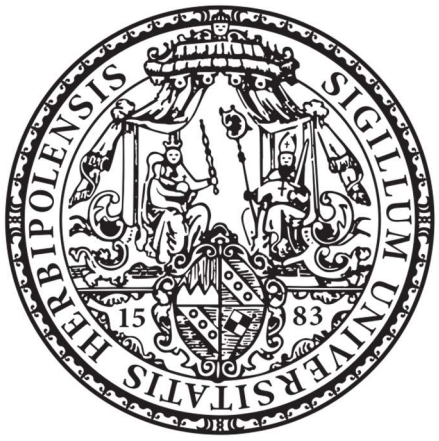

A novel mouse model for systemic cytokine release upon treatment with a superagonistic anti-CD28 antibody

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Ein neues Mausmodell zur Untersuchung der Zytokinfreisetzung nach Behandlung mit einem superagonistischen anti-CD28 Antikörper

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SUMMARY

The adaptive immune system is known to provide highly specific and effective immunity against a broad variety of pathogens due to different effector cells. The most prominent are $CD4^+$ T-cells which differentiate after activation into distinct subsets of effector and memory cells, amongst others T helper 1 (Th1) cells. We have recently shown that mouse as well as human Th1 cells depend on T cell receptor (TCR) signals concomitant with CD28 costimulation in order to secrete interferon γ (IFNγ) which is considered as their main effector function. Moreover, there is a class of anti-CD28 monoclonal antibodies that is able to induce T cell (re-)activation without concomitant TCR ligation. These so-called CD28-superagonists (CD28-SA) have been shown to preferentially activate and expand $CD4^+$ $Foxp3^+$ regulatory T (Treg) cells and thereby efficaciously conferring protection e.g. against autoimmune responses in rodents and non-human primates. Considering this beneficial effect, CD28-SA were thought to be of great impact for immunotherapeutic approaches and a humanized CD28-SA was subjected to clinical testing starting with a first-in-man trial in London in 2006. Unexpectedly, the volunteers experienced life-threatening side effects due to a cytokine release syndrome (CRS) that was unpredicted by the preclinical studies prior to the trial. Retrospectively, $CD4^+$ memory T cells within the tissues were identified as source of pro-inflammatory cytokines released upon CD28-SA administration. This was not predicted by the preclinical testing indicating a need for more reliable and predictive animal models. Whether mouse $CD4^+$ T cells are generally irresponsive to CD28-SA stimulation or rather the lack of a *bona fide* memory T cell compartment in cleanly housed specific-pathogen-free (SPF) mice is the reason why the rodent models failed to predict the risk for a CRS remained unclear. To provide SPF mice with a true pool of memory/effector T cells, we transferred *in vitro* differentiated TCR-transgenic OT-II Th1 cells into untreated recipient mice. Given that Treg cells suppress T cell activation after CD28-SA injection *in vivo*, recipients were either Treg-competent or Treg-deficient, wild type or DEREG mice, respectively. Subsequent CD28-SA administration resulted in induction of systemic pro-inflammatory cytokine release, dominated by IFNγ, that was observed to be much more pronounced and robust in Treg-deficient recipients. Employing a newly established *in vitro* system mirroring the *in vivo* responses to CD28-SA stimulation of Th1 cells revealed that antigen-presenting cells (APCs) amplify CD28-SA-induced IFNγ release by Th1 cells due to CD40/CD40L-interactions. Thus, these data are the first to show that mouse Th1 cells are indeed sensitive to CD28-SA stimulation *in vivo* and *in vitro* responding with strong IFNγ release accompanied by secretion of further pro-inflammatory cytokines, which is compatible with a CRS. In conclusion, this study will facilitate preclinical testing of immunomodulatory agents providing a mouse model constituting more “human-like” conditions allowing a higher degree of reliability and translationability.

ZUSAMMENFASSUNG

Das adaptive Immunsystem ermöglicht mittels hocheffektiver, antigen-spezifischer Mechanismen und unterschiedlicher Effektorzellen den Schutz vor einer nahezu unbegrenzten Vielfalt von Pathogenen. Die Hauptakteure stellen hierbei $CD4^+$ T-Zellen dar, welche nach Aktivierung distinkte Effektorpopulationen, unter anderem Th1 Zellen, bilden. Wir zeigten kürzlich, dass sowohl für Maus- als auch humane Th1-Zellen CD28-Kostimulation mit zeitgleicher T-Zellrezeptor (TZR)-Aktivierung essentiell für die Sekretion von Interferon γ (IFNγ), deren Haupteffektorfunktion, ist. Allerdings sind monoklonale anti-CD28 Antikörper bekannt, die auch ohne TZR-Signal T-Zellen aktivieren können. Diese sogenannten CD28 Superagonisten (CD28-SA) aktivieren und expandieren vorrangig $CD4^+$ $Foxp3^+$ regulatorische T-Zellen (Treg) und vermitteln wirksamen Schutz vor z.B. Autoimmunreaktionen in Nagern und Primaten. Um diesen erfolgversprechenden Effekt für immuntherapeutische Ansätze nutzen zu können, wurde 2006 in London eine erste klinische Erprobung eines humanisierten CD28-SA begonnen. Unerwarteterweise zeigten sich bei den Probanden lebensbedrohliche Nebenwirkungen, die Ausdruck eines Zytokin-Ausschüttungs-Syndroms (Cytokine Release Syndrome, CRS) waren, welches durch die vorangegangenen präklinischen Studien nicht vorhersagbar war. Rückblickend konnte die Sekretion pro-inflammatorischer Zytokine auf $CD4^+$ Gedächtnis-T-Zellen im Gewebe zurückgeführt werden, die so auf die Gabe des CD28-SA reagierten. Die unvorhersehbare Reaktion im Menschen zeigt deutlich, dass verlässlichere und prädiktivere Tiermodelle unverzichtbar sind. Ob Maus $CD4^+$-T-Zellen möglicherweise nicht durch CD28-SA stimulierbar sind oder dieser fehlgeleiteten Einschätzung über das mögliche Risiko eines CRS eher das Fehlen eines echten $CD4^+$ Gedächtnis-T-Zellen-Kompartiments in sauber gehaltenen spezifischen-Pathogen-freien (SPF) Mäusen zugrunde liegt, ist bisher ungeklärt. Um in SPF-Mäusen ein Gedächtnis-T-Zell-Kompartiment zu etablieren, wurden *in vitro*-differenzierte Th1 Zellen, die TZR-transgenen OT-II-Mäusen entstammen, in unbehandelte Empfängermäuse transferiert. Da bekannt ist, dass Treg-Zellen die Aktivierung von T-Zellen nach Anwendung von CD28-SA *in vivo* supprimieren, wurden Treg-kompetente (wildtypische) oder -defiziente (DEREG) Empfänger verwendet. Die anschließend erfolgte Injektion von CD28-SA löste die systemische Sekretion pro-inflammatorischer Zytokine aus, wobei eine stark erhöhter IFNγ-Konzentration im Serum zu beobachten war, welche deutlich ausgeprägter und robuster bei den Treg-defizienten Empfängern ausfiel. Ein neu etabliertes *in vitro*-System, welches die *in vivo* Antwort der Th1-Zellen auf CD28-SA-Stimulation widerspiegelt, identifizierte Antigen-präsentierende Zellen (APZs) als essentiellen Faktor für die erhöhte IFNγ-Sekretion der Th1-Zellen nach CD28-SA-Stimulation in Abhängigkeit von CD40/CD40L-Interaktionen. Zusammenfassend zeigt diese Thesis zum ersten Mal, dass Maus Th1 Zellen sowohl *in vivo* als auch *in vitro* durch CD28-SA

stimulierbar sind, wodurch eine starke IFNγ-Sekretion induziert wird, die von der gesteigerten Ausschüttung anderer pro-inflammatorischer Zytokine begleitet wird und in Abwesenheit von Treg einem CRS gleicht. Folglich kann diese Erkenntnis die präklinische Forschung bei der Erprobung neuer immuntherapeutischer Ansätze durch ein neues Mausmodell voranbringen, das dem menschlichen erfahreneren Immunsystem mehr als bisherige Modelle entspricht und somit verlässlichere Vorhersagen erlaubt und eine verbesserte Übertragbarkeit von Maus zu Mensch ermöglicht.

1 INTRODUCTION

The mammalian immune system has evolved throughout thousands of centuries providing an effective protection of the organism against invading pathogens*. Already lowermost protozoa and invertebrates exhibit protective systems functionally akin to cellular immunity of the mammalian immune system[1]. Nevertheless, the latter is highly complex, consists of various layers including numerous cell types as wells as humoral factors and involves different organs. These are organized in two branches, the innate and the adaptive immune system, that together are capable of mounting responses against all kinds of pathogens ranging from viruses and bacteria to parasitic worms and fungi. In order to provide efficacious protection one basic requirement is to distinguish between self and non-self allowing to maintain tolerance. Moreover, its function comprises the elimination of dead and apoptotic cells which is equally important for the maintenance of a healthy organism.

1.1 INNATE IMMUNITY

The innate immune system provides the first line of defense which is characterized by an immediate reaction. Several components and different mechanisms enable this hereditary system to fulfill its function. While chemical and physical barriers like epithelial tissues defend the organism mechanically against the invasion of pathogens cooperative interaction of humoral factors as well as a variety of cells combat already incorporated pathogens. The myeloid lineage-derived phagocytes like macrophages and neutrophils mediate protection via engulfment and digestion of microbes whereas eosinophils and basophiles, as well as mast cells release soluble factors. Natural Killer (NK) cells are of lymphoid origin and are capable of killing pathogens or infected cells. The innate immune cells sense invading pathogens via their pattern recognition receptors (PRRs) that are present within or on the surface of the cells. These receptors recognize pathogen-associated molecular patterns (PAMPs) like bacterial lipopolysaccharides, peptidoglycans or unmethylated CpG DNA which are common to many pathogens. These structures are conserved and, therefore, constitute an excellent target for detection of those pathogens. However, at the same time the dependence on these patterns limits the variability of the innate immune response. Engagement of these receptors induces the release of humoral mediators such as cytokines and chemokines that can activate cells of the adaptive immunity. Moreover, dendritic cells are antigen-presenting cells cooperating with the adaptive immune system as their key

* if not otherwise referenced, basics of immunology are summarized in
(1) *Murphy, K. & Weaver, C. Janeway's Immunobiology, 9th Edition. America (2017)* and
(2) *Abbas, A. K., Lichtman, A., & Pillai, S. Cellular and Molecular Immunology. (2014).*

function is the presentation of pathogen-derived peptide-antigens in the context of major histocompatibility complex (MHC) class I and class II to T-lymphocytes that form part of adaptive immunity. Furthermore, the innate immune system also comprises the complement system which forms another bridge to adaptive immunity. Finally, these properties facilitate an immediate immune response within minutes but lack the capacity to adapt.

1.2 ADAPTIVE IMMUNITY

The adaptive immune system is characterized by a highly specific response targeted against a large variety of antigens that is – compared to the innate response – delayed due to antigen-induced initiation but generates a long-term memory. The cellular component of the adaptive immunity is based on B and T lymphocytes bearing antigen receptors that are randomly assembled by somatic rearrangement of receptor-encoding gene segments providing a huge and highly diverse repertoire conferring protection against a large variety of pathogens. These antigen receptors are recombined during maturation that takes place in the bone-marrow in case of B cells while T cells mature in the thymus. Here, selection of recombined antigen receptors is a prerequisite as both successful defense against invading pathogens as well as maintenance and induction of tolerance are major task of the adaptive immune system. Thymocytes expressing successfully rearranged antigen receptors are positively selected while a non-functional antigen receptor causes death by neglect. Additionally, lymphocytes bearing autoreactive antigen receptors are negatively selected during maturation and die by apoptosis.

1.3 SECONDARY LYMPHOID ORGANS

Mature lymphocytes migrate into the secondary lymphoid organs, mainly spleen and lymph nodes where they encounter their cognate antigen. Upon activation through antigen recognition, they clonally expand in order to perform their effector functions. B cells activated by antigen can differentiate into antibody-producing plasma cells mediating the humoral component of adaptive immunity. Antibodies consist of two distinct regions: the constant region, including the Fc portion, is recognized by effector cells and determines its function while the variable region binds the antigen. Activated $CD8^+$ T cells differentiate into cytotoxic T lymphocytes (CTLs) being able to destroy infected cells while $CD4^+$ T cells become helper T cells which promote the immune response by activation of further immune cells, e.g. helping B cells to produce antibodies. A close interplay of B and T cells is key to a successfully mounted adaptive immune response.

1.4 T CELL ACTIVATION

T cell activation occurs in secondary lymphoid organs, i.e. primarily lymph nodes and spleen, where mature naïve circulating T cells encounter antigen-bearing dendritic cells. Before, the latter have internalized microbes or pathogens and processed their proteins to peptide-antigens which they export to the cell surface bound to MHC molecules. While peptides of engulfed pathogens or proteins are presented in the context of MHC class II which is solely expressed on professional antigen-presenting cells (APCs) like DCs or B cells, MHC class I is expressed on every nucleated cell within an organism and presents intracellular antigens, e.g. from a virus-infected cell. For activation through its cognate antigen, the T cell receptor (TCR) is depended upon a co-receptor to recognize the peptide-MHC-complex: the co-receptor CD4 pairs with MHC II whereas MHC I is bound by CD8 while both are expressed on helper T cells or CTLs, respectively. These co-receptors activate the intracellular kinase Lck which is essential for the initiation of the signal cascade upon activation through antigen-recognition. Once the co-receptor-associated kinase Lck is recruited it will phosphorylate further signaling molecules that finally lead to activation and nuclear translocation of transcription factors. These in turn induce transcription of the cytokine IL-2 that promotes proliferation and differentiation of the activated lymphocytes as well as enhanced survival. However, the TCR signaling itself is not sufficient to activate naïve T cells.

1.4.1 CD28 COSTIMULATION

In order to successfully activate a naïve T cell, engagement of a costimulatory receptor is required whose ligands are expressed on APCs. These costimulatory receptor signals are integrated into TCR signaling that is thereby amplified. Several costimulatory molecules have been identified among which the homodimeric glycoprotein CD28 is best understood and of major importance for activation of naïve T cells[2]. It is constitutively expressed on all rodent T cells and the vast majority of human $CD4^+$ T cells[3]. CD28 belongs to the Immunoglobulin (Ig) superfamily like inducible costimulator (ICOS), while other costimulatory receptors (4-1BB, CD27 or Ox40) are members of the TNF receptor superfamily. The ligands for CD28 on APCs are CD80 and CD86, also known as B7 molecules, whose expression is upregulated upon activation of the APC. According to the "Two signal hypothesis"[4], both signals are required to successfully activate a naïve T cell resulting in proliferation and differentiation while the mere TCR signaling in the absence of the costimulatory signal renders naïve T cells anergic, a state of growth arrest and inhibition of effector functions[5–7]. This mechanism supports the maintenance of tolerance by preventing activation of self-reactive T cells[8]. Although the original model was developed further throughout the last decades[9], it still remains controversial whether CD28 costimulation integrates into

TCR signaling in a quantitative or qualitative manner[10,11]. Importantly, CD28 costimulation enhances IL-2 production and induces the expression of the α subunit of the IL-2 receptor, CD25, thereby promoting T cell growth and differentiation. During the last decade, the hypothesis has been complemented and adapted including cytokines as a third signal[12] (Figure 1). The surrounding cytokine milieu mainly determines the differentiation of the activated T cells into a certain effector population. Besides cytokines, also antigen dose and type of APC as well as the kind of pathogen to combat determines the differentiation of a T cell upon priming[13].

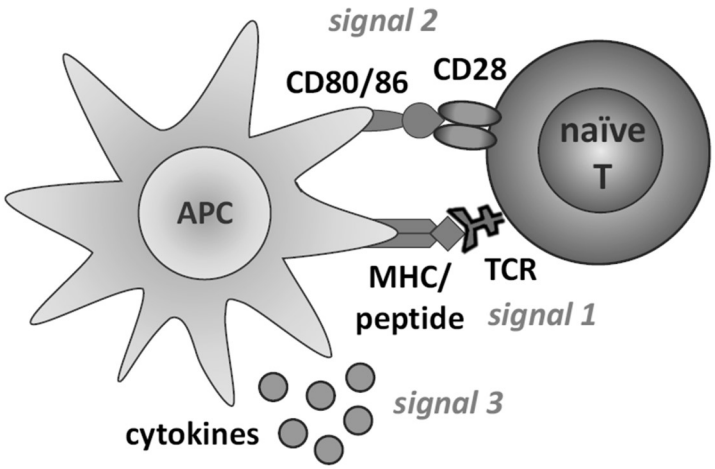


Figure 1: Three signal model of T cell activation

Successful activation of naïve T cells requires a series of signals: The first is given to the TCR by recognition of its cognate antigen loaded onto an MHC-complex. As a second signal the costimulatory receptor CD28 is engaged by ligation of CD80/86. Cytokines (signal 3) drive the differentiation of $CD4^+$ T cells into different T helper cell subsets.

However, the CD28 ligands CD80 and CD86 cannot only co-stimulate T cells resulting in activation but also inhibit T cells through the ligation of the inhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA-4, CD152) expressed on the same T cell or regulatory T (Treg) cells. CTLA-4 is upregulated on activated T cells[14] and constitutively expressed by Treg cells[15] and binds CD80/86 with much higher affinity than CD28[16]. Additionally, CTLA-4 binds its ligand bivalently resulting in even higher avidity thereby outcompeting the costimulatory receptor CD28, which is essential for contraction of immune responses, homeostasis and tolerance[17].

1.5 T CELL SUBSETS

Successful activation of naïve T cells results in their proliferation and differentiation into effector cells. Several distinct T cell subsets are known which are distinguished by their effector function and expression of cell surface markers. Subpopulations within $CD4^+$ T helper (Th) cells are characterized by their

cytokine as well as transcription factor profile and can be divided into Th1, Th2, Th17 and follicular Th cells[18–21].

