Technische Universität München
TUM School of Medicine and Health

A disease modifying treatment for psoriasis
-
anti-Interleukin-23 preferentially inhibits pathogenic T helper 17 cells

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Zusammenfassung

Die Psoriasis ist eine systemische Erkrankung, welche sich nicht nur an der Haut, sondern auch an den Nägeln sowie den Gelenken manifestieren kann. Abgesehen davon umfasst sie zahlreiche charakteristische Komorbiditäten und kann auf Grund ihrer auffälligen Hautmanifestationen zu Stigmatisierung und infolgedessen auch zur psychosozialen Beeinträchtigung der Patienten führen. Eine Auswertung von Sekundärdaten einer gesetzlichen Krankenversicherung im Jahr 2005 ergab eine Prävalenzrate von 2,5% in der deutschen Gesamtbevölkerung, wonach sich etwa 2 Millionen Deutsche auf Grund einer Psoriasis in ärztlicher Behandlung befanden. Demzufolge handelt es sich bei der Psoriasis um eine interdisziplinäre und von der Weltgesundheitsorganisation (WHO) als chronisch anerkannte Volkskrankheit, welche auch für unser Gesundheitssystem eine ökonomische Belastung darstellt und effizienter sowie innovativer Therapie bedarf (Schäfer 2011).

Forschungsergebnisse der letzten zwei Dekaden ordnen der Interleukin (IL)-23/ T Helfer 17 (T_H17) Achse und dem von den T_H17 Zellen produzierten IL-17 eine entscheidende Rolle in der Pathogenese der Psoriasis zu. Nicht zuletzt wurde dies durch die Effizienz neuartiger Biologika in der Psoriasis Therapie hervorgehoben. Hierbei handelt es sich um spezifische, gegen Zytokine gerichtete Antikörper, welche durch die Unterbrechung der Immunzellkommunikation die Autoimmunität unterbinden. Überzeugende Ergebnisse mit sogar bei sehr schwerer Krankheitslast nahezu erscheinungsfreier Haut und vollständiger Remission lieferten Tumornekrosefaktor (TNF)-α, IL-17 und IL-23 Inhibitoren. Letztere führten zu Ergebnissen bei Probanden, welche trotz Absetzen der Therapie beziehungsweise Verlängerung des Injektionsabstandes einen langanhaltenden Zustand der Vollremission aufrechterhielten. Die erstaunliche Wirksamkeit der IL-23 Inhibition und die Suche nach einer Erklärung für die langanhaltenden Effekte der Therapie im Sinne einer Krankheitsmodifikation gaben Anlass zu der Erarbeitung der Forschungsinhalte dieser Doktorarbeit. Schon zuvor gab es Publikationen, welche eine dichotome Natur der T_H17 Zelle thematisierten. Es wurde gezeigt, dass einerseits Subtypen existieren, welche ein pathogenes Zytokinmuster exprimieren und autoreaktive Mechanismen unterhalten. Andererseits wurden nicht-pathogene Subtypen beschrieben, welche durch die Sekretion von IL-10 in der Lage sind, die selbst-gerichtete Autoimmunantwort zu unterbinden.

Die Hypothese entstand, dass sich diese beiden Subtypen in einer Art Homöostase befinden, welche im Rahmen der Psoriasis-Pathogenese zugunsten der pathogenen T_H17 Zelle verschoben ist. Die langanhaltenden Effekte der IL-23 Inhibitoren könnten

somit möglicherweise durch eine therapieinduzierte Verschiebung der Balance in Richtung des nicht-pathogenen Schenkels der T_H17 Zellen erklärt werden.

Zur Überprüfung dieser Hypothese wurde in dieser Doktorarbeit die Auswirkung des IL-23 Entzugs auf die zwei verschiedenen T_H17 Phänotypen untersucht. Hierzu wurden sowohl Effektor- als auch naïve T-Zellen mehrerer Spender isoliert und in vitro unter IL-23 Entzug stimuliert beziehungsweise differenziert. Es konnte gezeigt werden, dass IL-23 essenziell für das das Überleben und die Stabilität beider T_H17 Subtypen ist, wobei nicht-pathogene Mitglieder der T_H17 Familie den IL-23 Entzug besser tolerieren und damit eine geringere Sensitivität für den IL-23 Entzug, verglichen mit dem pathogenen T_H17 Subtyp, aufweisen. Dies ließ sich durch eine geringere Reduktion der Zellzahlen im Verhältnis zur Ausgangspopulation zeigen. Darüber hinaus war es nicht möglich eine große Anzahl nicht-pathogener T_H17 Zellen aus naïven T-Zellen zu differenzieren, was darauf hindeuten könnte, dass nicht-pathogene Zellen ein Resultat von T-Zell Plastizität sind und aus ausdifferenzierten T_H17 Zellen entstehen.

Zusammenfassend kann man sagen, dass der Einsatz von IL-23 Inhibitoren in der Lage ist effizient pathogene T_H17 Zellen zu reduzieren. Hierbei lassen meine Ergebnisse darauf schließen, dass nicht-pathogene Subtypen eine höhere Widerstandfähigkeit haben, wodurch das Verhältnis der verschiedenen Phänotypen zueinander verändert wird.

Summary

Because of its manifestations beyond the skin, joints and nails and its association with numerous characteristic comorbidities, psoriasis represents a systemic disease which can lead to stigmatization and subsequent psychosocial impairment of the patients. An evaluation of statutory health insurance data in 2005 in the German population revealed a prevalence rate of 2.5%. It was reported that around 2 million Germans were receiving medical treatment for psoriasis. This makes psoriasis an interdisciplinary and chronic widespread disease recognized by the World Health Organization (WHO), which also represents an economic burden for our health system and requires efficient and innovative therapy (Schäfer 2011).

Research results of the last two decades assign a decisive role in the pathogenesis of psoriasis to the Interleukin (IL)-23/ T helper 17 (T_H17) axis and to IL-17 produced by T_H17 cells. This is highlighted by the efficacy of novel biologics recently approved for treatment of psoriasis. Here, specific antibodies are directed against cytokines that prevent autoimmunity by interrupting immune cell communication. Tumor necrosis factor (TNF)-α, IL-17 and IL-23 inhibitors provided convincing results with almost clear skin and complete remission even in very severe disease burden. The latter even led to a long-lasting state of complete remission in some patients despite discontinuation of therapy or prolongation of the injection interval. The high efficacy of IL-23 inhibition and the search for an explanation for the long-lasting effects of the therapy in terms of disease modification prompted the development of the research content of this doctoral thesis. There are already publications that address the dichotomous nature of T_H17 cells. It was shown that on the one hand there are subtypes that express a pathogenic cytokine pattern and maintain autoreactive mechanisms. On the other hand, non-pathogenic subtypes have been described which are able to suppress the self-directed autoimmune response by secreting IL-10.

The hypothesis arose that these two subtypes are in homeostasis which is shifted in favor of the pathogenic T_H17 cells within the pathogenesis of psoriasis. Thus, the long-lasting effects of IL-23 inhibitors could potentially not only be explained by a reduction of T_H17 cells in general, but rather by a therapy-induced shift of the balance in favor of the non-pathogenic branch of T_H17 cells.

To test this hypothesis, the effect of IL-23 withdrawal on the two different T_H17 phenotypes was investigated in this thesis. For this purpose, both effector T-cells and naïve T-cells were isolated from several donors and stimulated or differentiated *in vitro* under IL-23 deprivation. IL-23 was shown to be essential for the survival and stability of

both T_H17 subtypes, whereby non-pathogenic members of the T_H17 family tolerate IL-23 deprivation better and thus show a lower sensitivity for IL-23 deprivation compared to the pathogenic T_H17 subtype. This was demonstrated by a smaller reduction in cell numbers compared to the initial population. Furthermore, it was not possible to differentiate large numbers of non-pathogenic T_H17 cells from naïve T-cells, suggesting that non-pathogenic cells are a result of T-cell plasticity and arise from fully differentiated cells.

In conclusion, the use of IL-23 inhibitors is able to efficiently reduce pathogenic T_H17 cells. My results indicate that non-pathogenic subtypes have a higher resistance, which in turn changes the relation between the different phenotypes.

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List of Abbreviations

ADAMTSL5 ..... Disintegrin-like and metalloprotease domain containing thrombospondin type 1 motif-like 5
AIM ..... Apoptosis inhibitor of macrophage
BATF ..... Basic leucine zipper activating-transcription-factor-like transcription factor
BCA ..... Bicinchoninic acid
BSA ..... Bovine serum albumine
C. albicans ..... Candida albicans
CD ..... Cluster of Differentiation
CD5l ..... CD5 antigen-like
c-MAF ..... Musculoaponeurotic fibrosarcoma oncogene homolog
CO_2 ..... Carbon dioxide
DLQI ..... Dermatology life quality index
DMSO ..... Dimethylsulfoxid
DNA ..... Desoxyribonucleic acid
DPBS ..... Dulbecco's phosphate buffered saline
EDTA ..... Ethylendiaminetetraacetic acid
ELISA ..... Enzyme-linked immunosorbent assay
FCS ..... Fetal calf serum
FSC ..... Forward Scatter
GM-CSF ..... Granulocyte-macrophage colony-stimulating factor
Gpr65 ..... G protein-coupled receptor 65
$HIF1^{\alpha}$ ..... Hypoxia inducible factor 1α
hnRNP-A1 ..... Heterogeneous nuclear ribonucleoprotein A1
HS ..... Human serum
IBD ..... Inflammatory bowel diseases
IFN ..... Interferon
IFNAR1 ..... Interferon-alpha/beta receptor alpha chain
IFNAR2 ..... Interferon-alpha/beta receptor beta chain
IL ..... Interleukin
IL-12Rβ1 ..... Interleukin-12 receptor β1
IL-23R ..... Interleukin-23 receptor
iNOS ..... Inducible nitric oxide synthase
IRF4 ..... Interferon regulatory factor 4
JAK ..... Janus kinase
LB ..... Lysogeny broth
MACS ..... Magnetic activated cell sorting
mDC ..... Myeloid dendritic cells
MFI ..... Mean fluorescence intensity
mRNA ..... Messenger RNA
MS ..... Multiple sclerosis
MTX ..... Methotrexate
non-path. ..... Non-pathogenic polarization approach
PASI ..... Psoriasis Area and Severity Index
PASI 100 ..... Improvement of PASI by 100%
PASI 75 ..... Improvement of PASI by 75%
PASI 90 ..... Improvement of PASI by 90%
path. ..... Pathogenic polarization approach
PBMC ..... Peripheral blood mononuclear cell
pDC ..... Plasmacytoid dendritic cells
PHA ..... Phytohemagglutinin
PLA2G4D ..... Phospholipase A2 group IVD
Plzp ..... Promyelocytic leukemia zinc finger-like zinc finger protein
PMA ..... Phorphol 12-myristate 13-acetate
PROCR ..... Protein C receptor
PSORS ..... Psoriasis susceptibility genes
qRT-PCR ..... Quantitative real-time polymerase chain reaction
RA ..... Rheumatoid arthritis
RBC ..... Red blood cell
RBPJ ..... Recombinant signal binding protein for immunoglobulin kappa J region
RNA ..... Ribonucleic acid
ROR ..... Receptor-related orphan nuclear receptor
RPMI ..... Roswell Park Memorial Institute medium 1640
S. aureus ..... Staphylococcus aureus
SSC ..... Side Scatter
STAT ..... Signal transducer and activator of transcription
T-bet ..... T-box expressed in T-cells
TCR ..... T-cell receptor

T_{eff} ..... Culture of effector T-cells
Tfh ..... T follicular helper
TGF ..... Transforming growth factor
T_H1 ..... T helper 1
T_H17 ..... T helper 17
T_H2 ..... T helper 2
T_H22 ..... T helper 22
T_H9 ..... T helper 9
TIP-DCs ..... TNF/iNOS producing dendritic cells
TLR ..... Toll like receptor
TMB ..... Tetramethylbenzidine
$T_{naïve}$ ..... Culture of naïve T-cells
TNF ..... Tumor necrosis factor
T_{reg} ..... Regulatory T
w/o ..... Without
WHO ..... World Health Organization
YNB ..... Yeast Nitrogen Base

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1. Introduction

1.1. Psoriasis

1.1.1. Definition, epidemiology and clinical appearance

The skin is the largest organ of the body and represents a protective barrier against our environment. Generalized skin diseases such as psoriasis not only affect an important and large part of our body, but they are also visible for everyone. Consequently, psoriasis patients experience social stigmatization and discrimination, which causes a psychological burden additionally to the physiological troubles (Hawro et al. 2017; WHO 2013). In face of that, WHO has identified psoriasis as a major global health problem at the sixty-seventh World Health Assembly (WHO 2013).

Psoriasis is a chronic, auto-inflammatory skin disease that affects about 2% of the world population (Christophers 2001). Differences among geographic locations are noticeable. For example, countries closer to the equator are less affected than more distant ones (Parisi et al. 2013). Besides the geographic location, the prevalence rates of psoriasis depend on age and genetical background. Children are less affected than adults (Parisi et al. 2013). And regarding ethnical aspects, Africans and people from China or Japan have a lower incidence than people of European origin (Chandran and Raychaudhuri 2010). The broad field of psoriasis contains various subtypes and forms. According to the international psoriasis council, four main forms can be distinguished: psoriasis vulgaris, guttate psoriasis, pustular psoriasis and erythroderma (Griffiths and Barker 2007; Meglio et al. 2014).

With an estimated percentage of 85-90%, psoriasis vulgaris, also known as plaque-type psoriasis, is the most common form of these phenotypes (Nestle et al. 2009). It is characterized by the occurrence of erythematous and sharply demarcated plaques covered with adherent silvery scales (Figure 1). These lesions may manifest on any skin site, but the symmetric affection of predilection sites such as surfaces of forearms and shin, peri-umbilical, peri-anal and retro-auricular regions is characteristic (Boehncke and Schön 2015; Griffiths et al. 2007). Moreover, all types of psoriasis my affect fingernails and toenails, resulting in pits, yellowish discoloration and onychodystrophy (Reich 2009; Schön and Boehncke 2005) (Figure 1). Patients report that the papulosquamous skin lesions cause torturing itch, pain, bleeding and burning sensations as typical symptoms (Rapp et al. 1999; Dubertret et al. 2006). Beyond the visible lesions, this disease has a

negative impact on psychological and social dimensions, leading to an impairment of life quality that can be even greater than that caused by life-threatening illnesses (Rapp et al. 1999; Gelfand et al. 2004; Sampogna et al. 2012). In regard to the social aspect, a lower level of income and employment is observed compared to healthy individuals (Horn et al. 2007). Moreover, a psoriasis patient has a higher risk to suffer from typical comorbidities such as depression, anxiety, obesity, insulin resistance, cardiovascular diseases and more (Kovitwanichkanont et al. 2020). Considering that there is no cure for psoriasis, this chronic disease and its consequences lead to a heavy load for our health care systems.

The disease is diagnosed based on clinical findings obtained in the course of a detailed anamnesis and body examination, while severity is assessed using various scores like the most commonly used Psoriasis Area and Severity Index (PASI) (Boehncke and Schön 2015).

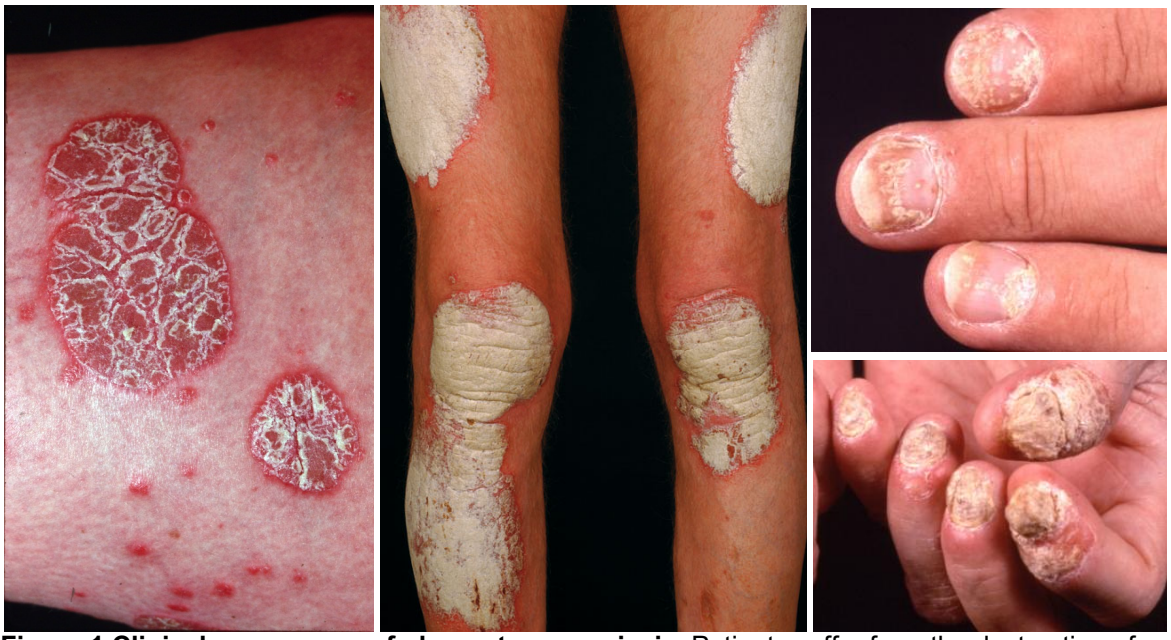


Figure 1 Clinical appearance of plaque-type psoriasis. Patients suffer from the destruction of nails and the occurrence of red well-demarcated scaly skin lesions, typically located on extensor surfaces.

1.1.2. Etiology

The etiology of psoriasis is not yet fully understood, but since it is a multifactorial disease, it can be assumed that there is a genetic predisposition in which exposure to environmental triggers leads to dysregulated immunological mechanisms (Meglio et al. 2014).

The idea of a genetic base of this disease was supported by the examination of relatives of first- and second-degree revealing a higher incidence than in the general population (Farber and Nall 1974; Lomholt 1963). Approximately 30% of all patients suffering from psoriasis vulgaris have an affected first degree relative (Andressen and Henseler 1982). The discovery of a higher disease concordance among monozygotic twins compared to dizygotic twins confirmed these findings as well (Farber et al. 1974; Brandrup et al. 1978; Duffy et al. 1993; Lønnberg et al. 2013). To further explain these associations, genetic analyses using genome-wide scans have identified many different psoriasis susceptibility genes (PSORS) that might be involved in the pathogenesis of psoriasis (Tsoi et al. 2012; Nair et al. 1997; Nair et al. 2000; Trembath et al. 1997; Veal et al. 2002).

This genetic background enables environmental triggers to give rise to inflammatory skin lesions. For example, streptococcal infections, smoking, alcohol abuse and certain systemic medications like β-blockers, antidepressants and antimalarials trigger psoriasis (Basavaraj et al. 2010; Abel et al. 1986; Perera et al. 2012). Within the framework of the Koebner phenomenon, non-specific physical trauma like pressure, scratching, piercing, tattoos or surgical incisions provoke characteristic plaques on body sites that appeared spotless before (Weiss et al. 2002).

1.1.3. Pathogenesis

The immunopathogenesis of psoriasis contains a complex inflammatory cycle of proinflammatory mediators that leads to typical histological alterations of skin architecture, characterized by profound thickening of the epidermis (acanthosis), hyperkeratosis and parakeratosis (Grän et al. 2020). Whereas earlier, psoriasis was supposed to be an exclusively keratinocyte-driven disease, today it is widely accepted that this chronic inflammation originates from a complicated crosstalk between different cell types including keratinocytes and cells of both the innate and the adaptive immune system (Bos et al. 2005; Griffiths and Barker 2007). Especially, the cell-mediated adaptive immune response shaped by IL-17 producing Cluster of Differentiation (CD) 4^+ T_H17 cells plays an essential role in this context (Hawkes et al. 2017; Kim and Krueger 2017).

The mechanism of the starting point in the pathogenesis of psoriasis is still not fully understood, but in the last years possible autoantigens that are potentially crucial for the initial development of psoriasis were described. Anti-microbiotic peptides such as Cathelicidin LL-37, β-defensins and S100 proteins like S100A7 (Psoriasin), but also keratin 17, melanocytic disintegrin-like and metalloprotease domain containing

thrombospondin type 1 motif-like 5 (ADAMTSL5), lipid antigens like phospholipase A2 group IVD (PLA2G4D) and heterogeneous nuclear ribonucleoprotein A1 (hnRNP-A1) have been discussed as such potential autoantigens (Ten Bergen et al. 2020; Grän et al. 2020; Cheung et al. 2016; Arakawa et al. 2015; Guarneri et al. 2018).

Cathelicidin LL-37 is the most-studied and first identified T-cell autoantigen, discovered in 2014 by Lande et al. who observed its correlation with psoriasis severity (Lande et al. 2014). A variety of immune cells such as neutrophils, antigen-presenting-cells, mast cells and Keratinocytes are representing a rich source of LL-37. When released, it binds free desoxyribonucleic acid (DNA)/ ribonucleic acid (RNA) fragments of damaged cells and the resulting LL-37/DNA complex activates in a Toll like receptor (TLR) dependent manner plasmacytoid dendritic cells (pDC) (Lande et al. 2014; Lande et al. 2007; Ganguly et al. 2009). In turn, pDC's secrete type-I Interferon and thereby activate dermal dendritic cells, which migrate to draining lymph nodes and induce IL-23 and IL-12 mediated differentiation of naïve T-cells towards T_H17, T helper 22 (T_H22) and T helper 1(T_H1) cells (Cella et al. 1999; Nestle et al. 2005).

These T helper cells are the key players that migrate to the skin and release proinflammatory cytokines, like IL-17, IL-22, Interferon (IFN)-γ and TNF-α, causing typical hallmark features of psoriasis by impairment of the skin architecture (Boehncke and Schön 2015). In detail, they cause uncontrolled proliferation of keratinocytes accompanied by a maturation deficiency, leading to thickening and scaling of the epidermis (Grän et al. 2020). Increased proliferation rate and shortened differentiation process of keratinocytes - which takes usually approximately 28 days and is reduced to 5-8 days in psoriatic skin lesions - result in reduced lipid secretion, loss of stratum granulosum and nuclei retention in corneocytes (Meglio et al. 2014).

In parallel, IL-17, IL-22, IFN-γ and TNF-α stimulate keratinocytes to release cytokines, chemokines and anti-microbiotic peptides to sustain and reinforce inflammation. For example, keratinocytes can secrete TNF-α and IL-23, amplifying the inflammatory immune response synergistically with members of IL-17 family (Locksley et al. 2001; Chiricozzi et al. 2011; Johnston et al. 2013). The crucial role of TNF-α in this context is underlined by the therapeutic efficacy of treatments targeting TNF-α (Kupper 2003; Leonardi et al. 2003; Chaudhari et al. 2001).

In addition to their antimicrobial function, antimicrobial peptides have also chemotactic abilities. Likewise chemokines, they can recruit immune cells to migrate to the inflammation site (Büchau and Gallo 2007). In this regard, chemotactic mechanisms allow cell types from the innate immune system like macrophages, mast cells and neutrophils to contribute to the persisting inflammation as well (Boehncke and Schön

2015; Boehncke and Brembilla 2018). It was shown that neutrophiles as well as mast cells are mostly underestimated regarding their ability to secrete IL-17 (Lin et al. 2011; Senra et al. 2016; Keijsers et al. 2014; Brembilla et al. 2017).

Proinflammatory mediators like IL-17 and other T_H17 products also activate myeloid dendritic cells (mDC) representing the main producers of IL-23 in the skin, as well as a certain mDC subtype called TNF/iNOS producing dendritic cells (TIP-DCs), which secrete high amounts of TNF-α and inducible nitric oxide synthase (iNOS), two critical mediators of inflammation (Lee et al. 2004; Lowes et al. 2005).

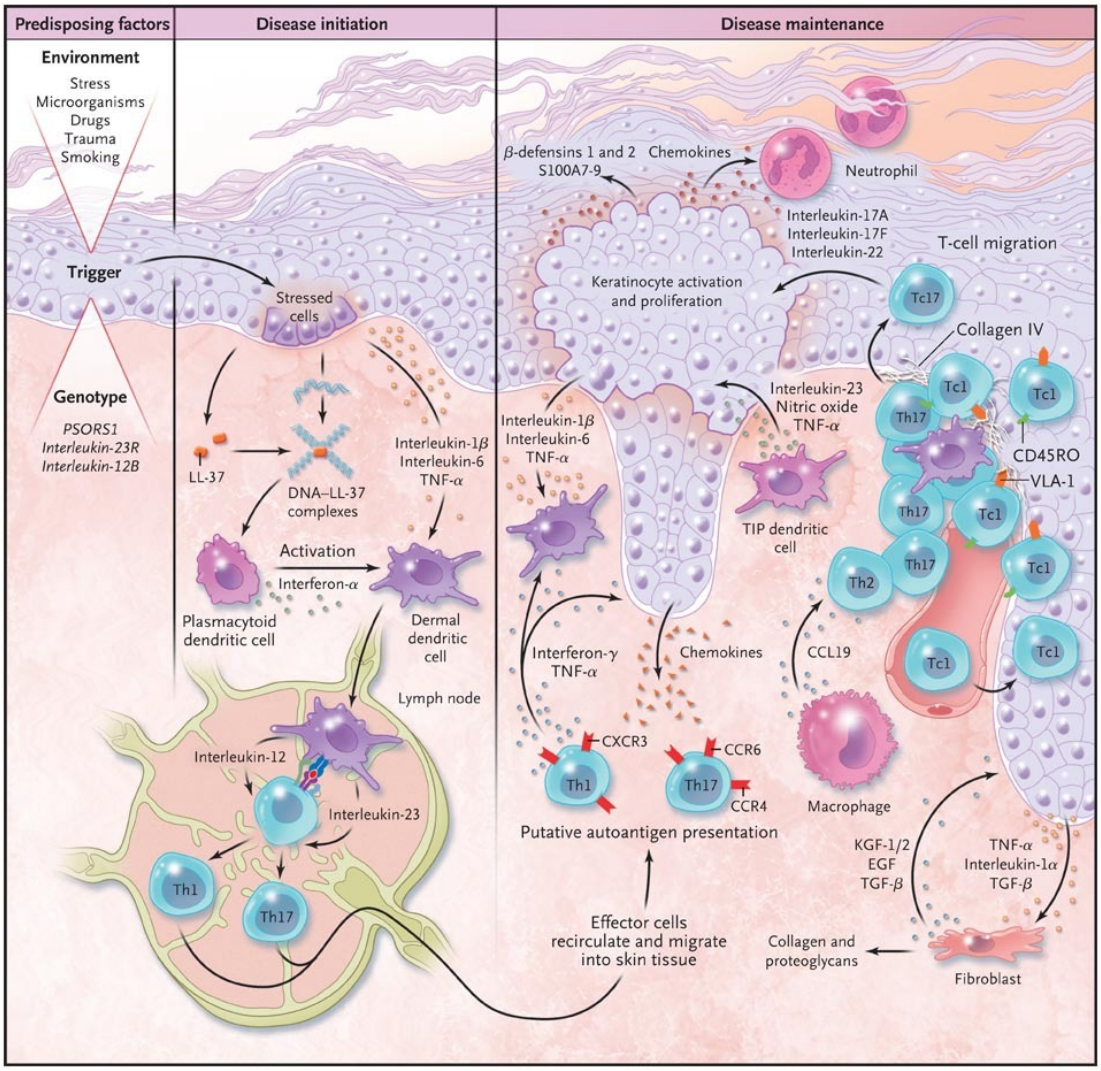


Figure 2 Pathogenesis of psoriasis. Physical trauma in combination with a genetic predisposition enables the induction of an overwhelming immune response leading to uncoordinated keratinocyte proliferation and inflammation. Stressed keratinocytes release DNA, RNA and anti-microbial peptides that activate pDC's via TLR-9. These, in turn, force Langerhans cells to migrate to tissue draining lymph nodes to initiate the IL-23/T_H17 axis. T_H17 cells migrate back to the skin and induce a continuous inflammation by secreting proinflammatory cytokines and creating inflammatory feedback loops in cooperation with mDC's and keratinocytes. Figure adapted from Nestle 2009.

In summary, the abundant production of proinflammatory cytokines and, in particular, subsequent overexpression of IL-23 by mDC's and keratinocytes generate feedback loops of tissue damage and sustain a vicious circle of inflammation (Boehncke and Schön 2015).

1.1.4. Therapy

1.1.4.1. Topical treatments

Because psoriasis is currently not curable, the success of treatment is measured by the reduction of the physical and psychological burden. Scores like PASI, measuring skin symptoms and their distribution on the body surface, or dermatology life quality index (DLQI), measuring life quality, are determined during treatment to evaluate its effectivity. The aim of therapy is to induce a PASI 75 response, meaning a reduction in PASI of at least 75 percent referred to the baseline value. Correspondingly, the DLQI score should reach values from 0 to 1, representing no impairment of life quality caused by the skin leasions. Numerous patients are not satisfied because their treatment is inappropriate to their suffering. That is why the treatment should always be adapted to the patient's disease burden and if it does not effectuate the desired lessening of symptoms it should be switched (Nast et al. 2017).

In mild forms of psoriasis, topical treatments like glucocorticosteroids, calcineurin inhibitors, dithranol, tazarotene and vitamin D3 analogues or vitamin D3 itself can be considered. In more severe affected patients, these therapies can be augmented by a combination with phototherapy or systemic therapies. Every kind of therapy should always be accompanied by a basic care of the skin (Nast et al. 2017). Since 2022, a topical aryl hydrocarbon receptor-modulator called tapinarof is approved for plaque psoriasis in adults in the United States and it showed superiority to the vehicle control in two phase three trials (Lebwohl et al. 2021).

1.1.4.2. Conventional systemic treatments and biologics

Systemic therapy is recommended in severe forms of psoriasis. On the one hand, there is access to conventional treatments using the retinoid acitretin or immunosuppressants like ciclosporin or methotrexate (MTX). When using MTX, attention should be paid to the co-medication with folic acid to avoid toxic side effects (Nast et al. 2017). On the other hand, there are novel antibody treatments available called biologics. These synthesized antibodies target key points in the proinflammatory signal cascades, leading to groundbreaking therapeutic successes (Table 1). Because of their effectivity, it was

considered to adjust the aim of therapy to PASI 90, meaning a 90% reduction in symptom burden (Nast et al. 2017). At this stage, the field of biologics for the treatment of psoriasis is focused on four groups of antibodies regarding their targets: TNF-α, IL-17, IL-23 and IL-12/IL-23 (Menter et al. 2019). In the latter case, the antibody targets the shared p40 subunit of IL-12 and IL-23.

Table 1 Currently approved biologics in the treatment of psoriasis

Target	Name	FDA approval
TNF-α	Entanercept	2004
	Infliximab	2006
	Adalimumab	2008
	Certolizumab	2018
IL-12/ IL-23	Ustekinumab	2009
IL-17	Secukinumab	2015
	Ixekizumab	2016
	Brodalumab	2017
	Bimekizumab	2021
IL-23	Guselkumab	2017
	Tildrakizumab	2018
	Risankizumab	2019

(Menter et al. 2019; U.S. Food and Drug Administration 2019; Rodrigues et al. 2022)

1.1.4.2.1. IL-23 agents

Guselkumab is the first approved IL-23 targeting antibody for the treatment of psoriasis vulgaris and targets the p19 subunit, which is exclusive to IL-23 (Oppmann et al. 2000).The efficacy of guselkumab was confirmed in several phase III trials: VOYAGE 1, VOYAGE 2, NAVIGATE, ECLIPSE, DISCOVER-1 and DISCOVER-2 (Al-Salama and Scott 2018; Reich et al. 2019; Deodhar et al. 2020; Mease et al. 2020). While the recent DISCOVER-1 and DISCOVER-2 studies revealed that guselkumab might be an effective treatment option for patients with active psoriatic arthritis, all other studies examined its efficacy in direct comparison to placebo controls and other approved antibody treatments in the context of plaque-type-psoriasis (Deodhar et al. 2020; Mease et al. 2020). X-plore, a 52-week lasting, dose-ranging phase II trial, already showed the high therapeutic

potential of guselkumab, approximately half of the patients that received guselkumab reached a PASI 100 after 40 weeks (Gordon et al. 2015). Following phase III trials, like the VOYAGE studies, confirmed this effectiveness by showing that after just 16 weeks of guselkumab treatment about three-fourths of the patients revealed PASI 90 (Blauvelt et al. 2017) (Figure 3). In comparison to adalimumab, a TNF-α antagonist, significantly greater improvements in DLQI and skin clearance from baseline were observed in patients treated with guselkumab (Blauvelt et al. 2017; Armstrong et al. 2019). Furthermore, head-to-head trials like the NAVIGATE and the ECLIPSE study demonstrated the superiority of guselkumab compared to ustekinumab and secukinumab regarding long-term efficacy (Langley et al. 2018; Reich et al. 2019). In this context, non-responders of ustekinumab treatment derived significant benefit from switching to guselkumab (Langley et al. 2018). In addition to that, long-time studies showed a maintained clinical response and a high safety profile under treatment with guselkumab over more than 150 weeks (Reich et al. 2020). Interestingly, long-time studies, including a study with a randomized withdrawal period after 28 weeks of guselkumab, revealed that some patients maintained PASI 100 score in the absence of treatment (Reich et al. 2017). This raised the question of whether IL-23 inhibition is capable of modifying disease activity in patients with sustained treatment success.

