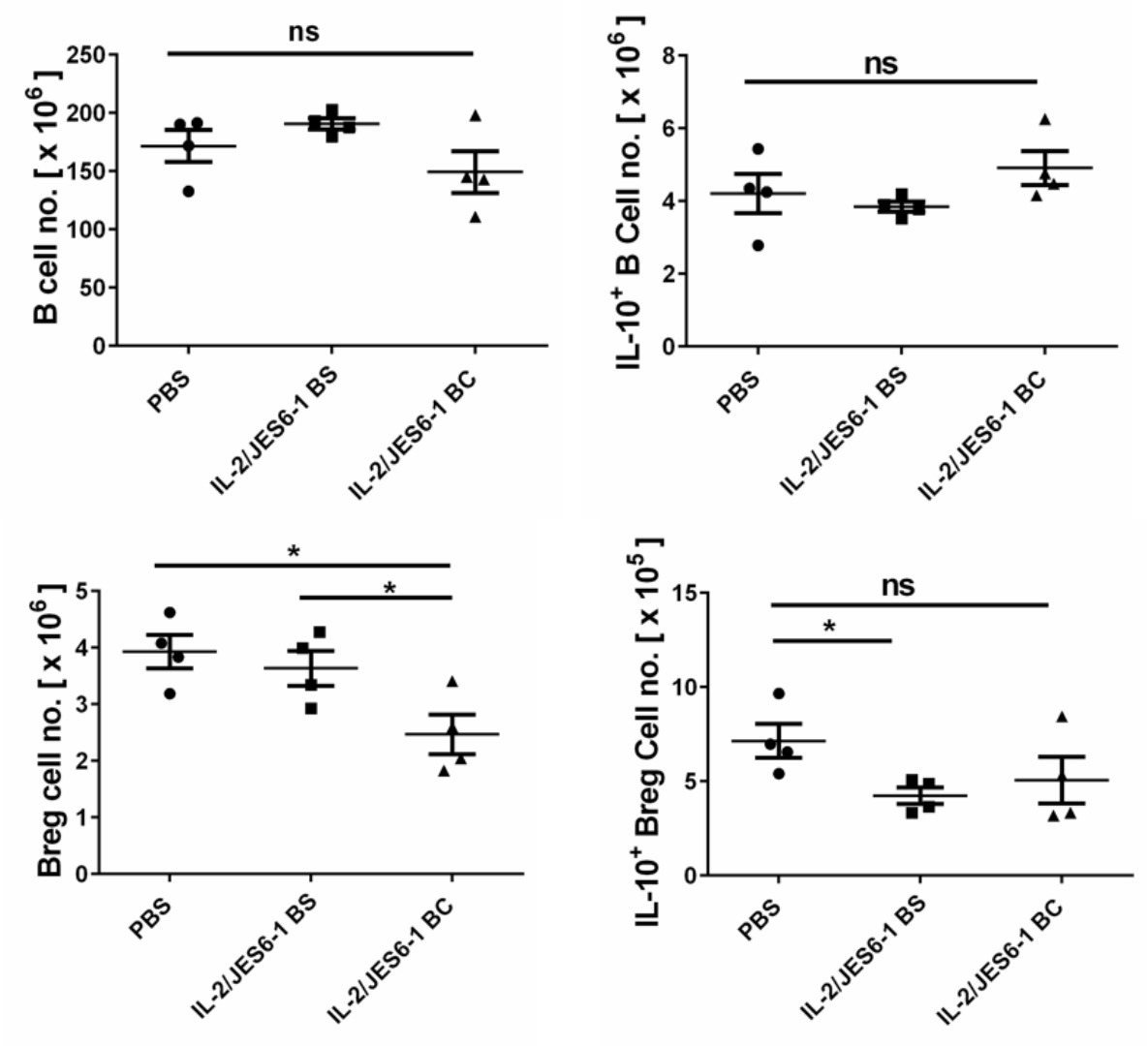

Figure 3.28: Reduced Breg numbers following IL-2/JES6-1 treatment. Mice were treated with IL-2/JES6-1 or PBS and CHS was induced. Breg cells were identified as $CD19^+$ $CD5^+$ $CD1d^{hi}$. Bars represent total $CD19^+$ B cell (upper row) and Breg (lower row) counts, and their IL-10 expression in spleen 24 hours post-challenge. Data are depicted as mean ± SD, n= 4 (two-tailed unpaired Student's t-test). *$P < 0.05$.