1.5.1 TH1 CELLS

The vast majority of bacteria as well as certain viruses and protozoa induce differentiation of $CD4^+$ T cells into Th1 cells. This is due to the presence of IL-12 and Interferon (IFN) γ that are secreted by innate immune cells early during infection like NK cells, DCs and macrophages[19,22]. Consequently, these cytokines activate the Januskinase/Signal Transducer and Activator of Transcription proteins (JAK/STAT) intracellular signaling pathway, more precisely STAT1 and STAT4, which leads to expression of the transcription factor T-box containing protein expressed in T cells (T-bet)[23]. This transcription factor is characteristic for Th1 cells, increases the expression of the IL-12 receptor and initiates secretion of the pro-inflammatory IFNγ which is known as signature cytokine for Th1 cells. In turn, IFNγ enhances IL-12 secretion by macrophages[24] and – through an autocrine feedback loop – also induces further Th1 differentiation which results in an amplification of the immune response[25]. At the same time, differentiation into the Th2 subset is prevented allowing a target-directed effective immune response[26]. In addition to IFNγ, Th1 cells produce TNF and IL-2[27] that activate macrophages and mediate B cell help for antibody secretion supporting defense against intracellular microbes even more. Although CD28 co-stimulation is essential for activation of naïve T cells and subsequent finally differentiated T cells were for a long time believed to be less dependent on CD28 signals[28]. However, we have recently shown that for mouse as well as human Th1 cells CD28 costimulation is crucial in terms of effector functions and cytokine secretion[29].

1.5.2 REGULATORY T CELLS

Treg cells were discovered and characterized as a suppressive population of $CD4^+$ T cells expressing CD25, the α subunit of the IL-2 receptor[30]. The characteristic transcription factor forkhead box protein 3 (Foxp3) is crucial for their suppressive function maintaining tolerance and homeostasis[31,32] as shown by Foxp3-deficient or mutated mice[33,34] and humans[35] developing severe lethal autoimmunity. $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells perform their suppressive function via several distinct mechanisms[36–38]: they secrete the anti-inflammatory cytokines IL-10 and transforming growth factor β (TGF-β); they constantly express CD25 and rapidly consume IL-2 thereby competing with conventional T cells for this cytokine which is essential for their survival; they secrete Granzyme B and Perforin resulting in cytolysis

of effector Th cells; they constitutively express the inhibitory receptor CTLA-4 that binds the costimulatory ligands CD80/86 and reduces their availability on the surface of activated APCs by trans-endocytosis preventing CD28 costimulation of conventional T cells[39].

While conventional $CD4^+$ T cells recognize foreign antigens, Treg cells leaving the thymus are in their majority autoreactive[40]. Consequently, they constantly receive TCR signals which concomitantly with CD28 co-stimulation is of major importance for their survival and functionality under homeostatic conditions. In fact, this is demonstrated by a drastically reduced number of thymic as well as peripheral Treg cells and impaired effector functions in mice deficient for either CD28 or CD80/86[41–45]. Additionally, CD28 signaling is essential for upregulated Foxp3 expression during Treg cell development[46–48]. Furthermore, Foxp3 suppresses IL-2 production by Treg cells which renders them dependent upon paracrine IL-2 secreted by conventional T cells[49]. During an immune response, activated T cells secrete huge amounts of IL-2 and expand. Thereupon, Treg cells, activated by self-antigens, start to proliferate. While the expanded effector population of T cells fight against the invading pathogen, Treg cell numbers and suppressive activity increase until they outcompete and suppress conventional T cells efficiently. Finally, numbers of conventional T cells and IL-2 levels are decreased declining the immune response and returning to homeostasis[50].

1.6 CD28 SUPERAGONISTS (CD28-SA)

Physiological CD28 costimulation can also be mimicked by monoclonal antibodies (mAb). The first mAbs against human[51], mouse[52] and rat[53] CD28 were found to be inducing activation of T cells only in synergy with TCR signals. This class of αCD28 mAbs are called conventional. Later on, functionally different CD28 mAbs were discovered that were able to induce proliferation of resting T cells without the need for simultaneous TCR ligation and hence named superagonistic[54]. It was elucidated that unequal binding sites account for this functional difference between the distinct antibody classes. While conventional CD28 antibodies bind monovalently close to the B7 motifs, CD28 superagonists (CD28-SA) bind laterally[55] and are able to cross-link multiple homodimers[56] (Figure 2). This also holds true for the superagonistic α-mouse CD28 mAb D665 used in this thesis[57]. Mechanistically, a dependency on constitutive proximal, latent, so called “tonic”, TCR signaling was observed which is amplified downstream of the TCR by CD28-SA[58]. More precisely, initiation of CD28 signaling upon superagonistic stimulation is dependent on Lck and tonic TCR signals are amplified further downstream by integration within the SLP-76 signalosome[58].

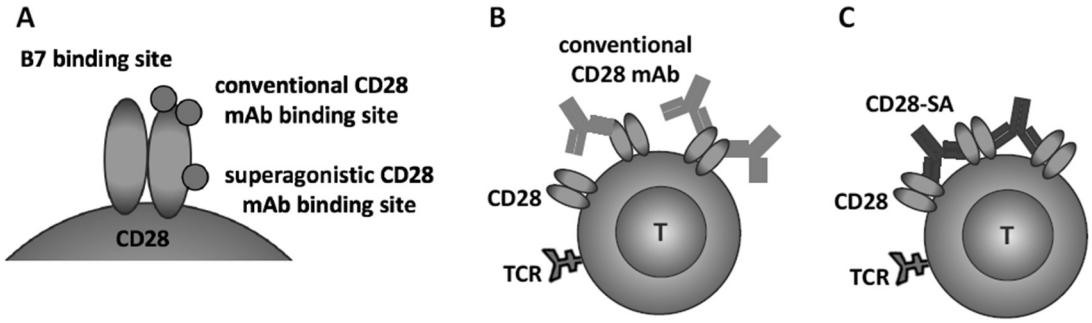


Figure 2: Epitope-function relationship of different αCD28 mAbs

(A) Conventional αCD28 mAbs bind their target close to the B7 motifs whereas superagonistic mAbs bind laterally. This results in **(B)** monovalent ligation of CD28 by conventional mAbs and **(C)** bivalent engagement by CD28-SA crosslinking multiple homodimers.

1.6.1 CD28-SA TREATMENT OF RODENTS

As Treg cells constantly receive tonic TCR signals through their autoreactive TCRs scanning peptide-loaded MHC molecules, superagonistic CD28 stimulation preferentially expands regulatory T cells over conventional T cells and the former are highly suppressive *in vitro*[54,59,60]. When the rat-specific CD28-SA JJ316 was tested *in vivo*, a dose-dependent and transient lymphocytosis, characterized by an increase in $CD4^+$ T cells, was observed[54], accompanied by an induction of the anti-inflammatory cytokine IL-10[61]. Subsequently, Treg cells were identified as the source of IL-10[60,62]. Moreover, Treg cell frequencies within the $CD4^+$ T cell compartment escalated from 5 % up to 20% three days post CD28-SA treatment while they declined to baseline seven days later[60,62]. These Treg cells exhibited a highly activated phenotype ($CD25^{high}$, CTLA-4^{high}) and were far more suppressive than their resting counterparts during *in vitro* suppression assays[60,62]. These results obtained in rats were confirmed in mice by similar effects upon administration of the mouse-specific CD28-SA D665[50,57]. Here, cytokine release was closely monitored revealing a slight increase of the anti-inflammatory cytokines IL-10 and IL-4 on day 3 after treatment while pro-inflammatory cytokine levels remained unchanged suggesting that the drastically increased number of Treg cells suppressed cytokine production by effector T cells efficiently[50]. Furthermore, CD28-SA-mediated Treg expansion, either preventive or therapeutic, proved to be protective in several rodent disease models of autoimmunity and graft rejection in rat and mice[60,63–67]. Adoptive transfer of *in vivo* CD28-SA-expanded Treg cells confirmed that the beneficial effects observed were mediated by the Treg cells themselves[60,68–70].

This preclinical data suggested a promising therapeutic potential for CD28-SA as a treatment for autoimmune or inflammatory diseases given the advantage of polyclonal Treg cell expansion regardless of TCR specificity which would allow application to a broad variety of autoimmune diseases.

1.6.2 CD28-SA TREATMENT OF HUMANS

In order to exploit the therapeutic potential of CD28-SA for treatment of human patients suffering from autoimmune or inflammatory diseases, a fully humanized CD28-SA was developed (TGN1412). This antibody was generated in mice being specific for human CD28 and was genetically engineered to be of the antibody subclass IgG_4 since this type of antibody is much less likely to recruit cytotoxic effector mechanism via their Fc portion[71,72]. It was intended to be a novel treatment option for rheumatoid arthritis and B cell chronic lymphocytic leukemia (B-CLL)[72]. For preclinical trials, non-human primates (cynomolgus macaques) were chosen as CD28 molecules exhibit 100 % sequence identity to the human counterpart resulting in comparable affinity of TGN1412 for CD28 of these animals. Furthermore, the binding of the IgG_4 antibody to various Fc receptors was found to be virtually identical between human and cynomolgus macaques. Subsequent administration of TGN1412 to the non-human primates was efficacious in expanding Treg cells while no adverse events were observed[72]. Even high doses (50 mg/kg body weight (BW)) were well tolerated. Additionally, treatment of human peripheral blood mononuclear cells (PBMCs) confirmed the beneficial effect of TGN1412 on Treg cell expansion[72].

Knowing this, a first in man clinical trial including six healthy male volunteers was started in 2006 in London[73]. As common for phase I clinical trials, a very low dose (0.1 mg/kg BW) of TGN1412 was first applied to evaluate toxicity. Surprisingly, this trial had to be stopped due to the unexpected and life-threatening side effects the volunteers experienced. Retrospectively, the observed cytokine release syndrome (CRS) was found to be induced by $CD4^+$ effector memory T cells (T_{EM}), which are numerously present in immunocompetent humans as a consequence of endured infections and vaccinations. Within an hour after antibody infusion, the volunteers' serum contained extremely high levels of pro-inflammatory cytokines like IFNγ and TNF[73]. Despite the in part severe adverse events all volunteers survived after being admitted to an intensive care unit and treated with methylprednisolone and an anti-IL-2-receptor antagonist.

Certainly, the cytokine release and the subsequent deterioration of the volunteers' health condition was not predicted by the preclinical data. In the meanwhile, several underlying reasons that were the basis of the unpredictability were identified[74]: Regarding the rodent data, the major difference between the two species seems to be the prevalence of T_{EM}. As mentioned above, they are prominent

in human tissues due to multiple infections and immunologic stimuli in the surroundings. In contrast, the prevalence of the murine counterparts is relatively low considering the clean housing facilities for specific pathogen-free (SPF) mice that are mostly used for immunological research. In these mice, CD28-SA-stimulated conventional T cell secrete IL-2 which is rapidly consumed by Treg cells that immediately quench proliferation and pro-inflammatory cytokine secretion by T_{EM} thereby preventing the establishment of a CRS[75]. Thus, in humans, Treg cells are simply overrun by T_{EM} which leads to the observed cytokine release and the according syndrome. Follow-up studies depleting Treg cells in mice prior to CD28-SA treatment demonstrated increased levels of pro-inflammatory cytokines (IL-2, IL-6, TNF), albeit lacking induction of IFNγ which is considered as a key factor of the CRS[69]. As mentioned earlier (1.5.1 and 1.5.2), conventional as well as Treg cells depend on engagement of their costimulatory receptor CD28 in order to perform their effector functions. Consequentially, CD28-SA activate both subsets of T cells. However, Treg cells are preferentially expanded over conventional T cells due to the fact that they constantly receive TCR signals through their autoreactive TCR while conventional T cells hardly receive any TCR signals as their TCRs are restricted to non-self peptides[58].

Human PBMC cultures in preclinical testing also did not predict the unintentional activation of conventional T cells and the resulting cytokine release[72]. Apparently, circulating T cells within PBMCs have lost their tonic TCR signal in the periphery due to less cell-cell interactions and consequently less scanning of MHC complexes as compared to their counterparts residing in tissues[76]. With hindsight, it was found that this unresponsiveness due to the lack of latent TCR signaling can be overcome by culturing the lymphocytes at high cell density restoring the T cell reactivity to soluble CD28-SA *in vitro*[77]. Furthermore, a dose-dependent activation of Treg cells over conventional T cells has been demonstrated[60,78,79]. Low-dose application of CD28-SA results in the appreciable effect of Treg cell expansion while preventing secretion of pro-inflammatory cytokines in mice and humans.

Regarding the dataset of non-human primates, the failure to predict CRS induction upon CD28-SA treatment is most likely due to a combination of a lack of CD28 expression by T_{EM} and an inherent unresponsiveness of central memory T cells (T_{CM}) in this respect which was unnoticed until then[74].

1.7 CLINICAL PERSPERCTIVES OF HUMAN CD28-SA TREATMENT

Once having gained knowledge about the reasons that led to the unpredictability of preclinical testing, new trials were started with the renamed CD28-SA TAB08/TGN1412. Taking into account the dose-dependent activation of Treg cells, doses of about a thousandth to a 15th of the initially applied amount

(0.1-7 µg/ kg BW) were administered in a phase 1a study which showed good tolerability in the absence of any adverse events while increased IL-10 levels were observed[79]. Thereupon, a phase 1b trial treating Rheumatoid Arthritis patients was initiated revealing the intended effect of CD28-SA mediating amelioration of disease symptoms[80]. Finally, a series of clinical trials aiming at therapeutic application of CD28-SA treatment for several diseases like Psoriasis, Systemic Lupus Erythematosus (SLE) as well as solid neoplasm has been started[81].

1.8 OBJECTIVE OF THE THESIS

Irrespective of the requirement for TCR and CD28 costimulation for activation of naïve T cells, fully differentiated T cells were thought to be less dependent on CD28 signals. However, we could recently show that mouse Th1 cells strongly rely on CD28 costimulation for efficient secretion of their key effector cytokine IFNγ. In humans, cytokine secretion by memory T cells *in vitro* is enhanced by CD28 costimulation. Furthermore, isolated CD28 stimulation by administration of saturating doses of the superagonistic anti-CD28 mAb TGN1412 to healthy volunteers resulted in an uncontrolled cytokine release culminating in a CRS causing severe complications during a clinical trial. In contrast, preclinical animal testing using rodents indicated efficacious protection from autoimmune and inflammatory diseases through expansion and activation of Treg cells irrespective of the applied dose leading to an underestimated risk of CRS induction in the human trial. Having shown that Th1 cells are not refractory to CD28 costimulation we addressed the question whether the failure of the used mouse models to predict the CRS in humans is explained by irresponsiveness of mouse effector/memory T cells to CD28-SA or rather by the lack of a *bona fide* memory T cell pool in cleanly housed mice. In order to provide SPF mice with an easily trackable effector/memory $CD4^+$ T cell compartment we adoptively transferred *in vitro* generated Th1 cells originating from TCR-transgenic mice carrying a congenic marker. Additionally, Treg cells were depleted as they are known to quickly suppress T cell activation upon CD28-SA treatment *in vivo*. This model provided a feasible method to test whether mouse Th1 cells are in principle capable of responding to isolated CD28 stimulation mediated by CD28-SA treatment *in vivo*. Furthermore, it allowed us to monitor serum cytokine concentrations as well as direct tracking and phenotypic characterization of the transferred Th1 cells. With this model, we intended to create an immunological setting mirroring the conditions in humans more closely and, thereby, providing an improved mouse model for evaluation of further immunotherapeutic agents.

2 MATERIALS

2.1 BIOCHEMICAL REAGENTS

Reagent	Manufacturer	Concentration
Brefeldin A (GolgiPlug)	Sigma (B7651-5MG)	10 µg/ml
Ionomycin	Sigma (I0634-1MG)	500 ng/ml
Mouse IL-12	R&D Systems	10 ng/ml
Normal mouse IgG (nmIgG)	Sigma Aldrich	20 µg/ml
Ovalbumin-peptide$_{327-339}$ (OVA)	Charité Berlin	1-2 µM
Penicillin	InfectoPharm Arzneimittel und Consilium GmbH	100 U/ml
Phorbol 12-myristate 13-acetate (PMA)	Sigma (16561-29-8)	5 ng/ml
Pro-leukin® (recombinant, human IL-2)	Novartis	0.1 µM
Streptomycin	AppliChem	100 µg/ml

2.2 CHEMICALS

Chemical	Manufacturer
2-mercaptoethanol	Invitrogen
Bovine serum albumin (BSA)	Biomol
Calcium dichloride ($CaCl_2$)	Merck
Disodium hydrogen phosphate (Na_2HPO_4)	Roth
Glucose	Roth
HEPES	Roth
L-Glutamine	Roth
Magnesium dichloride ($MgCl_2$)	Roth
Magnesium sulfate ($MgSO_4$)	Merck
Non-essential amino acids MEM	Gibco
Phenol red	Merck
Potassium chloride (KCl)	Roth
Potassium dihydrogen phosphate (KH_2PO_4)	Roth
Sodium azide (NaN_3)	Merck
Sodium chloride (NaCl)	Roth
Sodium hydroxide (NaOH)	Roth

2.3 BEADS, KITS AND DYES

Bead	Manufacturer	Dilution
CD90.1 Microbeads	Miltenyi Biotec (130-094-523)	1:10
CD90.2 Microbeads	Miltenyi Biotec (130-049-101)	1:10
Dynabeads® Pan Mouse IgG	Invitrogen (11041)	1:5
Streptavidin Microbeads	Miltenyi Biotec (130-048-101)	1:20

Kit	Manufacturer
Legendplex™ Multi-Analyte Flow Assay Kit Mouse Th Cytokine Mix and Match Subpanel	BioLegend
Foxp3 transcription factor staining Kit	eBioscience/Invitrogen (00-5523-00)

Dye	Manufacturer	Dilution
Fixable Viability Dye eFluor 780	eBioscience (65-0865-18)	1:750
Trypan Blue	AppliChem GmbH	0.04 %