Among the group of IL-23 agents, guselkumab is not unique regarding therapy success. Indeed, studies with the latest approved IL-23 antibody risankizumab showed similar or even better results regarding superiority to other biologics, achievement of symptom reduction and the high safety profile (Papp et al. 2017; Haugh et al. 2018) (Figure 3). Besides, early studies of risankizumab revealed long-lasting effects far beyond drug's half-life time, which also demonstrates that the IL-23 antibody class most likely has the potential for long-lasting disease modification (Krueger et al. 2015).

The superiority of IL-23 agents compared to the neutralization of other inflammatory mediators highlights the crucial role of IL-23 and the T_H17 pathway in the inflammatory process of psoriasis. Experiments in mouse models, showing that psoriatic like lesions are provoked by intradermal injection of IL-23, but not IL-12, support that IL-23 is probably the cytokine with the most important role in this auto-inflammatory disease (Nakajima et al. 2011; Lowes et al. 2013; Levin and Gottlieb 2014).

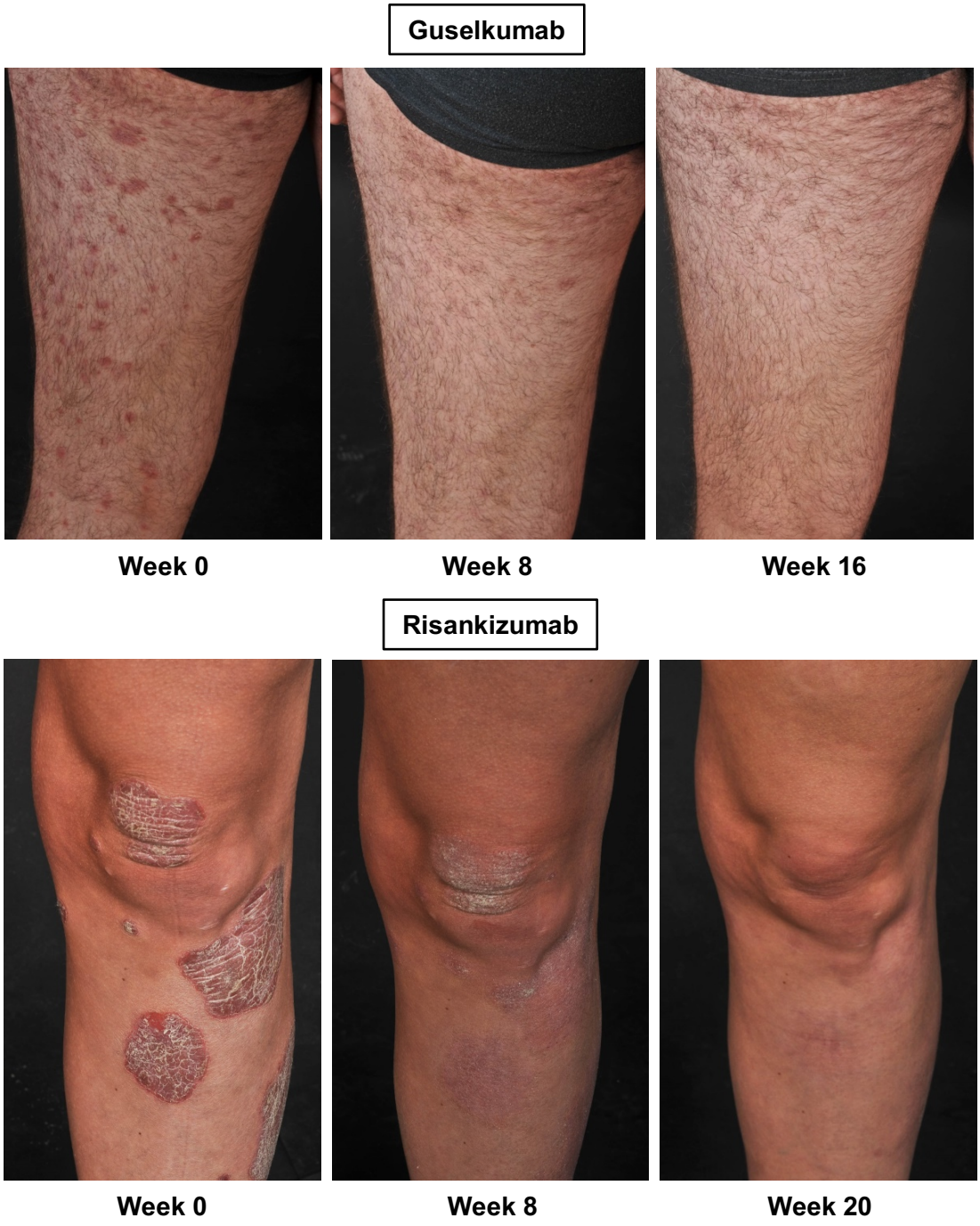


Figure 3 Efficacy of anti-IL-23 treatment. Both agents, guselkumab and risankizumab, are capable of relieving the patients within a few weeks from their entire disease burden.

1.2. T helper cell immunity in the context of psoriasis

1.2.1. T helper phenotypes

T-lymphocytes together with B-lymphocytes build up the cellular part of our adaptive immune system, which provides potent even though slightly delayed immune response to infections. On the other hand, the innate immune system with its actors reacts more quickly but rather unspecific facing foreign antigens. Together, they create a robust unit protecting our body from dangerous pathogens. In order to do this job successfully, these two units need a solid linkage and communication among their cell subtypes to orchestrate a proper immune response. One of these essential facilitators belonging to the family of T-lymphocytes are T helper cells, which are characterized by their T-cell receptor (TCR) and CD4 surface marker enabling them to interact with antigen presenting cells. In this function, they provide help to B-lymphocytes and $CD8^+$ cytotoxic T-cells and activate cells of the innate immune system (Shea et al. 2012). Furthermore, different subsets of $CD4^+$ cells can be distinguished regarding lineage-specific transcription factors, function and patterns of cytokine secretion: T_H1, T helper 2 (T_H2), T_H17, T_H22, T helper 9 (T_H9), T follicular helper (Tfh) cells and regulatory T-cells (T_{reg}) (Cosmi et al. 2014; Shen and Shi 2019; Eyerich et al. 2009). For orchestrating an immune response, particular T helper cell subtypes fulfill special functions. For example, T_H1 cells defend the host against intracellular pathogens including viruses, protozoa and bacteria, whereas T_H2 cells protect from infections by helminths and other extracellular parasites and respond to a plurality of allergens (Szabo et al. 2003; Pulendran and Artis 2012). The main role of T_H17 cells is to fight against extracellular bacteria and fungi, while T_H22 cells fulfill a crucial role in maintaining the mucosal barrier function (Cosmi et al. 2014; Eyerich et al. 2008). The decision which phenotype naïve cells gain during their differentiation to effector T-cells is predominantly determined by the cytokines in their microenvironment and by the strength of the interaction between the TCR and the antigen (Boyton and Altmann 2002; Zhou et al. 2009). Current findings indicate plasticity among these different phenotypes, especially for T_H17 cells, regarding the range of their cytokine production (Peck and Mellins 2010; O'Shea and Paul 2010; Guéry and Hugues 2015). The successful treatment of psoriasis, that was earlier assumed to be a T_H1 driven disease, with antibodies targeting IL-23, a well-known activator of T_H17 cells, clearly showed the predominant role of T_H17 cells in pathogenesis of psoriasis (Zheng et al. 2007; Tang et al. 2012; Di Meglio and Nestle 2010).

1.2.2. IL-23/T_H17 axis

T_H17 cells have an essential role in protective immunity against bacterial and fungal infections, but they are also involved in many auto-immune diseases like psoriasis, multiple sclerosis (MS), rheumatoid arthritis (RA) and inflammatory bowel diseases (IBD) (Curtis and Way 2009; Marwaha et al. 2012; Meller et al. 2015; Patel and Kuchroo 2015). The indispensability of IL-23 for the T_H17 lineage is for example shown by the fact that IL-17A and IL-17F inductions were completely abolished in IL-23p19 deficient mice (van der Fits et al. 2009).

IL-23 is a heterodimeric cytokine consisting of a p40 subunit shared by IL-12 and a p19 subunit that is exclusive to IL-23. In lesional skin, IL-23 is produced abundantly by dendritic cells and keratinocytes sustaining the inflammation process, continuously. According to the heterodimeric composition of IL-23, its cellular effect is mediated by a receptor complex composed of two subunits, namely IL-23 receptor (IL-23R) and Interleukin-12 receptor β1 (IL-12Rβ1) (Teng et al. 2015).

Most findings explaining the molecular mechanisms of T_H17 specific signal transduction originate from mouse models. The differentiation of human T_H17 cells is not completely elucidated, indeed substantial differences to the mouse model are assumed (Annunziato et al. 2009). Binding of IL-23R leads to expression of key cytokines like IL-17 and IL-22 predominantly by activation of transcription factors like the signal transducer and activator of transcription (STAT) 3 and transcription factors of the retinoic acid receptor-related orphan nuclear receptor (ROR) family (Marwaha et al. 2012; Chiricozzi et al. 2014; Castro et al. 2017). The IL-17 family consists of six isoforms, IL-17A-F, while T_H17 cells produce only IL-17A and IL-17F which are very homolog (Kolls and Lindén 2004). Which cytokines drive T_H17 differentiation is an extensively discussed topic that revealed partly contradictory findings. However, transforming growth factor (TGF) β, IL-6, IL-1β and IL-23 seem to be involved in the T_H17 cell lineage development. T_H17 cells can produce a range of cytokines. Besides the predominant expression of IL-17 and IL-22 they are also able to produce TNF-α, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-21, IL-9 and IL-10 (Bedoya et al. 2013). The secretion of IL-17 and IL-22 in the skin sites leads to proliferation of keratinocytes, production of proinflammatory cytokines and antimicrobial peptides, and to acanthosis by inducing epidermal hyperplasia and by inhibiting epidermal differentiation (Teunissen et al. 1998; Zheng et al. 2007; Chiricozzi et al. 2011; Rizzo et al. 2011; Adami et al. 2014; Ha et al. 2014; Baliwag et al. 2015).

1.2.3. Pathogenic and non-pathogenic T_H17 phenotypes

Due to the increased understanding of the co-expression of different cytokines additional to IL-17 within the past decade, the idea grew to distinguish T_H17 cells in different phenotypes. The hypothesis was proposed that the development of different T_H17 phenotypes depends on their environmental factors in the same way the microenvironment instructs the T helper cell lineage (Peters 2011). In context of the pathogenic role of T_H17 cells in different autoimmune diseases and their non-pathogenic role in the defense against opportunistic infections, a dichotomous nature of T_H17 cells at the molecular level was observed (Stockinger and Omenetti 2017).

On the one hand, T_H17 cells can show a pathogenic phenotype categorized by coproduction of proinflammatory cytokines like IFN-γ, GM-CSF and TNF-α (Annunziato et al. 2007; El-Behi et al. 2011; Lowes et al. 2008). On the other hand, a so-called non-pathogenic T_H17 cell, which represents a more regulatory subset co-expressing IL-10, is assumed to protect the body from overwhelming inflammation by regulating pathogenic T_H17 cells in a T_{reg} cell manner (McGeachy et al. 2007; Chaudhry et al. 2011; Huber et al. 2011). T_H17 cells and their phenotypes are assumed to play a crucial role in pathogenesis of diverse autoimmune diseases (Xinyu Wu et al. 2018; Bystrom et al. 2019). Apart from rheumatic diseases like RA or systemic lupus erythematosus, they are also contributing to the manifestation of type 1 Diabetes, severe asthma and neuroinflammatory diseases like MS (Ramakrishnan et al. 2019; Yasuda et al. 2019; Shao et al. 2020; Moser et al. 2020). They are even considered to participate in the pathogenesis of depression (Beurel and Lowell 2018). Moreover, T_H17 cells and their pathogenic activity are well known from elaborate studies in particular mouse models such as experimental auto-immune encephalomyelitis or collagen-induced arthritis. Since they are primarily located in intestinal and skin tissue, they fulfill their physiological task of barrier protection against invading pathogens and maintain balance between immune system and microbiome in the sense of tissue homeostasis. That is why a dysregulation in this relation can cause IBD or psoriasis, highlighting the outstanding role of T_H17 cells in development of these diseases (Huber et al. 2012; Stockinger and Omenetti 2017).

In context of shaping different T_H17 phenotypes, the direction of differentiation towards a beneficial or detrimental phenotype is guided by the cytokine milieu and by environmental stimuli via activation of distinct transcription factors (Stockinger and Omenetti 2017). Cytokine requirements of T_H17 differentiation are intensively under investigation. These investigations even provide controversial results, especially regarding the role of TGFβ and led to the assumption of differences between mice and

men (Peters et al. 2011; Ghoreschi et al. 2010; Acosta-Rodriguez et al. 2007; Mangan et al. 2006; Veldhoen et al. 2006; Revu et al. 2018). Most findings consistently hypothesize that IL-23, IL-6 and IL-1β give rise to the pathogenic T_H17 subset, in contrast to TGFβ and IL-6, which induce differentiation of naïve T-cells towards the non-pathogenic T_H17 cell (Revu et al. 2018; Ghoreschi et al. 2010; Langrish et al. 2005; Peters et al. 2011; McGeachy et al. 2007). Besides the important role of IL-1β within pathogenic T_H17 differentiation, Zielinski et al. could show a pathogen dependence. Namely, C. albicans induced a pathogenic T_H17 subset, while S. aureus led to a non-pathogenic phenotype in an *in vitro* co-culture system. (Zielinski et al. 2012)

Apart from indispensable key transcription factors necessary for all T_H17 subtypes like RORγt, basic leucine zipper activating-transcription-factor-like transcription factor (BATF) and interferon regulatory factor 4 (IRF4), examination of molecular mechanisms and gene expression revealed differences among the two different phenotypes (Ivanov et al. 2006; Brüstle et al. 2007; Schraml et al. 2009; Huber and Lohoff 2014). For example, it has been shown that IL-23 is able to induce T-box expressed in T-cells (T-bet) - a typically T_H1 related gene - in pathogenic subsets which leads to the production of IFN-γ and is suggested to increase expression of IL-23R creating a feed-forward loop (Gocke et al. 2007; Lee et al. 2012). Furthermore, IL-10 expression by non-pathogenic T_H17 cells is correlated with the upregulation of the transcription factor musculoaponeurotic fibrosarcoma oncogene homolog (c-MAF), while pro-inflammatory T_H17 cells lacking IL-10 production were associated with the inability to upregulate c-MAF (Aschenbrenner et al. 2018). It has also been shown that the recombinant signal binding protein for immunoglobulin kappa J region (RBPJ), a molecule belonging to the Notch signaling pathway, affects the development of T_H17 cells towards a pathogenic phenotype (Meyer Zu Horste et al. 2016). Interestingly, IRF is in fact necessary for T_H17 differentiation, but it modifies the T_H17 phenotype in a biphasic dose-dependent way (B. Wu et al. 2018). Furthermore, other molecules like hypoxia inducible factor 1^{α} ($HIF1^{\alpha}$), STAT 4 and protein C receptor (PROCR) were observed in this context revealing crucial impact on development of the T_H17 phenotype (Wu and Wan 2020).

Analysis and comparison of single-cell RNA-Seq identified new genes like g protein-coupled receptor 65 (Gpr65), promyelocytic leukemia zinc finger-like zinc finger protein (Plzp), Toso and CD5 antigen-like (CD5l), also known as apoptosis inhibitor of macrophage (AIM), governing heterogeneity and pathogenicity of T_H17 cells (Gaublomme et al. 2015).

1.3. Aim of the thesis

This thesis investigates the effect of IL-23 inhibition on T_H17 cells within the framework of different *in vitro* models. In more detail, the following questions were addressed:

1. What is the effect of *in vitro* IL-23 deprivation on the T_H17 phenotype?
2. Does anti-IL-23 cause different effects in different T_H17 subpopulations?
3. Regarding maintained treatment success in the area of psoriasis therapy, is the neutralization of IL-23 capable of shaping pathogenic towards non-pathogenic T_H17 cells in the sense of plasticity?

2. Material

2.1. Buffers, media and solutions

Table 2 Material used for manufacturing of buffers, media and solutions

Reagents	
Agar	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Ammonium chloride	Merck KGaA (Darmstadt, Germany)
Bovine serum albumine (BSA) solution 7,5%	Sigma-Aldrich (Saint-Louis, USA)
BSA lyophilized powder	Sigma-Aldrich (Saint-Louis, USA)
Citric acid monohydrate	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Disodium phosphate	Merck KGaA (Darmstadt, Germany)
Dimethylsulfoxid (DMSO) (unsterile)	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Dulbecco's phosphate buffered saline (DPBS), without (w/o) Ca and Mg	Gibco by Life Technologies (Carlsbad, USA)
UltraPure™ Ethylendiaminetetraacetic acid (EDTA) 0.5 M	Invitrogen by Life Technologies (Carlsbad, USA)
Ethanol (96% and 70%)	Otto Fischar (Saarbrücken, Ger- many)
Fetal calf serum (FCS)	GE Healthcare (Chalfont Saint Giles, United Kingdom (UK))
Glucose	MerckMillipore (Billerica, Massachusetts)
Glutamine	MerckMillipore (Billerica, Massachusetts)
Human serum (HS)	Sigma-Aldrich (Saint-Louis, USA)
Hydrochloric acid (5 N)	Merck KGaA (Darmstadt, Germany)
Hydrogenperoxide solution	Sigma-Aldrich (Saint-Louis, USA)
Lysogeny broth (LB) powder	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Non-essential amino acids solution (100X)	Gibco by Life Technologies (Carlsbad, USA)
Penicillin-Streptomycin (10 000 U/ml)	Gibco by Life Technologies (Carlsbad, USA)

Potassium chloride	Merck KGaA (Darmstadt, Germany)
Potassium dihydrogenphosphate	Merck KGaA (Darmstadt, Germany)
Potassium hydrogen carbonate	Merck KGaA (Darmstadt, Germany)
Roswell Park Memorial Institute medium 1640 (RPMI) (1X)	Gibco by Life Technologies (Carlsbad, USA)
Sodium azide	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Sodium hydroxide (10 N)	Merck KGaA (Darmstadt, Germany)
Sodium pyruvate (100 mM)	Gibco by Life Technologies (Carlsbad, USA)
Sodiumchloride	Merck KGaA (Darmstadt, Germany)
Sulphuric acid (2 N) as ELISA stop solution	Merck KGaA (Darmstadt, Germany)
Tetramethylbenzidine (TMB)	Sigma-Aldrich (Saint-Louis, USA)
Tween®20 detergent	MerckMillipore (Billerica, Massachusetts)
Yeast Nitrogen Base (YNB) powder	Sigma Aldrich

Table 3 Composition of manufactured buffers, media and solutions

Blocking buffer (ELISA)	▪ 1% BSA lyophilized powder in DPBS
Citrat buffer	▪ 2 l distilled water + 4.2 g Citric acid monohydrate ▪ Adjust pH to 6.0 with 10 N NaOH
Culture medium	▪ 455 ml RPMI + 25 ml HS + 5 ml glutamine + 5 ml P/S + 5 ml non-essential amino-acid solution + 5 ml sodium pyruvate ▪ Sterile filtration
Red blood cell (RBC) lysis buffer	▪ 9 ml ultrapure water + 1 ml 10x RBC lysis buffer
FACS buffer	▪ 500 ml DPBS + 26.25 ml FCS (5%) + 0.105 ml sodium azide (0,02%)
LB medium	▪ 500 ml VE water + 12.5 g LB powder
Substrate solution (ELISA)	▪ 5.5 ml Citrat buffer

	+ 2.55 µl Hydrogenperoxide solution + 55 µl TMB
Washing buffer (ELISA)	▪ 4,75 l VE water + 250 ml PBS 20x ▪ Adjust pH to 7.2 + 2.5 ml Tween®20 detergent
YNB agar	▪ 100 ml YNB medium + 2g Agar (2%)
YNB medium	▪ 500 ml ultrapure water + 3.35g YNB powder + 10 g glucose

2.2. Pipettes and tips

Table 4 Pipettes and tips used across all experiments

Single-channel pipettes	
Single-channel pipette Research plus 0.1-2.5 µl	Eppendorf (Hamburg, Germany)
Single-channel pipette Research plus 0.5-10 µl	Eppendorf (Hamburg, Germany)
Single-channel pipette Research plus 10-100 µl	Eppendorf (Hamburg, Germany)
Single-channel pipette Research plus 100-1000 µl	Eppendorf (Hamburg, Germany)
Single-channel pipette Reference 0.5-10 µl	Eppendorf (Hamburg, Germany)
Single-channel pipette Reference 10-100 µl	Eppendorf (Hamburg, Germany)
Single-channel pipette Reference 100-1000 µl	Eppendorf (Hamburg, Germany)
Single-channel pipette Research plus 1-10 ml	Eppendorf (Hamburg, Germany)
Single-channel pipette Reference 0,25-2,5 ml	Eppendorf (Hamburg, Germany)
Multi-channel pipettes	
Multi-channel pipette Transferpipette®-8 20-200 µl	Brand (Wertheim, Grmany)

Multi-channel pipette Transferpipette®-8 2,5-25 µl	Brand (Wertheim, Grmany)
Multi-channel pipette Transferpipette®-8 5-50 µl	Brand (Wertheim, Grmany)
Multi-channel pipette Transferpipette®S-8 30-300 µl	Brand (Wertheim, Grmany)
Tips	
Pipette tips 2-200 µl	Brand (Wertheim, Grmany)
Pipette tips epT.I.P.S. 0,5-10 ml	Eppendorf (Hamburg, Germany)
Pipette tips epT.I.P.S.0,25-2,5 ml	Eppendorf (Hamburg, Germany)
Pipette tips epT.I.P.S. 50-1000 µl	Eppendorf (Hamburg, Germany)
Pipette tips SurPhob 10 µl	Biozym Scientific (Oldendorf, Germany)
Pipette tips SurPhob 200 µl	Biozym Scientific (Oldendorf, Germany)
Pipette tips SurPhob 1250 µl	Biozym Scientific (Oldendorf, Germany)
Pipetboy and serological pipets	
Falcon® Pipet Controller	Corning (Corning, New York, USA)
Cellstar® Serological pipette 1 ml	Greiner Bio-One (Frickenhausen, Germany)
Cellstar® Serological pipette 5 ml	Greiner Bio-One (Frickenhausen, Germany)
Cellstar® Serological pipette 10 ml	Greiner Bio-One (Frickenhausen, Germany)
Cellstar® Serological pipette 25 ml	Greiner Bio-One (Frickenhausen, Germany)

2.3. Collection of blood samples and peripheral blood mononuclear cell (PBMC) isolation

Table 5 Material used for sample acquisition and PBMC isolation

Buffers, solutions and media
➔ see Table 3 Composition of manufactured buffers, media and solutions2.1Buffers, media and solutions
Consumables

Menzel™ Coverslips	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Octeniderm® farblos	Schülke & Mayr GmbH (Norderstedt, Germany)
Original Perfusor® Syringe 50 ml	B. Braun Melsungen AG (Melsungen, Germany)
Safety-Multifly® 21G	Sarstedt AG & Co. KG (Nümbrecht, Germany)
Venofix® Safety 19G	B. Braun Melsungen AG (Melsungen, Germany)
Zelletten®	Lohmann & Rauscher GmbH & Co. KG (Neuwied, Germany)
Devices	
Axiovert 25	Carl Zeiss AG (Oberkochen, Germany)
Marienfeld superior™ counting chamber Neubauer-improved	Paul Marienfeld GmbH & Co. KG (Lauda-Königshofen, Germany)
Polymax 1040	Heidolph Instruments GmbH & Co. KG (Schwabach, Germany)
Reagents	
Heparin 5000 U/ml	Biochrom GmbH for MerckMillipore
Lymphoprep™	Serumwerk Bernburg AG for Alere Technologies AS mittlerweile von Abott übernommen
Trypan Blue Stain (0,4%)	Gibco by Life Technologies (Carlsbad, USA)

2.4. Cell culture

Table 6 Material used in cell culture

Buffers, solutions and media	
➔ see Table 3 Composition of manufactured buffers, media and solutions	
Consumables	
Biosphere® SafeSeal tube 1.5 ml and 2.0 ml	Sarstedt AG & Co. KG (Nümbrecht, Germany)
Cellstar® tubes 15 ml and 50 ml	Greiner Bio-One International GmbH (Kremsmünster, Austria)

Falcon® 24-well plat non-tissue culture-treated, flat	Corning Inc. (Corning, New York, USA)
Falcon® 48-well plat non-tissue culture-treated, flat	Corning Inc. (Corning, New York, USA)
Falcon® 96-well microplate non-tissue culture-treated, flat	Corning Inc. (Corning, New York, USA)
Filtropur V50, 0.2 µm	Sarstedt AG & Co. KG (Nümbrecht, Germany)
Millex®-GP, 0,22 µm	MerckMillipore (Billerica, Massachusetts)
TC plate 96-well, round bottom	Sarstedt AG & Co. KG (Nümbrecht, Germany)
Tissue culture dish 100	TPP Techno Plastic Products AG (Trasadingen, Switzerland)
Devices	
Biological Safety Cabinet Heraeus™ HeraSafe™	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Freezer -20 ° C	Liebherr-International S.A. (Bulle, Switzerland)
Freezer HERAFreeze™ -80 ° C	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Heraeus™ Megafuge™ 1.0R	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Heraeus™ Megafuge™ 40R	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Heraeus™ Multifuge™ 1 L-R	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Incubator heracell®	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Refrigerator	Liebherr-International S.A. (Bulle, Switzerland)
Special-340	Phillipp Kirsch GmbH (Willstätt-Sand, Germany)
VacuuHandControl VHCpro	Vacuubrand GmbH & Co. KG (Wertheim, Germany)
Vacuum controller CVC 2000	Vacuubrand GmbH & Co. KG (Wertheim, Germany)
Vortex V-1 plus	BioSan SIA (Riga, Lettland)

VX-150	Systec GmbH (Linden, Deutschland)
Reagents and proliferation dyes	
CellTrace™ Violet	Invitrogen by Life Technologies (Carlsbad, USA)
DMSO for cell culture	PanReac AppliChem GmbH (Darmstadt, Germany)
Stimulants, cytokines and antibodies	
IL-1β	PromoCell (Heidelberg, Germany)
IL-2	Sigma-Aldrich (St. Louis, USA)
IL-23	PromoCell (Heidelberg, Germany)
IL-6	PromoCell (Heidelberg, Germany)
Mouse anti-human CD28 (clone CD28.2)	BD (Franklin Lakes, USA)
Mouse anti-human CD3 (clone UCHT1)	BD (Franklin Lakes, USA)
Phytohemagglutinin (PHA)	Sigma-Aldrich (St. Louis, USA)
TGF-beta 1	PromoCell (Heidelberg, Germany)
Tremfya®	Janssen-Cilag GmbH (Neuss, Germany)

2.5. Magnetic activated cell sorting (MACS)

Table 7 Material used for MACS

Devices	
Automacs® Pro Seperator	Miltenyi Biotec B.V. & Co. KG (Bergisch Gladbach, Germany)
Chill 50 tube rack	Miltenyi Biotec B.V. & Co. KG (Bergisch Gladbach, Germany)
Purelab® flex	ELGA LabWater/Veolia Water Technologies Deutschland GmbH (Celle, Germany)
MACS kits and MicroBeads	
CD14 MicroBeads	Miltenyi Biotec B.V. & Co. KG (Bergisch Gladbach, Germany)
CD4+ T Cell Isolation Kit human	Miltenyi Biotec B.V. & Co. KG (Bergisch Gladbach, Germany)

CD45RO MicroBeads	Miltenyi Biotec B.V. & Co. KG (Bergisch Gladbach, Germany)
Solutions and buffers	
Automacs® Running Buffer	Miltenyi Biotec B.V. & Co. KG (Bergisch Gladbach, Germany)
Automacs® Washing Solution	Miltenyi Biotec B.V. & Co. KG (Bergisch Gladbach, Germany)
EMSURE® Ethanol	MerckMillipore (Billerica, Massachusetts)

2.6. Proteinbiochemistry

Table 8 Material used for ELISA and BCA assay

Consumables	
Costar® Assay Plate 96-well, Flat bottom, high binding	Corning Inc. (Corning, New York, USA)
Weighing pan Rotilabo®	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Corning® Microplate Sealing Tape	Corning Inc. (Corning, New York, USA)
Devices	
Analytical balance	Kern & Sohn GmbH (Balingen-Frommern, Germany)
Epoch™ Microplate Spectrophotometer	BioTek Instruments Inc. (Winooski, USA)
Gen 5 Version 3.03	BioTek Instruments Inc. (Winooski, USA)
HydroSpeed™ microplate washer	Tecan Group AG (Männedorf, Switzerland)
Magnetic stirrer RCT basic	IKA®-Werke GmbH & Co. KG (Staufen, Germany)
Magnetic stirrer RH digital	IKA®-Werke GmbH & Co. KG (Staufen, Germany)
TLE 3002	Mettler-Toledo International Inc. (Columbus, USA)
Vortex-Genie™ 2	Scientific Industries, Inc. (Bohemia, New York, USA)
WTW® inoLab® pH 7110	Xylem Inc. (Rye Brook, USA)
Kits	

Human IL-17 DuoSet® ELISA	R&D Systems Inc. (Minneapolis, USA)
Human INF-γ DuoSet® ELISA (DY285B)	R&D Systems Inc. (Minneapolis, USA)
OptEIA™human IL-10 ELISA set	BD (Franklin Lakes, USA)
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Reagents, buffers and solutions	
➔ see chapter 2.1	

2.7. Flow cytometry

Table 9 Material used for flow cytometric analysis

Buffers, stimulants, solutions and reagents	
Aqua ad injectabile	Berlin-Chemie AG (Berlin, Germany)
FACS buffer	➔ see Table 3 Composition of manufactured buffers, media and solutions
Precision count beads™	BioLegend, Inc. (San Diego, USA)
Fixation/Permeabilization solution Cytofix/Cytoperm™	Beckton & Dickinson BD (Franklin Lakes, USA)
GolgiPlug™ (containing Brefeldin A)	BD (Franklin Lakes, USA)
GolgiStop™ (containing Monensin)	BD (Franklin Lakes, USA)
Ionomycin	Sigma-Aldrich (St. Louis, USA)
Perm/Wash™ buffer (10X)	BD (Franklin Lakes, USA)
Phorphol 12-myristate 13-acetate (PMA)	Gibco by Life Technologies (Carlsbad, USA)
Consumables	
FACS cluster tubes	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Falcon® tubes, 5 ml	Corning Inc. (Corning, New York, USA)
Devices	
BD FACSDiva Software, Version ??	BD (Franklin Lakes, USA)
Centrifuge MC 6	Sarstedt AG & Co. KG (Nümbrecht, Germany)

Flow cytometer, LSR Fortessa	BD (Franklin Lakes, USA)
FlowJo Software, Version 10	FlowJo LLC (Ashland, USA)
Purelab® flex	ELGA LabWater/Veolia Water Technologies Deutschland GmbH (Celle, Germany)
Vortex-Genie™ 2	Scientific Industries, Inc. (Bohemia, New York, USA)
Dyes	
LIVE/DEAD™ Fixable Dead Cell Stain Kit – Aqua	Invitrogen by Life Technologies (Carlsbad, USA)
LIVE/DEAD™ Fixable Dead Cell Stain Kit – Far red	Invitrogen by Life Technologies (Carlsbad, USA)

Table 10 Fluorescent antibodies used for flow cytometric staining

Target	Fluorochrome	Company
CD3	PeCF594	BioLegend, Inc. (San Diego, USA)
CD4	APC-Cy7	BD Bioscience (San Jose, USA)
CD45RA	V450	BD Bioscience (San Jose, USA)
CD45RO	Bv650	BioLegend, Inc. (San Diego, USA)
CD69	PeCF594	BD Bioscience (San Jose, USA)
IFNg	BV605	BD Bioscience (San Jose, USA)
IL10	Vio-515	Miltenyi Biotec B.V. & Co. KG (Bergisch Gladbach, Germany)
IL13	V450	BD Bioscience (San Jose, USA)
IL17A	PeCy7	BioLegend, Inc. (San Diego, USA)
IL22	eF660	eBioscience, Inc. (San Diego, USA)
IL23R	PE	R&D Systems Inc. (Minneapolis, USA)

Target	Fluorochrome	Company
IL4	BV711	BD Bioscience (San Jose, USA)
TGFbetaRII	PerCp	R&D Systems Inc. (Minneapolis, USA)
TNF	AF700	BD Bioscience (San Jose, USA)

2.8. Microbiology

Table 11 Material used for manufacturing of S. aureus and C. albicans lysates

Consumables	
Filtropur V50, 0.2 µm	Sarstedt AG & Co. KG (Nümbrecht, Germany)
Incubation loops	Sarstedt AG & Co. KG (Nümbrecht, Germany)
Nunc™ 14ml Round-Bottom Tube	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Petri dish	Sigma-Aldrich (Saint-Louis, USA)
Media	
➔ see chapter 2.1	
Devices	
Ecotron	Infors AG (Bottmingen, Switzerland)
Thermomixer® 5437	Eppendorf AG (Hamburg, Germany)

3. Methods

3.1. Cell culture

3.1.1. Collection of blood samples and PBMC isolation

The cells used for the following experiments were obtained from human peripheral venous blood. The blood was collected from voluntary healthy donors between the ages of 20 and 45.