4 DISCUSSION

4.1 Overview

In this study, I have demonstrated that injection of IL-2/JES6-1 complexes led to the expansion of both nTregs and pTregs in a model of Tc1 mediated CHS. The experimental therapy using different treatment regimens suppressed CHS-mediated skin inflammation, led to reduced hapten-specific IFN-γ^+ CD8 T cell frequencies and numbers, and to a lymphocyte-mediated reduced neutrophil influx into the affected skin. IL-2/JES6-1 treatment showed long-lasting suppressive effects, i.e. it attenuated the inflammatory reaction triggered by repeated challenges.

4.2 Tregs are required for IL-2/JES6-1 suppressive effects on skin inflammation

Previously, IL-2/JES6-1 treatment was shown to induce Treg expansion and improvement of multiple diseases with different pathogenic profiles, e.g. Th2-driven allergic asthma (140). It also renders mice resistant to autoimmune encephalomyelitis (123). Likewise, IL-2/JES6-1 inhibited development and progression of atherosclerosis (141, 142). However, to the best of my knowledge, it had not been formally shown that Treg expansion is indeed the crucial mechanism by which IL-2/JES6-1 treatment suppresses these inflammatory conditions. In the present study, the effect of IL-2/JES6-1 treatment on Tc1-induced CHS in which CD8 T cells and neutrophils are crucial effector cells was tested. Various IL-2/JES6-1 treatment regimens showed reduced ear swelling, decreased CD8 T cell and neutrophil infiltration into the inflamed skin, and suppressed inflammatory cytokine production. Depletion of Foxp3$^+$ Tregs in DEREG mice demonstrated that Tregs are responsible for these suppressive effects. In other words, IL-2/JES6-1 mediated *in vivo* expansion of endogenous Tregs impaired CHS effector mechanisms and inflammation. Whether or not IL-2/JES6-1 treatment can affect inflammatory conditions also by other mechanisms remains to be elucidated.

4.3 IL-2/JES6-1 treatment before sensitization resulted in defective hapten-specific CD8 T cell priming

Hapten-specific CD8 T cell priming is the critical step in the establishment of the sensitization phase during CHS. For this purpose, migration of DCs from the skin to draining LNs is essential. Priming of hapten-specific CD8 T cells takes place in LNs five to seven days after sensitization, and is measured by their IFN-γ production upon hapten re-stimulation (143). Treg expansion before sensitization led to suppressed CHS and reduced ear swelling. In order to inhibit this phase, Tregs might act directly on T cells or indirectly via DCs. Data presented in this thesis showed that IL-2/JES6-1 treatment applied before sensitization reduced the frequencies and numbers of hapten-specific CD8 T cells in a Treg dependent manner. In addition, adoptive transfer of LN cells from sensitized mice that were previously treated with IL-2/JES6-1 resulted in impaired CHS. These results demonstrate that CD8 T cell priming was impaired in these mice. Moreover, Tregs can down-regulate the expression of CD80 and CD86 on DCs (144). These molecules are critical for antigen-presentation, co-stimulation, and proper activation of effector T cells. Previous studies have shown that CTLA-4 plays an important role in the suppressive functions of Tregs, and its blockade leads to an abrogation of Treg functions (131, 145). The data presented here demonstrate that CTLA-4^+ $Foxp3^+$ Treg cell numbers were higher in IL-2/JES6-1 treated mice, these cells also show an up-regulated CTLA-4 expression compared with PBS controls. Tregs can attenuate T cell priming through inhibition of proper DC-T cell interactions (146). CTLA-4 expressing Tregs can capture and degrade CD86 from APCs resulting in impaired co-stimulation via CD28 (147). In the present study, DCs downregulated CD86 expression after sensitization in IL-2/JES6-1 treated mice. These data indicate that IL-2/JES6-1 induced Treg populations impair T cell priming in LNs by restraining hapten presentation by DCs during sensitization.