2.4 ANTIBODIES

Target	Conjugate	Clone	Isotype	Manufacturer	Dilution
CD11b	Biotin	M1/70	Rat IgG_{2b}, κ	BD	1:1000
CD11b	PE	M1/70	Rat IgG_{2b}, κ	BD	1:100
CD25	Biotin	7D4	Rat IgM, κ	BD	1:100
CD28	FITC	E18	Mouse IgG_{2b}, κ	BioLegend	1:100
CD28-SA	unconjugated	D665	Mouse IgG_1, κ	ExBio	150 µg/mouse 0.01-100 µg/ml
CD3ε	FITC	145-2C11	Armenian Hamster IgG_1, κ	BioLegend	1:100
CD4	Pacific Blue	RM4-5	Rat IgG_{2a}, κ	BioLegend	1:300
CD40L	unconjugated	MR1	Armenian Hamster IgG_3, κ	BD	1-10 µg/ml
CD40L	PE	MR1	Armenian Hamster IgG_3, κ	BD	1:50
CD45/B220	Biotin	RA3-6B2	Rat IgG_{2a}, κ	BD	1:1000
CD45/B220	FITC	RA3-6B2	Rat IgG_{2a}, κ	BioLegend	1:100
CD45/B220	AlexaFluor647	RA3-6B2	Rat IgG_{2a}, κ	BioLegend	1:2000
CD49b	Biotin	DX5	Rat IgM, κ	BD	1:1000
CD8a	Biotin	53-6.7	Rat IgG_{2a}, κ	BD	1:1000
CD8a	FITC	53-6.7	Rat IgG_{2a}, κ	BioLegend	1:300
CD8a	AlexaFluor700	53-6.7	Rat IgG_{2a}, κ	BioLegend	1:300

CD8a	Pacific Blue	53-6.7	Rat IgG_{2a}, κ	BD	1:100
Foxp3	APC	FJK-16s	Rat IgG_{2a}, κ	eBioscience	1:100
IFN-γ	PerCP/Cy5.5	XMG1.2	Rat IgG_1, κ	BioLegend	1:100
IFN-γ	PE	XMG1.2	Rat IgG_1, κ	BioLegend	1:750
IFN-γ	unconjugated	XMG1.2	Rat IgG_1, κ	BioXCell	268 ng/well
Isotype ctrl	unconjugated	MOPC-21	Mouse IgG_1, κ	BioXCell	100 µg/mouse
Isotype ctrl	PerCP/Cy5.5	RTK2071	Rat IgG_1, κ	BioLegend	1:100
Isotype ctrl	unconjugated	E36-239	Armenian Hamster IgG_3, κ	BD	10 µg/ml
mouse IgG	Biotin	polyclonal		Jackson ImmunoResearch	1:1000
rat IgG	Biotin	polyclonal		Jackson ImmunoResearch	1:1000
T-bet	PerCP/Cy5.5	4B10	Mouse IgG_1, κ	eBioscience	1:50
TER-119	Biotin	TER-119	Rat IgG_{2b},	BD	1:1000
IL-4	unconjugated	11B11	Rat IgG_1, κ	BioXCell	10 µg/ml
GATA-3	AlexaFluor647	16E10A23	Mouse IgG_{2b}, κ	BioLegend	1:50
CD90.1	FITC	OX-7	Mouse IgG_1, κ	BioLegend	1:300
CD90.1	PerCP/Cy5.5	OX-7	Mouse IgG_1, κ	BioLegend	1:100
CD90.2	PE-Cy7	53-2.1	Rat IgG_{2a}, κ	BioLegend	1:300

2.5 SOLUTIONS, BUFFER AND MEDIA

SOLUTIONS

BSS (Balanced Salt Solution I, II)

110 ml BSS I and 110 ml BSS II ad 1 L ddH_2O

BSS I: 50 g Glucose, 3 g KH_2PO_4, 11.9 g Na_2HPO_4, 0.5 g phenol red ad 5 L ddH_2O

BSS II: 9.25 g $CaCL_2$, 20 g KCl, 320 g NaCl, 10 g $MgCl_2$, 10 g $MgSO_4$ ad 5 L ddH_2O

BSS/BSA

BSS-Buffer containing 0.1 % BSA

1.8 % NaCl

18 g NaCl in 1 L ddH_2O

Trypan blue

1x PBS containing 0.04 % Trypan blue and 0.05 % NaN_3

BUFFER

FACS-Buffer

1x PBS containing 0.1 % BSA, 0.05 % NaN_3

Fixation/Permeabilization-Buffer (eBioscience)

75 % Fixation/Permeabilization Diluent and 25 % Fixation/Permeabilization Concentrate

PBS (Phosphate-buffered saline)

80.0 g NaCl, 11.6 g Na_2HPO_4, 2.0 g KH_2PO_4, 2.0 g KCl ad 1L ddH_2O, pH set to pH value of 7.4

Permeabilization-Buffer (eBioscience)

10 % 10x Permeabilization-Buffer and 90 % ddH_2O

MEDIA

RPMI

RPMI 1640 Medium (Gibco) containing 10 % FCS, 2 mM L-Glutamine, 10 mM HEPES, 50 µM 2-Mercaptoethanol, 50 µg/ml Streptomycin, 100 U/ml Penicillin

2.6 CONSUMABLES

Consumable	Manufacturer
Cell culture plates cellstar (6-, 48-, 96-well)	Greiner bio-one
Cell strainer EASYstrainer 70 µm	Greiner bio-one
Dispenser-Tips	Eppendorf and Brand
Eppendorf tubes 1.5 ml, 2 ml	Sarstedt
FACS bullets	A. Hartenstein GmbH
FACS tubes	Sarstedt
Falcons 15 ml, 50 ml	Greiner Labortechnik
Glas pipettes	Brand
Gloves SensiCareIce	Medline
LS and LD column	MiltenyiBiotec
Microtainer SST	BD
Needles 100 Sterican (26 and 27 G)	Braun
Pipette tips crystal	Sarstedt
Pipette tips yellow	A. Hartenstein GmbH
Pipette tips yellow, blue	Greiner bio-one

Serological Pipettes 5 ml, 1 0ml, 25 ml	Greiner bio-one
Syringes 1 ml	BD Plastikpak
Syringes Injekt 2 ml	Braun

2.7 INSTRUMENTS

Instrument	Manufacturer
Autoclave	Melag
Centrifuge 5415 C, 5804 R, 5810 R	Eppendorf
Counting chamber Neubauer	Laboroptik, Marienfeld
FACS AriaIII	Beckton Dickinson
FACS LSR II	Becton Dickinson
Freezer -20 °C	Bosch
Freezer -80 °C	Forma Scientific
Handy Step	Brand
Ice Machine AF 100	Scotsman
Incubator Hera cell	Heraeus
Laminar Flow Hood LaminAir HBB 2448	Heraeus
MacBook Pro	Apple
MACS Multistand	Miltenyi Biotec
MACS Separation Stand	Promega
Microscope Biomed	Leitz
Microscope Labovert FS	Leitz
Pipette controller accu-jet	Brand
Pipettes	Eppendorf
Refrigerator	Bosch
Scale SBA51	Scaltec
Stepper Multipipette plus	Eppendorf
Thermomixer comfort	Eppendorf
Ultrasound instrument	Electrosonic
Vortex Genie-2	Scientistic Industries
Water bath	Lauda
Water bath Thermostat Assistent 3180	Hecht
Water Purification System BarnsteadGenPure Pro	ThermoScientific

2.8 SOFTWARE

Software	Application
FACS Diva	FACS Acquisition
FlowJo Versions 9.9.6/10.5.3	Flow cytometry data analysis
GraphPad Prism 6.0	Graphical presentation of data and statistical analysis
LEGENDplex™ Data Analysis Software	Analysis of LEGENDplex Data
Mendeley v1.19.4	Reference Management
Microsoft Office for Mac 2015	Data management and manuscript preparation

3 METHODS

3.1 MICE

C57BL/6.OT-II, C57BL/6.Thy1.1$^{+/+}$, C57BL/6.Thy1.1$^{+/-}$, C57BL/6.Thy1.1$^{+/-}$ DEREG and WT littermates were bred and maintained in the specific pathogen-free animal facility of the Institute for Virology and Immunobiology of the University of Würzburg. Animals used for experiments were aged between six and 15 weeks.

3.1.1 C57BL/6.OT-II

These mice express a transgenic TCR consisting of the α-chain (Vα2) and β-chain (Vβ5) that pairs with the co-receptor CD4 on T cells and recognizes the Ovalbumin-Peptide 329-337 ($OVA_{329-337}$) presented in the context of MHC II[82]. This property allows an antigen-specific activation of the whole CD4$^+$ T cell population by the non-self-peptide OVA being a great advantage compared to wildtypic T cells which qualifies these cells for the study of T cell responses *in vitro* as well as in *vivo*. Due to its genetically predetermined TCR the T cell repertoire is dominated by CD4$^+$ cells while there is a marked reduction in CD8$^+$ T cells in this mouse line.

3.1.2 C57BL/6.THY1.1

Thy1.1, also known as CD90.1, is a constitutively expressed T cell-specific marker with a so far unknown function. While wildtypic mice naturally express the surface protein CD90.2, these mice carry the Thy1.1 allele[83]. This greatly facilitates the identification of T cells originating from these mice based on surface expression of the respective proteins.

3.1.3 C57BL/6. DEREG

C57BL/6 bacterial artificial chromosome (BAC)-transgenic DEREG (*De*leter of *reg*ulatory T cells) mice express a simian diphtheria toxin receptor as well as GFP (green fluorescent protein) under the control of the Foxp3 promotor[84]. Upon DT treatment, all Foxp3$^+$ regulatory T cells are transiently depleted in these mice. Recipient mice were either DEREG or WT littermates, whereas the latter showed no depletion of regulatory T cells upon DT treatment.

3.2 CELL ISOLATION FROM LYMPH NODES AND SPLEEN

Mice were sacrificed by CO_2 narcosis. Lymph nodes and spleen were grinded through 70 µm cell strainers in BSS/BSA to obtain single cell suspension. Subsequently, suspensions were centrifuged (1600 rpm, 4 °C, 5 min) and lymph node suspensions were resuspended in a defined volume for cell counting. Splenocyte suspensions were subjected to erythrocyte lysis due to hypo-osmotic shock by adding ddH_2O followed by NaCl (1.8 %) in equal volumes (each 3 ml per spleen) to the cell pellet while vortexing. Nucleated cells are resistant to the osmotic challenge and are thereby enriched in the supernatant whereas non-nucleated erythrocytes are lysed. The supernatant was separated from sedimented cell debris of erythrocytes and was washed with BSS/BSA. For counting, trypan blue exclusion was used for which samples of cell suspension were diluted in Trypan blue and counted in a Neubauer counting chamber under the microscope.

3.3 ISOLATION OF CONVENTIONAL T CELLS

Conventional $CD4^+$ $CD25^-$ T cells were isolated from single cell solutions by MACS technique based on negative selection. Biotinylated antibodies against surface markers (CD11b for monocytes, CD49b for NK cells, CD45R/B220 for B cells, CD8a for $CD8^+$ T cells, Ter119 for erythrocytes and CD25 for activated and regulatory T cells) were used to label undesired cells ($1 \cdot 10^8$ cells/ml, 15 min on ice). After washing with BSS/BSA, streptavidin-labelled magnetic beads were added (1:20 in 1 ml, 20 min on ice) to allow for magnetic separation. Subsequent passage through LS columns placed in a magnetic field resulted in $CD4^+$ $CD25^-$ cell solution in the flow-through. Purity of obtained $CD4^+$ $CD25^-$ T cells ranged from 70-95 %.

3.4 ISOLATION OF ANTIGEN-PRESENTING CELLS (APCS)

Antigen-presenting cells were obtained by MACS technique based on negative selection. $CD90^+$ T cells among splenocytes were depleted using anti-CD90-labeled magnetic beads and subsequent passage through LD columns. To do so, splenocytes ($2.5 \cdot 10^8$ cells/ml) were incubated with normal mouse IgG (1:50) for 15 minutes on ice to block unspecific binding followed by a 15-minute incubation with anti-CD90 labeled magnetic beads (1:10) on ice. Then, cells were washed before loading them onto an LD column in a magnetic field obtaining $CD90^+$ T cell-depleted splenocytes in the flow-through mainly consisting of antigen-presenting cells, i.e. B cells and monocytes. Purity of T cell-depleted splenocytes ranged from 90-100 %.

3.5 GENERATION OF TH1 CELLS

In order to polarize $CD4^+$ $CD25^-$ T cells into Th1 cells, $1 \cdot 10^6$ T cells were co-cultured with $1 \cdot 10^6$ APCs in complete RPMI Medium containing the OVA-peptide$_{329-337}$ (2 µM) as well as α-IL-4 (10 µg/ml) and the Th1-polarizing cytokine IL-12 (10 ng/ml) in a 48-well plate over 5 days (37 °C, 5 % CO_2). Cell cultures were split on day 3 (1:2) and harvested on day 5. Differentiation status was assessed by flow cytometric analysis based on intracellular expression of the transcription factors T-bet and Gata-3 as well as IFN-γ production. For intracellular cytokine staining, cells were restimulated with phorbol 12-myristate 13-acetate (PMA, 5 ng/ml) and Ionomycin (500 ng/ml) for two hours before adding Brefeldin A (10 µg/ml) for the last two hours to prevent cytokine secretion.

3.6 *IN VIVO* CD28-SA RESPONSES

$2 \cdot 10^6$ activated OT-II $CD4^+$ Th1 cells were transferred intravenously into C57BL/6. $Thy1.1^{+/-}$ DEREG or C57BL/6. $Thy1.1^{+/-}$ WT littermate recipient mice on day 0. In order to deplete Treg cells, 0.5 µg Diphteria toxin (DT) was administered intraperitoneally (i.p.) on the same and the following day to all animals, as well DEREG as wildtype (WT) littermates ruling out effects resulting from DT treatment itself. On day 3, 150 µg of CD28-SA or isotype control antibody (MOPC-21) were injected intraperitoneally. Blood samples were taken from the submandibular vein 2, 4, 6 and 24 hours post CD28-SA injection. Serum was separated by centrifugation (10 min, 14.000 rpm, RT) and stored at -80 °C until analysis. For cellular analysis, lymph nodes and spleens were taken 4 hours after superagonist administration and single cell suspensions were stained for flow cytometry analysis as described in 3.8.

3.7 *IN VITRO* CD28-SA RESPONSES

For secondary culture, Th1 cells, differentiated over 5 days, were allowed to recover for two days at $5 \cdot 10^5$ cells/ml density in RPMI Medium containing recombinant human IL-2 (0.1 µM). Subsequently, on day 7, cells were harvested, washed 3 times with BSS/BSA and stimulated over 24 hours with either CD28-SA (at the indicated concentrations) or OVA$_{329-337}$ (1 µM) to assess the ability of Th1 cells to respond with IFNγ production. Furthermore, either Dynabeads® Pan Mouse IgG, ratio 1:5 (Th1:beads), and/or freshly isolated, erythrocyte-depleted splenocytes – complete or selectively depleted by sorting – from a sex-matched congenic wildtype mouse (C57BL/6.$Thy1.1^{+/+}$), ratio 1:10 (Th1:WT), were added as indicated. On day 8, cells were stained for IFNγ production and analyzed via flow cytometry as described in 3.8.

3.7.1 CD40L BLOCKADE

To block CD40-CD40L-interactions during secondary cultures, an antagonistic α-CD40L mAb (1-10 µg/ml as indicated) was added to the secondary culture on day 7. As a control, an isotype-matched control antibody (Armenian hamster IgG_3,κ) was used.

3.8 FLOW CYTOMETRY

Cell surface markers were stained with the respective fluorochrome-labelled antibodies in the dark (30 min, 4 °C), washed with FACS-Buffer and resuspended in 50-100 µl FACS-Buffer. Samples were either directly used for flow cytometric analysis or fixed with Fixation Buffer (30 min, 4 °C) for later analysis.

For staining of intracellular targets, cells were fixed with Fixation Buffer (30 min, 4 °C), washed and permeabilized in Perm-Buffer before staining with respective antibodies (45 min, RT).

Cells were resuspended in 50-100 µl FACS Buffer for acquisition. Flow cytometric analysis was performed on a BD™ LSRII using the FACS DIVA Software. Data analysis was realized using FlowJo Software.

Intracellular Cytokine Staining

For intracellular cytokine staining, cells were incubated for 2 hours with Brefeldin A (10 µg/ml) to prevent cytokine secretion before staining as described above. For IFNγ staining, an isoclonic control was used, i.e. for one well of three replicates cells were permeabilized and incubated with excess amount of unconjugated α-IFNγ antibody (15 min, RT) followed by staining with the fluorochrome-conjugated antibody as described above. Δ % IFNγ^+ was calculated as follows:

$$\Delta\,\%\,\mathrm{IFN}\gamma^+ = \%\,\mathrm{IFN}\gamma^+(without\ isoclonic\ control) - \%\,\mathrm{IFN}\gamma^+(with\ isoclonic\ control)$$

3.8.1 CELL SORTING

For secondary cultures of Th1 cells, freshly isolated splenocytes were depleted of T cells ($CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$) or APCs ($CD11b^+$ and $CD45R/B220^+$) using a BD™ AriaIII. To do so, $2 \cdot 10^7$ cells were stained with the desired antibodies (30 min, 4 °C) in RPMI Medium and washed before being sorted by negative selection. Sorted cells were collected in RPMI Medium containing 50 % FCS. Unstained splenocytes were sorted to control for effects caused by mechanical stress during the sorting procedure.

3.9 ANALYSIS OF CYTOKINE CONCENTRATION IN SERUM AND CULTURE SUPERNATANT

Cytokine concentration in serum and supernatants was determined using the bead-based immunoassay LEGENDplex™ Mouse Th Cytokine Kit Mix and Match according to manufacturer's instructions.

Briefly, dilutions of standard containing predefined concentrations of each cytokine as wells supernatant (1:4) or serum (1:2) were incubated with a mixture of beads coated with antibodies against single cytokines and biotinylated detection antibodies (2 hours, shaking at RT). Thereafter, PE-conjugated streptavidin was added and shakingly incubated for 30 min at RT. After washing, the beads were resuspended in 50 µl of washing buffer and analyzed by flow cytometry. Distinct cytokine-specific beads varying in size and brightness in the APC-channel allowed to distinguish the cytokines analyzed. Absolute cytokine concentrations were calculated with LEGENDplex™ Data Analysis Software based on the MFI in the PE channel using standard curves.

3.10 STATISTICAL ANALYSIS

Data are presented as means ± SD. Statistical significance was analyzed by two-tailed unpaired *t*-test or two-way ANOVA followed by Sidak's or Dunnett's test as indicated using GraphPad Prism 6.0. Values of $p < 0.05$ were considered to be statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$).