- Prior to blood collection, 50 ml syringes were prepared with 100 µl Heparin to avoid blood coagulation
- Depending on the required cells in each experiment, the required blood volume was estimated and taken in 50 ml portions after local disinfection from a forearm vein of the donor
- The obtained blood was diluted 1:2 with DPBS
- After preparing 50 ml tubes with respectively 15 ml Lymphoprep, 25 ml of diluted blood was carefully layered above without mixing both solutions
- The tubes were centrifuged at room temperature for 15 minutes with 2,200 rpm and break switched off
- The PBMC bands of two 50 ml tubes were collected and merged into a new sterile 50 ml tube, washed with DPBS + 5 mM EDTA and centrifuged for 10 minutes at room temperature with 1,600 rpm
- The liquid was discarded without disturbing the PBMC pellet
- Two pellets were merged, washed with DPBS + 5 mM EDTA and centrifuged for 10 minutes at room temperature with 12,000 rpm
- If the pellet contained to many erythrocytes, an erythrocyte lysis was performed by incubating the cell pellet resuspended in RBC lysis buffer for 4 minutes at room temperature
- After an additional washing step, the cells were resuspended and counted
- Depending on the experiment, they were cultured or subsequently purified via MACS separation following the manufacturer's instructions when a single subpopulation was required

3.1.2. MACS® separation

The magnetic-activated cell sorting allows to separate cell populations depending on their surface antigens. Antibodies, targeting CD molecules and linked to superparamagnetic nanoparticles, are incubated with the samples. When the samples subsequently pass through a column in a strong magnetic field, tagged cells attach and get captured. Depending on which fraction is of interest, either the positive tagged cells or the untouched negative cells, a certain program must be chosen to obtain a high purity. Three different isolation kits were used: $CD4^+$ T-cell isolation kit, CD14 MicroBeads and CD45RO MicroBeads. Prior to the use of the CD45RO MicroBeads, always the $CD4^+$ cells were isolated. For example, to obtain naïve T-cells, purified $CD4^+$ cells were incubated with CD45RO MicroBeads and the negative ($CD45RA^+$) fraction was isolated by autoMACS® Pro Seperator, subsequently. In turn, when $CD45RO^+$ effector T-cells were required, the positive fraction was used (Figure 4).

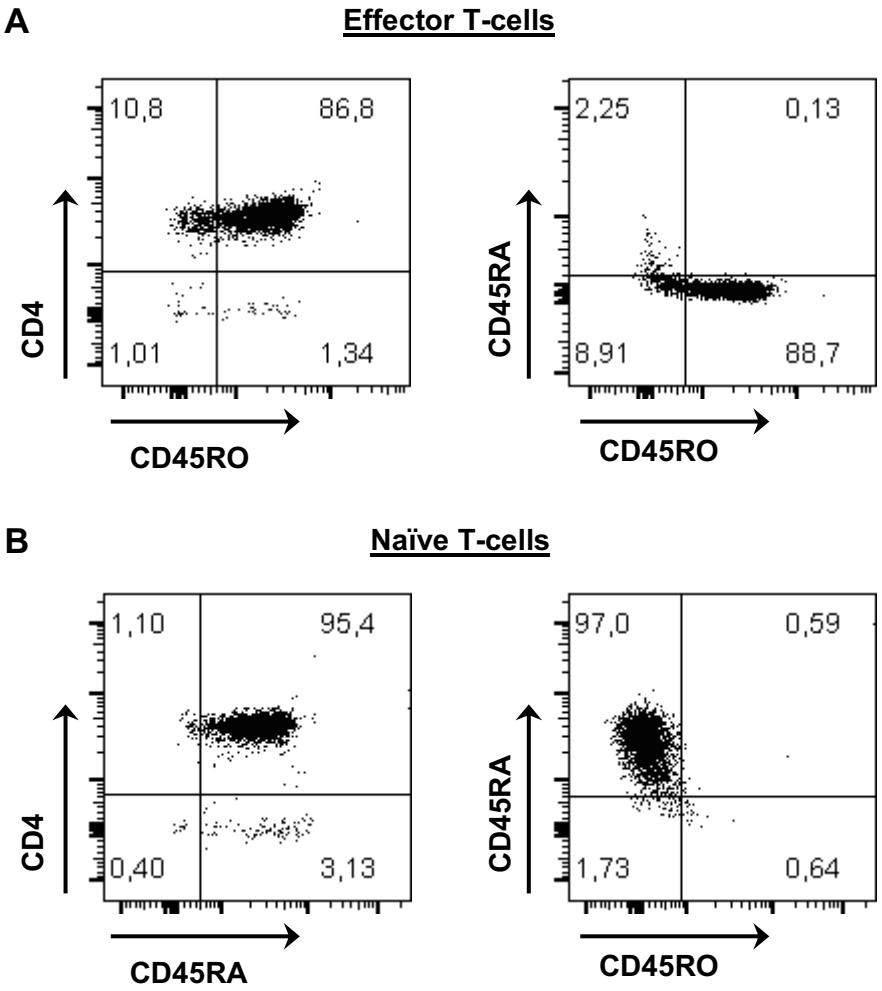


Figure 4 Purification of naïve and effector T-cells by MACS. Flow cytometric control of MACS efficacy. $CD4^+CD45RO^+$ effector (A) and $CD4^+CD45RO^{neg}$ naïve T-cells (B) were isolated from human PBMC's in a high purity.

3.1.3. Toxicity testing

To test whether guselkumab has toxic effects in an *in vitro* culture, PBMC's were incubated with increasing concentrations of guselkumab and analyzed by flow cytometry.

- PBMC suspension was adjusted to 3.0 x10^6 cells/ml in culture medium supplemented with 5% human serum (HS)
- 0.3 x10^6 cells (≙100 µl PBMC suspension) were seeded per well in a 96-well flat bottom plate
- Assaying in duplicates, 9 different guselkumab concentrations were diluted in culture medium + 5% HS, before 100 µl of Stimulus was added to each well to reach final concentrations of 0; 10; 30; 50; 70; 90; 100; 1,000 and 10,000 µg/ml
- After 72 hours of incubation at 37°C and 5% carbon dioxide (CO_2), the cells were stained with Aqua, a dead cell stain kit, and analyzed by flow cytometry

3.1.4. Stimulation of PBMC's

To determine an effective dose of guselkumab and IL-23 in the framework of an *in vitro* model for a following T-cell culture, PBMC's were stimulated with phytohemagglutinin (PHA) in the presence of different guselkumab and IL-23 concentrations.

- PBMC suspension was adjusted to 3.0 x10^6 cells/ml in culture medium
- 0.3 x10^6 cells (≙100 µl PBMC suspension) were seeded per well in a 96-well flat bottom plate
- Five different guselkumab concentrations [0; 5; 10; 50 and 1,000 µg/ml] were combined variously with four different IL-23 concentrations [0; 1; 10 and 100 ng/ml]
- The resulting mixtures were preincubated with PHA (10 µg/ml) for 30 minutes while shaking
- 100 µl of Stimulus mix was added to each well and the cells were incubated for 7 days at 37°C and 5% CO_2
- Every sample was assayed in duplicates
- After 7 days of stimulation, the supernatants were removed and stored at -80°C to perform an IL-17 ELISA, subsequently

3.1.5. *In vitro* T_H17 differentiation

Current publications provide scarce information about human *in vitro* T_H17 polarizations with the intention to stimulate the cells for a flow cytometric staining after differentiation. A promising protocol was published by Revu et al. in 2018 showing that T_H17 differentiation is inhibited by anti-CD28 (Revu et al. 2018). After several tests, titrations and improvements concerning an alteration of anti-CD3 or IL-23 concentration or the supplementation of the medium with different concentrations of human HS or fetal calf serum (FCS), a differentiation protocol was established based on the method of Revu et al. and consisting of two parts: a stimulation and a resting phase.

- $CD4^+$ T-cells were purified from PMBC's prior to their incubation with CD45RO beads
- In a further MACS separation $CD45RO^+$ cells were extracted and the naïve $CD45RA^+$ cells remained in the negative population
- 24-well, non-treated, flat bottom plates were coated with 5 µg/ml anti-CD3 for 1.5-2.0 hours at 37°C and 5% CO_2
- Several stimulation mixtures containing IL-23, IL-1β, TGFβ, IL-6 and guselkumab were prepared based on culture medium supplemented with 5% HS, 1 µg/ml anti-IFN-γ and 5 µg/ml anti-IL-4 to reach the following working concentrations when required:
    - IL-23: 10 or 50 ng/ml
    - IL-1β: 50 ng/ml
    - TGFβ: 1 ng/ml
    - IL-6: 30 ng/ml
    - Guselkumab: 50 µg/ml
- Stimulation mixtures were preincubated for 30 minutes at 37°C and 5% CO_2
- Cell suspensions were adjusted to 2.0 $x10^6$ cells/ml in culture medium + 5% HS and 1 ml was seeded out in every well of a 24 well plate
- 500 µl of 3-fold concentrated stimulus was added to each well to reach a final volume of 1,500 µl
- Samples were incubated for 6-7 days at 37°C and 5% CO_2
- After 6-7 days of stimulation, the cells were removed from anti-CD3 and transferred into a new 24-well plate. They obtained fresh culture medium + 5% HS supplemented with 50 U/ml IL-2 and the same cytokine mix they have been exposed to before

- Samples were incubated further 6-7 days at 37°C and 5% CO_2
- Medium changes were performed when necessary: half of the medium was carefully removed and was replaced by fresh culture medium + 5% HS supplemented with guselkumab when required
- After 6-7 days of resting the cells were counted and a part of them was restimulated in duplicates in culture medium + 5% HS supplemented with 0.75 µg/ml anti-CD28 for 3 days in a 96-well plate coated with 0.75 µg/ml anti-CD3 to perform an ELISA of the supernatants afterwards
- The rest of the cells was stimulated according to the GolgiStop stimulation protocol (3.3.2.1b) and stained before subsequent flow cytometric analysis

3.1.6. Culture of pan $CD4^+$ T-cells and $CD4^+$ effector T-cells

- PBMC's were isolated and $CD4^+$ cells were purified by MACS separation
- The $CD4^+$ population was split, one part was put on ice and the other one was incubated with CD45RO MicroBeads to obtain $CD4^+$ $CD45RO^+$ effector T-cells in a further MACS separation
- Both populations, $CD4^+$ and effector T-cells, were adjusted to 1.5-2.0 x 10^6 cells/ml in culture medium + 5% HS
- 96-well, non-treated, flat bottom plates were coated with 0.75 µg/ml anti-CD3 for 1.5-2.0 hours at 37°C
- Stimulation mixes were prepared, containing culture medium + 5% HS, IL-23, guselkumab, TGFβ and anti-CD-28 to reach final concentrations of 100 ng/ml IL-23, 50 µg/ml guselkumab and 6 ng/ml TGFβ, as well as 0.75 µg/ml anti-CD28
- Stimulation mixes were preincubated for 30 minutes and put on ice, subsequently
- 0.15-2.00 x10^6 cells were seeded per well and 100 µl stimulation mix was added, each sample was assayed in duplicates
- The samples were incubated at 37°C and 5% CO_2 for 3 days
- After 3 days of stimulation, the supernatants were removed and stored at -80°C to perform an ELISA. The cells were resuspended in culture medium + 5% HS containing 50 µg/ml guselkumab when required and transferred to a new anti-CD3 free, flat bottom 96-well plate
- The cells were incubated for 10 days without stimulation and with medium changes three times a week, including guselkumab when required to have time to rest prior to the stimulation for intracellular staining

- After 10 days of rest, the cells were stimulated with the GolgiStop and GolgiPlug protocol (3.3.2.1a), stained and analyzed by flow cytometry

3.1.7. Proliferation assay

In the course of this experiment, antigen pulsed autologous monocytes provided a natural stimulus for T-cells. The monocytes were pulsed with either C. albicans or S. aureus (see 3.2.2), instead of the control, which obtained no pathogen exposure. Both, naïve and effector T-cell populations were observed via flow cytometric analysis, also under IL-23 neutralizing conditions.

- After PBMC isolation MACS separations were performed as follows
    - Half of the cells underwent a separation with CD14 MicroBeads to obtain the monocytes
    - $CD4^+$ cells were purified out of the remaining part and subsequently CD45RO MicroBeads were used to divide them in naïve and effector T-cells in a further MACS run

1. Pulsing Monocytes

- Monocytes were adjusted to 1.0 $x10^6$ cells/ml in culture medium + 5% HS
- Prior to seeding 500 µl of this Monocyte suspensions in a flat bottom, non-treated 24-well plate, they were inoculated with 1 µg/ml of the pathogen lysates. The control did not obtain a stimulus
- All monocytes were pulsed for 3.5 hours at 37°C and 5% CO_2 and half an hour before expiration of this period 50 µg/ml guselkumab was added when required

2. Violet staining of T-cells

- According to the manufacturer's instructions the lyophilized violet dye was dissolved in 20 µl DMSO before a 1:1,000 dilution in DPBS
- Naïve and effector T-cells were counted, centrifuged and the supernatants were discarded
- Regarding that 1.0 $x10^7$ cells require 1 ml of violet solution, they were gently resuspended in a 15 ml falcon and incubated for 4 minutes at room temperature while shaking in the dark
- The labeling reaction was stopped by filling up the falcon to 15 ml with cold DPBS + 10% FCS

- The cells were centrifuged, resuspended in culture medium + 5% HS, counted and adjusted to a concentration of 1.0 x10^6 cells/ml

3. Coculturing

- 1 ml of T-cells (naïve or effector) was added to the pulsed monocytes and when required 5 µl of a 1:10 guselkumab dilution was added to reach the final concentration of 50 µg/ml
- Effector T-cells were incubated for 5-6 days at 37°C and 5% CO_2 and naïve T-cells for 12 days
- After 2 days all samples were supplemented with 20 U/ml IL-2
- When necessary medium changes were performed, also with guselkumab when required

4. Harvesting

- After incubation, the cells were harvested and stimulated according to the GolgiStop and GolgiPlug protocol (3.3.2.1a), before they were stained and analyzed by flow cytometry
- With attention to the violet labeling the steps of the procedure were performed in the dark

3.2. Proteinbiochemistry

3.2.1. Enzyme-linked immunosorbent assay (ELISA)

In order to measure IL-17, IFN-γ and IL-10 in the T-cell supernatant three different common ELISA sets were used in accordance with the manufacturer's instructions.

- Human IL-17 DuoSet ELISA (R&D)
- Human IFN-γ DuoSet ELISA (R&D)
- Human IL-10 OptEIA ELISA Set (BD)

The used reagents differed among the kits depending on the manufacturer, but the procedure was similar: after coating 96 well microtiter plates with a capture antibody diluted in a recommended buffer overnight and blocking free binding sites in the morning, the capture antibodies were able to bind the corresponding cytokine in the supernatant. The following addition of a detection antibody, specific for another cytokine epitope completed the sandwich ELISA and the linkage of this antibody to a peroxidase led to an

enzyme-mediated color change of a colorimetric substrate solution. By the measurement of the absorption at a certain wavelength compared with a standard curve, the cytokine concentrations could be determined. In the majority of cases, the samples had to be diluted to fit in the measuring range of the ELISA.

3.2.2. Manufacture of lysates from Staphylococcus aureus (S. aureus) and Candida albicans (C. albicans)

1. S. aureus

- Lysogeny broth (LB) powder was dispensed in demineralized water and autoclaved to obtain a 2.5% solution of LB medium
- A colony of S. aureus was picked and put in 3.5 ml LB medium in a 14 ml bacteria culture tube
- The miniculture was incubated for 5-6 hours at 37°C while shaking with 120 rpm
- 200 ml LB medium was inoculated with 100 µl of the miniculture in an Erlenmeyer flask and incubated over night at 37°C while shaking with 120 rpm
- The culture was heat inactivated by putting the Erlenmeyer flask for two hours in a water bath with 65°C
- The culture was divided up on four 50 ml falcons and centrifuged with 4,000 g for 15 min
- Pellets were merged and washed two times with DPBS before the final pellet was resuspended in 2 ml DPBS and divided up in aliquots of 100 µl each
- The aliquots underwent five times a freeze/thaw cycle with liquid nitrogen and a water bath at 37°C
- To confirm the successful inactivation of the bacteria the viability was checked with 50 µl lysate on blood agar
- The aliquots were stored at -80°C and the protein concentration was determined by a BCA assay

2. C. albicans

- Yeast nitrogen base (YNB) powder and Glucose were dispensed in ultra-pure water and filtered with a pore size of 0.22 µm to obtain YNB medium
- A colony of C. albicans was picked, inoculated in 10 ml YNB medium and incubated over night at 30°C while shaking with 200 rpm

- 300 ml YNB medium was inoculated with 10 ml of the miniculture for 3-4 days at 30°C while shaking with 200 rpm
- The culture was heat inactivated by putting the Erlenmeyer flask for two hours in a water bath with 65°C
- The culture was divided up on four 50 ml falcons and centrifuged with 4,000 g for 15 min
- Pellets were merged and washed two times with DPBS before the final pellet was resuspended in 2 ml DPBS and divided up in aliquots of 100 µl each
- The aliquots underwent five times a freeze/thaw cycle with liquid nitrogen and a water bath at 37°C
- To confirm the successful inactivation of the fungus the viability was checked with 50 µl lysate on YNB agar plates
- The aliquots were stored at -80°C and the protein concentration was determined by a BCA assay

3.2.3. Bicinchoninic acid (BCA) assay

A BCA assay was needed to determine the protein concentrations of both lysates to make their use comparable. The mechanism of this assay is based on the capability of peptides to reduce copper ions, concluding that the amount of proteins correlates with the amount of reduced copper ions. After their reduction the Cu^{+} ions are able to form bonds with bicinchoninic acids and the resulting colored complex absorbs light at a specific wavelength. This absorption can be measured and compared to a standard curve with distinct protein concentrations the protein concentrations of the samples can be identified. Therefore, a standard BCA assay kit was used according to the manufacturer's instructions.

3.3. Flow cytometry

3.3.1. Live/dead and surface staining

In order to detect the protein expression of the cells they were stained with fluorochrome conjugated antibodies and analyzed by flow cytometry. First of all, the cells were stained with amine reactive dyes (Aqua or Far Red) to distinguish living and dead cells. Afterwards, the antibodies were used to stain the cell surface before the cells were permeabilized to enable an intracellular staining. Amine reactive dyes interact with proteins, in living cells they cannot pass the cell membrane and are only able to bind

surface proteins. But due to their capability to penetrate the cell membrane of dead cells, they can form bonds with intracellular proteins, which causes a stronger fluorescence compared to living cells. Furthermore, they can also bind sodium acid, that's why dilutions and washings during live/dead staining is performed with DPBS instead of FACS buffer.

- The cell suspension was pipetted in a 96 well round bottom microtiter plate for staining
- A washing step followed, consisting of a resuspension with 200 µl cold DPBS, a subsequent centrifugation for 1 minute with 2000 rpm at room temperature and the removal of the supernatants
- After a further washing step, the supernatants were discarded again
- The cells were resuspended in Aqua (1:1,000 in DPBS) or Far Red (1:10,000 in DPBS) and incubated for 30 minutes at 4°C in the dark
- 100 µl cold DPBS was added, cells were centrifuged and supernatants were discarded

After accomplishing the previous live/dead staining FACS buffer could be used for the following surface staining. The FACS buffer contains FCS to keep the cells in a better condition during the staining procedure.

- The cells were washed with 200 µl FACS buffer, centrifuged and the supernatants were discarded
- 10 µl of antibody mix diluted in FACS buffer was added and the cells were stained for 30 minutes at 4°C in the dark
- Depending on the experiment, the following antibodies were used:
    - PeCF594 anti-CD3 (end dilution 1:50)
    - PeCF594 anti-CD69 (end dilution 1:50)
    - APC-Cy7 anti-CD4 (end dilution 1:20)
    - V450 anti-CD45RA (end dilution 1:50)
    - Bv650 anti-CD45RO (end dilution 1:100)
    - PE anti-IL-23R (end dilution 1:10)
    - PerCp anti TGF-βRII (end dilution 1:10)
- After a further washing step with 200 µl FACS buffer, a flow cytometric analysis or an intracellular staining was performed

3.3.2. Intracellular staining

To perform an intracellular staining, the cells have to be extensively activated, thereby the intracellular cytokines cumulate and a proper fluorescent signal can be obtained. When an intracellular staining is required, a stimulation previous to the live/dead and surface staining is necessary. The stimulation protocol depends on the experiments: most experiments contained a five-hour lasting stimulation protocol with GolgiPlug and GolgiStop for cytokine retention. For the T_H17 differentiation a stimulation protocol including only GolgiPlug was used adapted from Revu et al. (Revu et al. 2018). Both substances impair secretion process of cells and cause thereby an accumulation of intracellular proteins, but they differ in their mode of action. GolgiPlug contains brefeldin A that hinders vesicular transport from the endoplasmic reticulum to the cis golgi network, while GolgiStop containing monensin blocks transport between the cis and the trans golgi network. After stimulation and surface staining the cells get fixed. By treating the cells with a detergent, the cell membrane gets porous and the fluorochrome conjugated antibodies are able to reach the cytoplasm.

1. **Stimulation prior to live/dead and surface staining**

   a. **GolgiStop and GolgiPlug protocol – 96 well**

      - Approximately 0.15 x 10^6 cells were diluted in 100 µl of culture medium and plated into a 96 well
      - Stimulation mix was prepared consisting of PMA, ionomycin and GolgiStop in 50 µl RPMI for 1x96 well (final volume 150 µl)
        - 0.015 µl PMA (stock: 100 µg/ml; working conc.: 10 ng/ml)
        - 0.15 µl ionomycin (stock: 1 mg/ml; working conc.: 1 µg/ml)
        - 0.1 µl GolgiStop (end dilution 1:1,500)
        - Ad 50 µl RPMI
      - 50 µl of stimulation mix was added to each well and they were incubated for 2 hours at 37°C
      - GolgiPlug was diluted 1:100 in RPMI and 16.6 µl of this dilution was added to each well before they were incubated for another 3 hours

   b. **GolgiStop protocol (T_H17 differentiation) – 24 well**

      - Approximately 0.2 x 10^6cells were diluted in 100 µl of culture medium and plated into a 96 well

- 50 µl of stimulation mix was added to the cells consisting of RPMI supplemented with:
  - PMA (stock: 100 µg/ml; working conc.: 50 ng/ml)
  - Ionomycin (stock: 1 mg/ml; working conc.: 500 ng/ml)
  - GolgiPlug (end dilution: 1:1,000)
- Cells were incubated for 4 hours at 37°C

2. Permeabilization and intracellular staining

- Cells were transferred into a 96 well round bottom plate, centrifuged with 2,000 rpm at room temperature for one minute and supernatants were discarded
- After accomplishing live/dead and surface staining (see section 3.3.1), the cells were fixed with 100 µl Fix/Perm solution and incubated for 20 minutes at 4°C
- 100 µl FACS buffer was added, cells were centrifuged and supernatant was discarded
- After another washing step with 200 µl FACS buffer, 200 µl 1X Perm solution (10X Perm/Wash buffer 1:10 diluted in sterile water) was added to the cells
- Cells were again centrifuged and the supernatants were discarded
- 10 µl antibody mix diluted in 1X Perm solution was added to the cells and they were incubated for 30 minutes at room temperature in the dark while shaking
  - depending on the experiment the following antibodies were used
    - BV605 anti-IFN-γ (end dilution 1:100)
    - BV711 anti-IL-4 (end dilution 1:20)
    - Vio515 anti-IL-10 (end dilution 1:10)
    - V450 anti-IL-13 (end dilution 1:10)
    - PeCy7 anti-IL-17A (end dilution 1:200)
    - eF660 anti-IL-22 (end dilution 1:20)
    - AF700 anti-TNF-α (end dilution 1:100)
- Cells were washed again with 200 µl FACS buffer, centrifuged and supernatants were discarded
- Cells were resuspended in 200 µl FACS buffer and transferred into FACS cluster tubes for flow cytometric analysis

3.3.3. Acquisition and gating strategy

To detect the stained cells the flow cytometer LSR Fortessa (BD) was used. In the process of flow cytometric analysis, the cells are getting aspirated and aligned in a liquid stream carrying them almost separately through the illumination by different lasers. These lasers emit light of different wavelengths, which can specifically excite the fluorescent labeled antibodies and these, in turn, reemit the light. The dye-specific fluorescence as well as the measurement of forward- and side-scattered light by a detector unit provides information about size, granularity and protein expression of the cells. The fast processing of this information by a connected computer allows the analysis of many thousand cells in only one second.

The results of flow cytometry were analyzed by FlowJo software Version 10. The gating strategies of the different experiments showed little discrepancies, which is why they are showed in detail in the following.

3.3.3.1. Naïve T-cell differentiation culture

To obtain naïve T-cells, PBMC's were isolated and $CD4^{+}CD45RO^{neg}$ cells were purified by MACS (3.1.2). Subsequently, cells were seeded in 24-well plates and stimulated in different manners for six to seven days before they underwent a resting phase, which was modified as well (3.1.5). Similar to the prior experiment, the gating strategy was built up on the identification of living lymphocytes before $CD45RO^{+}$ effector T-cells were focused to observe IL-17 expression and co-expression of other cytokines (Figure 5).

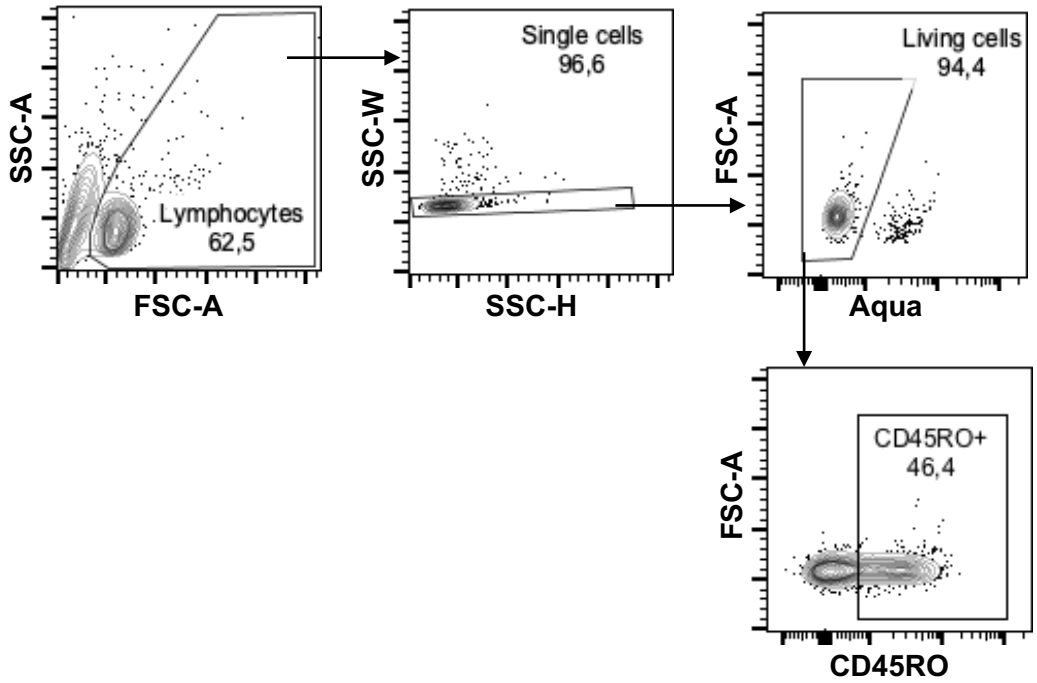


Figure 5 Gating strategy for analyzing T_H17 polarization. After gating on lymphocytes and exclusion of doublets and dead cells, effector T-cells expressing CD45RO were analyzed in regard to cytokine expression.

3.3.3.2. $CD4^+$ and effector T-cell culture

$CD4^+$ cells were isolated from human PBMC's of healthy donors by MACS and for the effector T-cell culture $CD45RO^+$ cells were isolated in a further sorting cycle, subsequently. The cells were stimulated with anti-CD3 and anti-CD28 for three days in the presence of TGFβ and IL-23 with or without guselkumab (3.1.6). TGFβ was added to the cultures because of its controversially discussed role in T_H17 differentiation. In this context, TGFβ was supposed to have beneficial effects on the development of non-pathogenic T_H17 cells (McGeachy et al. 2007). Subsequently, cells were rested for ten days before they were stimulated with PMA and ionomycin for intracellular staining (3.3.2). In the following flow cytrometric analysis lymphocytes likewise single cells were identified in the Forward Scatter (FSC) and the Side Scatter (SSC), initially (Figure 6A). Living cells were diminished by Aqua, an amine reactive dye, subsequently. Activated effector cells were put into focus by expression of CD69 and CD45RO before cytokine profile was examined (Figure 6B).

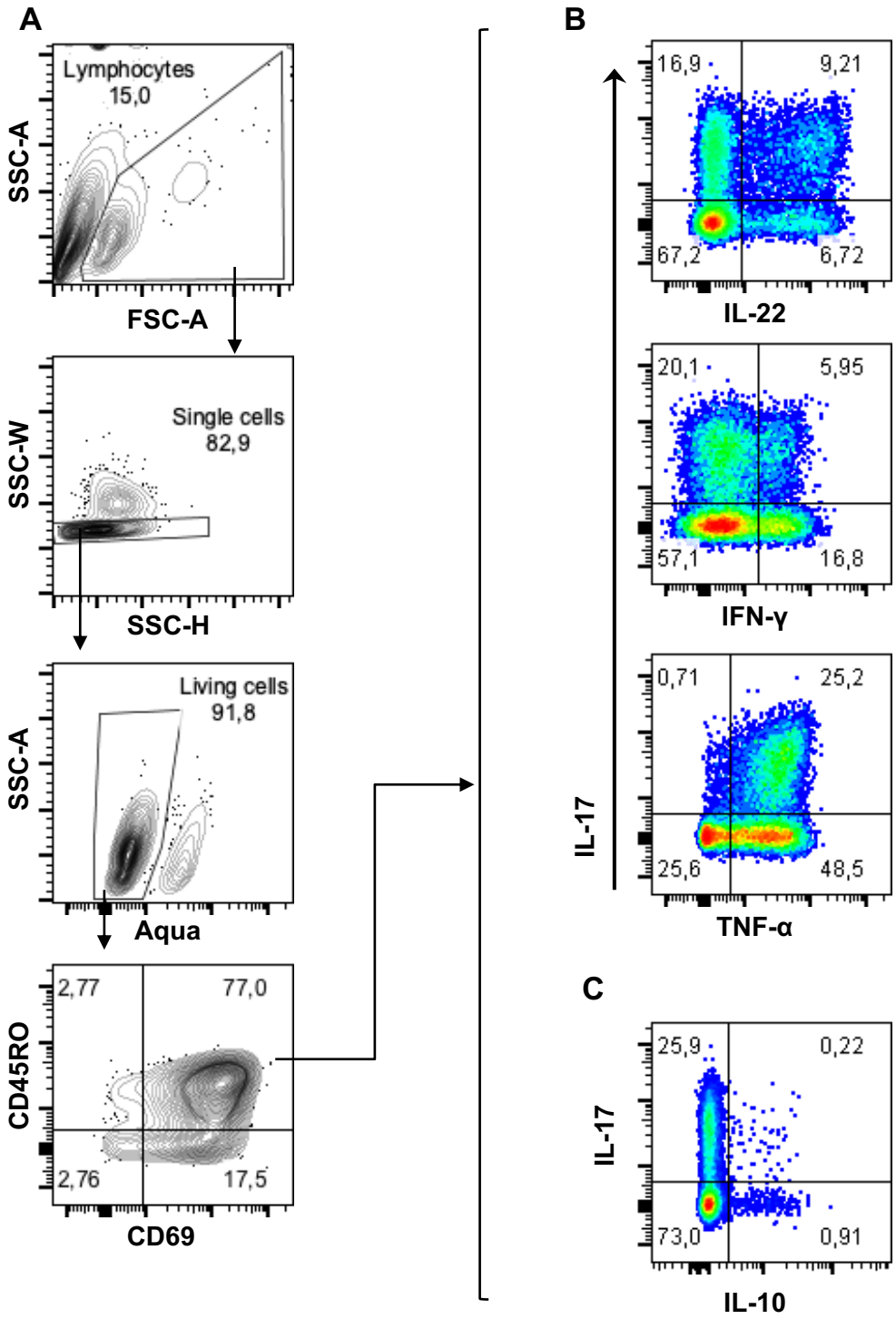


Figure 6 Gating strategy of CD4⁺ T-cell culture. Cells were analyzed by flow cytometry after stimulation with PMA and ionomycin and subsequent surface and intracellular staining. After gating on Lymphocytes and exclusion of doublets and dead cells, the CD45RO⁺ CD69⁺ population representing activated effector T-cells was gated (A). Among them, cytokine profiles of pathogenic (B) and non-pathogenic (C) T_H17 subsets were observed.