4.4 Effector phase inhibition via IL-2/JES6-1 expanded Tregs

After challenge, CD8 T cells and neutrophils accumulate in the inflamed skin. Previous studies have shown that depletion of neutrophils reduces CD8 T cell accumulation in the skin and impairs CHS, suggesting that initial neutrophil recruitment is essential for subsequent T cell infiltration (67). The experiments in this thesis demonstrated CHS suppression in IL-2/JES6-1 treated mice before challenge, this effect was Treg-dependent. Furthermore, neutrophil and hapten-specific CD8 T cell frequencies were decreased following IL-2/JES6-1 treatment. Since Treg intervention is expected to take place only during challenge in IL-2/JES6-1 BC group, Tregs might suppress CHS response in these mice using a different mechanism as in IL-2/JES6-1 BS group. Adoptive transfer of LN cells from sensitized mice into IL-2/JES6-1 treated mice resulted in reduced ear swelling. These results lead to the hypothesis that Tregs might have affected CHS effector phase through two possibilities: suppressing effector functions of primed T cells, or inhibiting the recruitment of these cells to inflamed skin. Lymphocyte derived IL-10 can block neutrophil migration and distribution within tissues (148). Given that increased IL-10^+ CD4 T populations were detected following IL-2/JES6-1 treatment, it is possible that IL-10 could play a role in blocking neutrophil influx into skin tissue after this treatment. Previous studies have found that Tregs inhibit reactive oxygen intermediates and cytokine production by neutrophils. Moreover, Tregs can promote neutrophil apoptosis via IL-10 and TGF-β (149), leading to the hypothesis that reduced neutrophil frequencies observed in blood and skin tissue following IL-2/JES6-1 treatment might be the consequence of a direct effect of Tregs on neutrophil functions and survival. In the skin, Richards *et al.* have shown that the inhibition of neutrophil accumulation and survival by Tregs was associated with decreased expression of the neutrophil chemoattractants CXCL1 and CXCL2 (150), mainly produced by keratinocytes (151), indicating that Tregs may prevent neutrophil infiltration in the skin in IL-2/JES6-1 treated mice, which limits the amplification of inflammatory responses at this site and thus results in reduced ear swelling. Of note, IL-2/JES6-1 treatment did not affect neutrophil influx

in the absence of lymphocytes in SCID mice during non-specific croton oil mediated skin irritation. These data are in accordance with the idea that IL-2/JES6-1 does not directly act on neutrophils, but mediates its suppressive effects on these cells through induction of Tregs. Hence, it is possible that Tregs control neutrophil accumulation within the inflamed skin, which subsequently affect T cell infiltration. On the other hand, it has been shown that Tregs are able to directly suppress CD8 T effector cells via T cell-T cell interaction (152). Therefore, a direct inhibition of CD8 T effector cell functions via T-T cell contact by Tregs cannot be excluded. In accordance with these hypotheses, increased Treg frequencies were detected in IL-2/JES6-1 treated mice not only in LNs but also in inflamed ears. Conversely, CD8 T cells were reduced in ears after IL-2/JES6-1 treatment. Upregulation of CXCL10 by keratinocytes leads to the recruitment of CD8 T cells into the inflamed skin in a CXCL10-CXCR3-dependent manner (95). As Treg intervention was reported to decrease the expression of CXCL10 and CXCR3 by keratinocytes and CD8 T cells, respectively (153), reduction of CD8 T cell infiltration into the skin after challenge in IL-2/JES6-1 treated mice might be due to the Treg inhibition of CXCL10 expression by keratinocytes and/or CXCR3 expression by CD8 T cells. In conclusion, Treg expansion may either suppress effector cell functions in the skin or inhibit their recruitment to the challenge site. These suppressive effects might be affecting hapten-specific CD8 T cells directly or indirectly by controlling neutrophil accumulation in the skin.