4 RESULTS

4.1 TH1 CELLS RESPOND WITH STRONG IFNγ PRODUCTION TO CD28-SA TREATMENT *IN VIVO*

$CD4^+$ $CD25^-$ T cells from C57BL/6 OT-II TCR-transgenic mice were differentiated *in vitro* into Th1 cells in the presence of the OVA-$peptide_{327-339}$ in Th1 polarizing medium. After a culture period of five days, cells were analyzed for Th1 cell characteristics, i.e. expression of the transcription factor T-box containing protein expressed in T cells (T-bet) and production of their signature cytokine IFNγ[23]. The flow cytometric analysis is exemplarily depicted in Figure 3. The intracellular staining of both parameters revealed a true Th1 phenotype of the differentiated $CD4^+$ T cells as the vast majority of the cells expressed high levels of T-bet compared to low levels of Gata-3 concomitantly with a strong IFNγ response to restimulation with PMA and Ionomycin.

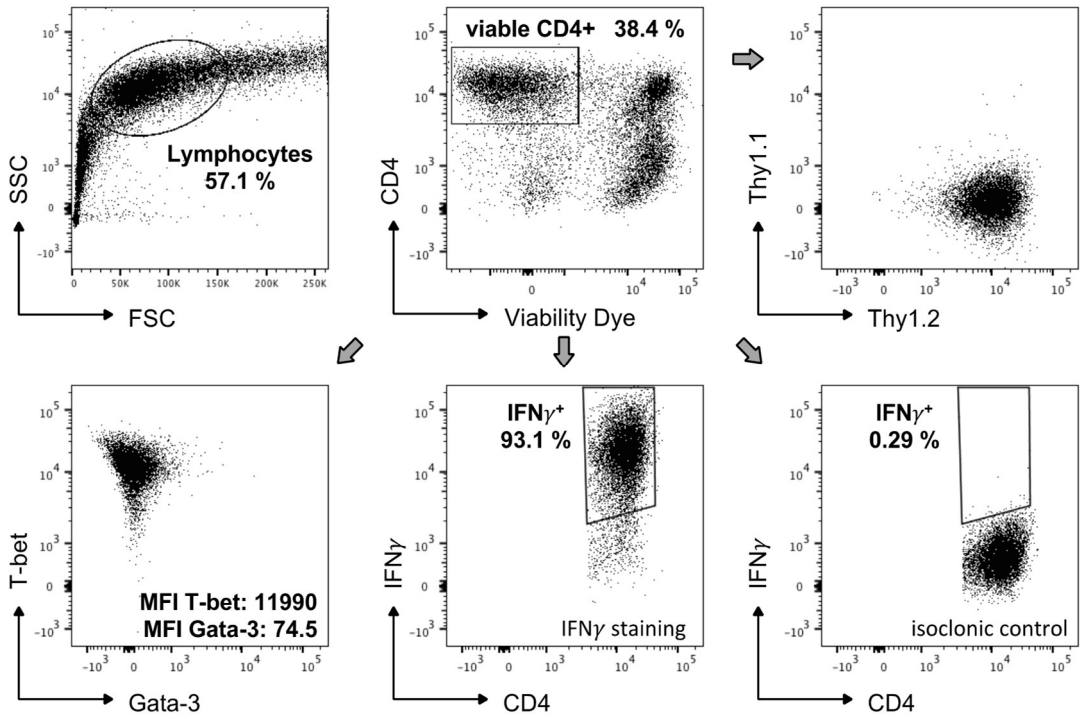


Figure 3: Th1 differentiated $CD4^+$ T cells exhibit characteristic Th1 features

$CD4^+$ $CD25^-$ T cells were differentiated in Th1 medium in the presence of OVA-$peptide_{327-339}$ and antigen-presenting cells. On day 5, $2 \cdot 10^5$ cells were restimulated with PMA and Ionomycin for 4 hours adding Brefeldin A for the last two hours before staining. Lymphocytes were discriminated based on Forward Scatter (FSC) and Side Scatter (SSC) signals (top left panel) followed by identification of viable $CD4^+$ T cells using a viability dye (top middle panel). Viable $CD4^+$ T cells were analyzed for congenic markers Thy1.1 and Thy1.2 (top right panel), for transcription factors T-bet and Gata-3 (lower left panel) showing the median fluorescence intensity (MFI) as well as for IFNγ (lower middle and right panel). Specificity of IFNγ staining was verified with an isoclonic control.

The *in vitro* generated Th1 cells were used for *in vivo* experiments addressing the responsiveness of Th1 cells to treatment with superagonistic monoclonal antibody (mAb) against CD28 in specific-pathogen-free (SPF) mice. For this purpose, $2 \cdot 10^6$ Thy1.2$^{+/+}$ Th1 cells were transferred intravenously into C57BL/6 Thy1.1$^{+/-}$ recipient mice, being either *Deleter of regulatory T cells* (DEREG) or wildtype (WT) littermates. On day 0 and day 1, all mice were treated with Diphteria toxin (DT) in order to deplete Treg cells in DEREG mice (Treg-depleted) whereas WT recipients were treated to control for effects based on DT administration itself. On day 3, mice were injected intraperitoneally with CD28-SA followed by blood sampling as indicated (Figure 4). For intracellular analysis of IFNγ production in transferred Th1 cells as well as in host derived T cells, spleens and lymph nodes were collected 4 hours after CD28-SA treatment.

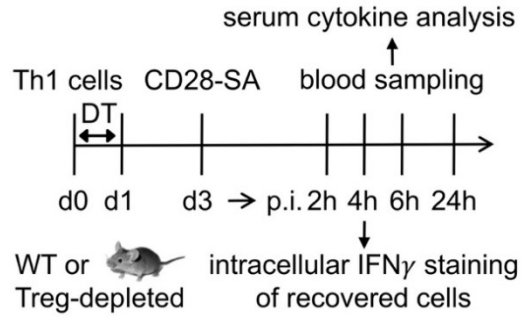


Figure 4: Experimental procedures for CD28-SA treatment *in vivo*

$2 \cdot 10^6$ *in vitro* differentiated Thy1.2$^{+/+}$ Th1 cells were intravenously transferred into C57BL/6 Thy1.1$^{+/-}$ DEREG or WT recipient mice on day 0. DT was administered on day 0 and 1 in order to deplete Treg cells. On day 3, 150 µg of CD28-SA was administered intraperitoneally before blood sampling. Cells from spleens and lymph nodes were isolated 4 hours after CD28-SA treatment and stained intracellularly for IFNγ expression.

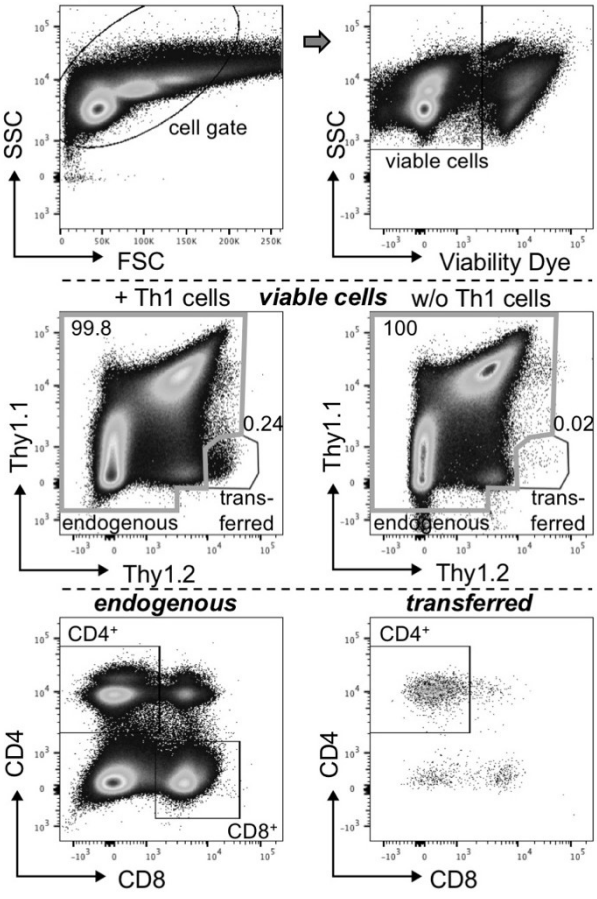


Figure 5: Gating strategy for *ex vivo* analysis of transferred Th1 and endogenous T cells

Viable splenocytes were selected based on negative viability dye staining and further identified as endogenous or transferred due to expression of the congenic markers Thy1.1 and Thy1.2. Different T cell populations within the endogenous pool of T cells were discriminated using the surface antigens CD4 and CD8.

The recipient mice expressed the congenic marker Thy1.1 heterozygously which facilitated distinguishing host-derived endogenous T cells from the transferred Th1 cells which are homozygous for Thy1.2 (Figure 5). Staining the isolated lymphocytes from spleens and lymph nodes for Foxp3 four hours post CD28-SA injection showed the successful depletion of Treg cells in DEREG recipients whereas the WT recipients still exhibited normal numbers and frequencies of Treg cells (Figure 6A, B). However, absolute numbers of Treg cells within spleens showed a significant increase after CD28-SA treatment in WT mice (Figure 6C) which was not caused by an increase of the overall cellularity of the spleens as total numbers of recovered cells from secondary lymphoid organs were not affected by CD28-SA treatment (Figure 7A).

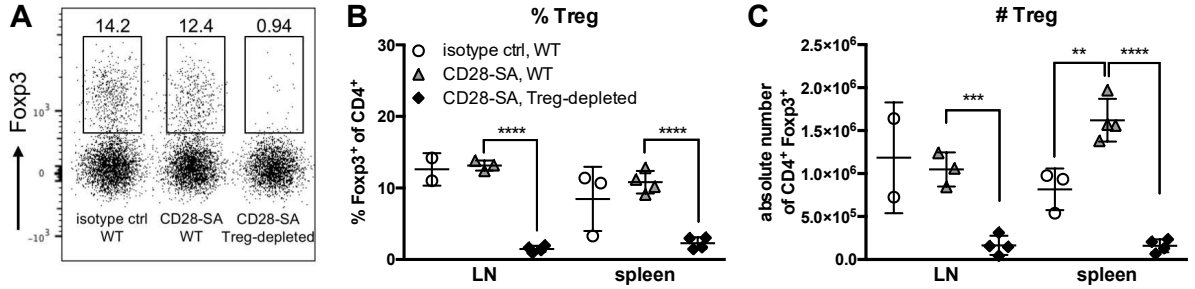


Figure 6: Treg cells are successfully depleted *in vivo* by DT administration.

Recipient mice were treated with 0.5 µg DT by intraperitoneal injection on day 0 and day 1. Lymph nodes and spleens were isolated on day 3, four hours post CD28-SA injection, and analyzed via flow cytometry. **(A)** Intracellular Foxp3 staining of recipients' $CD4^+$ T cells in lymph nodes (pregated on viable host-derived ($Thy1.1^+$ $Thy1.2^+$) $CD4^+$ T cells. **(B)** Treg cell frequencies among recipient $CD4^+$ T cells in lymph nodes (LN) and spleen. **(C)** Absolute numbers of $CD4^+$ $Foxp3^+$ Treg cells within lymph nodes (LN) and spleen. Data from individual mice are shown together with means ± SD. Results were pooled from two independent experiments with a total of n=2-4 mice per group and tested with a two-tailed unpaired *t*-test (**p<0.01; ***p<0.001; ****p<0.0001).

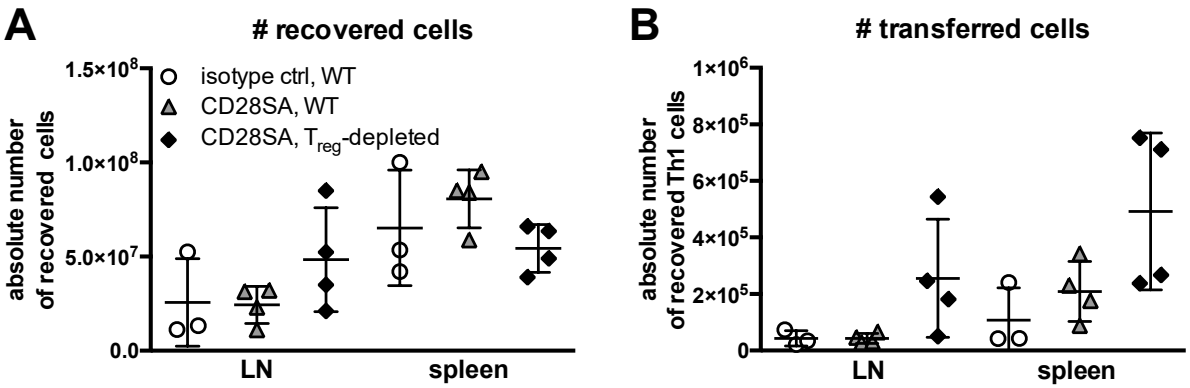


Figure 7: CD28-SA treatment does not affect overall cellularity of secondary lymphoid organs nor absolute numbers of recovered Th1 cells 4 hours after administration.

Lymphocytes from lymph nodes and spleens were counted and analyzed four hours post CD28-SA treatment via flow cytometry. **(A)** Absolute numbers of recovered cells from lymph nodes (LN) and spleens. **(B)** Absolute number of recovered Th1 cells within lymph nodes and spleen. Results were pooled from two independent experiments with a total of n=3-4 mice per group and tested with a two-tailed unpaired *t*-test (no significance).

The depletion of Treg cells in the recipient mice led to a marked, about four-fold, but statistically non-significant increase in the number of recovered Th1 cells (Figure 7B). Notably, CD28-SA treatment in the presence of Treg cells did not seem to impact on the expansion or survival of Th1 cells in the recipient mice.

Recovered cells from lymph nodes and spleens were intracellularly stained for IFNγ expression in order to analyze the response to CD28-SA treatment on a single cell level. This analysis revealed that the transferred Th1 cells were indeed producing IFNγ upon CD28-SA administration *in vivo* (Figure 8). The Th1 cells' IFNγ response was similar in both, spleen and lymph nodes, and the proportion of IFNγ-producing Th1 cells nearly tripled in the absence of Treg cells. However, in the presence of Treg cells the IFNγ response of Th1 cells to superagonistic CD28 stimulation was only moderately increased compared to isotype control-treated mice. In contrast, endogenous T cells, $CD4^+$ as well as $CD8^+$, were totally irresponsive to CD28-SA administration in terms of IFNγ production in both secondary lymphoid organs. The data, thus, indicate that CD28-SA treatment induces IFNγ production by Th1 cells *in vivo.*

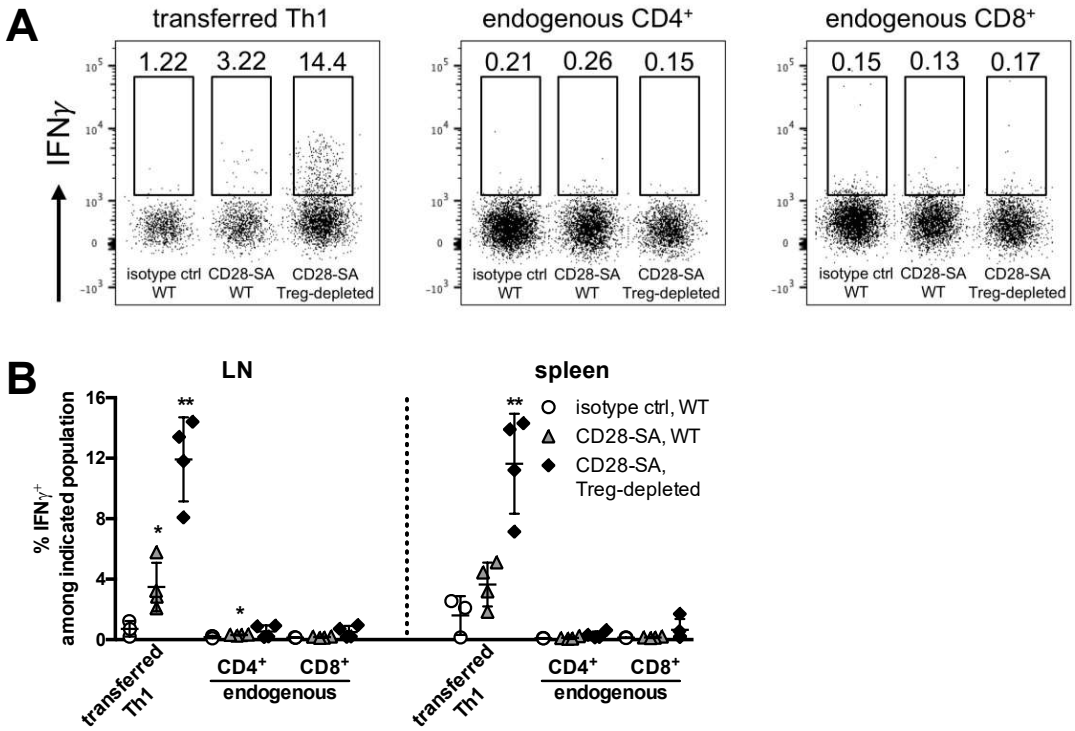


Figure 8: Th1 cells produce IFNγ in response to CD28-SA treatment *in vivo*

Recipient mice were treated with 150 µg CD28-SA on day 3. Four hours after treatment cells from lymph nodes (LN) and spleens were isolated and incubated with Brefeldin A for two hours prior to **(A)** intracellular staining for IFNγ. Cells from LN are shown and pregated as depicted in Figure 5. **(B)** Summary of % $IFN\gamma^+$ cells within transferred Th1 cells, endogenous $CD4^+$ and $CD8^+$ T cells, respectively. Data from individual mice and means ± SD are shown. Results were pooled from two independent experiments with a total of n=3-4 mice per group. Two-tailed unpaired *t*-test comparing CD28-SA with isotype control treatment (*p<0.05; **p<0.01).