3.3.3.3. T-cell and monocyte co-culture

Autologous monocytes were isolated and pulsed with S. aureus and C. albicans lysates. Simultaneously, CD4⁺CD45RO⁺ effector T-cells and CD4⁺CD45RO^{neg} naïve T-cells were isolated by MACS and stained with a cell trace dye, to monitor antigen specific

proliferation of these cells by dilution of the cell trace dye. Effector and naïve T-cells were co-cultured with autologous pulsed monocytes and afterwards stimulated for intracellular cytokine staining for flow cytometry. To analyze effects of IL-23 neutralization, anti-IL-23 was added during activation of monocytes and their co-culture with T-cells (3.1.6). The gating strategy of the flow cytometric analysis consisted of the following steps: after gating on lymphocytes and the separation of viable single cells, CD3 was used to define T-cells and exclude monocytes from cytokine analysis. Subsequently, proliferating T-cells were identified by gating on cell trace^{neg} cells before cytokine profile was analyzed (Figure 7).

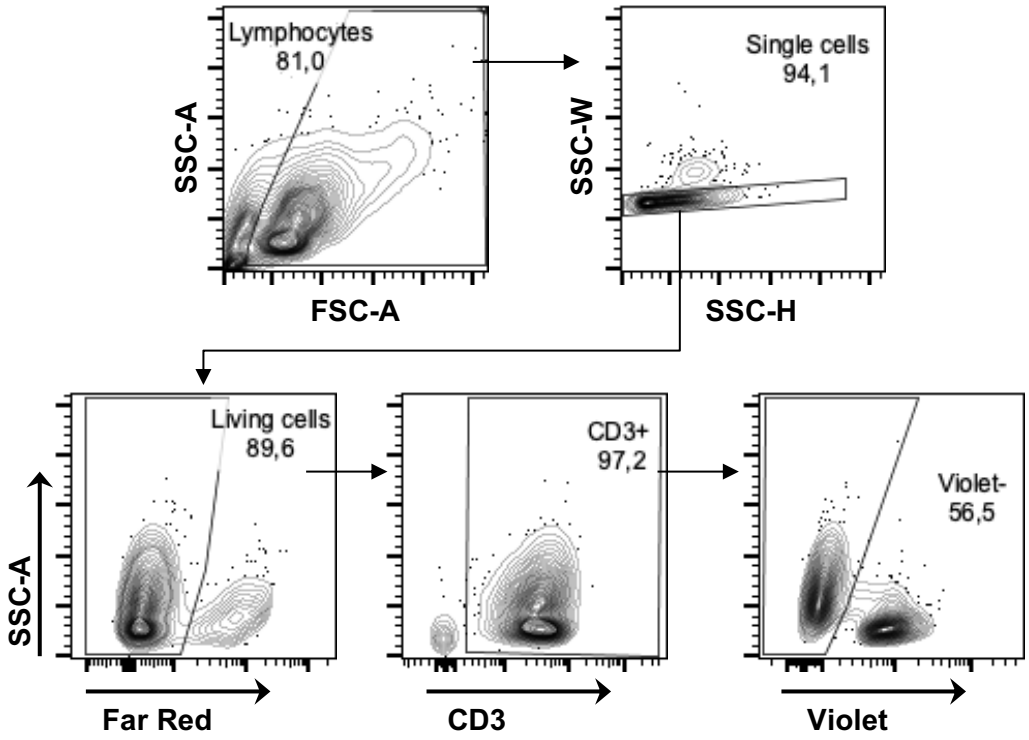


Figure 7 Detection of proliferating CD4^{+} effector and naïve T-cells specific for S. aureus and C. albicans via flow cytometry. The gating strategy consisted of successive gates on lymphocytes, single cells, living cells, CD3^{+} cells and violet^{neg} cells representing proliferating Lymphocytes specific for the respective lysate.

3.3.3.4. Acquisition of absolute cell numbers

After determination of cell frequencies, additional counting beads in the samples were required to determining absolute cell numbers. The FACS software counts the cells, that are soaked up, but it cannot refer this number to the whole sample. Soaking up different volumes causes different cell counts. To make the cell numbers counted by the FACS software useful, a tracer has to be added to the sample to find out how much of it is soaked up. Therefore, counting beads were used, which are able to be detected in almost every staining channel (Figure 8). A distinct number of beads was added to each

sample and by identifying how many of them were measured by the FACS software a factor was calculated. Multiplying this factor with the cell counts of interest gave the opportunity to refer these counts to the whole sample.

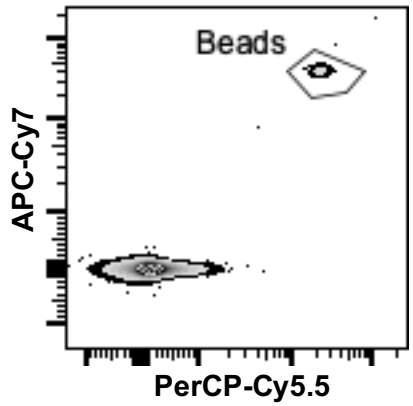


Figure 8 Acquisition of counting beads via flow cytometry. Counting beads show high fluorescence intensity. For example, the beads were identified as a brightly fluorescent population emitting light with the same wavelength as the dyes APC-Cy7 and PerCP-Cy5.5

3.4. Statistical analysis

The software GraphPad Prism 8 and Microsoft Office 2019 were used to process, illustrate and analyze all results. Effects of anti-IL-23 or certain stimuli were detected regarding cytokine specific cell frequencies, cell counts and concentrations. Due to the small number of donors, a testing of gaussian distribution was not suitable. A non-gaussian distribution was assumed and in consequence of that a non-parametric test was considered. According to this, the comparison of two matched samples per donor required a Wilcoxon signed-rank test for paired samples.

4. Results

4.1. Adapting the use of guselkumab to an *in vitro* model

4.1.1. High doses of guselkumab do not show toxic effects

Guselkumab, a therapeutic IL-23 antibody, was used to test the effects of IL-23 inhibition in cell cultures. Because therapeutic antibodies are not made for use in cell culture, we had to exclude toxic effects on *in vitro* cells. Therefore, human PBMC's were incubated for 72 hours with increasing concentrations of guselkumab from 10 to 10,000 µg/ml and analyzed by flow cytometry (3.1.3). After gating on lymphocytes and excluding the doublets, living and dead cells were distinguished by a previously performed Live/Dead staining (3.3.1/ Figure 9A). None of the tested doses had a notable effect on cell viability with 94-96% of living lymphocytes in all conditions which was comparable to untreated cells (ctrl) (Figure 9B).

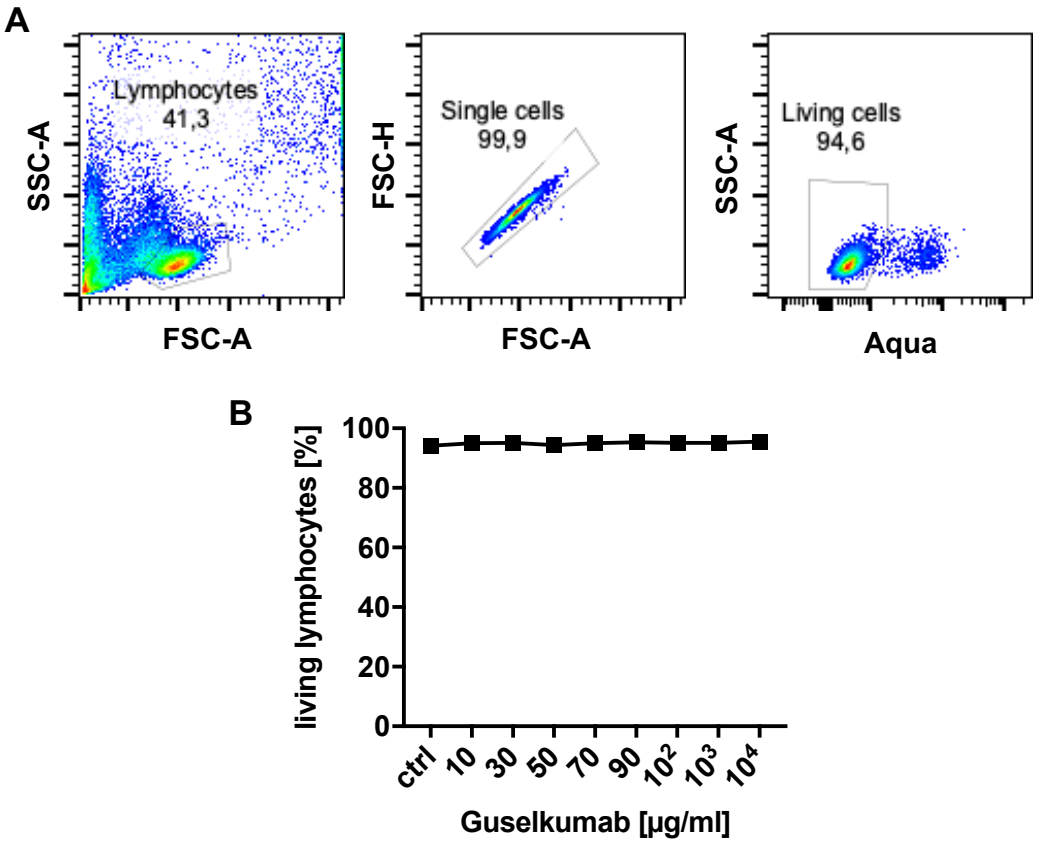


Figure 9 Toxicity testing of guselkumab. PBMC's were cultured *in vitro* in the presence of increasing concentrations of guselkumab. Cells were stained with Aqua, a Live/Dead dye, before flow cytometric analysis. The gating strategy consisted of the isolation of Lymphocytes and single cells before gating on Aqua^{neg} cells (A). The percentage of living cells was compared to exclude toxic side effects (B).

4.1.2. Guselkumab inhibits IL-23 efficiently in vitro

Since guselkumab is used in therapeutic doses of 100 mg/ml, it was important to adapt the concentration efficiently neutralizing IL-23 to an *in vitro* cell culture model. For this purpose, IL-17, as the effector cytokine of the IL-23/IL-17 axis, was measured by ELISA after stimulating PBMC's with PHA in the presence of IL-23 (3.1.4). 100 ng/ml IL-23 was required to induce increased IL-17 secretion with a mean of 905 pg/ml (Figure 10A). In order to test the efficacy of guselkumab, it was added in increasing concentrations to the IL-23 supplemented medium. Already 5 µg/ml of guselkumab efficiently suppressed IL-23 mediated IL-17 induction to a mean level of 556 pg/ml, while high concentrations such as 1,000 µg/ml reverted this effect (Figure 10B). Based on these results, a concentration of 100 ng/ml IL-23 and 50 µg/ml guselkumab was used for the following experiments.

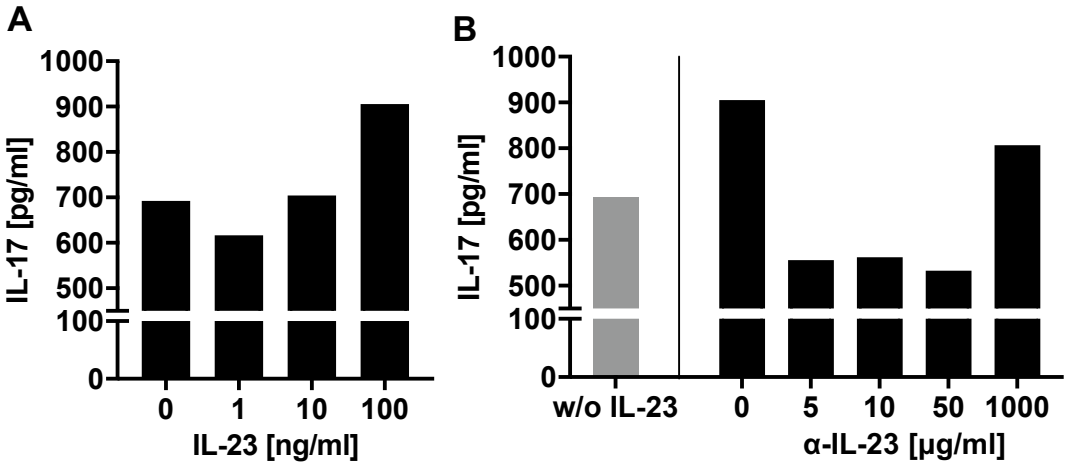


Figure 10 Stimulation of PBMC's in the presence of IL-23 and guselkumab. PBMC's were stimulated for 7 days with PHA and different concentrations of IL-23 to induce an IL-17 response (A). After potent induction of IL-17, PBMC's were stimulated with PHA and 100 ng/ml IL-23 in the presence of rising α-IL-23 concentrations to reverse this effect and to ensure efficient IL-23 blocking (B). IL-17 concentrations were measured by ELISA.

4.2. Anti-IL-23 in context of an *in vitro* differentiation of naïve T-cells towards a T_H17 like phenotype

4.2.1. Anti-CD3, IL-23 and IL-1β induce a sufficient T_H17 differentiation

After guselkumab was confirmed to be a potent inhibitor of IL-23 *in vitro* without a detectable toxicity to *in vitro* cells, we started investigating IL-23 inhibition in different cell culture settings. Starting with T_H17 cell life cycle, we aimed to investigate IL-23

deprivation during T_H17 differentiation from naïve T-cells, initially. For this purpose, a T_H17 polarization procedure had to be established. Several T_H17 polarization protocols can be found in literature with quite different combinations of cytokines required for differentiation. Moreover, protocols are rather heterogenous in terms of incubations times, use of culture media and serum. In addition, there is a rather controverse discussion about the requirement of TGFβ that seems to be different in mice and men (Annunziato et al. 2009; Peters et al. 2011; Sallusto et al. 2012; Romagnani et al. 2009). Literature was screened to establish a working T_H17 polarization protocol in the human system that would also serve the readout of intracellular cytokine staining for flow cytometric analysis, as well as cytokine analysis in cell culture supernatants after TCR stimulation. Protocols were modified in order to establish most efficient T_H17 polarization. In this context flow cytometric analysis of IL-17$^+$ cell frequencies as portion of CD45RO$^+$ differentiated effector T-cells was used as a parameter to assess polarization efficiency.

The most promising protocol was deduced from a publication of Revu et al. which showed that anti-CD28 inhibits naïve T-cell differentiation towards T_H17 and, accordingly, differentiation without anti-CD28 leads to higher frequencies of IL-17$^+$ T-cells (Revu et al. 2018). This protocol, which required only IL-23 and IL-1β, was compared to another one which includes all cytokines known to be involved in T_H17 differentiation and an already existing in-lab protocol that successfully induced high IL-17 levels in cell culture supernatants measured by ELISA. This initial differentiation experiment is summarized in Figure 11 revealing as expected an effective induction of IL-17$^+$ cell frequencies by the protocol from Revu et al. compared to the respective control and higher IL-17$^+$ inductions than the other protocols (Revu et al. 2018). Since the frequency of IL-17$^+$ cells obtained with the protocol from Revu et al. comprising stimulation with 5 µg/ml anti-CD3, 50 ng/ml IL-1β and 50 ng/ml IL-23 was clearly induced, but still rather low, this protocol was further modified (Revu et al. 2018).

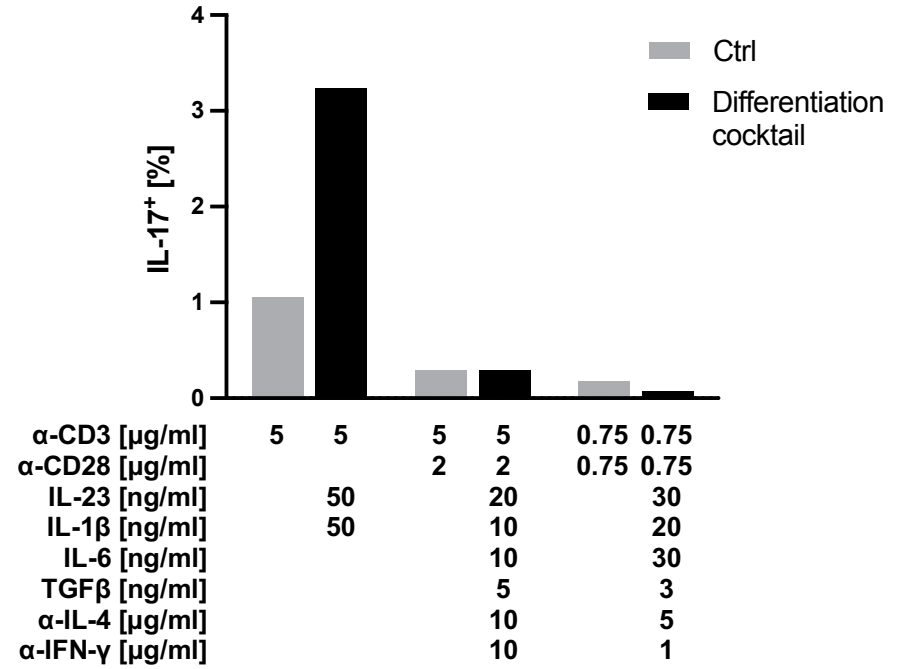


Figure 11 Testing of different *in vitro* T_H17 differentiation protocols. After isolation of human PBMC's and purification of $CD4^+CD45RO^{neg}$ naïve T-cells by MACS, cells were differentiated *in vitro* according to the respective protocol. Differentiated IL-17^+ cells were detected as percentage of $CD45RO^+$ cells by flow cytometric analysis. One protocol containing IL-1β, IL-23 and α-CD3 was deduced from a publication of Revu et al. (Revu et al) (left), the second one containing all well-known inducers of T_H17 differentiation and also a high level of a-CD3 and α-CD28 (middle) was compiled after literature screening, and the third one containing standard α-CD3/28 levels (right) was an in-lab protocol which already showed IL-17 induction in ELISA (black bars). To check whether cytokine combination induced a naïve differentiation towards T_H17, frequencies of IL-17^+ T-cells (black bars) were compared to a control for each protocol consisting of the respective α-CD3/28 concentrations (grey bars).

Next, variations of IL-23 and plate-bound anti-CD3 were examined, as well as effects of supplementing medium changes during resting phase with IL-23 and IL-1β. Neither variation of anti-CD3 concentrations nor supplementation of medium changes with cytokines further enhanced IL-17 production (

Figure 12A). Also supplementing media with increasing concentrations of IL-23 did not show higher IL-17^+ cell rates (

Figure 12B).

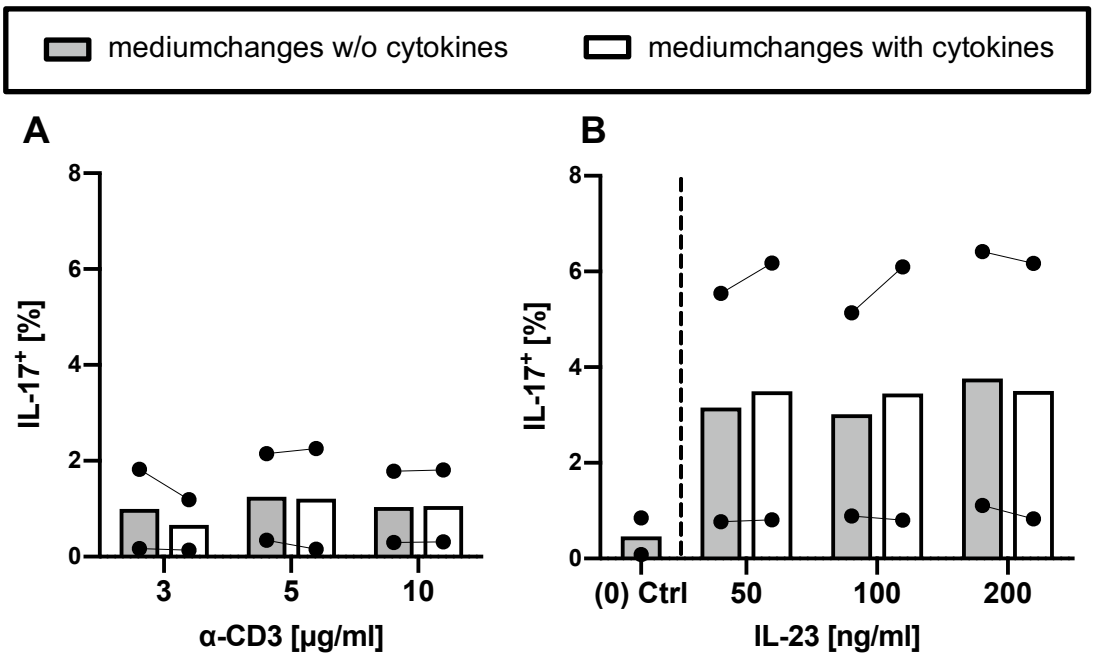


Figure 12 Examining dose-dependent effects of anti-CD3 and IL-23 on *in vitro* T_H17 differentiation. Naïve T-cells were differentiated *in vitro* for 6-7 days in the presence of 5 µg/ml plate-bound α-CD3, 50 ng/ml IL-23 and 50 ng/ml IL-1β diluted in T-cell medium + 10% FCS before undergoing a resting period that began initially in the presence of 50 ng/ml IL-23, 50 ng/ml IL-1β and 50 U/ml IL-2. During this resting phase, medium was changed as needed and either supplemented with 50 ng/ml IL-23 and 50 ng/ml IL-1β (white bars) or not (grey bars). Subsequently, T-cells were stimulated with PMA/ionomycin to perform intracellular and surface staining for flow cytometry. IL-17$^+$ cell frequencies were analyzed as portion of CD45RO$^+$ cells by flow cytometry. Each graph represents a separate experiment.

During the prior differentiation experiments, T-cell medium supplemented with 10% FCS was used. Since cells showed a high demand and consumption of nutrition by culture medium, due to a hypothetically strong state of activation different, concentrations of HS and FCS were tested. Anti-IL-4 and anti-IFN-γ antibodies were added to the samples supplemented with HS to avoid effects of preexisting cytokines in the HS on differentiation of naïve T-cells towards T helper phenotypes other than T_H17. Moreover, cells were analyzed by flow cytometry after six days of differentiation prior to their resting phase as well. Interestingly, using culture medium supplemented with 5% HS resulted in remarkably highest IL-17$^+$ cell frequencies (Figure 13A). Considering that *in vitro* differentiated T_H17 yield shows a strong donor variance IL-17$^+$ rates up to 29.6% were reached (Figure 13B). Also, the resting phase is essential to observe an efficient polarization towards T_H17 since after six days of differentiation a strongly diminished IL17$^+$ frequency was obtained compared to additional six days of resting (Figure 13A).

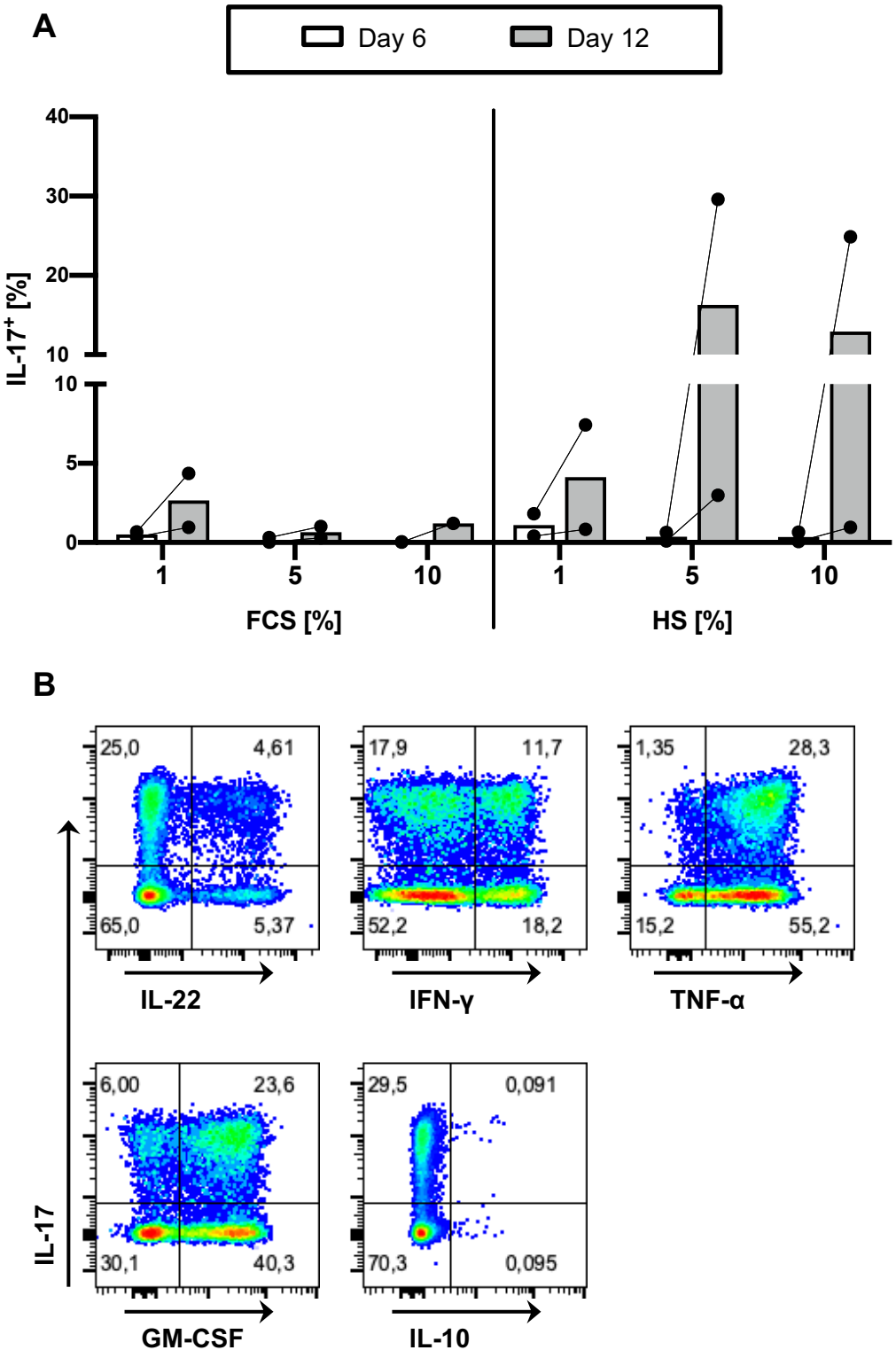


Figure 13 Optimization of *in vitro* T_H17 differentiation. Flow cytometric analysis of *in vitro* differentiated naïve T-cells was performed after they were stimulated for 6 days with α-CD3, IL-23 and IL-1β for (white bars). Subsequently, the cells rested for 6 days in the presence of IL-2, IL-23 and IL-1β and were analyzed again (grey bars). During these two periods, supplementation of T-cell medium with different sera was tested, from left to right 1%, 5% and 10% FCS and 1%, 5% and 10% HS (A). Dot plots of differentiated $IL17^+$ cells co-expressing IL-22, IFN-γ, TNF-α, GM-CSF, and IL-10 are exemplified in (B).

Based on these findings, the optimized differentiation protocol consisted of 6-7 days of stimulation with 5 µg/ml plate-bound anti-CD3 in T-cell medium supplemented with 5%

HS, 1 µg/ml anti-IFN-γ, 5 µg/ml anti-IL-4, 50 ng/ml IL-23 and 50 ng/ml IL-1β. Within the following resting period of 6-7 days, cells were incubated in the presence of 50 U/ml IL-2, 50 ng/ml IL-23 and 50 ng/ml IL-1β. After 12-14 days, cells were restimulated with anti-CD3 and anti-CD28 and cytokine concentration in the supernatant was measured by ELISA. Simultaneously, cells were activated for intracellular staining and subsequent flow cytometric analysis.

4.2.2. Analyzing T_H17 phenotype under different polarizing conditions

In context of T_H17 differentiation, there is a controversial discussion about the role of involved cytokines such as TGFβ, IL-6 and IL-1β driving naïve cells towards T_H17. Furthermore, human and mouse models show differences concerning the cytokine requirements during T_H17 differentiation. Based on our optimized differentiation protocol and by using different cytokines in different combinations and concentrations, we attempted to create different polarization approaches to guide T_H17 differentiation towards pathogenic or non-pathogenic subsets. Here, cytokines used during the seven-day resting period were adapted to the respective cytokines of the previous stimulation phase.

As expected, the polarization approach of our optimized protocol led to a high yield of pathogenic T_H17 cells coproducing IL-22, IFN-γ or TNF-α due to its high amounts of IL-23 and IL-1β. These two cytokines were already mentioned in several publications to be potent inducers of the pathogenic T_H17 subsets (McGeachy et al. 2009; Wilson et al. 2007; Langrish et al. 2005; Zielinski et al. 2012).

In contrast, studies in mouse models revealed that TGFβ and IL-6 were potent inducers for development of non-pathogenic IL-10 expressing T_H17 cells (McGeachy et al. 2007). Based on the crucial role of TGFβ during differentiation of IL-10 producing T_{reg} cells, this cytokine is an obvious candidate to be considered to play a role in the differentiation of non-pathogenic T_H17 cells, as well. In line with this, a non-pathogenic polarization protocol was created containing 1 ng/ml TGFβ and 30 ng/ml IL-6, but no IL-1β. Additionally, this approach was supplemented with a low concentration of IL-23 (10 ng/ml) to maintain survival of T_H17 cells, while avoiding pathogenic polarization driven by the presence of high IL-23 doses. A low dose of TGFβ was chosen because it was shown that TGFβ can act as an inhibitor of human T_H17 differentiation in general (Acosta-Rodriguez et al. 2007; Wilson et al. 2007) but might be still important for non-pathogenic T_H17 cell polarization.

Since preliminary experiments confirmed a suppressive effect of TGFβ on human T_H17 differentiation, another polarization protocol was considered by adding IL-1β to the culture to harness its proliferative capacity on naïve T-cells, potentially offsetting the inhibitory effects of TGFβ on proliferation. Thus, the culture medium of this polarization protocol was supplemented with 10 ng/ml IL-23, 1 ng/ml TGFβ and 50 ng/ml IL-1β. But supplementation with IL-1β creates a cytokine environment that in combination with IL-23 can also give rise to pathogenic T_H17 cells. On this basis, this polarization approach was considered an intermediate polarization protocol that promotes the differentiation of both pathogenic and non-pathogenic T_H17 cells (Figure 14).

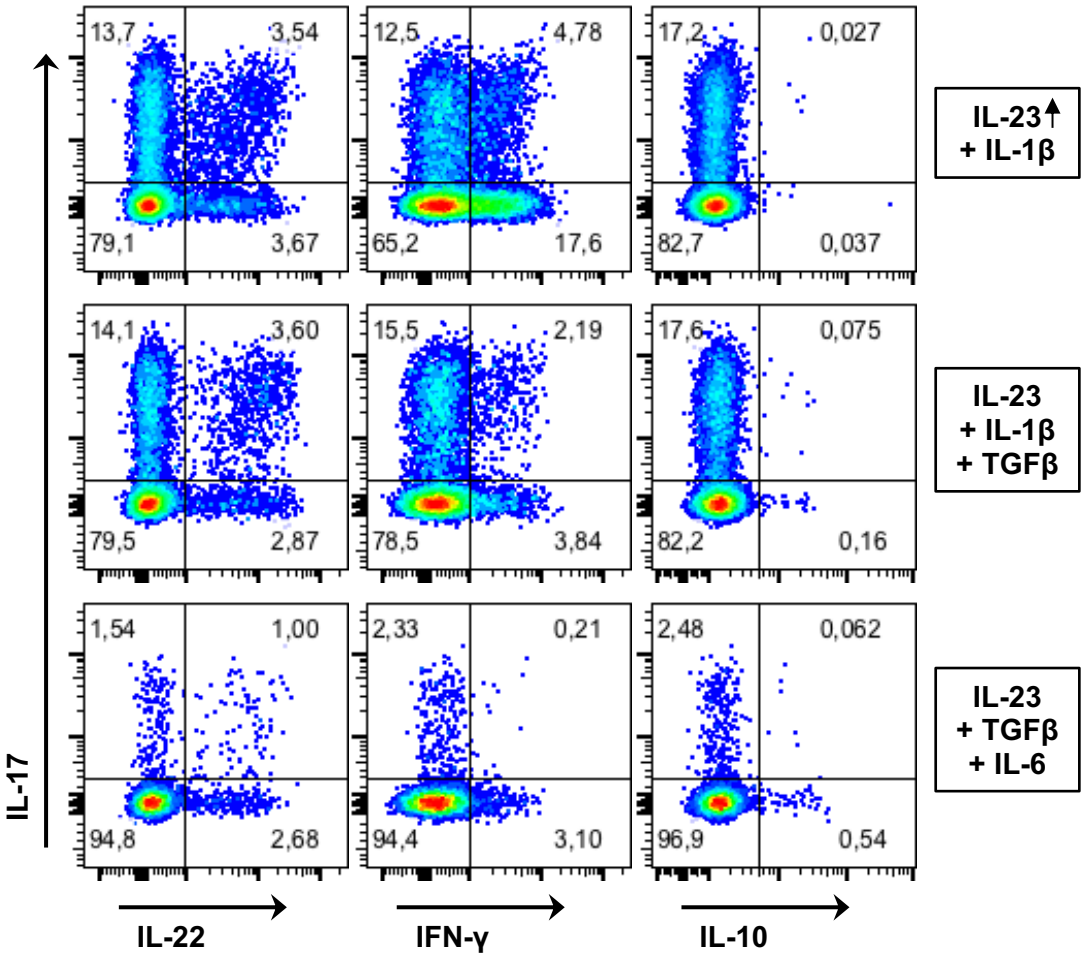


Figure 14 Evaluation of three different polarization cocktails. Following a standardized *in vitro* T_H17 polarization of $CD45RA^+$ naïve T-cells isolated by MACS, three different cytokine conditions were used to examine their influence on the cytokine pattern of differentiated T_H17 cells representing pathogenic ($IL\text{-}17^+/IL\text{-}22^+$ and $IL\text{-}17^+/IFN\text{-}\gamma^+$) or non-pathogenic subsets ($IL\text{-}17^+/IL\text{-}10^+$). A cytokine combination of IL-1β and high levels of IL-23 was used to drive naïve T-cells towards the pathogenic T_H17 phenotype (top). Another cytokine combination based on TGFβ, IL-6 and a low IL-23 concentration (bottom) aimed to induce an anti-inflammatory environment, while a combination of IL-1β, TGFβ and a low dose of IL-23 was designed to create an intermediate cytokine milieu (middle).