4.5 IL-2/JES6-1 complexes affect T cell memory and induce long-term CHS suppression

In vivo expansion of Tregs by injection of IL-2/JES6-1 complexes either before sensitization or before challenge resulted in CHS suppression. Ear swelling was still reduced even upon a second challenge in IL-2/JES6-1 treated mice. This long-lasting suppressive effect was still observed when the second challenge was applied at a previously unchallenged ear, implying that the beneficial effect of IL-2/JES6-1 treatment is not restricted to the site of original inflammation. Additionally, IL-2/JES6-1 seemed specific to the hapten administered by the time of the IL-2/JES6-1 treatment.

The results presented in this thesis indicate that IL-2/JES6-1 treatment similarly expands nTreg and pTreg populations. It has been previously shown that injections of IL-2/JES6-1 in the presence of antigen lead to the migration of antigen-specific Tregs to the site of antigen localization (154). Moreover, a recent study reported that IL-2 is required to generate a population of induced "memory Tregs" upon immunization which are maintained for more than 40 days (155). Hence, this opens the possibility that IL-2/JES6-1 treatment could induce the formation of "memory Tregs". Accordingly, in groups treated with IL-2/JES6-1 before or after sensitization, increased Treg frequencies were observed again after a second challenge in the absence of repeated IL-2/JES6-1 treatment. However, whether IL-2/JES6-1 treatment induces "memory Tregs" or not remains to be elucidated.

The fact that the long-lasting suppression of CHS responses wasn´t restricted to the site of original inflammation suggests that the effects Tregs have on CHS pathogenesis is systemic. This might be related to the systemic expansion seen of Treg populations upon IL-2/JES6-1 treatment, implicating that skin resident Tregs may not be the main cells that are responsible for these suppressive effect. The increase of Tregs in IL-2/JES6-1 treated mice after a second challenge was only detected in skin draining LNs and not in spleen, which strengthen the

hypothesis of a possible generation of antigen-specific Tregs that were activated by the second challenge. Such advantage can be relevant for a potential use of IL-2/mAbs complexes as a therapy for the human allergic contact dermatitis, which can be chronic for patients having a daily contact with chemical sensitizers because of their occupation.

An alternative explanation for the long-lasting effect of IL-2/JES6-1 treatment could be the impairment of hapten-specific memory CD8 T cells. Recent findings have demonstrated that Tregs can mediate the quiescence of memory CD8 T cells through a CTLA-4 dependent mechanism (156). The experiments in this thesis showed an impaired hapten-specific CD8 T cell priming in IL-2/JES6-1 treated mice. Moreover, CHS suppression by IL-2/JES6-1 was specific to the antigen that was introduced during the treatment. This latter suppression was also persistent after a second challenge application, supporting the idea that restraining a proper CD8 T cell priming can blunt the recall response. It is possible that such Treg mediated control of CD8 T cell memory reactivation also contributes to the long-lasting and antigen-specific suppression of CHS.

4.6 Relevance in allergic contact dermatitis

Treg induction by treatment with low levels of IL-2 showed its therapeutic potential for the treatment of several inflammatory diseases in clinical trials (113, 157). However, IL-2 potentially stimulates both Tregs and effector T cells, bearing the risk of unwanted amplification of pathogenic T cell populations, while IL-2/JES6-1 treatment is considered to mediate selective stimulation of Tregs. The data presented here demonstrate that induction of the IL-2 signaling via IL-2/JES6-1 treatment results in Treg expansion and long-lasting suppression of inflammation in a murine model of CHS. Therefore, it would be interesting to study the therapeutic potential of an equivalent human IL-2/anti-IL-2 complex comprising similar properties as the murine IL-2/JES6-1 and compare its efficiency and safety with that of low level IL-2 therapy. The findings described in this thesis suggest that triggering the IL-2

pathway may also reduce inflammation in patients with allergic contact dermatitis, highlighting IL-2 as an intriguing therapeutic option for this disease.