4.2 CD28-SA TREATMENT OF TH1 CELL RECIPIENTS INDUCES SYSTEMIC CYTOKINE RELEASE *IN VIVO*

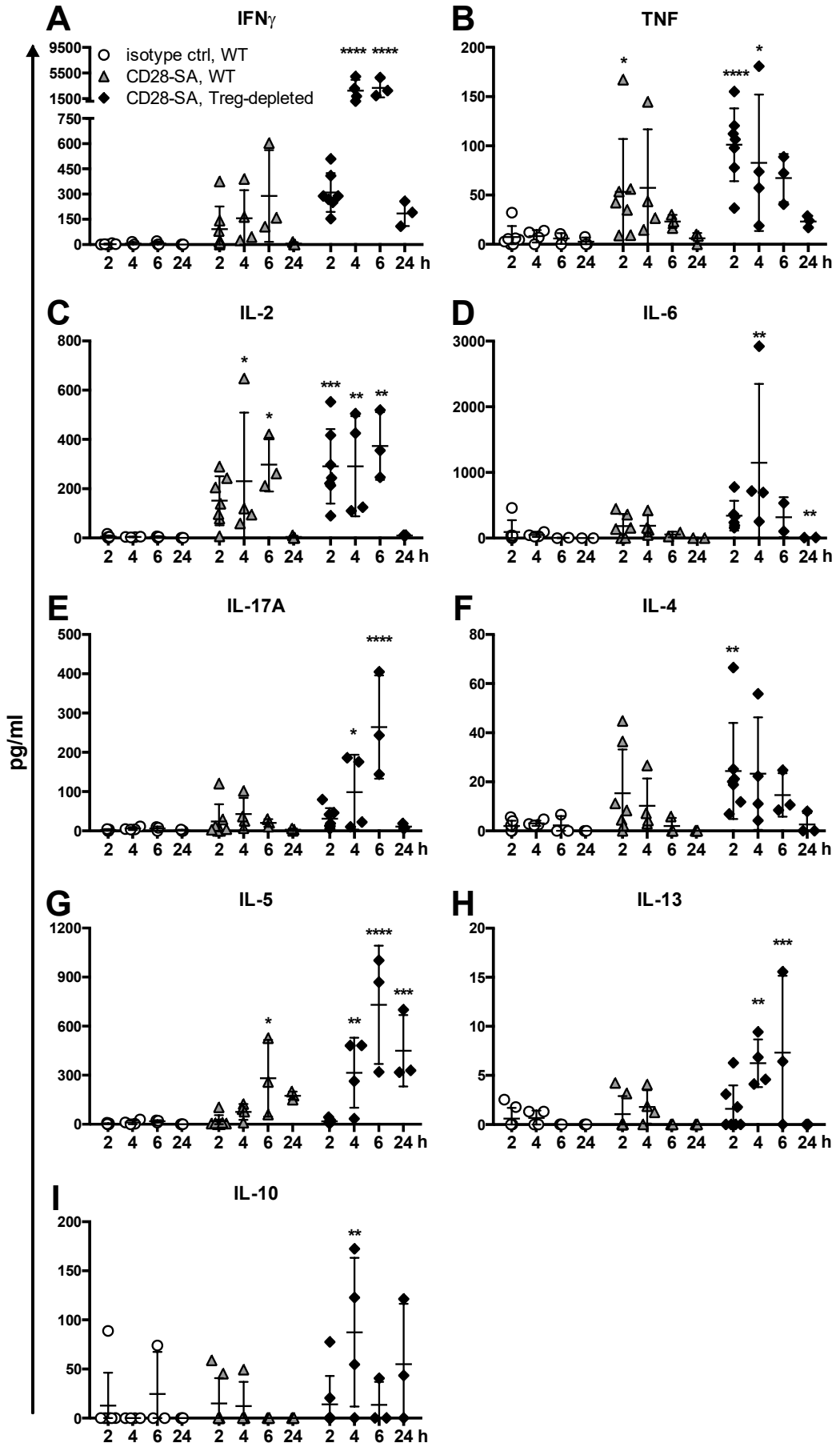
The application of the anti-human CD28-SA TGN1412 during a first-in-man clinical trial resulted in a severe CRS in all six healthy volunteers, which was retrospectively found to be induced by secretion of enormous amounts of cytokines from unintentionally activated effector/memory T cells.[73,77] CRS is characterized by an acute increase in the concentrations of pro-inflammatory cytokines like IFNγ, TNF, IL-2 and IL-6 in the serum[85].

In order to determine cytokine concentrations in the serum of CD28-SA-treated mice, blood samples were collected for up to 24 hours post treatment and analyzed using a bead-based multiplex assay as described in 3.9. All analyzed cytokines were observed to be significantly increased upon CD28-SA treatment in the absence of Treg cells (Figure 9). This overall systemic release of cytokines being compatible with CRS was mainly dominated by a sharp, about 10-fold increase in the concentration of IFNγ (Figure 9A). Moreover, TNF, IL-2 and IL-6 were systemically secreted in the treated Treg-deficient mice (Figure 9B-D). Additionally, IL-17A and the type 2 cytokines IL-4, IL-5 and IL-13 were observed to be elevated (Figure 9E-H), while the concentration of the anti-inflammatory cytokine IL-10 remained nearly unchanged (Figure 9I). Strikingly, in Treg-sufficient recipients the systemic cytokine release was markedly reduced. However, regarding the concentration of TNF, IL-2 and IL-5 there was still a significant increase observed compared to the untreated WT littermates. Like IL-6, which is one of the most prominent CRS drivers, IL-17A, IL-4, IL-13 and IL-10 levels were hardly elevated in Treg-sufficient mice. Obviously, Treg cells in our recipient mice efficiently suppressed the CRS-like cytokine release induced by CD28-SA treatment.

CD28-SA-treated Treg-deficient mice that were not adoptively transferred with Th1 cells only showed slightly elevated concentrations of pro-inflammatory cytokines (Figure 10). The response of Th1 cell recipients was considerably more homogenous and stronger, especially in terms of IFNγ secretion, confirming Th1 cells as the main source of the systemically secreted IFNγ as response to CD28-SA administration.

Figure 9: Concentration of pro-inflammatory cytokines are strongly increased after CD28-SA treatment of Treg-deficient Th1 cell recipients

Serum cytokine concentrations of **(A)** IFNγ, **(B)** TNF, **(C)** IL-2, **(D)** IL-6, **(E)** IL-17A, **(F)** IL-4, **(G)** IL-5, **(H)** IL-13 and **(I)** IL-10 as determined by multiplex assay. Data for individual mice are shown together with means ± SD. Results were pooled from two to four independent experiments with a total of n=3-7 mice per group and tested with a two-way ANOVA followed by Dunnett's test (comparison isotype controls versus CD28-SA at different time points) (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).


A
IFNγ
isotype ctrl, WT
CD28-SA, WT
CD28-SA, Treg-depleted
B
TNF
C
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D
IL-6
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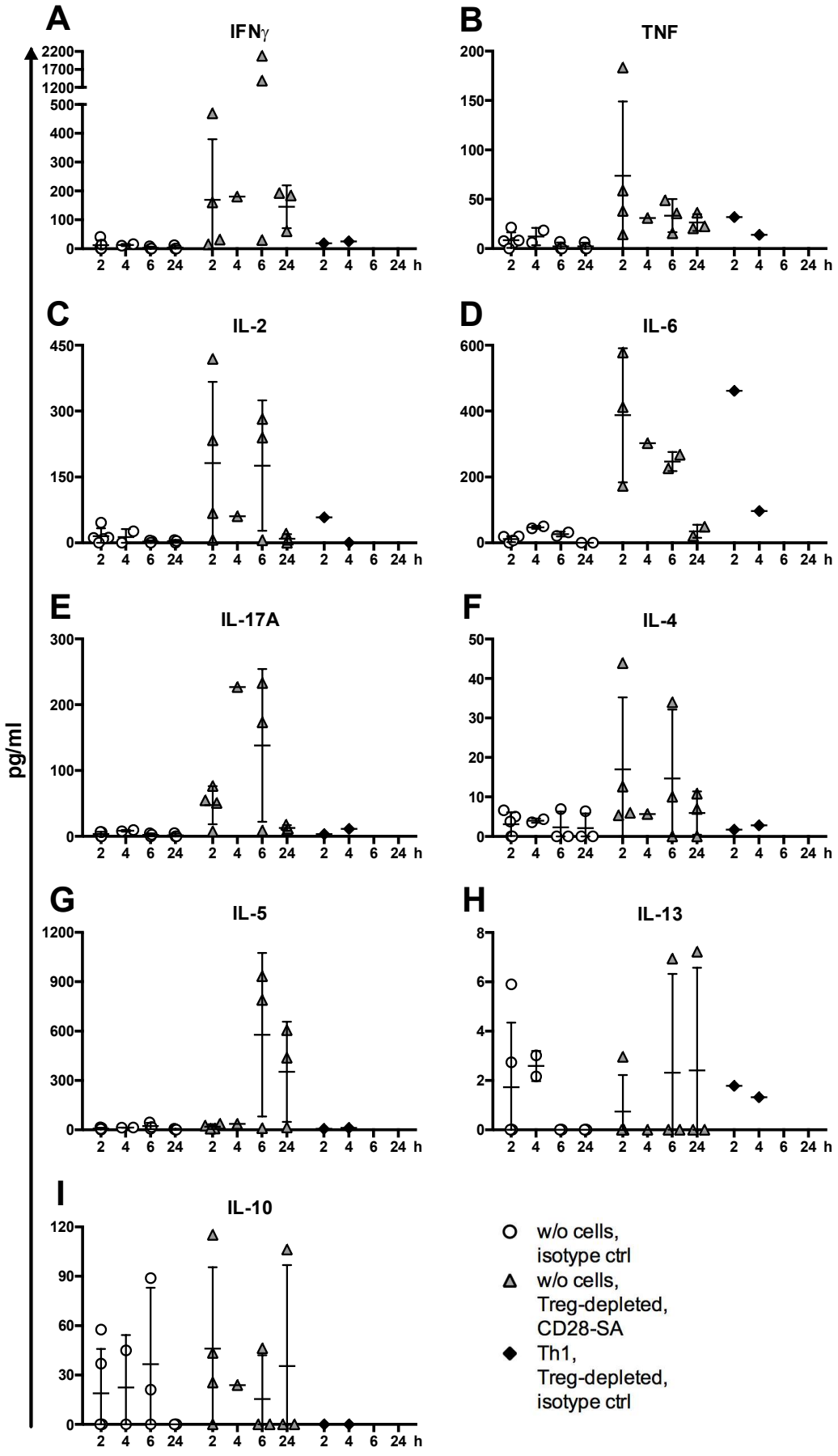
A
IFNγ
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w/o cells, isotype ctrl
w/o cells, Treg-depleted, CD28-SA
Th1, Treg-depleted, isotype ctrl

Figure 10: Concentrations of pro-inflammatory cytokines are transiently and only moderately elevated upon CD28-SA administration without Th1 transfer

Serum cytokine concentrations of **(A)** IFNγ, **(B)** TNF, **(C)** IL-2, **(D)** IL-6, **(E)** IL-17A, **(F)** IL-4, **(G)** IL-5, **(H)** IL-13 and **(I)** IL-10 as determined by multiplex assay. Data for individual mice are shown together with means ± SD. Results were pooled from one to three independent experiments with a total of n=1-4 mice per group.

As commonly observed for a broad variety of immunomodulating agents, also in case of the anti-human CD28-SA TGN1412/TAB08 a dose-response relationship was discovered. It revealed the beneficial effect of efficient mobilization of Treg cells over conventional T cells at low dosages of TAB08 (7 µg/kg BW), which might confer amelioration in autoimmune diseases and inflammatory conditions in humans instead of induction of a CRS.[79] Consequently, we also addressed this dose-response relationship in our newly established mouse model by reducing the CD28-SA-dose applied to the mice from 150 µg to as low as 1.5 µg. Similar to the response of human T cells, an about 10-fold higher dose of the CD28-SA was required to escalate from minimal cytokine release (at 15 µg/mouse) to a fulminant pro-inflammatory cytokine response *in vivo* (150 µg/mouse) (Figure 11) demonstrating very close dynamics of our mouse model to the human situation. However, in terms of absolute amount of CD28-SA, in the mouse model an about 60-fold higher dose (6 mg/kg) was required to induce CRS-like cytokine release as compared to humans (0.1 mg/kg).

Taken together, Th1 cell transfer and concomitant Treg cell depletion resulted in IFNγ-dominated cytokine release upon CD28-SA treatment *in vivo*. This approach, thus, provides a suitable preclinical model to study responses to T cell modulating agents *in vivo* under more “human-like” conditions.

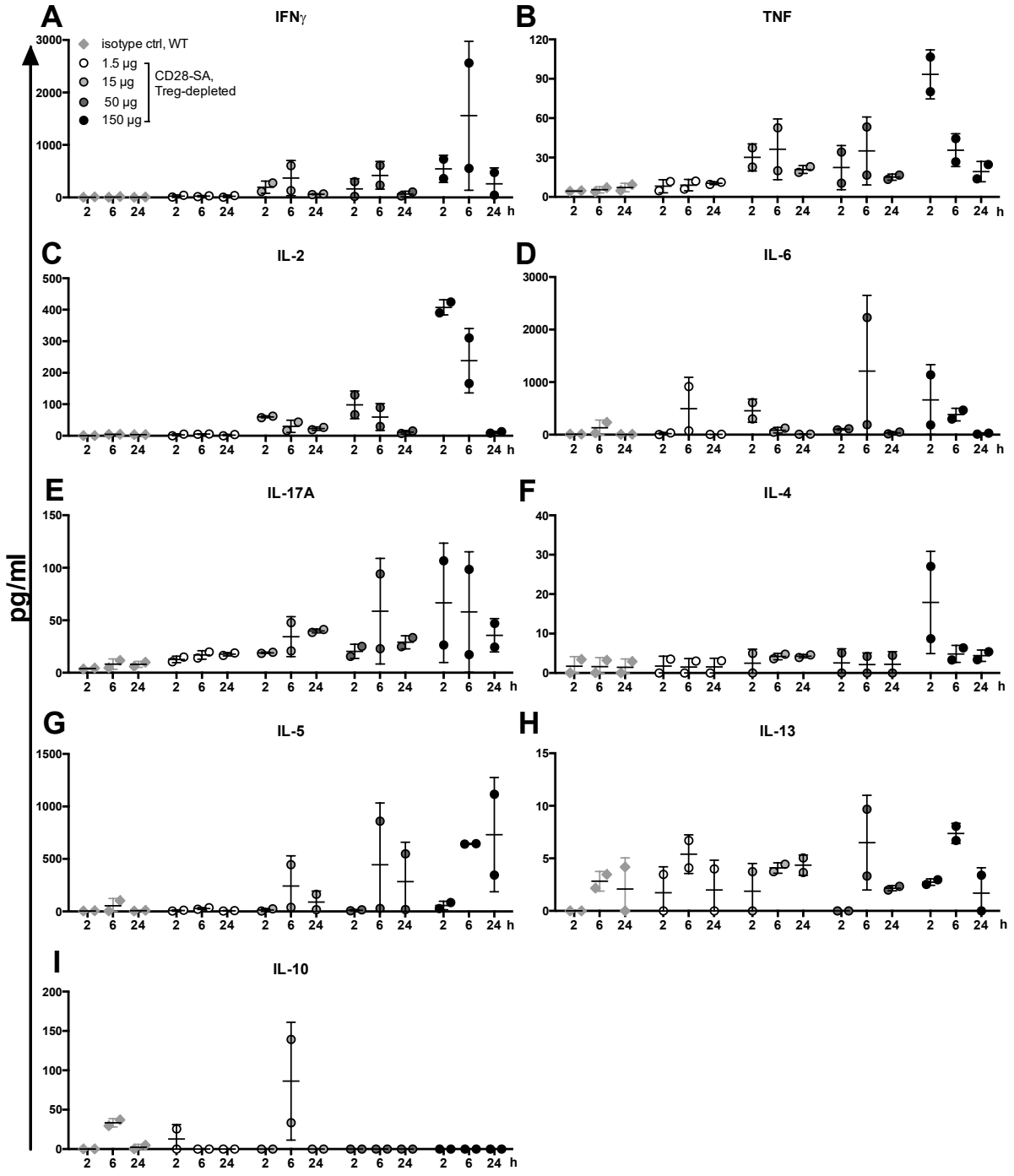

Figure 11: About 60-fold more CD28-SA/kg body weight than in human is required to induce cytokine release in Treg-deficient Th1 cell recipient mice

Serum cytokine concentrations of **(A)** IFNγ, **(B)** TNF, **(C)** IL-2, **(D)** IL-6, **(E)** IL-17A, **(F)** IL-4, **(G)** IL-5, **(H)** IL-13 and **(I)** IL-10 as determined by multiplex assay. Data for individual mice are shown together with means ± SD. Results from one experiment with a total of n=2 mice per group are shown.

4.3 *IN VIVO* RESPONSE OF TH1 CELLS TO CD28-SA STIMULATION IS REPRODUCED *IN VITRO*

To investigate the response of Th1 cells to CD28-SA stimulation in more detail, we established a novel *in vitro* assay mimicking the *in vivo* cytokine release dominated by secretion of IFNγ. For this purpose, Th1 cells were allowed to recover for two days in IL-2-containing medium past differentiation (Figure 12). Subsequently, Th1 cells were stimulated with CD28-SA in different concentrations in co-culture with either freshly isolated splenocytes from a sex-matched homozygous Thy1.1 WT mouse and/or Dynabeads® Pan Mouse IgG. The Dynabeads are able to bind mouse antibodies of the isotype IgG via ther Fc part on their surface thereby crosslinking the superagonistic antibody molecules added to the culture.

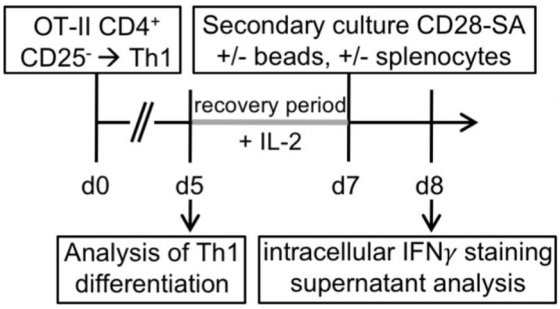

Figure 12: Setup of *in vitro* CD28-SA stimulation

OT-II Th1 cells were rested for two days in the presence of IL-2 prior to secondary culture with splenocytes, freshly isolated from a sex-matched Thy1.1$^{+/+}$ WT mouse, ratio 1:10 (Th1:WT), and/or Dynabeads® Pan Mouse IgG, ratio 1:5 (Th1:beads) and stimulated with CD28-SA (0.1/1 µg/ml) on day 7. On day 8, Brefeldin A was added to the culture for two hours in order to analyze intracellular cytokine content via flow cytometry. Furthermore, the culture supernatant was analyzed regarding secreted cytokines using a bead-based multiplex assay.