Flow cytometric analysis of these different polarization protocols revealed that the pathogenic polarization approach (path.) (50 ng/ml IL-1β and 50 ng/ml IL-23) and the

intermediate polarization approach (50 ng/ml IL-1β, 10 ng/ml IL-23 and 1 ng/ml TGFβ) induced with 11.37% and 11.33% (P=0.0312; 0.0625) IL-17$^+$ cells pronounced differentiation towards the T_H17 phenotype compared to the control showing 2.69% IL-17$^+$. In contrast, the non-pathogenic polarization approach (non-path.) (1 ng/ml TGFβ, 30 ng/ml IL-6 and 10 ng/ml IL-23) comprised only 2.47% IL17$^+$ cells with no significant increase compared to the control with 2.69% (Figure 15A/ Figure 14). Analysis of absolute cell numbers confirmed these effects by showing an increase from 5.266 x10^6 IL-17$^+$ cells in the control to 23.948 x10^6 under pathogenic (P=0.0312) and 15.008 x10^6 under intermediate conditions (P=n.s.) (Figure 16A). Also cytokine levels of IL-17 measured by ELISA after 72 hours of restimulation with anti-CD3/28 (3.1.5/ 3.2.1) showed most efficient IL-17 induction under IL-1β/IL-23 containing pathogenic conditions from 630.04 pg/ml in the control to 4,004.05 pg/ml, while the IL-1β/IL-23/TGFβ containing intermediate cytokine mix induced lower IL-17 levels at 1,295.96 pg/ml (Figure 17).

Neither concerning cell frequencies nor absolute cell numbers IL-22$^+$ cells showed significant differences comparing the control and the three differentiation conditions (Figure 15A/ Figure 16A). As expected, IFN-γ$^+$ cells were induced under pathogenic differentiation conditions with IFN-γ$^+$ frequencies increasing on average from 12.62% (control) to 17.75% (P=n.s.), corresponding to an absolute cell number increasing from 24.785 x10^6 cells (control) to 36.077 x10^6 cells (P=n.s.). They decreased under non-pathogenic differentiation conditions, showing 3.89% IFN-γ$^+$ cells (P=0.0312) or respectively 3.427 x10^6 cells (P=0.0312). The intermediate approach did not increase IFN-γ$^+$ cell frequencies above the existing level in the control and rather decreased IFN-γ$^+$ cell numbers from 24.785 x10^6 (control) to 9.547 x10^6 (P=0.0312) (Figure 15A/ Figure 16A). At the cytokine level, strong IFN-γ induction from 2,711.14 pg/ml to 4,655.31 pg/ml was observed under pathogenic conditions, whereas no IFN-γ induction occurred under intermediate (1,549.16 pg/ml) and non-pathogenic (172.74 pg/ml) conditions compared to the control (Figure 17).

TNF-α$^+$ cell frequencies increased slightly in a pathogenic environment from 38.01% (control) to 48.51% (P=n.s.) and significantly within the intermediate differentiation approach to 60.9% (P=0.0312), whereas TNF-α$^+$ cell frequency of the non-pathogenic approach did not substantially differ from the control (Figure 15A). Cell counts indicated a decrease from 76.953 x10^6 cells in the control to 40.441 x10^6 in the non-pathogenic approach (P=0.0312), but they did not show significant differences between the control, the pathogenic and the intermediate condition (Figure 16A).

In contrast, even though IL-10$^+$ cell frequency did not demonstrate a difference between pathogenic approach and control, an induction under intermediate and non-pathogenic

conditions was observed at rather low cell frequency level with frequencies increasing to 0.38% and 0.45% compared to 0.16% in the control (P=0.0312; 0.0312) (Figure 15A). However, concerning absolute cell numbers, no differences were observed in IL-10$^+$ cells compared to the control under all conditions (Figure 16A). At cytokine level, the pathogenic and the non-pathogenic cytokine milieus did not alter IL-10 levels, but IL-10 was induced under intermediate conditions (1,882.96 pg/ml) compared to the control (1,172.79 pg/ml) (Figure 17).

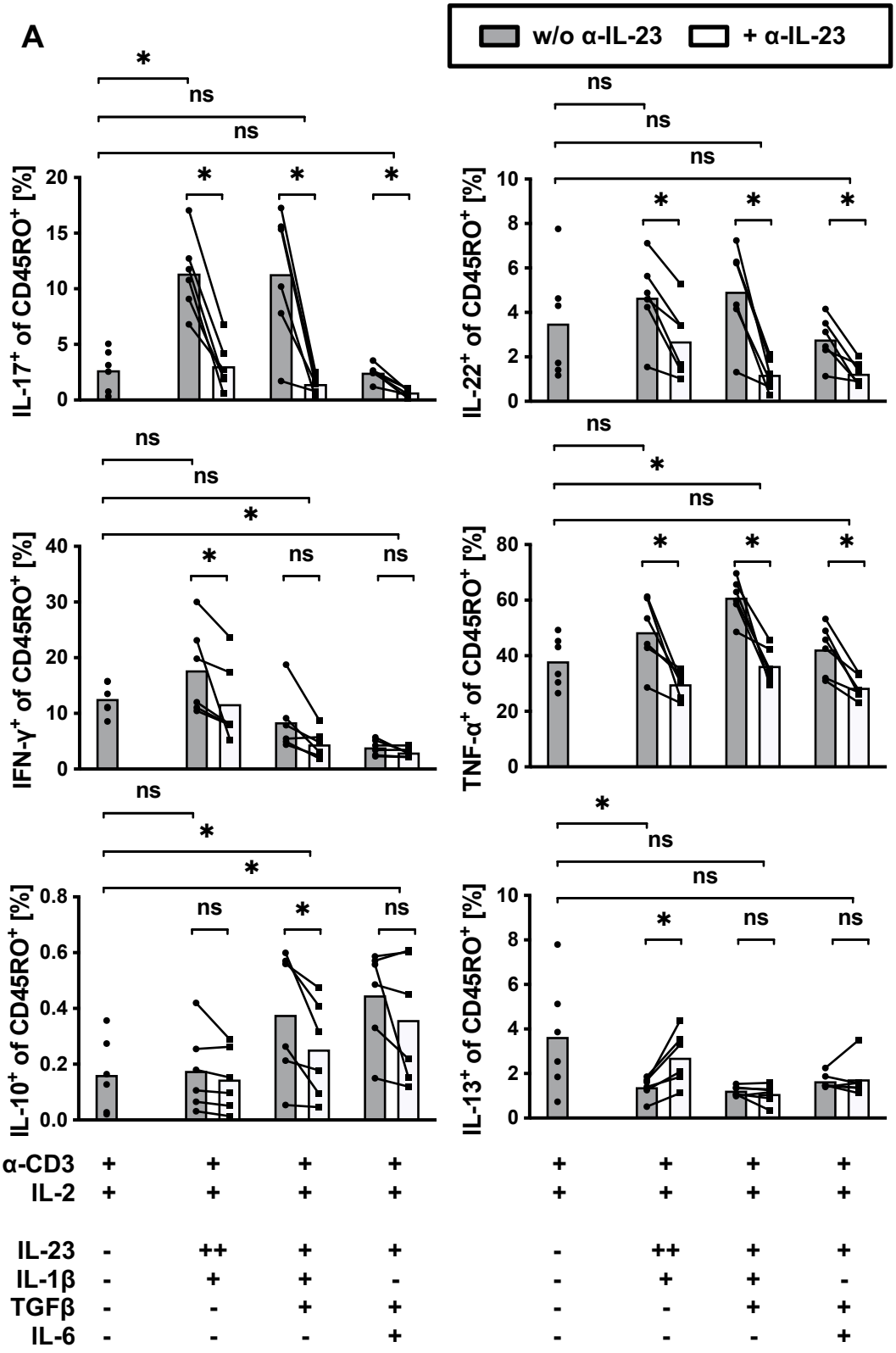
While frequencies of IL-13$^+$ cells did not strikingly differ among the three differentiation conditions, they all showed a decrease to 1.39% (path.), 1.23% (intermediate) and 1.65% (non-path.) compared to the control (3.65%) (Figure 15A). Absolute cell numbers reflected the decrease in the IL13$^+$ population, showing a reduction of 4.643 x10^6 cells in the pathogenic approach (P=0.0312), 5.972 x10^6 cells in the intermediate approach (P=0.0312) and 5.726 x10^6 in the non-pathogenic approach (P=0.0312), from a total number of 7.300 x10^6 cells in the control (Figure 16A).

Analyzing different T_H17 entities defined by their co-expression of different cytokines revealed that under pathogenic conditions pathogenic T_H17 cells were induced compared to the control, as indicated by the increase of IL-17$^+$/IL-22$^+$ from 0.81% to 2.15% (P=0.0312) and from 1.587 x10^6 to 4.612 x10^6 cells (P=0.0312) in absolute cell numbers. IL-17$^+$/IFN-γ$^+$ also increased from 0.49% to 2.93% (P=0.0312) and from 0.904 x10^6 to 6.283 x10^6 cells (P=0.0312). IL-17$^+$/TNF-α$^+$ cells increased from 2.38% to 9.89% (P=0.0312) and from 4.674 x10^6 to 20.768 x10^6 cells (P=0.0312). Also, within the intermediate approach both pathogenic T_H17 cell frequencies and numbers increased compared to the control but without statistical significance. In contrast, frequencies and numbers of the pathogenic T_H17 phenotype were not altered under non-pathogenic conditions compared to the control (Figure 15B/ Figure 16B).

The non-pathogenic counterpart of T_H17 cells co-expressing IL-17 and IL-10 was induced at a low level by all three differentiation conditions, reaching 0.046% under pathogenic (P=0.0312), 0.09% under intermediate (P=0.0312) and 0.04% under non-pathogenic stimulating conditions (P=0.0312) compared to the control showing 0.01% (Figure 15B). Absolute cell numbers showed similar effects with an increase on average from 22,003 cells in the control to 91,983 cells in the pathogenic (P=0.0312), 83,004 cells in the intermediate (P=0.0312) and 36,888 cells in the non-pathogenic approach (P=0.0312) (Figure 16B).

To exclude that the different polarization conditions used here had any undesirable effects on the T_H2 compartment IL4$^+$/IL13$^+$ T-cells were analyzed, as well. Apart from a

slight decrease of T_H2 cells under all polarization conditions compared to the control no significant differences were observed (Figure 15B/ Figure 16B).



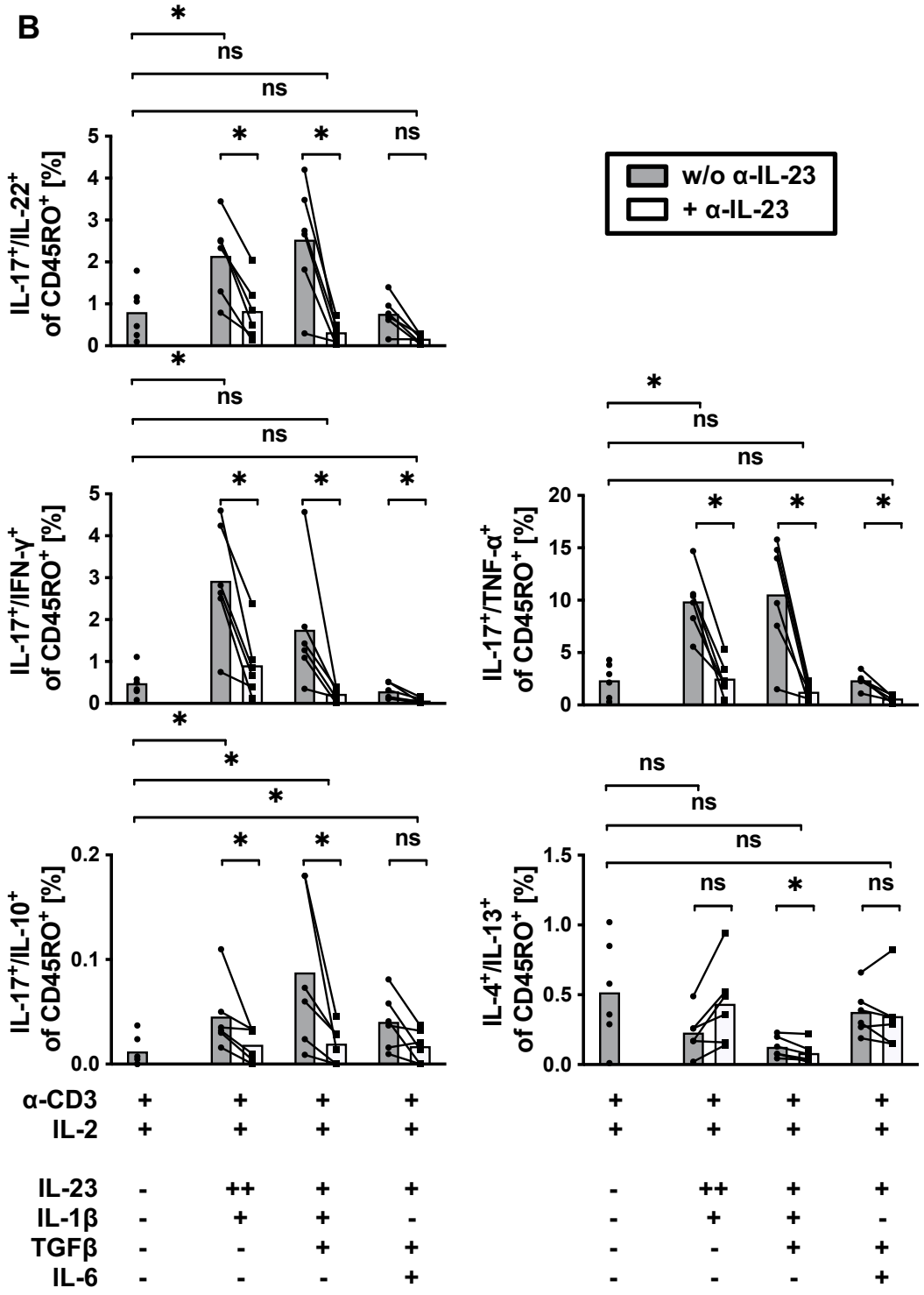
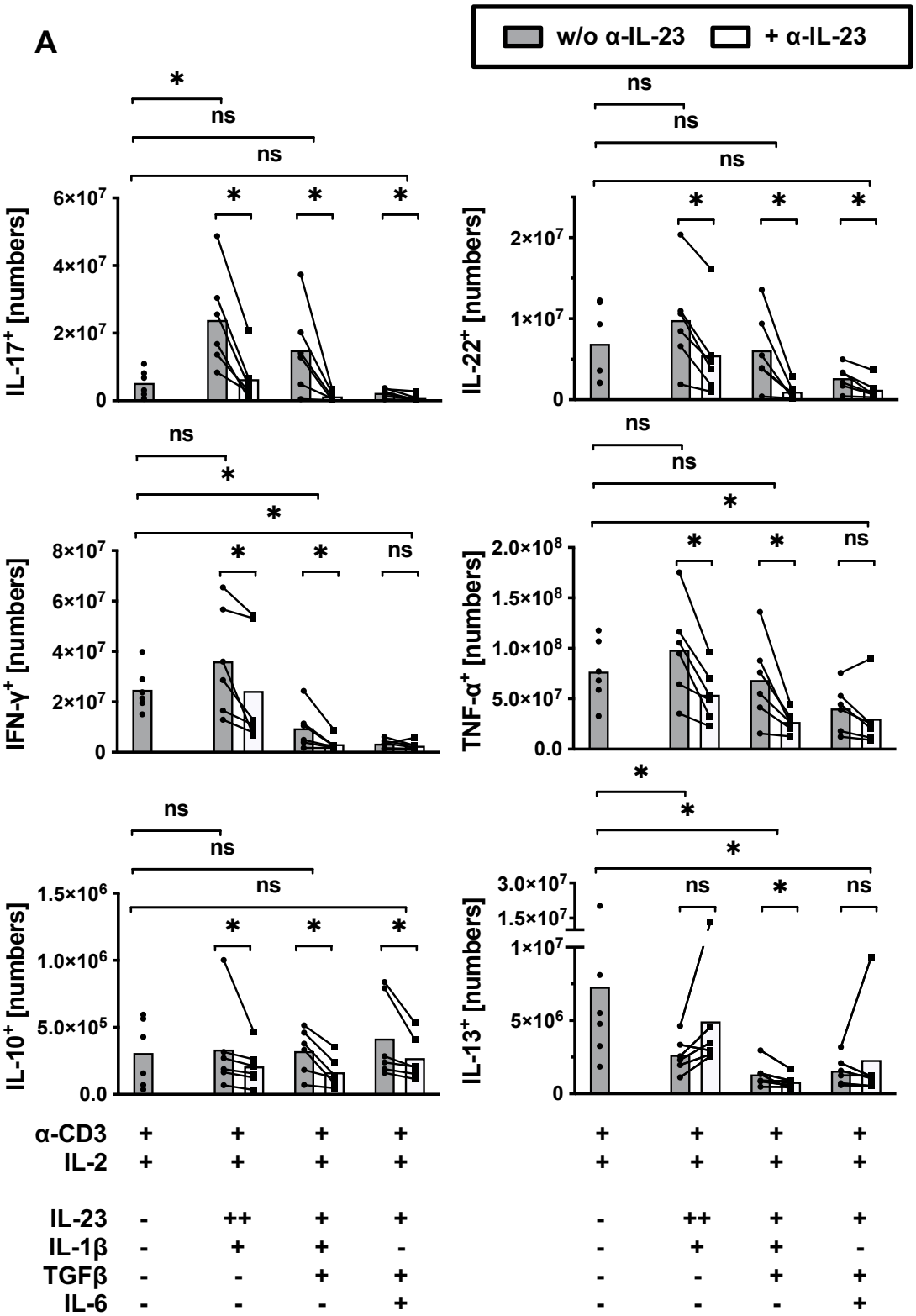


Figure 15 Flow cytometric analysis of cell frequencies under IL-23 deprivation. Naïve T-cells were isolated from human PBMC's and polarized for seven days *in vitro* with plate-bound α-CD3 and under three different polarization conditions (from left to right: control, pathogenic, intermediate and non-pathogenic approach). After the differentiation phase, cells were removed from α-CD3 and supplemented with IL-2 and the respective cytokine cocktail for further seven days. The pathogenic approach (middle-left) consisted of IL-1β and 50 ng/ml IL-23, the intermediate approach contained IL-1β, TGFβ and 10 ng/ml IL-23 (middle-right) and the non-pathogenic polarization cocktail included TGFβ, IL-6 and 10 ng/ml IL-23 (right). The control was not supplemented with cytokines (left). Furthermore, cells were treated with an IL-23 neutralizing monoclonal antibody (white bars). Cell frequencies were analyzed by flow cytometry at the single positive cell level regarding expression of IL-17, IL-22, IFN-γ, TNF-α, IL-10 and IL-13 (A). At the double positive cell level, cell frequencies of pathogenic and non-pathogenic T_H17 subsets as well as T_H2 cells expressing IL-4 and IL-13 were analyzed (B).


A
w/o α-IL-23
+ α-IL-23
IL-17+ [numbers]
IL-22+ [numbers]
IFN-γ+ [numbers]
TNF-α+ [numbers]
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α-CD3
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IL-23
IL-1β
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IL-6

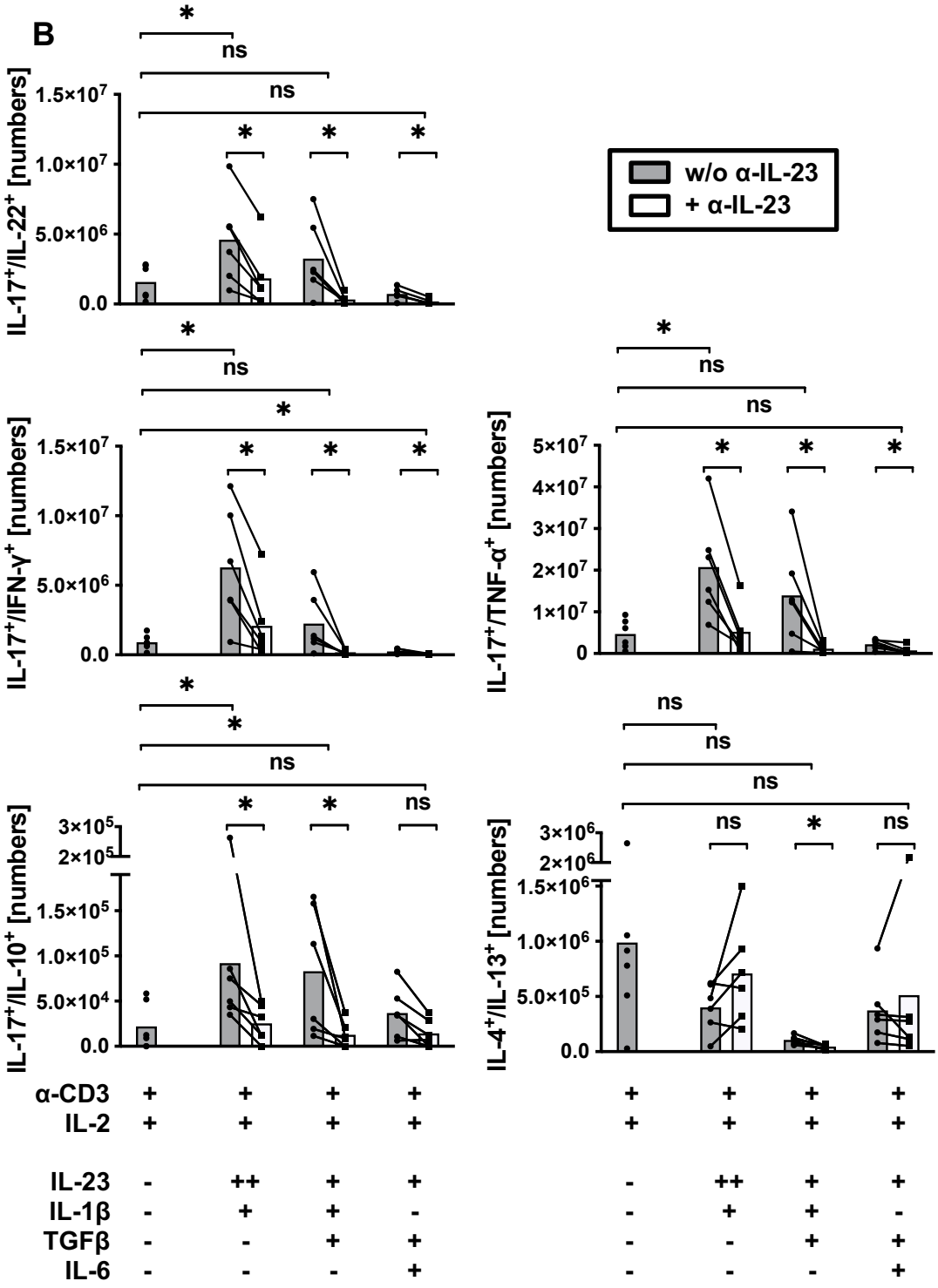

Figure 16 Cell counts of different T helper phenotypes under IL-23 neutralizing conditions in *in vitro* differentiation of T_H17 cells measured by flow cytometry. After *in vitro* polarization of naïve T-cells towards T_H17 under different cytokine conditions supplemented with α-IL-23 (white bars) or not (grey bars), cell numbers were analyzed by flow cytometry using multifluorescent counting beads. The pathogenic cytokine cocktail contained IL-1β and 50 ng/ml IL-23 (middle-left), the intermediate IL-1β, TGFβ and 10 ng/ml IL-23 (middle-right) and the non-pathogenic cocktail contained TGFβ, IL-6 and 10 ng/ml IL-23 (right). Differentiated $CD45RO^+$ cells were analyzed regarding their expression of IL-17, IL-22, IFN-γ, TNF-α, IL-10 and IL-13 (A) as well as their co-expression of IL-22, IFN-γ and TNF-α with IL-17 or their co-expression of IL-4 and IL-13 (B).

4.2.3. IL-23 is indispensable for T_H17 differentiation

In contrast to mouse models, working in the human system does not provide the opportunity to inactivate the IL-23 gene like possible in knockout-mice. Nevertheless, an *in vitro* T_H17 differentiation model is essential to investigate the influence and position of distinct cytokines in the process of T_H17 cell development in the human system. To clarify the role of IL-23 in this manner, guselkumab -an IL-23 neutralizing antibody- was used in the context of the previously described *in vitro* T_H17 differentiation and IL-23 dependent effects on T_H17 differentiation were assessed (4.2.2). To ensure constant IL-23 deprivation, all cytokine mixes that were added during differentiation and resting phase were preincubated with anti-IL-23 (3.1.5).

Regarding IL-17$^+$ cell frequencies, neutralization of IL-23 induced a remarkable decrease from 11.37% to 3.05% (P=0.0312) under pathogenic conditions, containing IL-1β and IL-23. Within the intermediate approach consisting of IL-1β, IL-23 and TGFβ, they decreased from 11.33% to 1.44% (P=0.0312) and within the non-pathogenic approach containing, IL-6, TGFβ and IL-23 from 2.47% to 0.70% (P=0.0312) (Figure 15A). Same effects could be observed regarding absolute cell numbers, where IL-23 deprivation caused a significant decrease from 23.948 $x10^6$ cells to 6.375 $x10^6$ cells (path.; P=0.0312), from 15.008 $x10^6$ cells to 1.293 $x10^6$ cells (intermediate; P=0.0312) and from 2.345 $x10^6$ cells to 0.811 $x10^6$ cells (non-path.; P=0.0312) (Figure 16A). Withdrawal of IL-23 during the differentiation process also caused a decrease of IL-17 levels in all differentiation approaches from 4,004.05 pg/ml to 1,369.96 pg/ml (pathogenic), 1,295.96 pg/ml to 343.22 pg/ml (intermediate) and 214.92 pg/ml to 38.28 pg/ml (non-pathogenic) (Figure 17).

Besides IL-17$^+$, also IL-22$^+$ cells showed a decrease under IL-23 neutralizing conditions from 9.828 $x10^6$ to 5.501 $x10^6$ (path.; P=0.0312), from 6.104 $x10^6$ to 1.026 $x10^6$ (intermediate; P=0.0312) and from 2.662 $x10^6$ to 1.258 $x10^6$ (non-path.; P=0.0312). Similarly, TNF-α$^+$ cells decreased due to IL-23 withdrawal from 98.632 $x10^6$ to 54.005 $x10^6$ (path.; P=0.0312), from 68.673 $x10^6$ to 27.045$x10^6$ (intermediate; P=0.0312) and from 40.442 $x10^6$ to 30.274 $x10^6$ (non-path.; P=n.s.) (Figure 16A). Cell frequencies showed similar effects inducing a decline of IL-22$^+$ cell frequencies from 4.67% to 2.70% (path.; P=0.0312), from 4.93% to 1.20% (intermediate; P=0.0312) and from 2.78% to 1.24% (non-path.; P=0.0312). TNF-α$^+$ cell frequencies decreased from 48.51% to 29.79% (path.; P=0.0312), from 60.90% to 36.33% (intermediate; P=0.0312) and from 42.27% to 9.05% (non-path.; P=0.0312) (Figure 15A).

Analysis of IFN-γ^+ cells revealed an anti-IL-23 mediated decrease from 17.75% to 11.70% (path.; P=0.0312) and from 8.43% to 4.45% (intermediate; P=n.s.) in terms of cell frequencies. Regarding absolute cell umbers, INF-γ^+ cells decreased from 36.077 x10^6 cells to 24.341 x10^6 cells (path.; P=0.0312) and from 9.547 x10^6 cells to 3.300 x10^6 cells (intermediate; P=0.0312). Within the non-pathogenic approach, IL-23 deprivation showed no change on average, neither concerning cell frequencies nor cell counts (Figure 15A/ Figure 16A). Regarding IFN-γ levels measured by ELISA, IL-23 neutralization led to a decrease in the pathogenic approach from an average of 4,655.31 pg/ml to 1,927.83 pg/ml, whereas it remained unaltered under intermediate and non-pathogenic conditions (Figure 17).

Compared to IL-17$^+$, IL-22$^+$ or TNF-α$^+$ cells IL-10$^+$, cell frequencies and cell numbers, on average, showed a lower decrease from 0.177% to 0.145% (path.; P=n.s), from 0.377% to 0.253% (intermediate; P=0.0312) and from 0.447% to 0.359% (non-path.; P=n.s) regarding frequencies. Absolute cell numbers showed a decrease from 0.336 x10^6 cells to 0.208x10^6 cells (path.; P=0.0312), from 0.323 x10^6 cells to 0.165 x10^6 cells (intermediate; P=0.0312) and from 0.418 x10^6 cells to 0.270 x10^6 cells (non-path.; P=0.0312) regarding absolute cell numbers (Figure 15A/ Figure 16A). Regarding cytokine levels measured by ELISA, a decrease of IL-10 levels in the intermediate approach from 1,882.96 pg/ml to 853.71 pg/ml and in the non-pathogenic approach from 964.50 pg/ml to 511.30 pg/ml was observed after IL-23 neutralization. Thereby, the IL-10 level of individual donors partially increased under pathogenic conditions, resulting in an increase of the mean value from 1,282.41 pg/ml to 2,115.59 pg/ml (Figure 17).

Apart from an anti-IL-23 mediated increase of IL-13$^+$ cells under pathogenic conditions from a mean of 1.39% to 2.71% in frequency (P=0.0312) and from 2.655 x10^6 cells to 4.943 x10^6 cells in absolute cell number (P=n.s) and a slight decrease in absolute cell number from 1.328x10^6 cells to 0.805 x10^6 cells (P=0.0312) within the intermediate approach, no differences were observed with respect to the non-pathogenic approach. (Figure 15A/ Figure 16A).

At the double positive cell level, all cells representing pathogenic T_H17 subtypes, namely IL-17$^+$/IL-22$^+$, IL-17$^+$/TNF-α$^+$ and IL-17$^+$/IFN-γ$^+$ decreased notably in frequency and number when anti-IL-23 was added. IL-17$^+$/IL-22$^+$ cell frequency declined from 2.15% to 0.83% (path.; P=0.0312), from 2.54% to 0.32% (intermediate; P=0.0312) and from 0.77% to 0.17% (non-path.; P=n.s.), while numbers decreased from 4.612 x10^6 cells to 1.819 x10^6 cells (path.; P=0.0312), from 3.258 x10^6 cells to 0.300 x10^6 cells (intermediate; P=0.0312) and from 0.722 x10^6 cells to 0.172 x10^6 cells (non-path.; P=0.0312) (Figure 15B/ Figure 16B). Also, IL-17$^+$/TNF-α$^+$ cells decreased from 9.89% to 2.52% (path.;

P=0.0312), from 10.57% to 1.27% (intermediate; P=0.0312) and from 2.36% to 0.64% (non-path.; P=0.0312) in frequency and from 20.768 x10^6 cells to 5.212x10^6 cells (path.; P=0.0312), from 13.962 x10^6 cells to 1.151 x10^6 cells (intermediate; P=0.0312) and from 2.223 x10^6 cells to 0.748 x10^6 cells (non-path.; P=0.0312) in absolute cell number. IL-17^+/IFN-γ^+ showed a decrease from 2.93% to 0.91% (path.; P=0.0312), from 1.76% to 0.23% (intermediate; P=0.0312) and from 0.29% to 0.07% (non-path.; P=0.0312) in frequency and from 6.283 x10^6 cells to 2.085 x10^6 cells (path.; P=0.0312), from 2.241 x10^6 cells to 0.190 x10^6 cells (intermediate; P=0.0312) and from 0.254 x10^6 cells to 0.054 x10^6 cells (non-path.; P=0.0312) in numbers (Figure 15B/ Figure 16B).

Although IL-17^+/IL-10^+ cells represented a rather rare cell population in our *in vitro* differentiation model, an anti-IL-23 mediated decline in non-pathogenic T_H17 cells was observed in all differentiation approaches, both in terms of frequency and absolute cell numbers (Figure 14). Under pathogenic condition, these cells decreased from 0.05% to 0.02% (P=0.0312) and from 0.092 x10^6 cells to 0.025x10^6 cells (P=0.0312). Under intermediate conditions, they declined from 0.09% to 0.02% (P=0.0312) and from 0.083 x10^6 cells to 0.013x10^6 cells (P=0.0312) and under non-pathogenic conditions from 0.04% to 0.02% (P=n.s.) and from 0.037 x10^6 cells to 0.014 x10^6 cells (P=n.s.) (Figure 15B/ Figure 16B).