5 OUTLOOK

The results generated in this thesis determine the effect of IL-2/JES6-1 complexes on nTreg and pTreg populations, and show the suppressive effects these complexes have on sensitization and effector phases of CHS. The impact IL-2/JES6-1 has on neutrophils and hapten-specific CD8 T cells was analyzed. The duration of this suppressive effects was also investigated. Tregs were shown to be required for the IL-2/JES6-1 mediated CHS suppression, and their influence on DCs during sensitization and its consequences on T cell priming was examined. It would be interesting to show if the long-lasting effect is due to the formation of persisting allergen-specific Tregs. For this purpose, mice could be treated with IL-2/JES6-1 and CHS to allergen "A" should be induced. Later, long after treatment, Tregs would be isolated and transferred into mice sensitized with allergen "A" or a different and unrelated allergen. This experiment would determine if IL-2/JES6-1 affects CD8 T cell memory or induces allergen-specific Treg memory, which both could be responsible for long-term CHS suppression observed in my experiments.

It would also be interesting to investigate the effect of IL-2/JES6-1 on mast cells. These cells have been shown to control neutrophil recruitment to the skin after allergen-challenge. So it would be interesting to understand if the effect observed on neutrophils was actually mediated by mast cells. Flow cytometry analysis of mast cells in ear tissue could be performed to investigate whether IL-2/JES6-1 alters the frequency and the activation status of these cells. Further experiments can be done using c-kit deficient mice which lack mast cells.

Another interesting experiment would be to analyze the expression of chemokines such as CXCL1 and CXCL2 in the inflamed skin, with and without IL-2/JES6-1 treatment. These chemokines are important for neutrophil recruitment to the skin in CHS. This experiment could explain the impairment of CHS detected following adoptive transfer of LN cells from sensitized mice into IL-2/JES6-1 treated mice. Reduced local CXCL1 and CXCL2 expression could explain the reduced recruitment of transferred effector cells.

OUTLOOK

Finally, it would be interesting to find out which Treg subpopulation is responsible for these suppressive effects. Adoptive transfer of either nTregs or pTregs into Treg depleted mice (DEREG mice) can be performed to elucidate this question.

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ABBREVIATIONS

APC	Antigen presenting cell
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
BLIMP1	B lymphocyte-induced maturation protein 1
Bregs	Regulatory B cells
CHS	Contact hypersensitivity
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAMPs	Damage-associated molecular patterns
DBP	Dibutyl phthalate
DC	Dendritic cell
DEREG	Depletion of regulatory T cells
DT	Diphtheria toxin
DTR-eGFP	Diphtheria toxin receptor-enhanced green fluorescent protein
ELISPOT	Enzyme-linked immunospot
fasL	Fas ligand
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
Foxp3	forkhead box P3
H&E	Hematoxylin and Eosin
HA	Hyaluronic acid
i.p.	Intraperitoneally
ICAM-1	intercellular adhesion molecule 1
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
IL-2R	IL-2 receptor
JAK-STAT	Janus kinase-signal transducer and activator of transcription pathway
LAG-3	Lymphocyte activation gene 3
LAP	Latency-associate peptide
LN	Lymph nodes
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex

mAbs	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MAPKs	Mitogen-activated protein kinases
NF-κB	Nuclear factor-κB
NK cells	Natural killer cells
NLRs	NOD-like receptors
Nrp-1	Neuropilin-1
nTregs	Natural regulatory T cells
PAMPs	Pathogen-associated molecular patterns
PMA	Phorbol 12-myristate 13-acetate
pTregs	Peripheral regulatory T cells
ROS	Reactive oxygen species
TGF-β	Transforming growth factor β
TLRs	Toll-like receptors
TLSP	Thymic stromal lymphopoietin
TNBS	2,4,6-Trinitrobenzenesulfonic acid
TNCB	2,4,6-trinitrochlorobenzene
TNF	Tumor necrosis factor
Tregs	regulatory T cells
TSDR	Treg-specific demethylated region

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