For optimal IFNγ production upon CD28-SA stimulation *in vitro*, Dynabeads® Pan Mouse IgG and by-stander splenocytes were required (Figure 13A). More precisely, the combination of both, WT splenocytes as well as Dynabeads resulted in the maximum response of about 70 % of IFNγ-producing Th1 cells in both applied concentrations of the CD28-SA whereas OT-II Th1 cells alone were irresponsive to CD28-SA stimulation. Moreover, quantification of the IFNγ response (as net frequencies of IFNγ$^+$ cells using an isoclonic control) throughout multiple experiments revealed a highly significant effect of WT splenocytes during CD28 SA stimulation of Th1 cells increasing the proportion of IFNγ$^+$ cells within the Th1 population from one fourth to about two thirds (Figure 13B). We used antigenic recall with OVA-peptide to control for overall responsiveness of the Th1 cells. In more detail, the used OT-II T cells are TCR-transgenic, meaning all CD4$^+$ T cells express exactly the same TCR recognizing the OVA-peptide$_{329\text{-}337}$. This property was used to activate all CD4$^+$ T cells OVA-specifically during the 5-day Th1 differentiation. Consequently, differentiated, functional OT-II Th1 cells are able to get re-activated by presentation of their cognate OVA-peptide via APCs, which results in IFNγ production. As expected, we observed IFNγ production in response to the OVA-peptide exclusively in the presence of splenocytes that are capable of presenting the peptide to OT-II Th1 cells. This underlined the purity of the used

Th1 cells showing that these populations were indeed devoid of APCs. Obviously, both stimuli – the CD28-SA as well as the antigenic recall - induced similar proportions of Th1 cells to produce IFNγ.

As tested *in vivo*, the dose-response relationship of the established *in vitro* model employing Dynabeads was investigated escalating the CD28-SA concentration from as low as 0.01 µg/ml to 100 µg/ml in the presence or absence of WT splenocytes. Here, a bell-shaped dose-response curve showing its

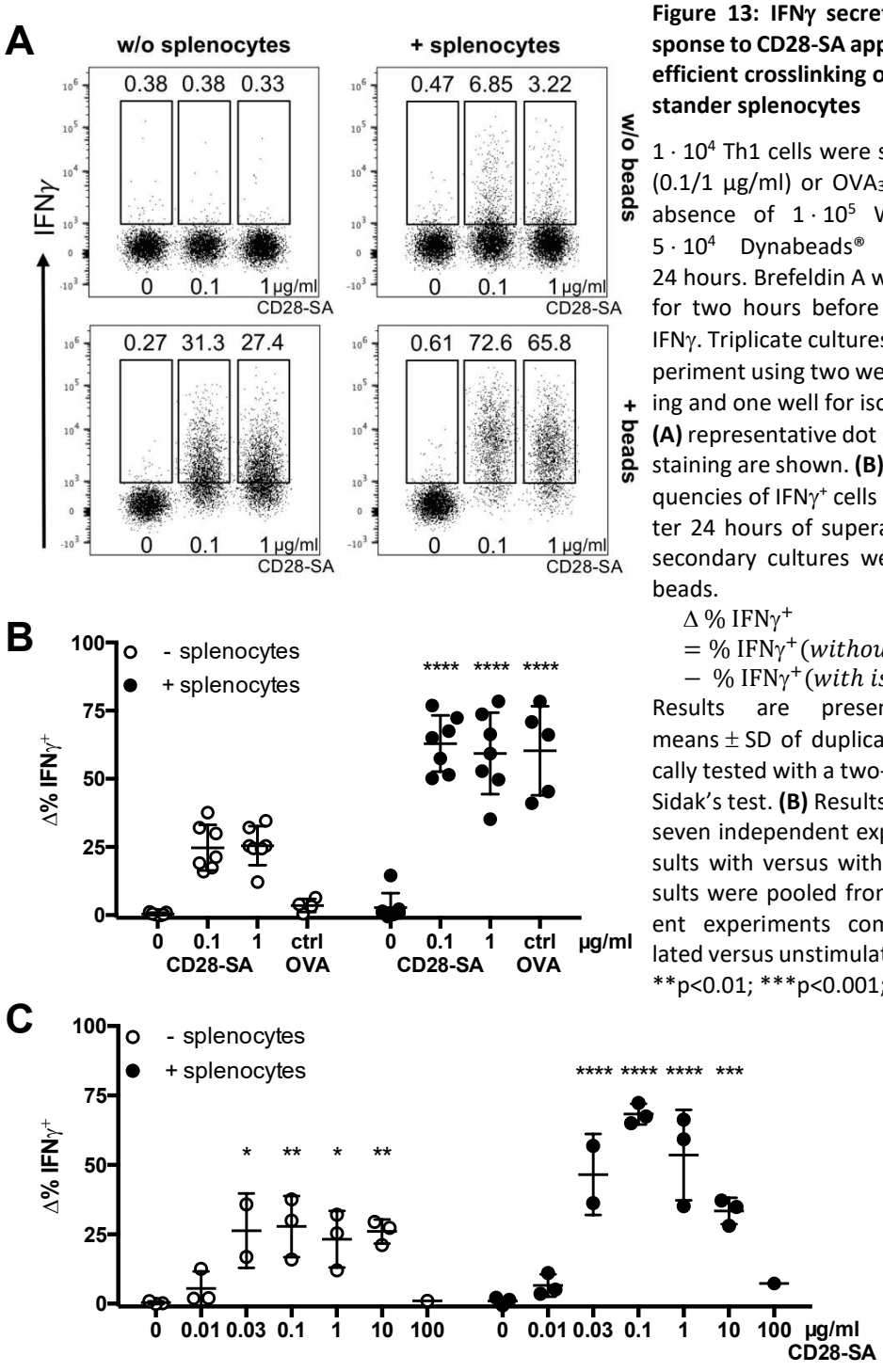

Figure 13: IFNγ secretion by Th1 cells in response to CD28-SA application *in vitro* requires efficient crosslinking of CD28-SA as well as bystander splenocytes

$1 \cdot 10^4$ Th1 cells were stimulated with CD28-SA (0.1/1 µg/ml) or $OVA_{327\text{-}339}$ in the presence or absence of $1 \cdot 10^5$ WT splenocytes and/or $5 \cdot 10^4$ Dynabeads® Pan Mouse IgG for 24 hours. Brefeldin A was added to the cultures for two hours before intracellular staining of IFNγ. Triplicate cultures were set up in every experiment using two wells for specific IFNγ staining and one well for isoclonic control.
(A) representative dot plots of intracellular IFNγ staining are shown. **(B)+(C)** Summary of net frequencies of $IFN\gamma^+$ cells among OT-II Th1 cells after 24 hours of superagonistic stimulation. All secondary cultures were supplied with Dynabeads.

$$\Delta\ \%\ IFN\gamma^+ = \%\ IFN\gamma^+(without\ isoclonic\ control) - \%\ IFN\gamma^+(with\ isoclonic\ control)$$

Results are presented as summarized means ± SD of duplicate analyses and statistically tested with a two-way ANOVA followed by Sidak's test. **(B)** Results were pooled from up to seven independent experiments comparing results with versus without splenocytes. **(C)** Results were pooled from up to three independent experiments comparing CD28-SA-stimulated versus unstimulated samples (0). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001;

optimum around 0.1 µg/ml of CD28-SA was observed (Figure 13C). Due to the lateral binding properties of the superagonistic antibody to CD28, the best crosslinking appears to be realized under these conditions. Like *in vivo*, a dose ten-fold below this optimum (0.01 µg/ml) already induced IFNγ secretion. Additionally, doses above the clear optimum led to decreasing proportions of IFNγ$^+$ cells among the Th1 cells indicating suboptimal stoichiometry probably due to saturation of the available CD28 molecules on the surface of the Th1 cells. Most likely, the characteristic lattice formation of CD28-SA[56] is not possible at high CD28-SA concentrations which results in less signal strength finally leading to a reduced IFNγ response.

To complement the intracellular analysis of IFNγ production, cytokine content of the secondary cultures' supernatant was measured (Figure 14). In line with the former findings, IFNγ concentrations were roughly doubled when Th1 cells were stimulated with CD28-SA in the presence of splenocytes (Figure 14A). However, TNF and IL-6 contents remained similarly high irrespective of the presence of splenocytes indicating that crosslinking was sufficient for induction of these cytokines' secretion (Figure 14B-C). Looking at IL-2 concentrations even a trend to decreased amounts was observed

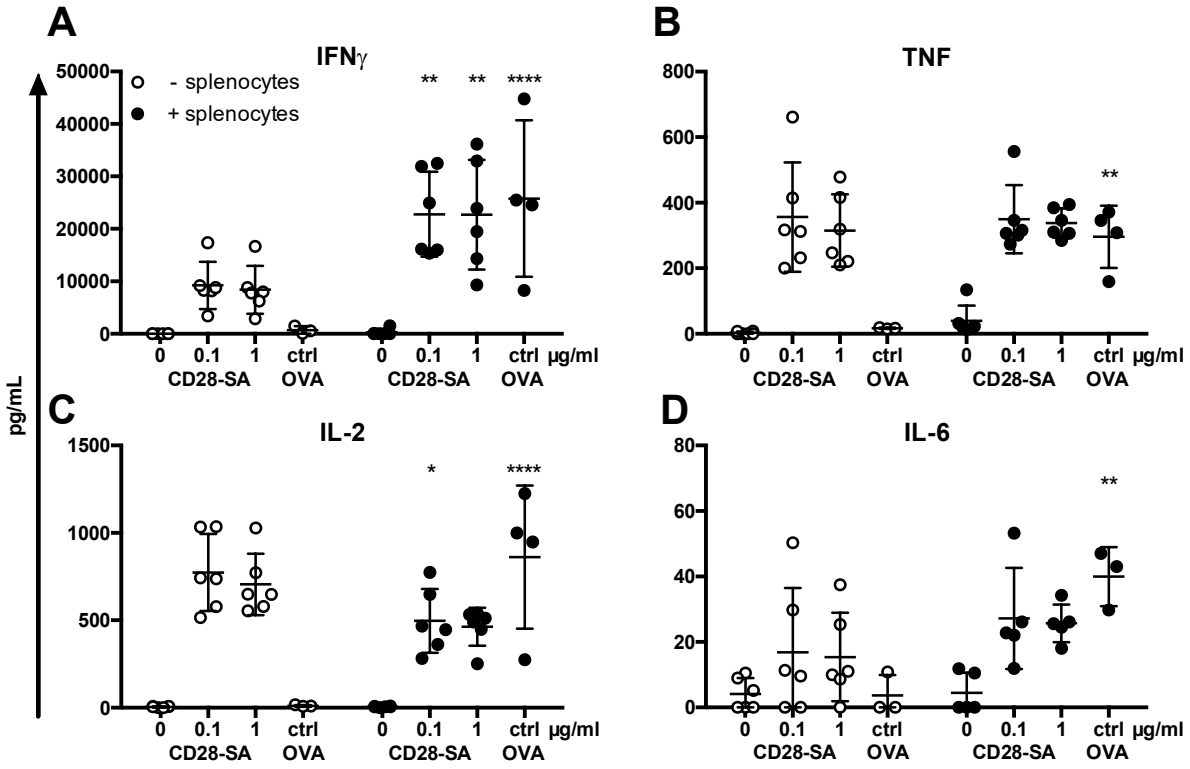

Figure 14: Bystander splenocytes enhance the secretion of IFNγ whereas TNF, IL-2 or IL-6 remain unaffected

Supernatants from secondary cultures containing Dynabeads® Pan Mouse IgG (results from flow cytometry shown in Figure 13) were analyzed after 24 hours for concentrations of **(A)** IFNγ, **(B)** TNF, **(C)** IL-2 and **(D)** IL-6. Results were pooled from up to six experiments showing means ± SD and statistically tested with a two-way ANOVA followed by Sidak's test comparing with versus without splenocytes. *p<0.05; **p0.01; ****p<0.0001.

when CD28-SA stimulation was performed in the presence of WT splenocytes suggesting that bystander splenocytes possibly consumed the IL-2 produced by Th1 cells. Generally, there was no cytokine production induced in the OVA-peptide control in the absence of splenocytes unlike to cultures supplied with splenocytes supporting the concept that the Th1 cell preparations were devoid of functional APCs. Thus, our newly established *in vitro* culture system elucidated that bystander splenocytes selectively enhanced IFNγ secretion by Th1 cells upon CD28-SA stimulation *in vitro*.

4.4 APCs SUBSTANTIALLY AMPLIFY IFNγ SECRETION BY CD28-SA STIMULATED TH1 CELLS

To further dissect the enhancing effect of bystander splenocytes to the Th1 cells' IFNγ response towards CD28-SA stimulation, we selectively depleted other T cells or APCs from the splenocytes prior to secondary cultures. Depletion of T cells did not affect the proportions of IFNγ-producing cells, while APC depletion led to a reduction to a fourth of $IFN\gamma^+$ cells resulting in frequencies of $IFN\gamma^+$ cells (Figure 15A) as low as in the cultures lacking splenocytes, altogether (Figure 13B).

The analysis of the supernatant reflected this reduced IFNγ secretion with unchanged levels of TNF and IL-6 as expected (Figure 15B-E). In line with earlier observations, IL-2 concentrations were raised in the absence of APCs supporting the hypothesis of IL-2 consumption by added splenocytes (Figure 15D). Considering the statistically insignificant but visible trend to reduced net amounts of IL-2 in the T cell-depleted cultures identified splenic T cells as contributors to IL-2 secretion, while the APCs seemed to be the IL-2-consuming population within the splenocytes. Obviously, the drop of all cytokines to the baseline in the OVA-control validated the true APC depletion for the sorted splenocytes. Consequently, APCs within the splenocytes, mainly being B cells and monocytes, contribute potently to enhanced IFNγ release by Th1 cells in response to CD28-SA stimulation *in vitro.*

4.5 CD40-CD40L INTERACTION DRIVES IFNγ PRODUCTION BY TH1 CELLS UPON CD28-SA STIMULATION

As CD40L has been reported to increase IFNγ production by Th1 cells[86], we determined the expression of the ligand on the surface of Th1 cells and, in the same way, its receptor CD40 on B cells in order to uncover the underlying mechanism of the APCs' beneficial effect on the IFNγ secretion.

In response to CD28-SA stimulation as well as to antigenic recall, Th1 cells upregulated CD40L and, as expected, the activation marker CD69 on their surface (Figure 16A). On the other side, activated, CD86-positive B cells were induced to express CD40L after either of the stimulations (Figure 16B).

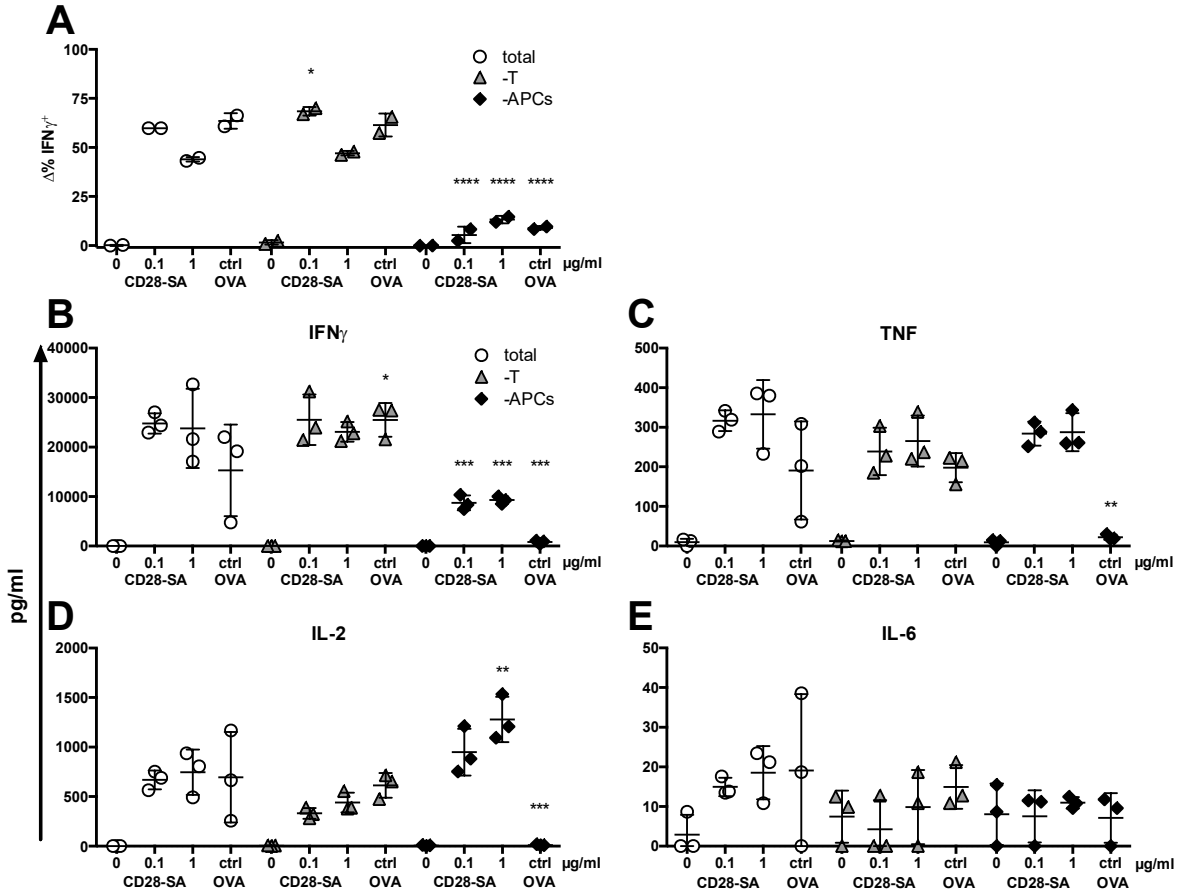

Figure 15: APCs increase the production of IFNγ by Th1 cells in response to CD28-SA stimulation *in vitro*

OT-II Th1 cells were co-cultured in triplicates with sorted splenocytes: either without cell depletion (total) to control for sorting-associated stress or depleted for T cells ($CD3^-$ $CD4^-$) or APCs ($CD11b^-CD45R/B220^-$). **(A)** Net frequencies of $IFN\gamma^+$ cells among Th1 cells 24h after stimulation with CD28-SA or OVA peptide as control. Data of one out of three representative experiments are shown. **(B-E)** Supernatants of cultures shown in (A) were analyzed for **(B)** IFNγ, **(C)** TNF, **(D)** IL-2 and **(E)** IL-6 content. Values from triplicate cultures of one representative experiment are shown.

$$\Delta\ \%\ \mathrm{IFN}\gamma^+ = \%\ \mathrm{IFN}\gamma^+(without\ isoclonic\ control) - \%\ \mathrm{IFN}\gamma^+(with\ isoclonic\ control)$$

Results are presented as means ± SD and were tested using a two-way ANOVA and Dunnett's test (comparison non-depleted 'total' versus depleted splenocytes for every stimulus/concentration analyzed). *p<0.05; **<0.01; ***p<0.001; ****p<0.0001.