Notably, under pathogenic conditions IL-23 neutralization showed a trend to increase not only the frequencies of IL-4^+/IL-13^+ expressing T-cells from 0.23% to 0.44% (P=n.s.) but also their actual number from 0.403 x10^6 cells to 0.709 x10^6 cells (P=n.s.). Under intermediate conditions, a significant decrease in both number and frequencies from 0.13% to 0.08% (P=0.0312) and from 0.107x10^6 cells to 0.046x10^6 cells (P=0.0312) was observed. The IL-4^+/IL-13^+ compartment remained unaltered by IL-23 neutralization under non-pathogenic conditions (Figure 15B/ Figure 16B).

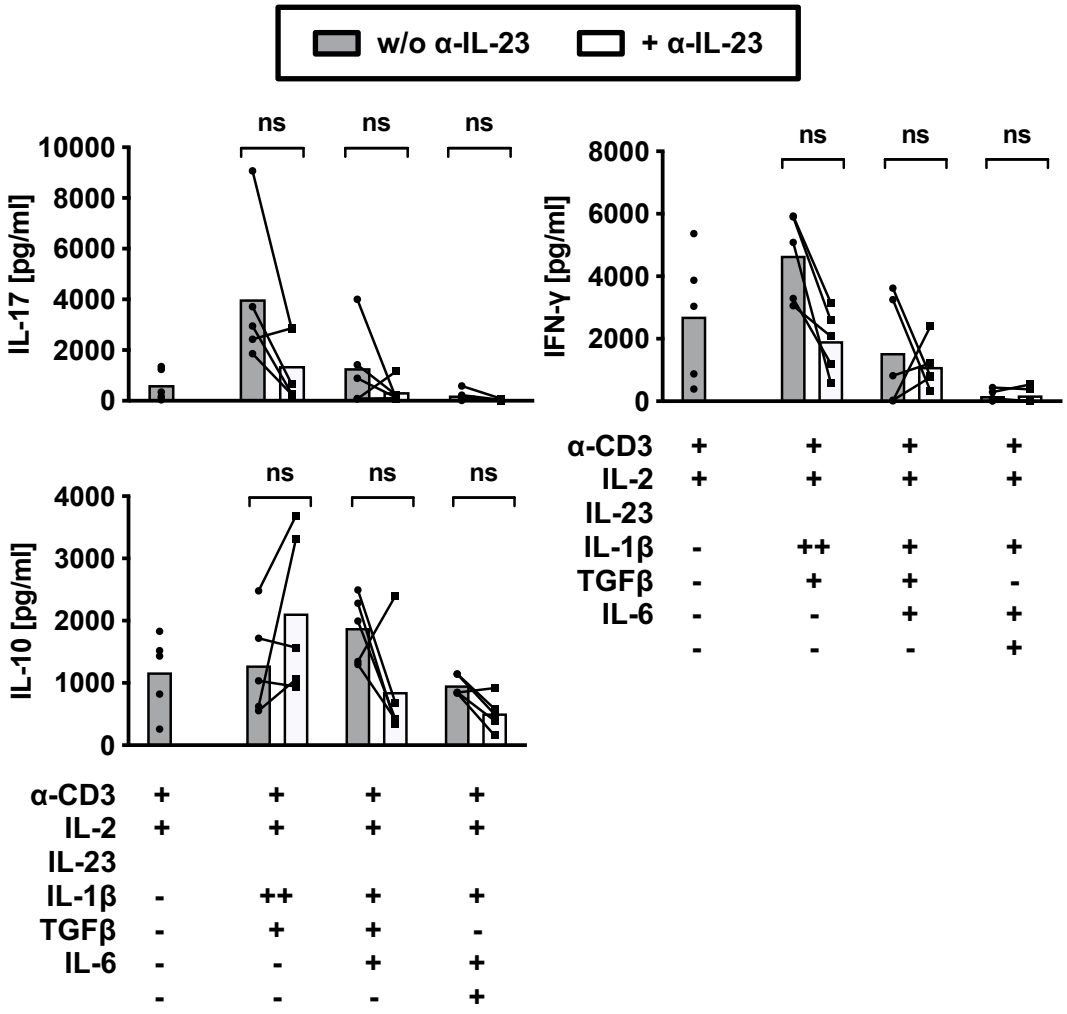

Figure 17 Effect of anti-IL-23 on cytokine levels in T_H17 polarized *in vitro* cultures. IL-17 (top), IFN-γ (middle) and IL-10 (bottom) were measured by ELISA in supernatants of restimulated *in vitro* polarized T_H17 cultures. *In vitro* differentiation was conducted under three different conditions ranging from more pathogenic to more non-pathogenic (left to right) (grey bars). Moreover, α-IL-23 was supplemented to analyze effects of IL-23 deprivation (white bars).

4.3. Effect of IL-23 inhibition on $CD4^+$ T-cells

4.3.1. Anti-IL-23 reduces the relative number especially of pathogenic T_H17 cells in $CD4^+$ T-cell culture

After observing an indispensable role of IL-23 on T_H17 differentiation in general, we wanted to elucidate whether the requirement of IL-23 for T_H17 differentiation is also manifested in the effector stage of T_H17 cells. Therefore, we analyzed the CD4 compartment as a whole with respect to the dependence of its IL-17 expression against IL-23. As expected, IL-23 inhibition caused a potent decrease from 9.85% to 5.22% IL-17^+ (P=0.0005) and from 11.19% to 4.87% IL-22^+ cells (P=0.0005) in $CD4^+$ T-cell culture (Figure 18A). The frequencies of other proinflammatory cytokines such as IFN-γ and

TNF-α showed a significant decrease from 24.22% to 20.48% (P=0.0342) and from 51.69% to 40.99% (P=0.0005), while IL-10$^+$ cell frequency increased over all donors by 0.33% (P=0.0024). Also, IL-13 increased slightly from 2.38% to 2.81% when neutralizing IL-23 (P=0.0498) (Figure 18A). Focusing on T_H17 cells, defined by their expression of IL-17, IL-23 inhibition showed strong effects on the pathogenic T_H17 cell subset co-expressing IL-22, IFN-γ and TNF-α, as evidenced by a decrease in frequency from 4.15% to 1.74% for IL-17$^+$/IL-22$^+$ cells (P=0.0005), from 3.15% to 1.33% for IL-17$^+$/IFN-γ$^+$ cells (P=0.0005) and from 9.03% to 4.52% for IL-17$^+$/TNF-α$^+$ cells (P=0.0005) (Figure 18B). On average, frequencies of the non-pathogenic subset co-expressing IL-10 and IL-17 remained unaltered (Figure 18B). Some donors responded to withdrawal of IL-23 with an increase in this population (Figure 19B). However, the IL-17$^+$/IL-10$^+$ subset showed much lower cell frequencies than its pathogenic opponent (IL-17$^+$/IL-10$^+$= 0.11±0.08% vs. IL-17$^+$/IFN-γ$^+$= 2.24±2.20%; IL-17$^+$/TNF-α$^+$= 6.77±7.18% and IL-17$^+$/IL-22$^+$= 2.95±3.11) (Figure 6B/C, Figure 18B). Nevertheless, even under IL-23 neutralizing conditions, the IL-10$^+$ fraction represented a distinct population in this model (Figure 19B).

Considering the change in cell frequencies due to IL-23 inhibition by normalizing them to the untreated control, there was an average decrease in IL-17$^+$ and IFN-γ$^+$ cell frequencies of 52.11% (P=0.0005) and 15.39% (P=0.0425), respectively, whereas the frequency of IL-10$^+$ cells increased by 36.32% (P= 0.0068) compared to the untreated control (Figure 19A). The frequency of pathogenic IL-22 and IFN-γ co-expressing T_H17 cells decreased by as much as 61.13% (P=0.0005) and 61.8% (P=0.0005), while their non-pathogenic IL-17/IL-10 expressing counterpart did not show significant alterations of its cell frequency compared to the untreated control, with large interindividual differences (Figure 19A).

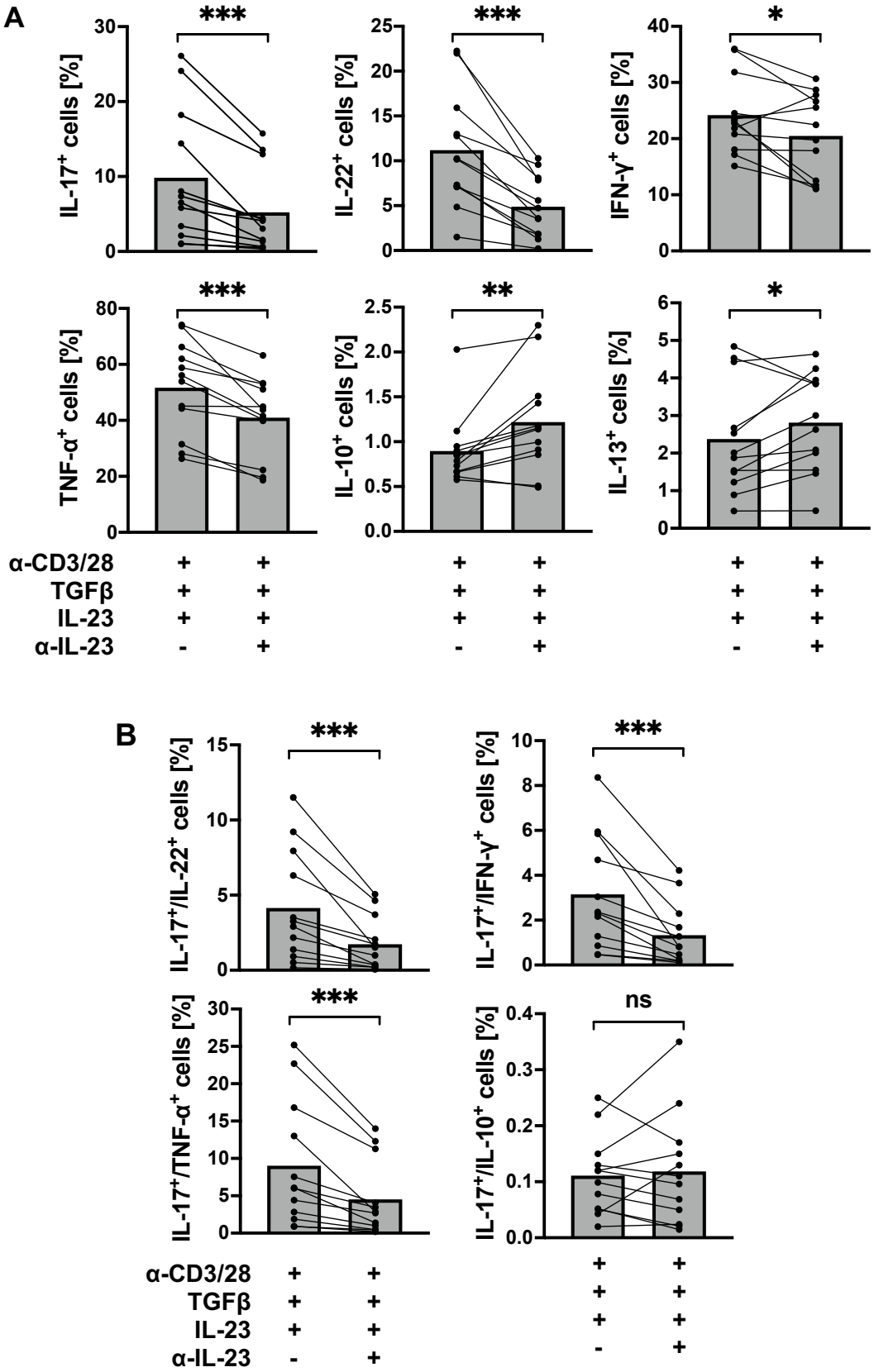

Figure 18 Activation of CD4⁺ cells in presence or absence of IL-23. CD4⁺ cells were isolated from human PBMC's by MACS and stimulated for three days with α-CD3/28, TGFβ, IL-23 (left bar) and α-IL-23 (right bar). After a subsequent ten-day lasting resting phase, they were stimulated and stained for flow cytometric analysis. Effects of IL-23 inhibition were examined based on alterations of cell frequencies which are illustrated as percentage of CD45RO⁺/CD69⁺ cell population. Cell frequencies were analyzed at the single positive cell level concerning cytokine expression of IL-17, IL-22, IFN-γ, TNF-α, IL-10 and IL-13 (A) and at the double positive cell level focusing co-expression of IL-17 (B).

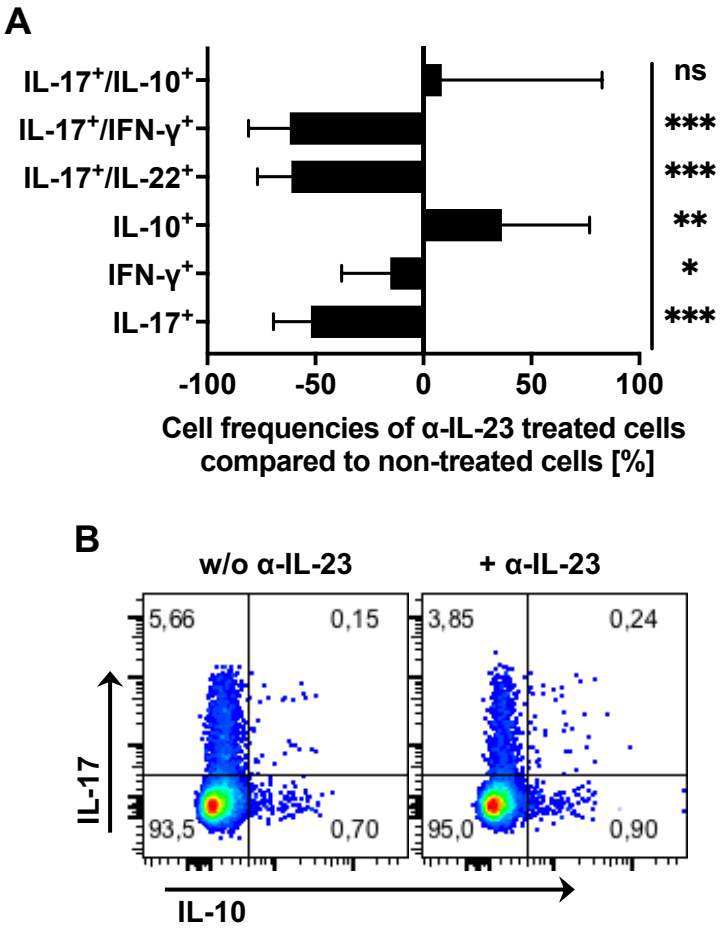

Figure 19 Influence of IL-23 inhibition on cell frequencies in a CD4⁺ T-cell culture recorded by flow cytometry. After stimulating CD4⁺ cells in the presence of TGFβ, IL-23 and α-IL-23, cytokine expressing cells were analyzed regarding their cell frequency as a portion of CD45RO⁺/CD69⁺ cells. The cell frequencies of α-IL-23 treated samples were normalized to the respective control without IL-23 neutralizing conditions, revealing either an α-IL-23 mediated growth (bars to the right) or a depletion (bars to the left) of the observed population (A). The effect of IL-23 inhibition on the non-pathogenic subset is exemplified by two-dimensional flow cytometric plots in (B).

4.3.2. IL-23 deprivation reduces absolute cell count of all T_H17 cells in CD4⁺ T-cell culture

Analyzing cell frequencies showed that neutralization of IL-23 has a suppressive effect exclusively on pathogenic subtypes (Figure 18B). To investigate potential alteration in T_H17 compartment in more detail, the data was analyzed not only at the level of relative numbers but also at the level of absolute cell count.

Therefore, a distinct number of counting beads was added to each sample. By analyzing the recorded number of beads, cell populations could be assigned to their real count within the sample (3.3.3.4).

Cell counts of all measured populations significantly decreased by adding anti-IL-23, with the exception of IL-13$^+$ cells, which did not show striking alterations on average (Figure 20A). IL-17$^+$ CD4$^+$ T-cells diminished from 4,841 to 1,047 cells (P=0.0039), IL-22$^+$ cells from 4,833 to 1,032 cells (P=0.0039), IFN-γ$^+$ cells from 10,433 to 5,356 cells (P=0.0078) and TNF-α$^+$ cells from 22,412 to 8,971 cells (P=0.0039). Even the absolute cell number of IL-10$^+$ cells decreased by about 140 cells from 381 to 241 (P=0.0117). Nevertheless, taking a closer look on the quantity of cells expressing characteristic T_H17 cytokine profiles revealed that the pathogenic subtype showed a stronger decrease than the non-pathogenic one (Figure 20B). The IL-17$^+$/IL-22$^+$ cell population decreased from 1,899 to 358 cells (P=0.0039), as did the cells with IL-17$^+$/IFN-γ$^+$ profile from 1,441 to 316 cells (P=0.0039) and the IL-17$^+$/TNF-α$^+$ compartment from 4,445 to 868 cells (P=0.0039). Although the IL-17$^+$/IL-10$^+$ representatives of the non-pathogenic subtype showed a significant decrease from an average of 59 to 23 cells (P=0.0273), the decrease was not as severe, relatively speaking, as for the pathogenic T_H17 cells. Moreover, comparison of both opponents showed that pathogenic T_H17 cells, which reached cell numbers of up to almost 14,000 cells, were much more abundant than its counterpart representing a population size less than 250 cells (Figure 20B).

Assessment of the reduction in cell numbers mediated by anti-IL-23 as a proportion of the untreated control of each sample revealed that IL-17$^+$ cells showed a greater decrease, 69.82% (P=0.0039), than IFN-γ$^+$ cells, 45.68% (P=0.0078) and IL-10$^+$ cells, 31.41% (P=0.0273). Furthermore, pathogenic T_H17 entities, defined by their expression of IL-17/IL-22 and IL-17/IFN-γ, decreased by 76.54% (P=0.0039) and 74.94% (P=0.0039), respectively, in a much more pronounced manner than non-pathogenic ones, which declined by 47.66% (P=0.0117) (Figure 21).

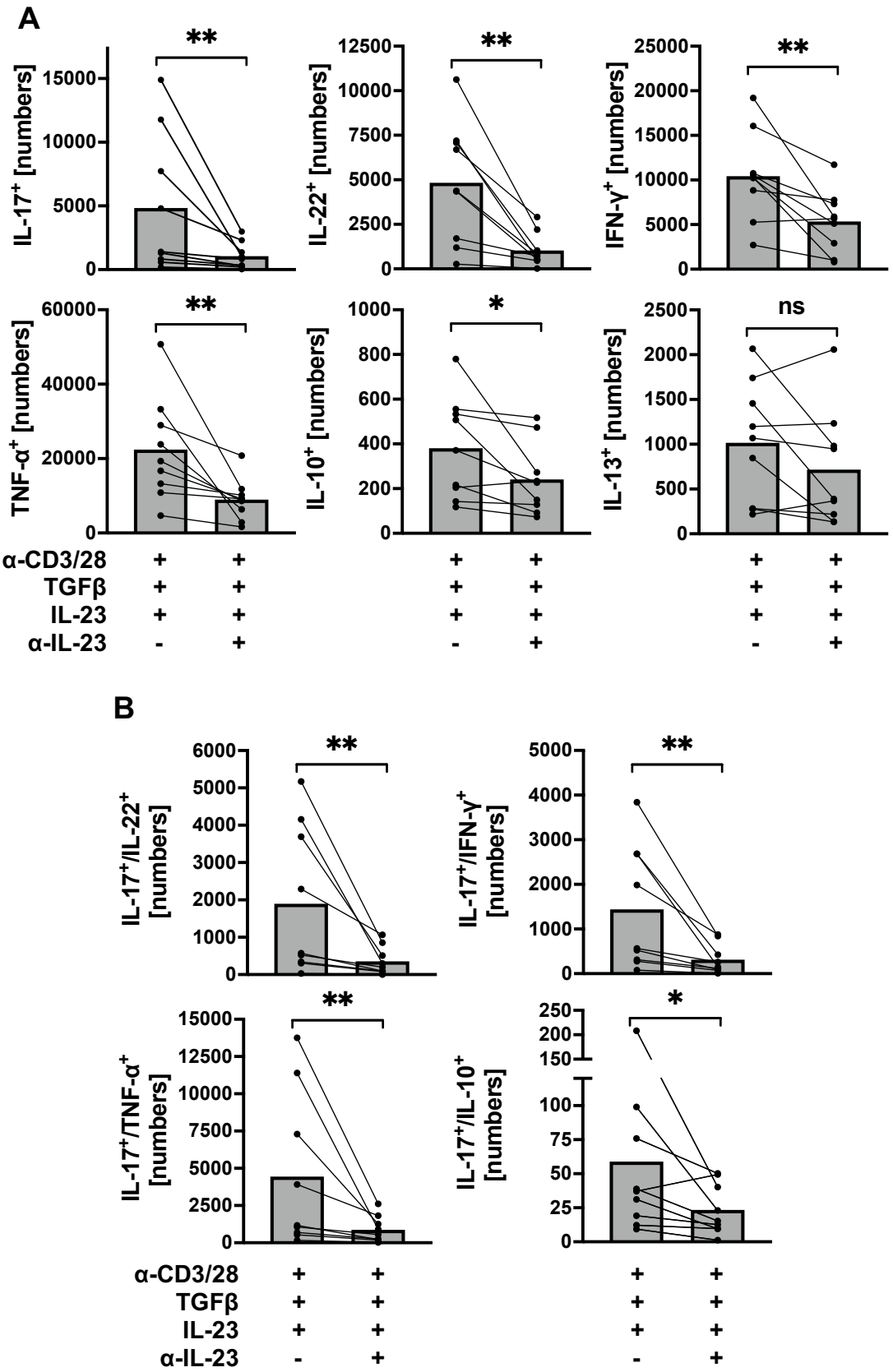

Figure 20 Detection of absolute cell numbers via flow cytometry in a CD4⁺ T-cell culture. CD4⁺ cells were stimulated with α-CD3/CD28, TGFβ and IL-23 (left bar) and additionally supplemented with α-IL-23 (right bar). Counting beads were used to analyze the influence of α-IL-23 at the level of absolute cell number. These multifluorescent beads can be detected by their bright fluorescence in multiple channels. Cytokine profiles of all cells were analyzed regarding singular expression of a cytokine (A), as well as simultaneous co-expression with IL-17, representing characteristic cytokine profiles of T_H17 phenotypes (B).

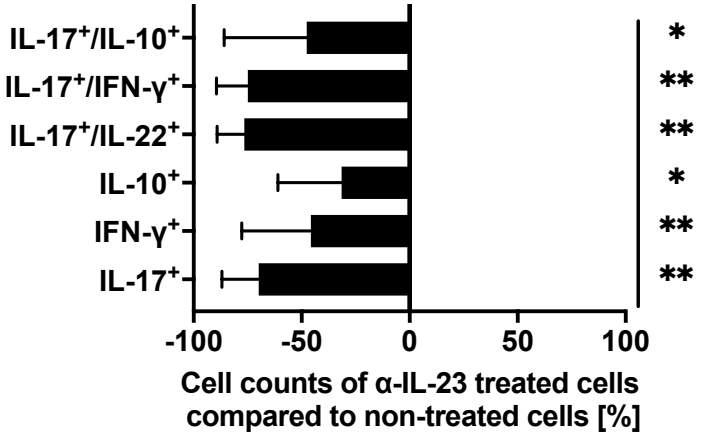

Figure 21 Examination of anti-IL-23 mediated effects on absolute cell numbers in a CD4$^+$ T-cell culture. CD4$^+$ cells were purified by MACS and stimulated for 3 days with α-CD3/28, TGFβ, IL-23 and α-IL-23. After a subsequent resting phase, cells were stimulated with PMA/ionomycin, and surface and intracellular staining was performed. Cell numbers of CD45RO$^+$/CD69$^+$ cells were determined by flow cytometric analysis using multifluorescent counting beads. Bars show the effects of α-IL-23 treatment on the expression of various cytokines normalized to the respective control without α-IL-23.

4.3.3. IL-17 and IL-10 levels are reduced in CD4$^+$T-cell culture by use of anti-IL-23

To validate the flow cytometric data and quantify cytokine expression in the previously shown setting, PBMCs were stimulated simultaneously with anti-CD3/anti-CD28 for three days and cytokines were measured in supernatants by ELISA. Consistent with the cell counts, the quantity of cytokines showed mean suppression of IL-17 from 1,274.44 pg/ml to 998.91 pg/ml (P=0.0122), as well as a reduction of IL-10 from 2,759.3 pg/ml to 2,220.26 pg/ml mediated by IL-23 neutralization (P=0.0161). However, no significant differences in IFN-γ levels could be observed, but compared to IL-17 and IL-10 some donors reached extremely high IFN-γ concentrations up to 20,272 pg/ml (Figure 22A).

To investigate whether cells alter the quantity of the cytokine production at the single cell level, mean fluorescence intensity (MFI) of the respective cytokine staining was analyzed. Neither IL-17$^+$, IFN-γ$^+$ nor IL-10$^+$ cells showed altered MFIs when supplemented anti-IL-23, indicating, on average, unaffected cytokine production at the single cell level (Figure 22B).

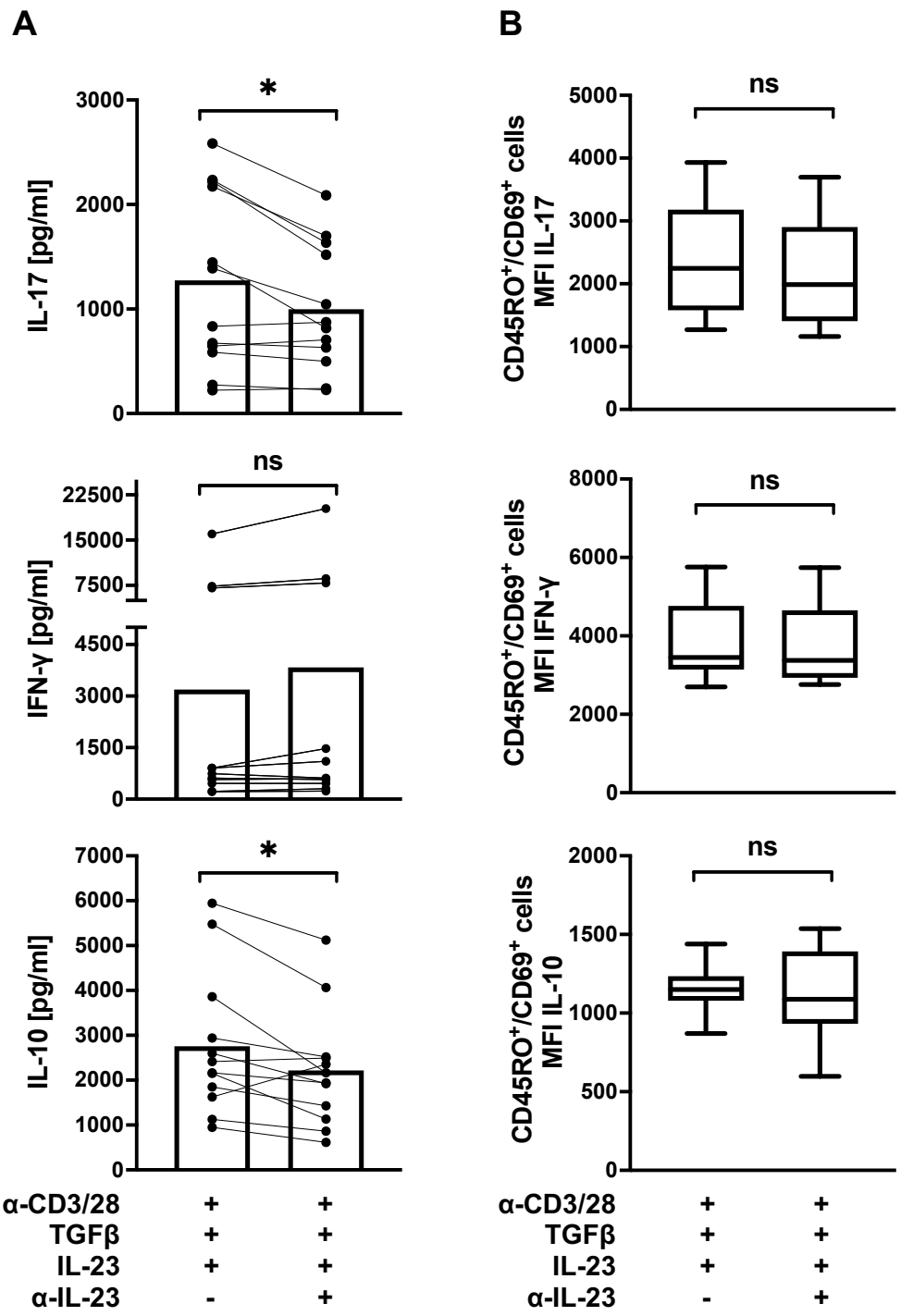

Figure 22 Cytokine expression and secretion of T-cells after treatment with IL-23 inhibitor. Cytokine concentrations of supernatants were measured by ELISA after three days of stimulation with α-CD3/28, TGFβ and IL-23 (A). Remaining cells were stimulated and stained for flow cytometric analysis after 10 days of rest to measure MFI of IL-17$^+$, IFN-γ$^+$ and IL-10$^+$ cell populations (B).

4.4. Role of IL-23 inhibition for effector function of $CD4^+$ cells

4.4.1. Anti-IL-23 increases frequencies of non-pathogenic T_H17 cells in effector T-cell culture

To address IL-23 dependent effects more specifically on the effector function of $CD4^+$ T-cells, we performed an IL-23 inhibition experiment exclusively on effector T-cells isolated from the $CD4^+$ T-cell compartment. Here, $CD4^+$ $CD45RO^+$ cells were purified by MACS to obtain effector $CD4^+$ cells (3.1.2). After they were purified to a percentage of 95-97%, cells were treated equivalently to the experiment examining the $CD4^+$ cell population: Cells were stimulated for three days with anti-CD3 and anti-CD28 in the presence of TGFβ, IL-23 and if necessary anti-IL-23. Subsequently, supernatants were collected for ELISA and cells were allowed to rest for ten days prior to for flow cytometric analysis. (3.1.6). To make results comparable, data were analyzed in the same manner as $CD4^+$ T-cells, including the same gating strategy for flow cytometric data (3.3.3.2).

The effect of IL-23 inhibition showed on the $CD4^+$ cell population were reproducible in the effector subset. At the single positive cell level, anti-IL-23 altered the proinflammatory milieu towards an anti-inflammatory milieu by decreasing the frequency of cell subsets expressing IL-17 from 11.02% to 4.72% (P=0.0005), IL-22 from 10.15% to 2.45% (P=0.0005), IFN-γ from 16.86% to 10.56% (P=0.0068) and TNF-α from 49.50% to 32.68% (P=0.0005), whereas the frequency of $IL\text{-}10^+$ cells increased from 1.41% to 2.56% (P=0.0029) (Figure 23A). Also, frequencies of $IL\text{-}13^+$ cells partially increased, showing an increase from 1.57% to 2.49% on average (P=n.s.). As expected, looking at the double positive cell level, a remarkable decrease of pathogenic T_H17 representatives from 3.63% to 0.87% ($IL\text{-}17^+/IL\text{-}22^+$; P=0.0005), from 2.46% to 0.71%% ($IL\text{-}17^+/IFN\text{-}\gamma^+$; P=0.0005) and from 9.85% to 3.48% ($IL\text{-}17^+/TNF\text{-}\alpha^+$; P=0.0005) was observed (Figure 23B/C), while the frequency of their non-pathogenic equivalent partly increased resulting in an increase in the mean value from 0.21% to 0.26% (P=n.s.) (Figure 23B).