Knowing this, we addressed the role of CD40L-CD40 interactions functionally by blockade of the ligand adding an inhibitory anti-CD40L antibody during secondary cultures (Figure 17). Both concentrations used, 1 and 10 µg/ml, completely nullified the IFNγ-response-enhancing effect of the added splenocytes when stimulated with 0.1 µg/ml CD28-SA. A similarly diminished, but not as low a proportion of $IFN\gamma^+$ cells was observed using the tenfold higher dose of CD28-SA (1 µg/ml). The response to the antigenic recall control, in contrast, was unaffected and remained strong showing about two thirds of Th1 cells producing IFNγ. Ultimately, the data identified the surface molecule CD40L as the key player for Th1 cells for successful induction of IFNγ secretion in response to CD28-SA stimulation *in vitro*.

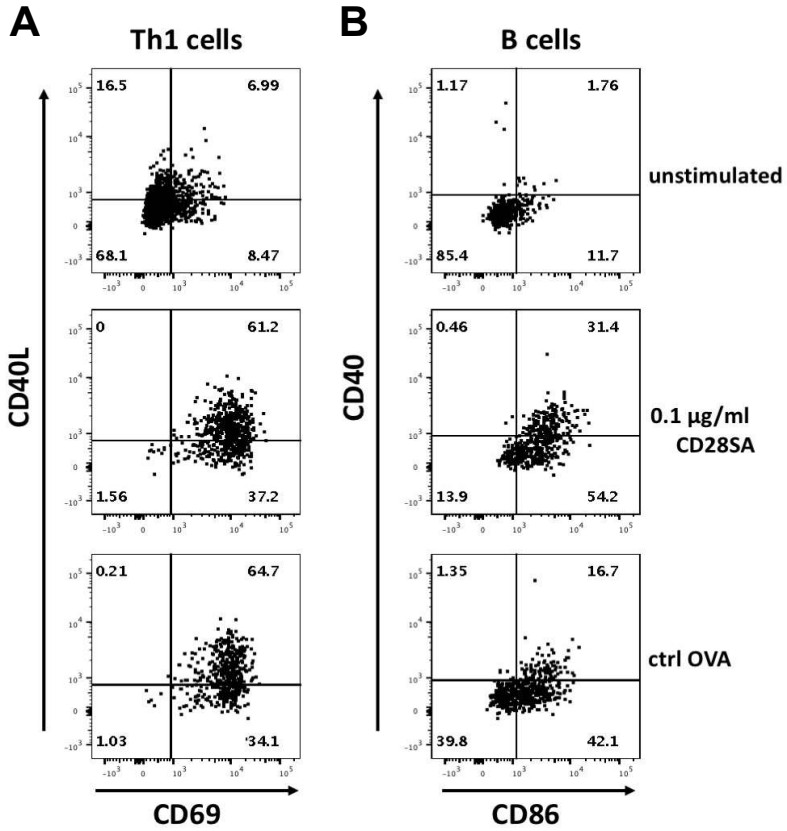

Figure 16: CD28-SA stimulation induces T cells to express CD40L while B cells upregulate CD40 surface expression

Th1 cells together with splenocytes were stimulated for 24 hours with either CD28-SA or OVA peptide as a control in the presence of Dynabeads® Pan Mouse IgG prior to extracellular analysis of **(A)** CD40L and CD69 expression by OT-II Th1 cells. CD40L-positive cells were identified based on CD40L isoclonic control staining (not shown). **(B)** CD40 and CD86 expression by B cells (identified within the viable cell population as $B220^+$).

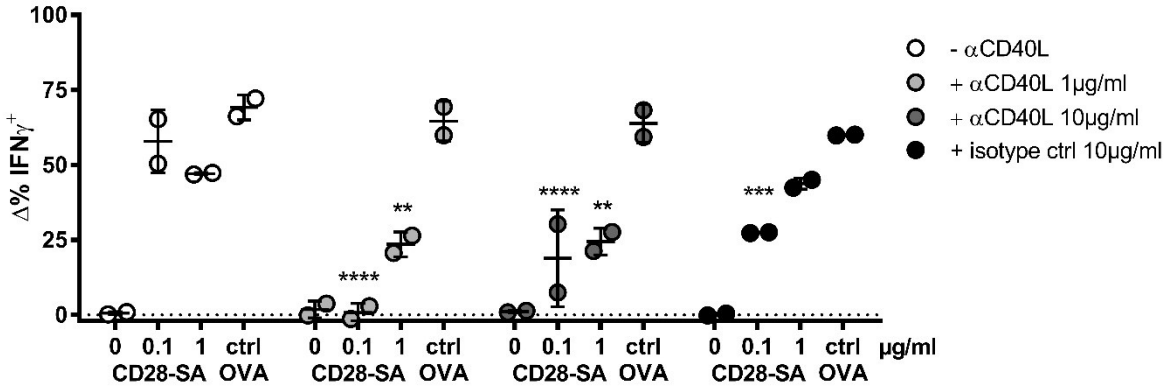

Figure 17: CD40L blockade abrogates the enhancing effect of APCs on IFNγ response by CD28-SA stimulated Th1 cells

Th1 cells were cultured in triplicates in the presence of splenocytes (white). CD40L blockade was performed by adding a blocking αCD40L mAb at 1 µg/ml (light grey) or 10 µg/ml (dark grey). To control for unspecific effects due to antibody addition an isotype control (10 µg/ml) was added (black).

$$\Delta\ \%\ \mathrm{IFN}\gamma^+ = \%\ \mathrm{IFN}\gamma^+(without\ isoclonic\ control) - \%\ \mathrm{IFN}\gamma^+(with\ isoclonic\ control)$$

Results of culture duplicates of one representative experiment are shown as means ± SD and were tested with a two-way ANOVA followed by Dunnett's test (comparison: without versus with αCD40L/isotype control for every concentration/stimulus analyzed). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

5 DISCUSSION

The first-in-man testing of the human superagonistic CD28 antibody TGN1412 in London in 2006 had unexpectedly to be stopped due to unexpected adverse events in all six healthy volunteers[73]. The observed cytokine release syndrome was not predictable from the earlier preclinical testing where rats, mice and cynomolgus macaques were treated with CD28 superagonists resulting in expansion of Treg cells mediating protection from pro-inflammatory processes like autoimmune diseases. With hindsight, memory T cells which are numerously present in immunocompetent humans but mostly lacking in cleanly housed animals, were found to be dose-dependently activated over Treg cells finally leading to the observed cytokine release and the resulting side effects[74,77]. In line with follow-up studies revealing the dose-dependent undesired activation of (tissue-resident) memory T cells besides the beneficial Treg cell activation[78,79], the human CD28-SA, now called TAB08, was tested at much lower doses (0.1-7 µg/ kg BW) in a phase 1a study with healthy volunteers showing a good tolerability without any symptoms of a CRS while IL-10 levels were elevated[79]. This indicated an induction of the appreciable Treg cell activation and suggested a similar mode of action of the CD28-SA at lower dosages as earlier shown in several animal and *in vitro* models[60,68,77,79]. In the subsequent phase 1b trial Rheumatoid Arthritis (RA) patients were treated and a marked improvement of disease symptoms was observed, e.g. about 90% of the treated patients experienced an improvement of at least 20% regarding one of the key symptoms tender and swollen joints[80]. As the expected therapeutic potential of the CD28-SA is not only limited to RA several distinct clinical studies have been started within the past decade, i.e. for treatment of Psoriasis (phase 1, 2016), SLE (phase 2, 2016) and solid neoplasm (phase 1b, 2017)[81].

The failed London trial showed once more the requirement for reliable animal models which allow testing of immunomodulating agents for treatment of patients under conditions resembling the human immune system. Subsequent studies reinvestigating the used preclinical mouse model failed to show that effector/memory T cells were able to react to CD28-SA stimulation with IFNγ secretion[69], which was one of the hallmarks of the human CRS[73] and later also reported for human T cells *in vitro*[77]. In contrast, we could show that, with regard to IFNγ secretion, mouse Th1 cells are indeed comparably responsive towards CD28-SA stimulation *in vivo* and *in vitro* as their human counterparts. Furthermore, we identified the CD40L-CD40 interaction of T cells and APCs as strongly enhancing the IFNγ response of CD28-SA-stimulated Th1 cells *in vitro*.

5.1 SYSTEMIC CYTOKINE RELEASE *IN VIVO* IS COMPATIBLE WITH HUMAN CRS WHILE NO CLINICAL SYMPTOMS ARE OBSERVED

The healthy volunteers within the London trial complained about worsening symptoms like headache, nausea, vomiting and diarrhea within a few hours after administration of the CD28-SA TGN1412/TAB08 developing into a full-blown CRS within less than 24 hours, characterized by hypotension, tachycardia, fever followed by severe respiratory difficulties, lymphopenia and monocytopenia[73]. Blood samples taken 8 hours after infusion showed an abnormal increase in pro-inflammatory cytokines, predominantly IFNγ, TNF, IL-6 and IL-2, indicating an ongoing CRS.

Similarly, our Treg cell-deficient Th1 cell recipient mice showed a considerable, IFNγ-dominated increase in cytokine levels in the serum shortly after CD28-SA treatment (Figure 9 + Figure 11) which is compatible with a CRS. However, these mice seemed clinically unaffected regarding parameters like body weight and general behavior that were monitored on a daily basis (data not shown). This is, of course, in contrast to humans treated with saturating amounts of mAb TGN1412/TAB08[73], dogs treated with 1C6[87], inbred mice born to a "dirty" foster mothers[88] or pigs receiving a newly developed CD28-SA (unpublished data of the Beyersdorf lab). We hypothesize that this difference might be due to a missing pre-activation of innate immunity in our cleanly-housed laboratory mice compared to immunologically well-trained humans, dogs, "dirty" mice and pigs. During a CRS, monocytes were found to secrete catecholamines which were identified as an important and clinically decisive amplification loop involving macrophages[89]. Not having measured their concentration in the serum, we assume that in our model catecholamine levels were not increased or, even if, did not aggravate symptoms since we did not observe any clinical symptoms in CD28-SA-treated mice. There are two mechanisms known to initiate a CRS: while type 1 is target cell-mediated where its secretion of cytokines is induced following binding of the agonist to its respective receptor, type 2 is Fc receptor-dependently induced by binding of an antibody-opsonized target cell[90]. Accordingly, the CD28-SA-induced CRS was originally classified as type 1 as clinical symptoms arise due to the release of cytokines from the targeted T cells and not due to activation of APCs in consequence of Fc receptor crosslinking by the CD28-SA. Our *in vivo* model conforms with a type I initiated CRS despite lacking clinical symptoms. Hence, we assume that stronger Fc receptor-mediated activation of APCs might be required for CD28-SA to cause clinically overt CRS (type 1 and 2).

5.2 TREG CELL DEPLETION IS CRUCIAL IN SPF MICE TO REVEAL EFFECTOR/MEMORY TH1 CELL RESPONSES

In line with an earlier report[69], in the newly described *in vivo* model for CD28-SA treatment, Treg cell depletion was shown to be decisive for successful induction of systemic cytokine release (Figure

9, Figure 10, Figure 11). Considering the Treg cell compartment of laboratory mice kept under SPF conditions, the most obvious difference to the human counterpart is its relative size. While in humans, and for example also pigs, it constitutes less than 5 % of all $CD4^+$ T cells (in blood and sLOs)[91,92], in SPF mice 10-15 % are Treg cells (Figure 6). Besides a numerous superiority of mouse Treg cells compared to human proportions they might also be more suppressive than their human counterparts. Thus, Treg cell depletion is necessary in order to provide an *in vivo* mouse model resembling more "human-like" conditions.

Contrary to the *in vivo* treatment, Treg cell depletion had no impact on *in vitro* responses of Th1 cells to CD28-SA (data not shown): Treg cells within the added splenocytes were depleted which did neither affect IFNγ production by Th1 cells measured intracellularly nor the secretion of cytokines determined in the culture supernatants. Most likely, this might result from the mere CD28 stimulation which is insufficient to initiate Treg cell suppression *in vitro* without TCR ligation[59]. In addition to less cell-cell contact *in vitro*, Treg cells encounter a comparatively low abundance of autoantigens *in vitro* that deliver tonic TCR signaling *in vivo* – a prerequisite for CD28-SA stimulation. Furthermore, the isolation procedure in order to obtain splenocytes might probably impair Treg cell functions.

5.3 DOSE-RESPONSE RELATIONSHIPS *IN VIVO* AND *IN VITRO* DIFFER IN MICE AND HUMANS

For a suitable animal model, a dose-response relationship closely mirroring the reaction of the human immune system is desirable. In the newly described animal model for CD28-SA treatment of mice, the observed dose-response relationship seemed to resemble the human curve: an about tenfold rise of the applied amount of CD28-SA was sufficient to escalate from minimally detectable increases in systemic cytokines to very high concentrations in the serum (Figure 11). However, considering the absolute dose of CD28-SA inducing cytokine release, the human and mouse system appear quite divergent: while during the London trial 0.1 mg/kg BW led to a critical CRS[73] we applied 150 µg/mouse resulting in an IFNγ-dominated cytokine release. This corresponds to an estimated dose of about 6 mg/kg BW supposing an average weight of 25 g BW per mouse. In fact, this is a 60-fold higher dose which is required for the induction of a CRS-like immune reaction to the mouse CD28-SA as compared to the human. Most likely, this difference originates from the different affinities both CD28-SA display for Fc receptors. The human CD28-SA TGN1412/TAB08 belongs to the isotype class of IgG_4, exhibiting a much higher affinity for Fc receptors than the mouse CD28-SA D665 which is of the IgG_1 isotype[93]. This assumption is supported by the observation that the *in vitro* activation of CD28-SA-stimulated Th1 cells, marked by IFNγ production, was much more pronounced in the presence of Dynabeads® Pan

Mouse IgG suggesting a requirement for enhanced crosslinking to induce optimal activation by the CD28-SA.

Considering the dose-response curve in the *in vitro* system supplied with splenocytes, a bell-shaped curve was observed with the maximum IFNγ production around 0.1 µg/ml (Figure 13C). Concretely, concentrations below or above the optimum resulted in a less pronounced IFNγ response. Regarding the lower concentrations, this demonstrates the appreciable effect the application is aiming at: No or only minor amounts of cytokines are released while sustained activation of Treg cells is achieved as has been shown for cells from RA patients *in vitro*[79,80]. In the *in vitro* system there was a similar effect of low-level cytokine release when relatively high concentrations of CD28-SA were applied. Most likely, this observation is based on stoichiometry: while the usage of intermediate doses of the SA achieves an apparently perfect crosslinking of numerous CD28 surface molecules by bivalent binding of the CD28-SA, leading to lattice formation, higher or lower concentrations lead to insufficient crosslinking due to saturation issues (Figure 18).

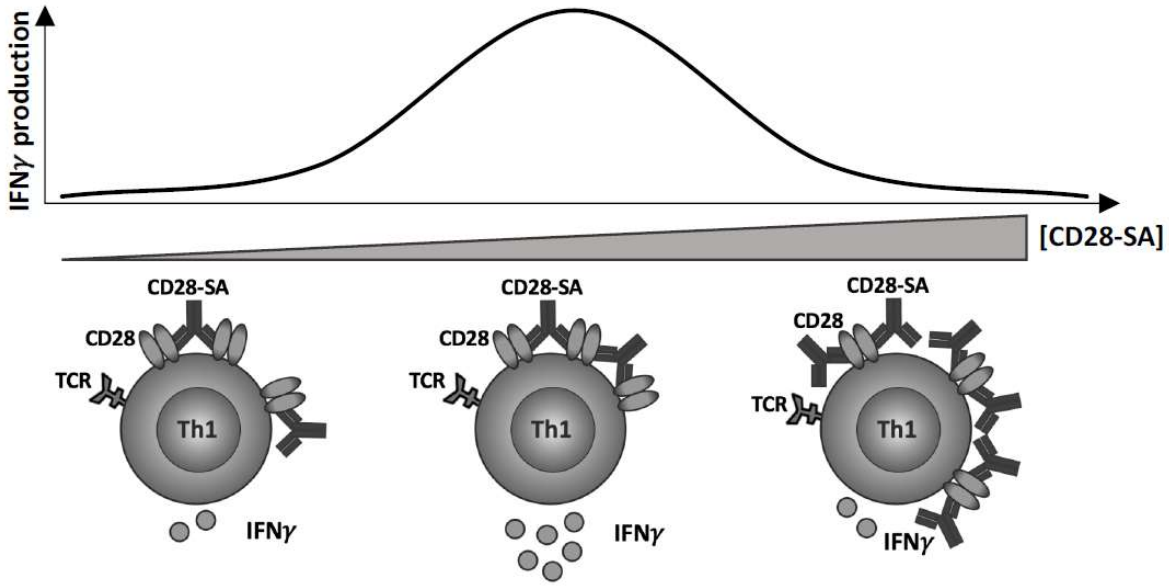

Figure 18: Dose-response curve of CD28-SA stimulation of Th1 cells *in vitro* shows a bell-shaped relationship

Th1 cells respond with IFNγ secretion to superagonistic CD28 stimulation *in vitro* in a dose-dependent manner. While relatively low as well as high concentrations of the SA induce only a moderate IFNγ response, intermediate concentrations trigger the maximum response by Th1 cells, probably due to stoichiometry issues.

In case of low dosage application only few CD28 molecules are bound resulting in less signal strength transmitted intracellularly and hardly any cytokine release. Otherwise, if saturating concentrations of the CD28-SA were used, single CD28-SA molecules would probably bind the costimulatory receptor monovalently resulting in reduced signal strength transmitted intracellularly and decreased cytokine release, consequently.

5.4 KINETICS OF IFNγ RESPONSE ARE DELAYED DURING *IN VITRO* STIMULATION AS COMPARED TO *IN VIVO* CD28-SA APPLICATION

While *in vivo* the cytokine release peaked at around four to six hours after CD28-SA treatment, *in vitro* there is a delayed IFNγ response observed that reached its maximum at around 24 hours after the stimulation with CD28-SA was started. Furthermore, an optimal response to CD28-SA induced *in vitro* required addition of splenocytes, more precisely APCs, since Th1 cells on their own were much less responsive in terms of IFNγ production and pro-inflammatory cytokine release (Figure 13 and Figure 14). In fact, adding splenocytes increased the proportion of IFNγ-producing cells among the Th1 cells from around 25% to up to 75% (Figure 13). Notably, splenocytes alone were not capable to react upon CD28-SA stimulation for 24 hours with cytokine secretion (data not shown) suggesting that the increased cytokine secretion in the presence of splenocytes is rather a consequence of their supporting influence on Th1 cells than due to a reaction of the splenocytes themselves. Additionally, for a maximum IFNγ response to CD28-SA by Th1 cells *in vitro*, sufficient crosslinking of CD28-SA on the cell surface was essential. This prerequisite was provided by Dynabeads Pan Mouse ® which were added after having observed that this function could only weakly be accomplished by freshly added splenocytes. These discrepancies between the *in vitro* system and the *in vivo* model might probably be based on several issues. The isolation procedure might affect the composition of cell populations and the integrity of their cellular surface as well as the conformation and functionality of the present receptors and ligands. For instance, the Fc receptors on the surface of the isolated splenocytes might be impacted which could possibly explain why added splenocytes were not able to provide enough crosslinking in contrast to *in vivo*. Of note, DCs express the Fc receptor FcγRIII, which binds IgG_1,[93] and thereby presumably contribute significantly to crosslinking of CD28-SA *in vivo*. Certainly, DCs are extremely sensitive to mechanical stimuli and hence might be mostly lost during isolation procedures when spleens are grinded through a cell strainer. The mechanical procedure might cause harm to the sensitive dendrites characteristic for DCs thereby impairing their functions or even losing the cells within the splenocytes. To obtain an intact population of DCs from spleens more refined isolation protocols are required[94]. Finally, this results in an altered composition and function of isolated

splenocytes which could account for the lacking crosslinking properties as well as the delayed kinetics. To test whether this assumption holds true, Th1 cell secondary cultures could be provided with *in vitro* differentiated bone marrow-derived DCs[95] instead of or in addition to splenocytes. As these DCs are shown to be physiologically functional, sufficient crosslinking for an optimal IFNγ response by Th1 cells like *in vivo* is expected which would be reflected by faster kinetics and reduced requirement for Dynabeads *in vitro*.

Moreover, the influence of the innate immune system should not be disregarded considering that it is a major actor initiating a cytokine release syndrome. For instance, macrophages secrete IL-6 and TNF that trigger the liver to produce acute-phase-protein which play a major role in CRS[96]. In the established *in vitro* assay, neither the role of the innate immune system nor the role of further organs like the liver are taken into account.

5.5 CD40L-CD40 INTERACTION IS ESSENTIAL FOR INDUCTION OF IFNγ RESPONSE OF TH1 CELLS *IN VITRO*

As described above (4.4), APCs within the splenocytes were the key player for successful induction of an optimal IFNγ response by Th1 cells stimulated with CD28-SA *in vitro*. Several mechanisms were considered as the underlying molecular basis. The most obvious seemed to be an MHC-II-dependent amplification of the tonic TCR signaling by the added splenocytes. To test this hypothesis, distinct approaches were used (data not shown): On the one hand MHC-II-deficient mice were employed as donor of the splenocytes while on the other hand the direct interaction with the co-receptor CD4 was disabled by the usage of an MHC-II-blocking mAb (Clone Y3P). The latter approach was performed in different settings: blockade of the interaction by addition of either the whole antibody or a Fab fragment directly into the secondary culture as well as blockade of surface MHC-II with a blocking mAb prior to secondary culture. Furthermore, a CD86-dependent manner of the enhanced IFNγ response upon CD28-SA application was supposed due to its capability to bind CD28 as a ligand. Accordingly, CD86 was functionally neutralized as CD28-ligand by application of a blocking mAb (Clone GL1) either prior to or directly within the secondary culture. None of the mentioned approaches reduced the capacity of splenic APCs to increase IFNγ secretion by Th1 cells upon CD28-SA stimulation.

Experiments targeting the CD40-CD40L-interaction revealed a crucial role during the induction of Th1 cells' IFNγ response to CD28-SA treatment *in vitro*. CD40L is expressed on activated T cells[97–99] and has been shown to induce IFNγ production in Th1 cells[86]. When adding an antibody (clone MR-1) blocking the capability of CD40L to bind to its receptor expressed on APCs the beneficial effect of splenic APCs was abolished resulting in basal levels of IFNγ production by Th1 cells equally observed in the absence of splenic APCs or total splenocytes (Figure 15 and Figure 13). In line with the aforementioned

report[86], secretion of the pro-inflammatory cytokine IL-6 remained unchanged irrespective of the presence of APCs within the splenocytes (Figure 15) while IFNγ levels were increased only in the presence of APCs supporting the key role of CD40L signaling in the newly established *in vitro* model.

Furthermore, it was revealed that mouse $CD4^+$ memory T cells, including Th1 cells, contain preformed CD40L in lysosomal vesicles that allows rapid translocation to the cell surface upon antigen recognition where it can interact with CD40 on APCs, which holds true for *in vivo* as well as *in vitro* responses[100,101]. Interestingly, for human PHA-stimulated PBMCs CD40L-CD40 interactions have been identified as a major enhancer for IFNγ release induced by CD28 stimulation[102]. These findings support the CD40L-dependent manner of the increased IFNγ response to CD28-SA stimulation observed in our study.

Within the scope of this work there is no data available regarding the expression of CD40L on the transferred Th1 cells *in vivo*, its relevance for the *in vivo* response upon CD28-SA treatment can be assumed to be highly decisive. In fact, this hypothesis is supported by reporting that CD40L-deficient mice exhibit impaired antigen-specific T cell responses revealing the indispensability of CD40L for *in vivo* priming of $CD4^+$ T cells[103]. Consistent with our results, CD40L expression on activated T cells was reported to start about two hours post activation, peaking about six hours, and is reduced to basal levels about 24 hours after activation[104]. However, whether CD40L signaling is as essential for the *in vivo* response to CD28-SA treatment in the newly established Th1 cell transfer model as observed *in vitro* remains elusive. To address this question, *in vivo* blockade of CD40L by injection of the anti-CD40L mAb MR-1 used *in vitro* could be performed. However, as already indicated by several clinical trials targeting this ligand in order to ameliorate autoimmune diseases, thromboembolic complications are a major concern here[105–109]. This is believed to be based on platelet aggregation and resulting thrombosis since CD40L is not exclusively expressed by activated T cells but also on platelets together with the FcγRIIa (CD32), which most likely are both bound by anti-CD40L mAbs on adjacent platelets[110,111]. This Fc-dependent adverse event can be circumvented by the usage of either Fc-silenced or IgG_4 mAbs, which induce less Fc receptor-dependent effector mechanisms, or Fab fragments. These formats of CD40L-blocking mAbs are currently tested in several clinical trials and appear to be promising therapeutics for treatment of autoimmune diseases, i.e. SLE[112], RA[113]. Administration of a CD40L-antagonist during CD28-SA responses *in vivo* would elucidate the role of CD40L for the IFNγ response of Th1 cells shown in this study.

Taken together we propose bidirectional interactions upon CD28-SA treatment leading to the observed IFNγ response (Figure 19): The superagonistic mAb triggers (re-)activation of Th1 cells via CD28 engagement inducing an IFNγ dominated cytokine release concomitantly with increasingly expressed CD40L signals – due to the ligation of its receptor found on APCs – that amplify the initiated response marked by further enhanced IFNγ secretion.

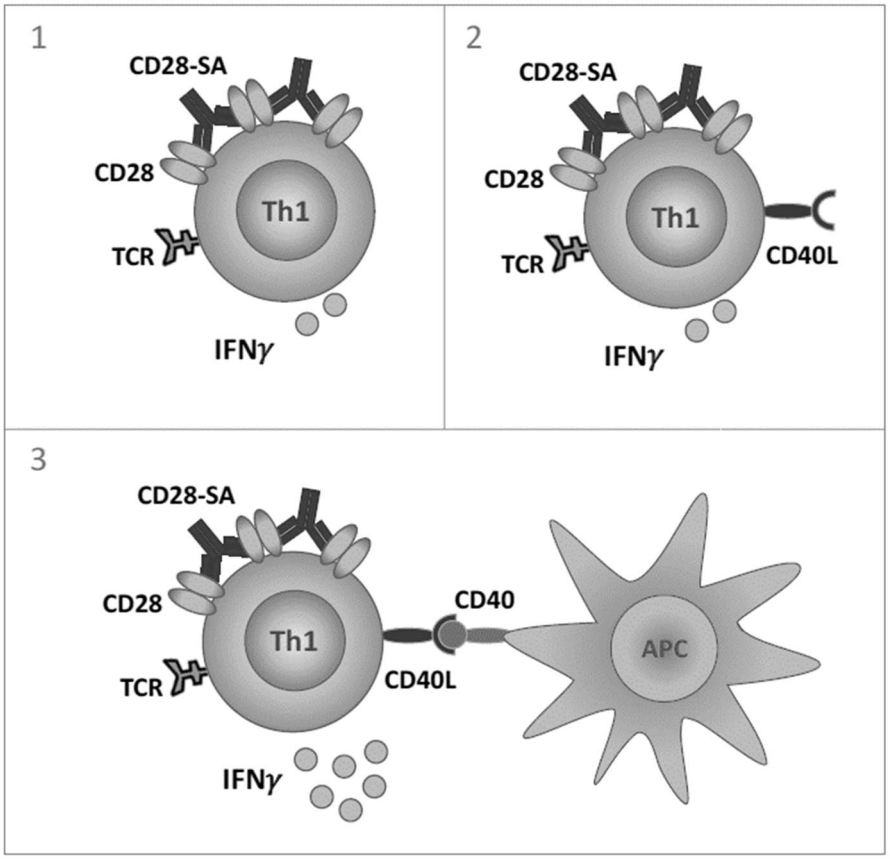

Figure 19: CD40L signaling enhances IFNγ response by Th1 cells upon superagonistic CD28 stimulation

Predicted model depicting the role of CD40-CD40L interactions on Th1 cells during CD28-SA stimulation leading to an enhanced IFNγ response.

(1) Crosslinking of CD28 via CD28-SA induces Th1 cells to secrete several cytokines, predominantly IFNγ. **(2)** Consequently, activated Th1 cells upregulate the surface expression of CD40L. **(3)** The receptor CD40, expressed constitutively on the added splenic APCs, is engaged by its ligand CD40L inducing intracellular signaling via the ligand into the presenting Th1 cell resulting in an increased secretion of IFNγ. Illustration of the molecular processes are simplified and Dynabeads Pan Mouse IgG® were not depicted for the sake of clearness despite their indispensable function for crosslinking of the CD28-SA.

5.6 DOES THIS NEWLY ESTABLISHED MODEL PROVIDE A SUITABLE PRECLINICAL MODEL?

The unexpected course of the London trial where the humanized CD28-SA TGN1412/TAB08 was administered to healthy volunteers highlighted that preclinical animal testing required more refinement. For immunotherapeutic approaches, cleanly housed, immunologically untrained mice were demonstrated to be inadequate. Consequently, we reconsidered the so far used animal model in mice and addressed the issue of how to improve a preclinical model in terms of reliability of predictions.

Since (tissue-resident) $CD4^+$ memory and effector T cells were identified as the main source of the cytokines released upon CD28-SA treatment of the volunteers, the lack of a true memory/effector $CD4^+$ T cell compartment in cleanly housed rodents represents a major immunological difference between the species. In order to provide the immunologically unexperienced SPF mice with a memory/effector $CD4^+$ T cell compartment, Th1 cells were transferred that were antigen-specifically differentiated using *in vitro* cell culture (Figure 4) followed by CD28-SA administration and subsequent blood sampling. This newly described mouse model for CD28-SA application reproduced the immediate and tremendous cytokine release that was observed during the London trial. Compared to earlier employed models, this new Th1 cell transfer-based model resembles the human reaction more closely and is, therefore, more predictive displaying a major advantage. However, the absence of clinical symptoms in the mice is a drawback when it comes to prediction and assessment of tolerance and undesired side effects prior to a clinical trial. In order to obtain a totally predictive model showing identical reactions comparing the two species even more refinement is required.

As the *in vivo*-model is based on adoptive transfer of *in vitro*-generated Th1 cells originating from OT II TCR-transgenic donor mice, the established memory/effector $CD4^+$ T cell compartment in the recipient mice is exclusively monoclonal. Considering that in immunocompetent individuals the present helper T cells are polyclonal, the transferred Th1 cell-based memory/effector compartment might appear artificial. To overcome this disadvantage of the model, polyclonally activated T cells could be transferred, then maybe also comprising further Th cell subsets, i.e. Th2 and Th17 cells, which would result in a much more human-like immune system than the one present in cleanly housed laboratory mice. Another conceivable possibility would be *in vivo* priming, e.g. using innocuous pathogens or superantigens. While this priming of the immune system would be less artificial, it is associated with further issues regarding pathogen containment within the facility, involvement of further immune reactions not necessarily being induced by the agent to be tested but the priming. Although the adoptive transfer itself might be considered as artificial, one major advantage of the adoptive cell transfer is that it constitutes a reliable method to induce a non-naïve immune system in the mice. Induction of a

diverse T cell memory *in vivo* could also be achieved by treatment with an anti-CD3 mAb resulting in a long-term T cell memory formation[114].

A further approach to adapt laboratory mice to humans was taken creating so-called "wildlings" by transferring embryos originating from SPF mice into wild mice caught in horse stables[88]. These wildlings were also treated with CD28-SA and resembled the human cytokine release similarly to the Th1 transfer model. The authors did not comment on any other results of their experiments regarding clinical symptoms. In terms of validity, reproducibility and translationability these two approaches provide an essential progress for immunological research and preclinical studies. However, one major advantage of the adoptive cell transfer is an easy checkable and manageable immune status of the mice allowing to keep the recipients and the whole colony in the same facility which is not practicable with wildlings due to the risk of pathogen spreading.

The *in vitro* model mimics quite closely the *in vivo* situation allowing to study further aspects in more detail. As *in vitro* models of human cells are of major importance for preclinical studies, the newly established model could provide even more preclinical data when performed with human material. To do so, Th1 cells could be expanded from isolated human PBMCs as described earlier[29] using PPD as a Th1 cell stimulating antigen[115]. Subsequently, during secondary culture human Th1 cells will be stimulated with CD28-SA and cytokine production could be determined to allow evaluation of the translationability of this model compared to the formerly proposed model using high density preculture[77].

In summary, the newly established mouse *in vivo* and *in vitro* model for CD28 stimulation by a superagnostic mAb revealed that mouse Th1 cells are in principle as responsive towards CD28-SA stimulation as human T cells. In the future, employing the new models will allow to study the potential of novel immunotherapeutic agents under more "human-like" conditions enabling researchers to predict human immune reactions more reliably. Confidently, application of the models and the gained insights will facilitate the development of the lately more and more emerging and promising immunotherapeutic approaches for a variety of diseases employing the patients' own immune system by either stimulating or silencing the required mechanisms.

6 TABLE OF FIGURES

7 ABBREVIATIONS

°C	grade Celsius
ANOVA	analysis of variance
APC	antigen presenting cell
APC	Allophycocyanin
BAC	bacterial artificial chromosome
BLL	B cell chronic lymphocytic leukemia
BSA	bovine serum albumin
BSS	balanced salt solution
BW	body weight
CD	cluster of differentiation
CRS	cytokine release syndrome
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
ctrl	control
Cy	Cyanine
DC	dendritic cell
ddH_2O	demineralized water
DEREG	Depleter of regulatory T cells
DT	Diphtheria toxin
et al	lat. *et alii*, and others
e.g.	lat. *exempli gratia*, for example
FACS	fluorescence activated cell sorting, flow cytometry
Fab	antigen-binding fragment of an antibody
FITC	Fluorescein Isothiocyanate

FSC	forward scatter
g	gram
g	gravitational acceleration
Gata-3	GATA-binding protein *3*
h	hour
i.e.	lat. *id est*, that is
IFNγ	interferon γ
Ig	immunoglobulin
IL	Interleukin
JAK	Januskinase
mAb	monoclonal antibody
MFI	median fluorescence intensity
MHC	major histocompatibility complex
min	minute
ml	milliliter
NK	natural killer cell
nmIg	normal mouse Immunoglobulin
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PD	programmed death
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll
PMA	Phorbol 12-myristate 13-acetate

PRR	pattern recognition receptor
RA	Rheumatoid Arthritis
rpm	revolutions per minute
RT	room temperature
SA	superagonist
SD	standard deviation
SLE	Systemic Lupus Erythematosus
sLO	secondary lymphoid organ
SPF	specific-pathogen-free
SSC	side scatter
STAT	Signal Transducer and Activator of Transcription proteins
T-bet	T-box containing protein expressed in T cells
T_{CM}	central memory T cells
TCR	T cell receptor
T_{EM}	effector memory T cells
TF	transcription factor
TGF-β	transforming growth factor β
Th	T helper cells
TNF	tumor necrosis factor
VD	viability Dye
WT	wild type
α	alpha; anti
µg	microgram
µl	microliter
µM	micromolar

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9 APPENDIX

9.1 CURRICULUM VITAE

Affidavit

I hereby confirm that my thesis entitled “A novel mouse model for systemic cytokine release upon treatment with a superagonistic anti-CD28 antibody” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I verify that this thesis has not yet been submitted as part of another examination process neither in identical nor similar form.

Place, Date

Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Ein neues Mausmodell zur Untersuchung der Zytokinfreisetzung nach Behandlung mit einem superagonistischen anti-CD28 Antikörper“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum

Unterschrift