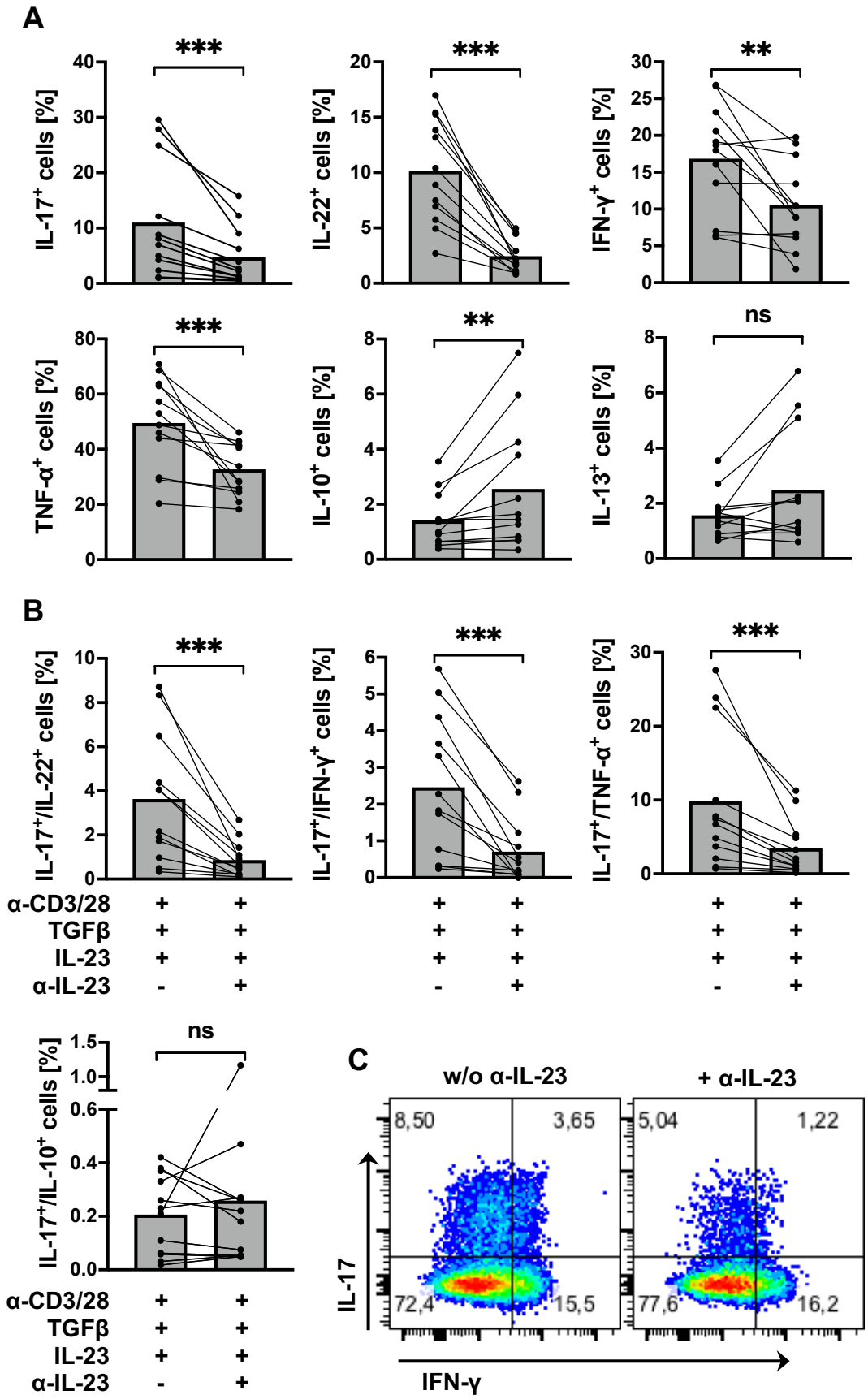

Figure 23 Flow cytometric analysis of effector T-cell frequencies before and after anti-IL-23 treatment. CD45RO$^+$ effector T-cells isolated from human PBMC's by MACS were incubated for 3 days with α-CD3/28, TGFβ and IL-23 before they underwent a 10-days lasting resting-period. Also, an IL-23 neutralizing antibody was added to the cultures (right bar) to compare its effects to a respective control (left bar). In order to detect cytokine expression, cells were stimulated with PMA/ionomycin prior to an intracellular staining. Cell frequencies of populations expressing IL-

17, IL-22, IFN-γ, TNF-α, IL-10 and IL-13 were observed at the single cell level (A). At the double positive cell level, frequencies of cells co-expressing IL-17 and thus representing T_H17 subtypes were analyzed (B). The effect of IL-23 inhibition on the pathogenic subset is exemplified by two-dimensional flow cytometric plots in (C).

Normalization of cell frequencies of samples treated with anti-IL-23 with the corresponding untreated control provides another way to compare anti-IL-23 mediated alterations of cytokine expression. Neutralizing IL-23 reduced frequencies of the IL-17$^+$ and the IFN-γ$^+$ effector T-cell population by 58.84% (P=0.0005) and 32.25% (P=0.0049) compared to the control, while it increased the frequency of the IL-10$^+$ cell population by 65.5% (P=0.0029). In addition, comparing different T_H17 entities revealed an anti-IL-23 mediated decrease of IL-17$^+$/IL-22$^+$ and IL-17$^+$/IFN-γ$^+$ cells by 74.74% (P=0.0005) and 70.11% (P=0.0005) compared to the control, whereas IL-17$^+$/IL-10$^+$ cells showed an increase by 47.62% on average, with a large interindividual variability between donors (P=n.s.) (

Figure 24A).

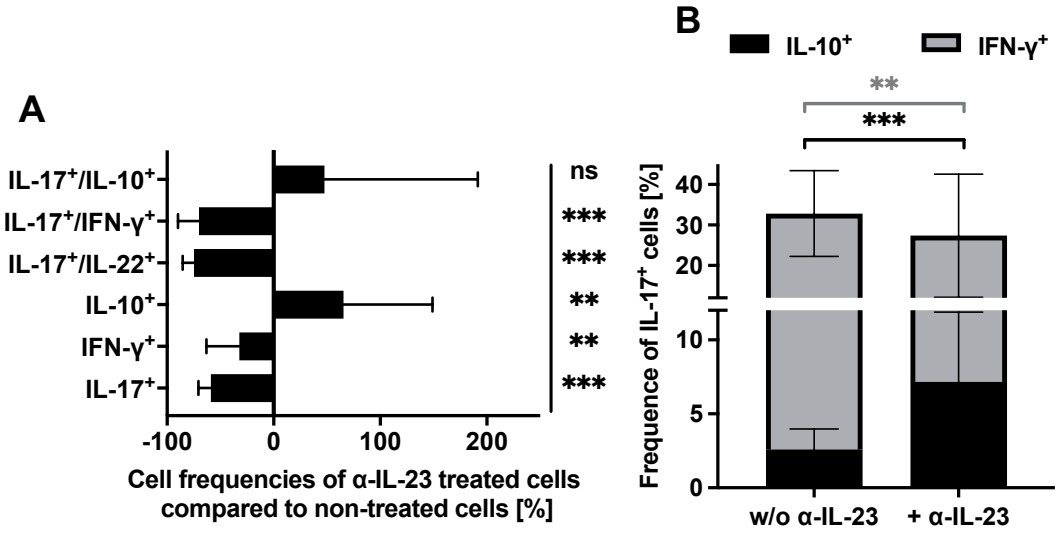

Figure 24 Comparing the effect of anti-IL-23 on frequencies of different T_H17 phenotypes analyzed by flow cytometry. After purified effector T-cells were cultured with or without IL-23 neutralizing conditions, they were stimulated and stained for flow cytometric analysis. Cytokine expression of cells was determined as percentage of CD45RO$^+$/CD69$^+$ cells. The effect of IL-23 was illustrated as IL-23 induced growth (Bars to the right) or depletion (Bars to the left) of distinct subpopulations (A) by normalizing the difference in cell frequency to the untreated control. To observe effects within IL-17$^+$ cell population, cell frequencies of IFN-γ$^+$ (grey) and IL-10$^+$ cells (black) as percentage of IL-17$^+$ cells were observed with (right bar) or without (left bar) α-IL-23 supplementation (B).

Given the fact that an IL-23 treatment tends to reduce all T_H17 entities more or less, a different gating strategy was used to point out the effect of anti-IL-23 treatment within the T_H17 population rather than looking at the different T_H17 subpopulation as part of all

$CD4^+$ T-cells. After gating on $CD45RO^+CD69^+$ and IL-17^+ cells, cells positive for the respective cytokines were analyzed as frequencies of all IL-17^+ cells showing remarkably the capability of anti-IL-23 to modify the proportion of pathogenic and non-pathogenic T_H17 cells for the benefit of the latter (

Figure 24B).

4.4.2. IL-23 deprivation induces a stronger decrease of pathogenic than of non-pathogenic T_H17 cells in effector T-cell culture

According to the analysis of the data from $CD4^+$ cells (4.3), absolute cell numbers were also addressed in the context of $CD4^+$ effector T-cells. Consistent with the $CD4^+$ cell results, the number of all effector cells expressing IL-17, IL-22, IFN-γ, TNF-α or IL-10 dropped down significantly, while numbers of IL-13^+ cells remained almost unaltered (Figure 25A). On average, the cell population of IL-17^+ cells was reduced from 4,372 to 581 cells (P=0.0039), of IL-22^+ cells from 4,375 to 545 cells (P=0.0039), of IFN-γ^+ cells from 6,276 to 2,799 cells (P=0.0195), of TNF-α^+ cells from 19,436 to 7,486 cells (P=0.0078) and of IL-10^+ cells from 393 to 192 cells (P=0.0039). Double positive cells co-expressing IL-17 and the respective cytokines decreased, as well (Figure 25B). Again, withdrawal of IL-23 had a greater impact on pathogenic (Figure 25C) than on non-pathogenic (Figure 25D) T_H17 cells, as indicated by the mean reduction from 73 to 19 for non-pathogenic T_H17 cells (P=0.0391), while pathogenic T_H17 cells showed a decrease from 1,618 to 165 cells for IL-17^+/IL-22^+ cells (P=0.0039), from 890 to 105 cells for IL-17^+/IFN-γ^+ cells (P=0.0039), and from 3,859 to 459 cells for IL-17^+/TNF-α^+ cells (P=0.0039) (Figure 25B). It is worth mentioning that the number of pathogenic T_H17 cells decreased across all donors by anti-IL-23-mediated, whereas the number of IL-10 expressing T_H17 cells increases even in single individuals.

Normalization of anti-IL-23 treated samples compared to their corresponding control clarified the massive anti-IL-23 mediated reduction in IL-17^+/IL-22^+ and IL-17^+/IFN-γ^+ cell counts by 85.82% (P=0.039) and 83.46% (P=0.039), respectively, whereas their non-pathogenic counterpart decreased by only 35.84% on average (P=n.s.) (Figure 26).

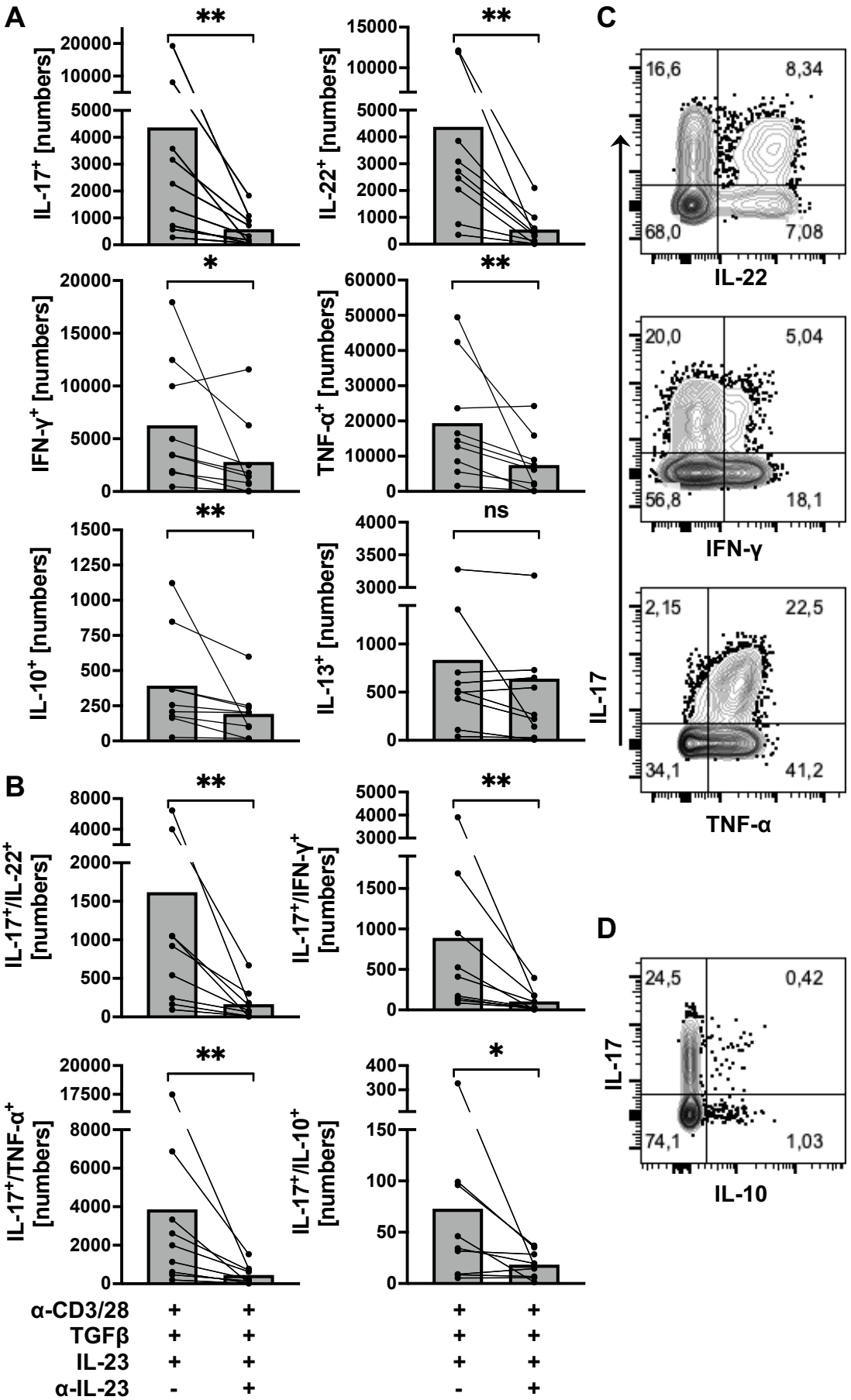

Figure 25 Absolute cell numbers of effector T-cell culture measured by flow cytometry. CD45RO+ cells were isolated and stimulated for 3 days with α-CD3/28, TGFβ and IL-23 before they rested 10 days. Subsequently, they underwent a stimulation and staining for flow cytometric

analysis including multifluorescent. Furthermore, counting beads were added to the samples to calculate their absolute cell numbers. In addition to the stimulation mix, cells were cultured either with (right bar) or without α-IL-23 (left bar). Size of populations expressing either IL-17, IL-22. IFN-γ, TNF-α, IL-10 or IL-13 were examined in (A). At the double positive cell level, typical cytokine expression patterns of pathogenic (IL-17$^+$/IL-22$^+$, IL17$^+$/IFN-γ$^+$ and IL-17$^+$/TNF-α$^+$) and non-pathogenic (IL-17$^+$/IL-10$^+$) T_H17 cells were examined (B). The cytokine patterns of the pathogenic and the non-pathogenic T_H17 subtype are exemplified by two-dimensional flow cytometric plots in (C) and (D).

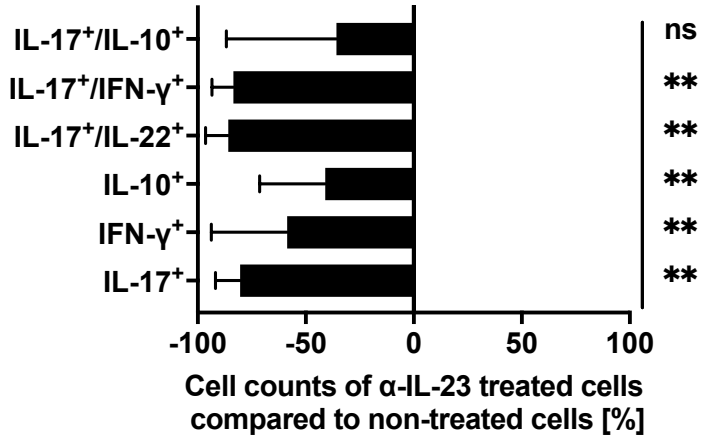

Figure 26 Measurement of anti-IL-23 mediated effects in an effector T-cell culture by flow cytometry. Effector T-cells were isolated from PBMC's of human healthy donors by MACS, stimulated with α-CD3/28 in the presence of TGFβ, IL-23 and α-IL-23 and subsequently analyzed by flow cytometry. Cell numbers of α-IL-23 treated samples measured by multifluorescent counting beads are illustrated normalized to a respective untreated control revealing a depletion of the respective cell populations.

4.4.3. Neutralization of IL-23 has no effects on cytokine secretion levels in effector T-cell cultures

To validate the flow cytometric data, cytokines in supernatants after three days of stimulation were analyzed by ELISA. On average, neither quantity of IL-17, IFN-γ nor IL-10 showed significant differences between the IL-23 neutralizing conditions and the control (

Figure 27A). To investigate whether the cells change the amount of their cytokine secretion under IL-23 neutralizing conditions, the MFI of each cytokine staining was determined. However, no significant differences were detected with respect to the MFI of IL-17, IFN-γ and IL-10 in the respective populations (

Figure 27B).

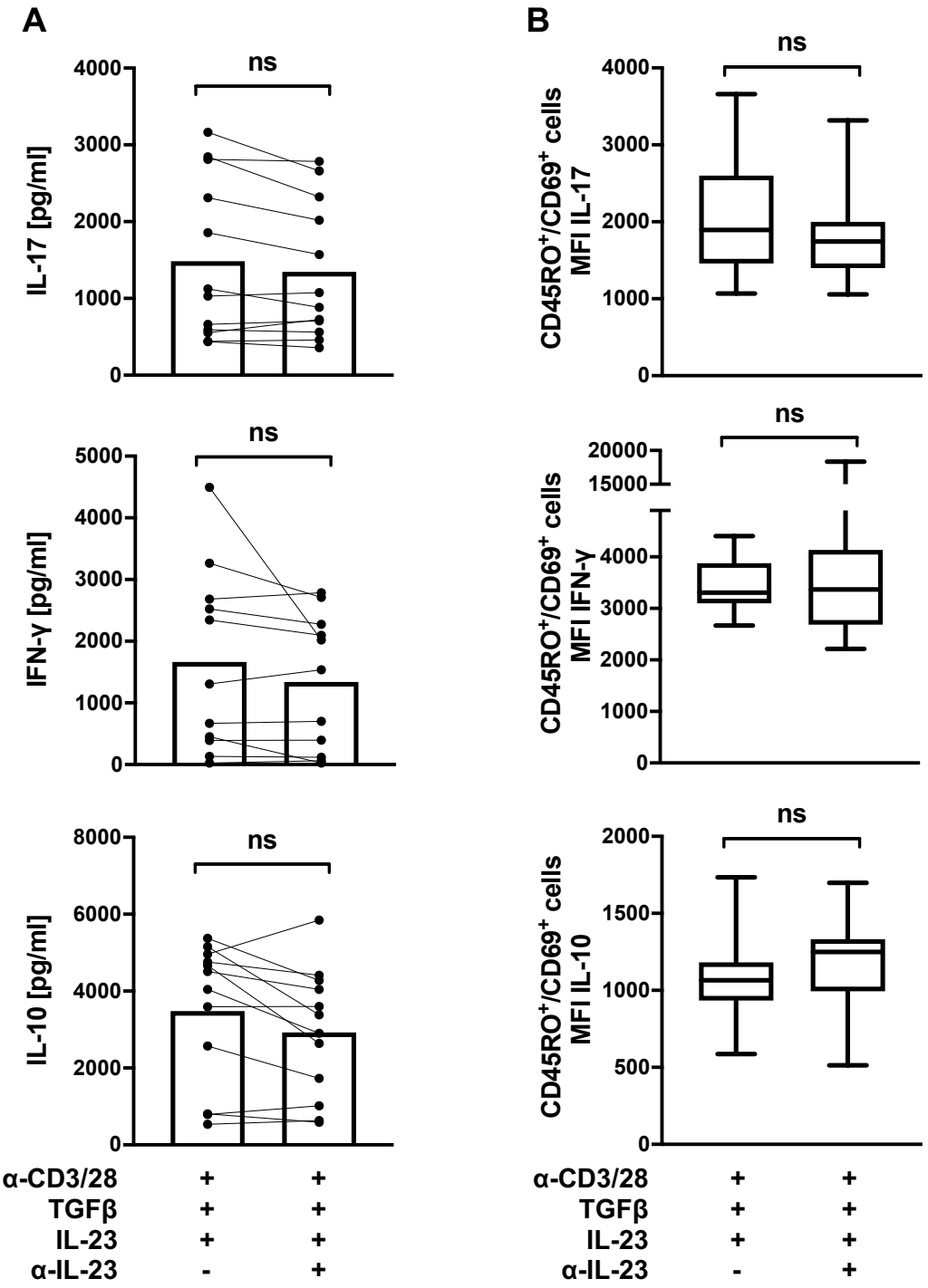

Figure 27 Analysis of cytokine quantity and MFI in a stimulated effector T-cell culture. Cytokine concentrations of IL-17, IFN-γ and IL-10 were measured by ELISA in supernatants obtained after 3 days of stimulation with α-CD3/28, TGFβ, IL-23 and α-IL-23 (A). Remaining cells rested 10 days before they were stimulated with PMA/ionomycin and stained for flow cytometric analysis to measure MFI of IL-17$^+$, IFN-γ$^+$ and IL-10$^+$ cells (B). IL-23 neutralizing conditions (right bar) are compared to a respective control (left bar).

4.5. Effect of IL-23 inhibition in a model of pathogen induced T_H17 activation and differentiation

After analyzing the effect of IL-23 neutralization in an anti-CD3/28 activated effector T-cell culture and in an *in vitro* differentiation setting, we examined these effects in a more physiologically relevant setting by monocyte mediated activation of T-cells. Moreover, as we were not able to differentiate non-pathogenic T_H17 from naïve T-cell sufficiently, we could not properly investigate IL-23 neutralization during development of non-pathogenic T_H17 cells. Since Zielinski et al. could induce non-pathogenic T_H17 cells in a co-culture of T-cells and Monocytes pulsed with S. aureus, we wanted to use this method to investigate IL-23 neutralization during non-pathogenic T_H17 differentiation. Additionally, they could show that C. albicans induces more pronounced pathogenic T_H17 cells. That is why lysates of C. albicans and S aureus were used as antigen pool to activate $CD4^+$ effector and naïve T-cells via autologous pulsed monocytes.

To ensure efficient neutralization of IL-23, anti-IL-23 was added throughout the co-culture of monocytes and T-cells. Anti-IL-23 was also added during the last 30 minutes of pulsing monocytes with C. albicans and S. aureus to neutralize IL-23 potentially secreted by monocytes during this phase (3.1.6).

Strong proliferation of cells specific for C. albicans and S. aureus was observed (Figure 28A-D) for $CD4^+$ effector (Control: 1.21±1.14%; C. albicans: 38.93±23.53%; S. aureus: 34.91±5.72%) (Figure 28C/D), as well as naïve T-cells (Control: 14.36±8.78%; C. albicans: 73.13±29.64%; S. aureus: 64.76±31.88%) (Figure 28A/B). Of note, IL-23 neutralization did not alter proliferative capacity of T-cells in response to C. albicans and S. aureus lysates (Figure 28 A/C).

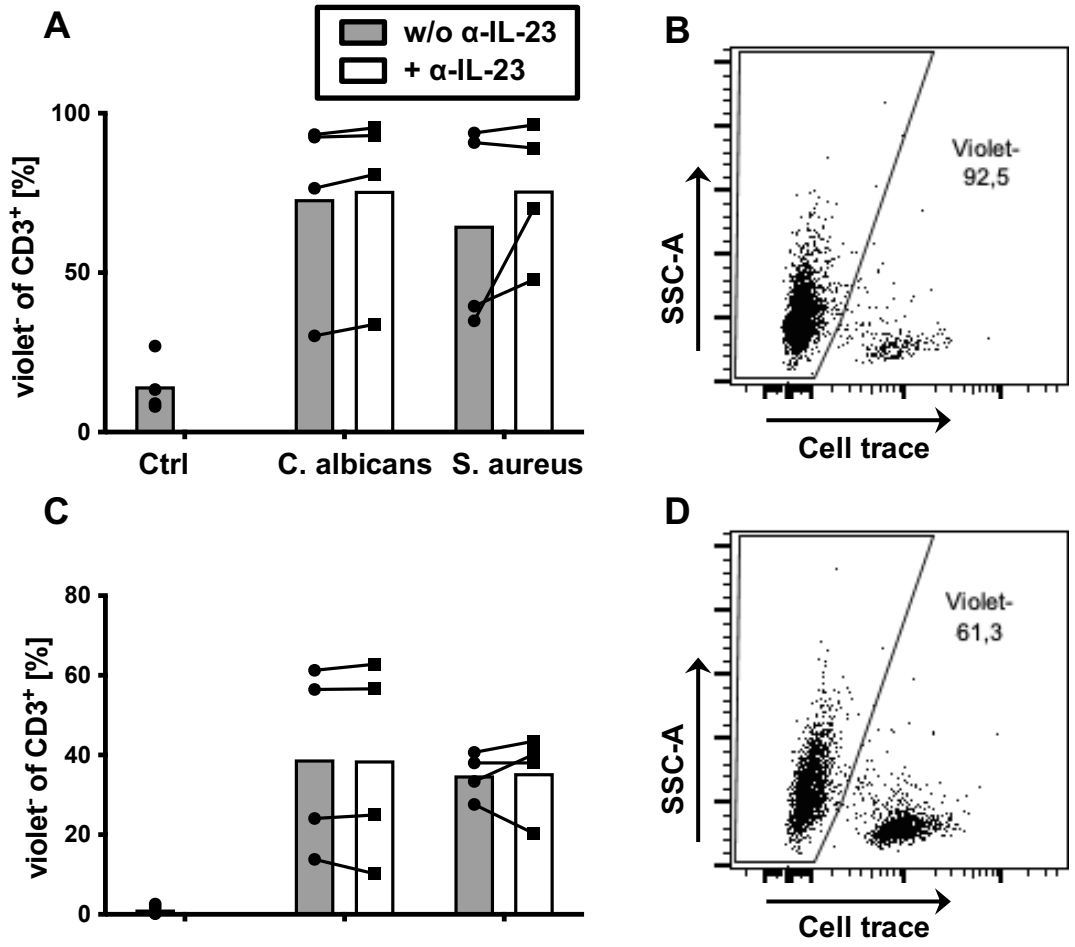

Figure 28 Detection of proliferating CD4⁺ effector and naïve T-cells specific for S. aureus and C. albicans via flow cytometry. Monocytes were pulsed with lysates from C. albicans and S. aureus before they were co-cultured with $CD4^+CD45RO^{neg}$ naïve (A/B) and $CD4^+CD45RO^+$ effector T-cells (C/D). Subsequently, cells were stimulated, stained and analyzed by flow cytometry. Proliferation rates (grey bars) of a control (left) were compared to proliferation rates inducted by lysates of C. albicans (middle) and S. aureus (right). Co-culture of pulsed Monocytes and T-cells was also conducted under IL-23 neutralizing conditions by supplementing α-IL-23 (white bars).

4.5.1. S. aureus and C. albicans induce T_H17 activation

Gating on "Cell trace"^{neg} cells enabled to analyze the cytokine profile of proliferating cells specific for S. aureus or C. albicans. Both pathogens are known to induce a potent IL-17 response. Also, in this model proliferating cells specific for S. aureus and C. albicans, respectively, showed a predominantly T_H17 phenotype defined by their expression of IL-17. In effector culture, 34.46±9.99% of proliferating cells specific for S. aureus were IL-17^+ and 37.64±8.76% of proliferating cells specific for C. albicans were IL-17^+ (Figure 29A). Starting from naïve T-cells, proliferating cells specific for S. aureus and C. albicans showed a less dominant but still pronounced T_H17 related immune response, 10.65±9.01% and 17.58±12.38%, respectively (Figure 31A).

Comparable to the IL-17 response, IFN-γ response was stronger with 42.6±16.8% (C. albicans) and 38.98±8.74% (S. aureus) IFN-γ^+ cells of all proliferating cells in the culture of effector T-cells (T_{eff}) than in the culture of naïve T-cells ($T_{naïve}$), where 18.7±12% of C. albicans- and 25.32±13.66% of S. aureus-specific cells were IFN-γ^+ **(**Figure 29A**,** Figure 31A**)**. TNF-α^+ proliferating cells reached high frequencies in both cultures when stimulated with C. albicans (T_{eff}: 63.98±2.58%, $T_{naïve}$: 62.57±18.97%), as well as with S. aureus (T_{eff}: 65.45±4.81%, $T_{naïve}$: 67.01±13.22%) **(**Figure 29A**,** Figure 31A**)**. In contrast to the expression of IL-17, IFN-γ and TNF-α, frequencies of IL-10^+ cells in the proliferating population were much lower, showing 0.97±1.11% of C. albicans-specific and 0.49±0.47% of S. aureus-specific cells in the effector culture and 0.55±0.48% C. albicans-specific and 0.49±0.39% S. aureus-specific cells in the culture of naïve T-cells **(**Figure 29A**,** Figure 31A**)**. In general, no differences were observed regarding the cytokine profile induced by C. albicans or S. aureus.

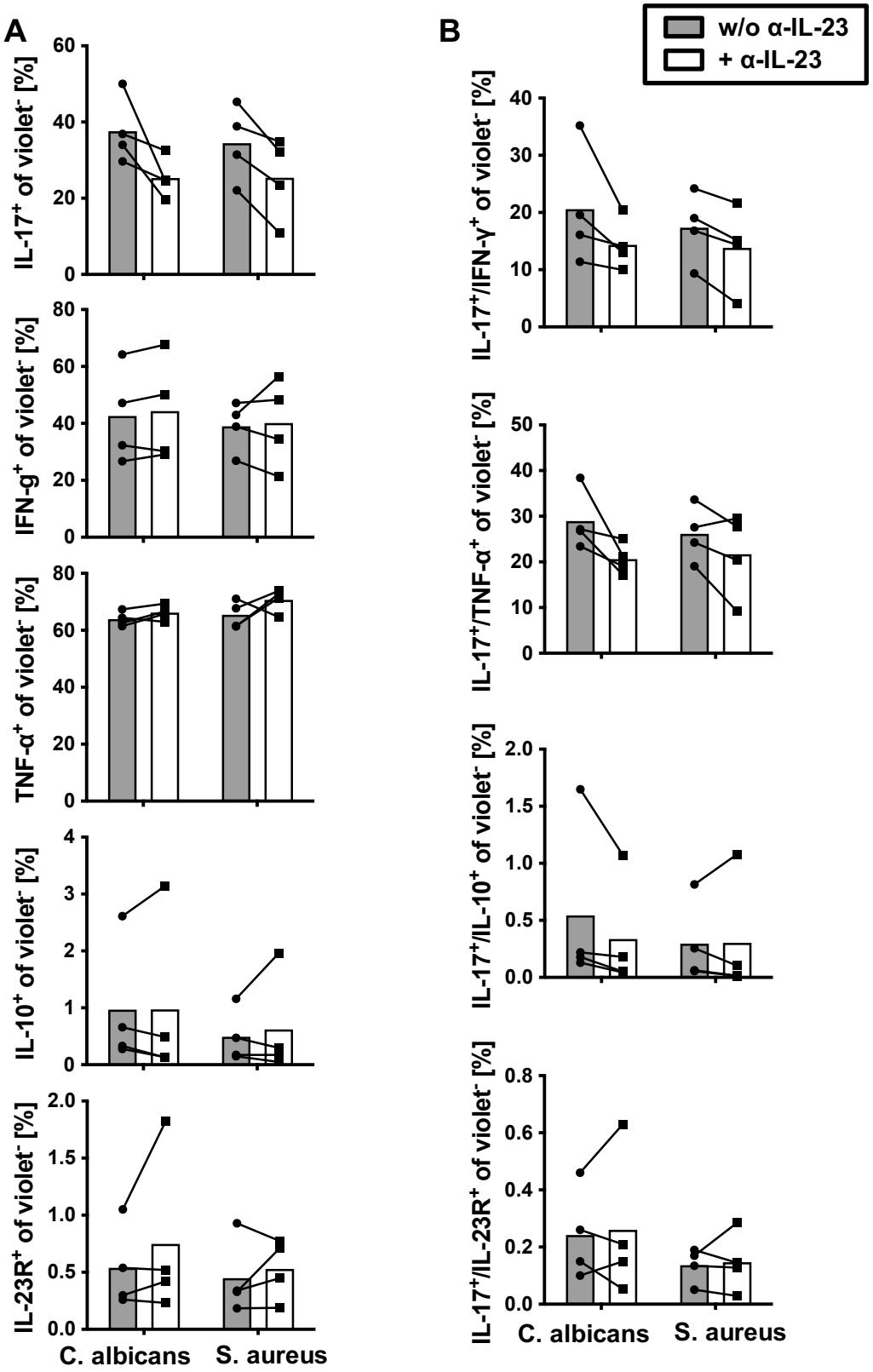

Figure 29 Cell frequencies of pathogen mediated activation of effector T-cells. $CD4^+CD45RO^+$ effector T-cells and monocytes were isolated from human PBMC's by MACS. Monocytes were pulsed with pathogens from lysates of C. albicans (left bars) and S. aureus (right bars) before they were co-cultured with autologous $CD4^+CD45RO^+$ effector T-cells (grey bars). Also, α-IL-23 was added to the cultures to observe effects of IL-23 neutralization (white bars). Cell frequencies of cells expressing IL-17, IFN-γ, TNF-α, IL-10 and IL-23R were determined as percentage of all proliferating cells by flow cytometry (A). In addition, frequencies of cytokine patterns representing different T_H17 entities and co-expression of IL-17/IL-23R were observed at the double positive cell level (B).

Since the IL-23 receptor plays a critical role in the IL-23/IL-17 axis, IL-23 receptor staining was performed (Figure 30). IL-23 receptor staining revealed a low frequency of IL-23R expressing cells in both cultures, C. albicans and S. aureus, namely 0.53±0.36% and 0.45±0.33% in the effector and 0.21±0.13% and 1.35±2.07% in the naïve T-cell culture, respectively **(**Figure 29A**,** Figure 31A**)**.

Both lysates induced a distinct pathogenic IL-17^{+}/IFN-γ^{+} response in effector T-cell cultures of 20.58±10.31% cells specific for C. albicans and 17.35±6.17% cells specific for S. aureus and an IL-17^{+}/TNF-α^{+} response of 28.95±6.53% C. albicans-specific and 26.15±6.1% S. aureus-specific cells (Figure 29B). This pathogenic response was less dominant in naïve T-cell cultures. 2.94±3.37% of C. albicans-specific and 5.34±3.58% of S. aureus-specific cells were IL-17^{+}/IFN-γ^{+}, while 10.05±8.82% cells specific for C. albicans and 15.74±11.29% cells specific for S. aureus were IL-17^{+}/TNF-α^{+} (Figure 31B). In contrast to the predominant pathogenic T_H17 subtypes, the non-pathogenic IL-17^{+}/IL-10^{+} expressing phenotype represented a relatively small portion of T_H17 cells specific for C. albicans, 0.55±0.74% cells (T_{eff}) and 0.14±0.17% cells ($T_{naïve}$). Similarly, 0.3±0.36% (T_{eff}) and 0.17±0.15% ($T_{naïve}$) of cells specific for S. aureus were IL-17^{+}/IL-10^{+} **(**Figure 29B**,** Figure 31B**)**.

In both cultures, cells co-expressing IL-17 and IL-23R were rare, but they were clearly present at 0.24±0.16% (C. albicans) and 0.14±0.06% (S. aureus) in effector T-cell cultures and 0.02±0.01% (C. albicans) and 0.22±0.3% (S. aureus) in naïve T-cell cultures **(**Figure 29A**,** Figure 31A**)**.

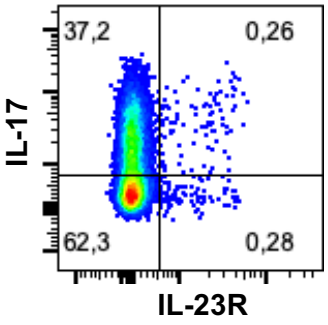

Figure 30 Detection IL-23 receptor on cell surface via flow cytometry. PBMC's were isolated from human blood and separated by MACS to obtain a high purity of T-cells. Naïve and effector T-cells were activated by autologous pulsed monocytes and analyzed by flow cytometry. This figure illustrates IL-23 receptor staining exemplified by an effector T-cell population stimulated by monocytes pulsed with lysate of C. albicans.

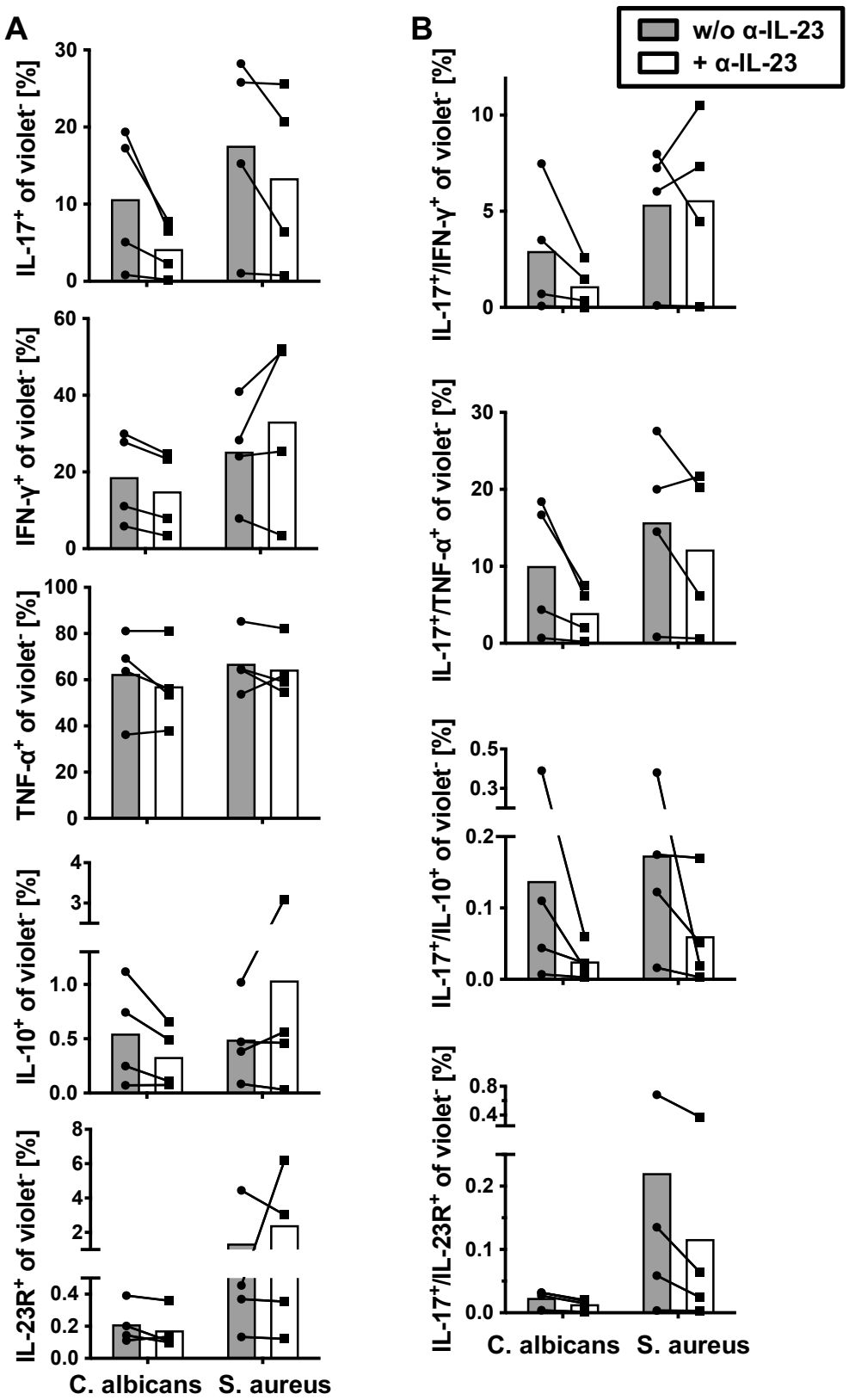

Figure 31 Cell frequencies of pathogen mediated activation and differentiation of naïve T-cells. Naïve T-cells were isolated and stimulated *in vitro* by autologous monocytes pulsed with lysates of C. albicans (left bars) and S. aureus (right bars) before. After differentiation, a flow cytometric analysis of T-cells was performed to determine cytokine or IL-23R expressing cells as portion of proliferating cells (A). At the double positive cell level, the expression pf cytokines and IL-23R simultaneously to IL-17 was examined (B). With regard to monocyte mediated activation of T-cells (grey bars), respective co-cultures were conducted under IL-23 neutralizing conditions, as well (white bars).

4.5.2. T_H17 response in a pathogen mediated T-cell activation model is IL-23 dependent

Neutralization of IL-23 diminished the T_H17 response in both cultures, although the decrease was more pronounced in effector T-cell cultures: IL-17$^+$ cells specific for C. albicans decreased from 37.64±8.76% to 25.34±5.36% in effector T-cell cultures and from 62.57±18.97% to 57.22±17.87% in naïve T-cell cultures, while T_H17 cells specific for S. aureus decreased from 34.46±9.99% to 25.41±10.81% (T_{eff}) and from 67.01±13.22 to 64.49±12.22% ($T_{naïve}$) **(**Figure 29A**,** Figure 31A**)**.

While blocking IL-23 did not cause differences on IFN-γ expression of neither C. albicans- nor S. aureus-specific effector T-cells, a decrease of C. albicans specific proliferating IFN-γ$^+$ cells from 18.7±12% to 14.87±10.81% in naïve T-cell cultures was observed, whereas S. aureus specific proliferating IFN-γ$^+$ cells partially increased from 25.32±13.66% to 33.09±23.33% **(**Figure 29A**,** Figure 31A**)**.

With regard to the average cell frequencies of TNF-α or IL-23R expressing cells, neutralization of IL-23 did not result in any striking alterations in either the effector or naïve T-cell cultures **(**Figure 29A**,** Figure 31A**)**.

IL-23 neutralization in naïve T-cell cultures caused a decrease in frequency of C. albicans-specific IL-10$^+$ cells from 0.55±0.48% to 0.33±0.29%, while the frequencies of S. aureus-specific IL-10$^+$ T-cells partly increased from 0.49±0.39% to 1.04±1.39%. **(**Figure 29A**,** Figure 31A**)**.

Remarkably, in effector T-cell cultures IL-23 neutralization diminished the pathogenic T_H17 response against C. albicans from 20.58±10.31% regarding IL-17$^+$/IFN-γ$^+$ cells to 14.37±4.39% and from 28.95±6.53% regarding IL-17$^+$/TNF-α$^+$ cells to 20.65±3.45%, as well as the response against S. aureus from 17.35±6.17% with respect to IL-17$^+$/IFN-γ$^+$ cells to 13.8±7.24% and from 26.15±6.1% regarding IL-17$^+$/TNF-α$^+$ cells to 21.72±9.25% (Figure 29B**,** Figure 31B, Figure 32**)**. In naïve T-cell cultures, neutralization of IL-23 induced a decrease of C. albicans-specific IL-17$^+$/IFN-γ$^+$ cells from 2.94±3.37% to 1.10±1.17%. C. albicans-specific IL-17$^+$/TNF-α$^+$ cells decreased from 10.05±8.82% to 3.95±3.43%. S. aureus-specific IL-17$^+$/IFN-γ$^+$ cells partly increased, whereas IL-17$^+$/TNF-α$^+$ cells also decreased from 15.74±11.29% to 12.20±10.42% (Figure 29B**,** Figure 31B, Figure 32**)**.

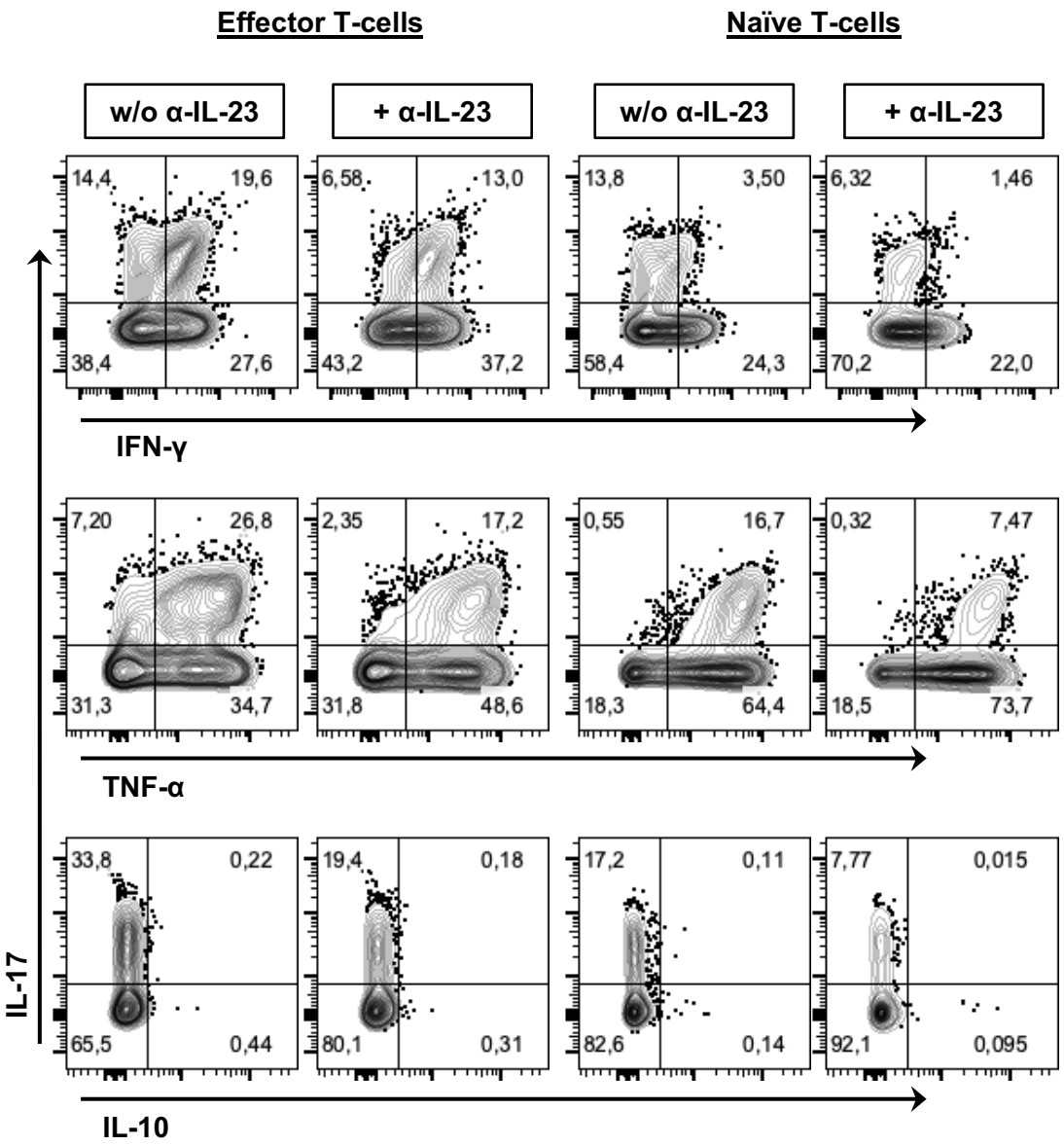

Figure 32 Effect of anti-IL-23 on T_H17 phenotypes in a pathogen induced immunoreaction. Pathogen mediated activation of $CD4^+CD45RO^+$ effector T-cells (left) and a Pathogen mediated *in vitro* differentiation of $CD4^+CD45RO^{neg}$ naïve T-cells (right) by lysates of C. albicans are exemplified. T-cells were stimulated by pulsed monocytes either under IL-23 neutralizing conditions or not. Typical cytokine patterns of pathogenic T_H17 cells, namely simultaneous co-expression of IL-17 and IFN-γ (top) or IL-17 and TNF-α (middle), and non-pathogenic T_H17 cells expressing simultaneously IL-17 and IL-10 (bottom), were analyzed by flow cytometry as percentage of proliferating cells.

Under IL-23 neutralizing conditions, the cell frequencies of $IL\text{-}17^+/IL\text{-}10^+$ effector T-cells proliferating in the presence of C. albicans partially decreased, resulting in a decrease from an average of 0.55±0.74% to 0.34±0.49%, whereas cells stimulated with S. aureus remained almost unaltered. In naïve T-cell cultures, C. albicans-specific $IL\text{-}17^+/IL\text{-}10^+$ cells diminished from 0.14±0.17% to 0.03±0.02% and those responding to S. aureus declined from 0.17±0.15% to 0.06±0.08% after anti-IL-23 treatment **(**Figure 29B**,** Figure 31B, Figure 32**)**.

Regarding IL-23R expression, treatment with anti-IL-23 in effector T-cell cultures did not result in alterations in the frequency of C. albicans- or S. aureus-specific IL-17^{+}/IL-23R cells. However, in cultures of naïve T-cells, neutralization of IL-23 resulted in a decrease in IL-17^{+}/IL-23R^{+} cells specific for C. albicans from 0.02±0.01% to 0.01±0.01% and in IL-17^{+}/IL-23R^{+} cells specific for S. aureus from 0.22±0.31% to 0.11±0.17% **(**Figure 29B**,** Figure 31B**)**.

5. Discussion

Over the last 15 years, the influence of different cytokines on T_H17 cell differentiation was analyzed, leading to the discovery of distinct T_H17 phenotypes. In particular, two distinct subtypes were defined: A pathogenic IFN-γ co-expressing subset with a detrimental character and a non-pathogenic subset with protective features by co-expressing IL-10 (McGeachy et al. 2007; Ghoreschi et al. 2010). Furthermore, subsequent studies showed, that apart from cytokines like IL-23, IL-6, IL-1β and TGFβ, also nutritional and environmental factors like fatty acids and hypoxia can influence the T_H17 phenotype (Stockinger and Omenetti 2017). In addition, the subsets differ at molecular level by showing differences regarding their gene expression and their regulation of transcription factors (1.2.3).

This study focused on the role of IL-23 in context of shaping different T_H17 phenotypes and more in detail on investigating selective effects of anti-IL-23 on pathogenic versus non-pathogenic T_H17 cells. The aim of this study was to analyze IL-23 deprivation in context of different stages of T_H17 cell life cycle. Therefore, we established different *in vitro* models to investigate the impact of guselkumab treatment on T_H17 cell biology. Guselkumab, an IL-23 inhibitor approved for the treatment of psoriasis, was successfully transferred for use in cell culture models. Here we showed efficient blocking of IL-23 by guselkumab, while maintaining cell viability and ruling out toxic effects (4.1/ Figure 9).

5.1. Investigating cytokine requirements of human T_H17 differentiation

First, we used cells of healthy, voluntary human donors to establish a T_H17 polarization protocol and to analyze the polarizing potential of anti-IL-23 at the beginning of T_H17 cell life cycle. A flow cytometric detection of intracellular stained cytokines serves as an important readout method to detect different T_H17 subtypes. However, this staining and flow cytometric analysis of *in vitro* differentiated human T_H17 cells from naïve T-cells was known in literature to be difficult. Here, different protocols were run to optimize differentiation and subsequent staining procedure. Adapted to findings by Revu et al., we considered activation of TCR only with anti-CD3, a resting phase after activation and a shortened and high concentrated stimulation for intracellular staining to be important to achieve a high yield of T_H17 cells in flow cytometry (3.1.5/4.2.1) (Revu et al. 2018).

There are many different pathways described in literature to transdifferentiate human T_H17 cells from naïve precursor cells. Moreover, some cytokines are considered to affect T_H17 phenotype and its pathogenicity. Major cytokines commonly mentioned in this context are IL-23, IL-1β, IL-6 and TGFβ. Various combinations of these four cytokines have been tested in the literature, some of which successfully induced T_H17 cells despite contradictory findings.

In particular, the need for TGFβ during T_H17 differentiation led to a rather controversial discussion. Initially, it was proposed in mice models to be critical for T_H17 development (Mangan et al. 2006). Later in 2007, Acosta-Rodriguez et al. could show that human T_H17 cells can be differentiated by IL-1β and IL-6 in the absence of TGFβ. They showed even dose-dependent inhibitory effects of TGFβ on T_H17 differentiation (Acosta-Rodriguez et al. 2007). Wilson et al. demonstrated that combination of IL-6 and TGFβ is unable to differentiate human T_H17 cells, but combination of IL-23 and IL-1β is (Wilson et al. 2007). In contrast, a year later, Yang et al. showed that TGFβ is required for human T_H17 differentiation, and Volpe et al., as well as Manel et al. postulated that TGFβ, IL-23, IL-6 and IL-1β are necessary for human T_H17 differentiation (Yang et al. 2008; Volpe et al. 2008; Manel et al. 2008).

Besides expected differences in cytokine requirement of *in vitro* T_H17 differentiation in mice and men, a dose-dependent effect of TGFβ on T_H17 differentiation, acting in an inhibitory manner in high doses, could explain these contradictory findings. As early as 2008, Zhou et. al demonstrated in a mouse model that TGFβ at low concentrations had synergistic effects on promoting IL-23R expression, while high doses suppressed IL-23R expression (Zhou et al. 2008).

Beyond that, it was shown that some of these cytokines affect the T_H17 phenotype and its pathogenicity. In agreement with the 2007 publication of McGeachy et al., which demonstrated the ability of TGFβ in combination with IL-6 to induce IL-17/IL-10 co-expressing cells, and the 2007 results of Acosta-Rodriguez et al., which showed a dose-dependent inhibition of IL-17/IFN-γ co-expressing cells, it was suggested that TGFβ plays a crucial role in determining T_H17 phenotype to be either pathogenic or non-pathogenic (McGeachy et al. 2007; Acosta-Rodriguez et al. 2007).

On the other hand, cytokines like IL-1β and IL-23 were shown to drive differentiation towards a pathogenic phenotype, for example Langrish et al. showed that IL-23 dependent $CD4^+$ cells have a high pathogenic potential (Langrish et al. 2005). Wilson et al. demonstrated in 2007 that combination of IL-1β and IL-23 induces human T_H17 cells expressing among others IL-17, IL-22 and IFN-γ and Zielinski et al. postulated later that

IL-1β inhibits IL-10 production of T_H17 cells, but it is essential to prime IL-17/IFN-γ co-expressing cells (Wilson et al. 2007; Zielinski et al. 2012).

Based on these findings, three different cytokine mixes for T_H17 polarization were established to differentiate either specifically pathogenic or non-pathogenic T_H17 cells. The first one consisted of IL-1β and IL-23 to prime differentiation towards pathogenic cells. The second one contained TGFβ and a lower concentration of IL-23, while the third one was based on TGFβ, IL-6 and a low concentration of IL-23 to potentially drive differentiation towards non-pathogenic T_H17 cells. However, by using human serum in these polarization culture, it cannot be excluded that various cytokines from the human system are present at low concentrations under these *in vitro* conditions.

The pathogenic and the intermediate polarization protocol containing IL-1β and IL-23 reached highest frequencies of IL-17^+ cells and pathogenic T_H17 cells co-expressing IL-22, IFN-γ or TNF-α compared to the non-pathogenic polarization protocol (Figure 15). Also, absolute cell numbers showed that the pathogenic approach induced most IL-17^+ cells (Figure 16) which was also confirmed by ELISA, showing highest IL-17 levels (Figure 17). Furthermore, IL-23 and IL-1β (path.) induced the highest number of pathogenic T_H17 cells expressing IL-17 and IFN-γ. Surprisingly, the non-pathogenic approach did not significantly induce IL-17^+ cells and thus was not sufficient to drive differentiation towards T_H17 in general. However, analyzing flow cytometric data and absolute cell numbers showed that the pathogenic and the intermediate approach induced a small but significant increase of non-pathogenic T_H17 cells.

Regarding T_H17 cells in general, by comparison of these three differentiation-approaches we could demonstrate that combination of IL-1β and IL-23 induces T_H17 differentiation most efficiently. In contrast, culturing naïve T-cells in presence of TGFβ and IL-6 (non-path.) failed to induce T_H17 cells, supporting the hypothesis that primarily IL-23 and IL-1β, but not TGFβ, are crucial for the development of human T_H17 cells. Additionally, this polarization approach was highly specific and showed no effects on cell numbers of T_H2 cells co-expressing IL-4 and IL-13. Based on the data, showing very low levels of non-pathogenic T_H17 cells, we hypothesize that potentially non-pathogenic T_H17 cells cannot be differentiated from naïve T-cells and their phenotype is rather a result of T_H17 plasticity then original differentiation. This is supported by the fact that there exists no literature for *in vitro* differentiation of human non-pathogenic T_H17 cells from CD45RO^{neg} naïve T-cells by supplementing recombinant cytokines. Most evidence for non-pathogenic T_H17 cells was collected in mouse models (McGeachy et al. 2007; Bellemore et al. 2015; Wang et al. 2015) and the rare investigations in human models mostly did not contain naïve *in vitro* differentiations. Differentiation of human T_H17 cells in general

was shown, but indeed they were not able to induce or even detect IL-10 by flow cytometry (Yang et al. 2008). The most promising results for differentiation of human non-pathogenic T_H17 cells were shown by Zielinski et. al., but even there the successful development of non-pathogenic T_H17 cells was mediated by pulsed monocytes and not by addition of particular cytokines (Zielinski et al. 2012). Due to this persisting lack of evidence for the specific *in vitro* differentiation of human non-pathogenic T_H17 cells it seems likely that they probably do not derive from naïve cells and are rather a result of T_H17 plasticity.

5.2. IL-23 is essential for differentiation of both pathogenic and non-pathogenic T_H17 cells

Cua et al. already proposed in 2003 that IL-23 is critical in autoimmune inflammation (Cua et al. 2003). Furthermore, Langrish et al. described in 2005 an IL-23 dependent, highly pathogenic type of $CD4^+$ T-cells and they assigned IL-23 the importance of a crucial mediator of autoimmune inflammation (Langrish et al. 2005). In the following time, other authors like McGeachy et al. confirmed the essential role of IL-23 in pathogenicity and terminal differentiation of T_H17 cells (Peters et al. 2011; McGeachy et al. 2007; McGeachy et al. 2009). To confirm the IL-23 mediated pathogenicity of T_H17 cells during differentiation and to investigate its role in differentiation of non-pathogenic T_H17 cells, we added anti-IL-23 to our different polarization approaches.

Supplementing polarization cultures with anti-IL-23 caused a pronounced decrease of absolute IL-17 expressing cell numbers (Figure 16) and IL-17 cytokine levels in cell culture supernatants (Figure 17). As expected, also the numbers of pathogenic IL-17^+/IFN-γ^+ cells decreased strikingly due to IL-23 inhibition in the pathogenic and intermediate approach, underlining the essential role of IL-23 during T_H17 differentiation with respect to the pathogenicity of T_H17 cells. As shown for effector and $CD4^+$ T-cell culture, non-pathogenic T_H17 cells co-expressing IL-10 decreased under IL-23 neutralizing conditions during the differentiation process, as well. This shows that even though non-pathogenic cells cannot efficiently be differentiated under these polarization conditions, they show a clear dependency on IL-23.

The importance of IL-23 for T_H17 cells and its mode of action was controversially discussed. Based on the initial data, IL-23 was thought to play an important role for survival or cell expansion of T_H17 cells but was not required for de novo generation of T_H17 cells (Mangan et al. 2006; Veldhoen et al. 2006; Bettelli et al. 2006). Stritesky et al. proposed that it is not necessary for survival or cell expansion, but it maintains IL-17

secretion and they confirmed that it does not promote T_H17 differentiation (Stritesky et al. 2008). Surprisingly, our data indicates that IL-23 is necessary for T_H17 differentiation and that generation of both pathogenic and non-pathogenic T_H17 cells rely on IL-23. We cannot exclude that there is an unknown variable in the physiological system which is not described yet and is able to differentiate T_H17 cells in the absence of IL-23. But within our *in vitro* model IL-23 was essential for differentiation of T_H17 cells.

5.3. S. aureus and C. albicans efficiently induce a T_H17 response and neutralization of IL-23 reverses this effect

Zielinski et al. have shown a pathogen mediated approach for inducing pathogenic IL-17^+/IFN-γ^+ and non-pathogenic IL17^+/IL10^+ cells. Here, C. albicans was rather triggering pathogenic T_H17 cells, while S. aureus enhanced non-pathogenic T_H17 cells (Zielinski et al. 2012). In order to analyze pathogenic and non-pathogenic T_H17 cells and their dependency on IL-23 in a more physiological relevant setting and to differentiate non-pathogenic T_H17 cells from naïve cells, efficiently, we used the protocol published by Zielinski et al. based on pathogen induced T_H17 differentiation.

Pathogen mediated differentiation and proliferation led to a prominent T_H17 response in context of C. albicans, as well as for S. aureus (Figure 29/ Figure 31). Both pathogens caused similar effects within effector and naïve T-cell cultures. In these cultures, both pathogens induced high frequencies of proliferating cells expressing IL-17, IFN-γ and TNF-α. Of note, although pathogenic T_H17 cells could be induced in a similar extent with both pathogens an IL-10 co-expressing population was not efficiently differentiated (Figure 32). Indeed, Zielinski et al. could detect IL-10 expression only transiently after restimulation of the co-cultures starting on day five, with the IL-10 signal not being present before that day and also vanishing a few days later. Nevertheless, neutralization of anti-IL-23 in this context decreased IL-17 expressing and more specifically also pathogenic T_H17 cells, clearly emphasizing IL-23 to be crucial in T_H17 pathogenicity in a physiologically relevant model of T-cell activation.

5.4. Do non-pathogenic T_H17 cells directly differentiate from naïve T-cells or are they a consequence of plasticity?

In general, we could show highest frequencies of a distinct non-pathogenic IL-17^+/IL-10^+ cell population in effector T-cell cultures. Together with the results from T_H17 polarization, where specific non-pathogenic T_H17 differentiation could not be efficiently induced, these data suggest an establishment of the non-pathogenic T_H17 phenotype rather through plasticity of differentiated T_H17 cells than through differentiation itself. Also, the fact that T_H17 cells in general are suspected to show a high grade of plasticity among the different T-cell lineages emphasizes that there is also a high grade of plasticity among the T_H17 subtypes (Peck and Mellins 2010; Mazzoni et al. 2019).

Investigating T_H17 cells, Zielinski et al. could only transiently show IL-17/IL-10 co-expression after restimulation of pathogen induced T-cell cultures starting with highest levels on day five and day nine, which then quickly decreased before completely disappearing again until day 20. Also, at day five a prominent population of IL-10^+ cells was shown but almost no cells expressing IL-17. During restimulation IL-17^-/IL-10^+ cells decreased after day nine, while IL-17^+/IL-10^- cells increased until the ratio reverted resulting in a large IL-17^+/IL-10^- and a small IL-17^-/IL-10^+ population. Predominantly IL-17/IL-10 co-expressing cells appeared on day five and nine and were almost absent before and after this period. Also, this highlights the IL-17^+/IL-10^+ phenotype as a transient phenotype and its appearance could be interpreted as a result of T_H17 plasticity. Since it has been shown that T_H17 cells can transdifferentiate to T_{reg} cells and in respect of this they can transiently appear *in vitro*, the non-pathogenic T_H17 cell expressing IL-17 and IL-10 could represent an intermediary state of a T_H17 cell transdifferentiating into a T_{reg} cells (Gagliani et al. 2015).

Recent profiling of T_H17 transcriptomes gave new insights in characteristic features of pathogenic and non-pathogenic T_H17 cells and revealed differences regarding their gene expression (1.2.3). In particular, investigations of CD5l/AIM from Wang et al. identifying it as a critical regulator of T_H17 phenotype supported the hypothesis that non-pathogenic T_H17 cells could more likely derive from pathogenic T_H17 cells than differentiate from naïve T-cells. CD5l/AIM, a member of the scavenger receptor cysteine-rich superfamily, is a critical regulator of T_H17 phenotype, which is expressed in non-pathogenic cells but is not detectable in pathogenic cells. CD5l/AIM has no influence on T_H17 de novo differentiation, but its deficiency converts non-pathogenic into pathogenic T_H17 cells. It

alters intracellular fatty acid composition and reduces cholesterol synthesis. Thereby, CD5l/AIM reduces the source of endogenous ligands for RORγt acting as a major regulatory switch for T_H17 phenotype. Additionally, IL-23R signaling rapidly suppresses CD5l/AIM expression and can readily convert non-pathogenic into pathogenic T_H17 cells. (Wang et al. 2015; Xinyu Wu et al. 2018)

This is just one example proving the concept that there is a high grade of plasticity not only among T helper cells but especially in T_H17 subtypes. (Zhou et al. 2009; Peck and Mellins 2010) More evidence was given earlier by experiments with cell fate reporter mice, revealing distinct plasticity in different inflammatory settings (Hirota et al. 2011). With the help of these fate-mapping mouse models the capability of changing phenotype the other way around from a pathogenic to a regulatory cytokine profile could also be shown (Gagliani et al. 2015). In this context, targeting T_H17 plasticity via biologics instead of only inhibiting a certain phenotype may provide an effective modification of chronic inflammatory disease course potentially with even a curable therapy intention.

5.5. Anti-IL-23 preferentially inhibits pathogenic T_H17 cells and thereby massively affects T_H17 subtype balance in an anti-inflammatory manner

We could show that regarding cell frequencies IL-23 inhibition caused a substantial decrease of pathogenic T_H17 cells in pan $CD4^+$ and effector $CD4^+$ T-cell cultures, while frequencies of non-pathogenic T_H17 cells even slightly increased (Figure 18B and Figure 23B). In general, blocking IL-23 caused a decrease of cell frequencies expressing inflammatory cytokines like IL-17, IL-22, IFN-γ and TNF-α, whereas frequencies of cell populations expressing IL-10 increased. Furthermore, use of anti-IL-23 increased the percentage of cells expressing IL-13 unrelated to T_H17 highlighting the selective effect of IL-23 deprivation on T_H17 cells (Figure 18A/ Figure 23A).

Additionally, we analyzed the expression level of IL-17, IFN-γ and IL-10 in the cell populations positive for these cytokines by assessing the MFI of respective signals by flow cytometry. We could not detect any differences between anti-IL-23 treated and untreated samples (Figure 22/

Figure 27), showing that anti-IL-23 treatment is changing cell numbers of the respective T_H17 phenotypes but not the expression levels of the respective cytokines. The measurement of the corresponding cytokines in cell free supernatants by ELISA did not reveal major differences between the conditions regarding concentration of IL-17, IFN-γ and IL-10.

These unexpected and small effects on the secretion of the cytokines could be caused by different treatment protocols for flow cytometric analysis and analysis of cytokine level in the cell supernatant. Cells which were prepared for flow cytometric analysis underwent an additional ten-day lasting resting phase containing a resting medium supplemented with anti-IL-23. Thus, cells analyzed by flow cytometry underwent a prolonged phase under IL-23 neutralizing conditions compared to cells which were directly activated for three days to obtain supernatants for cytokine measurement. It is possible that three days of IL-23 deprivation is not sufficient and a longer incubation under IL-23 neutralizing conditions is needed to cause major effects.

Furthermore, stimuli differ depending on the read out potentially, leading to different results. For measuring secreted cytokine levels by ELISA, cells were stimulated for three

days with anti-CD3 and anti-CD28 mimicking TCR stimulation, while for flow cytometric analysis, cells were stimulated for five hours with PMA/ionomycin, GolgiPlug and GolgiStop (3.1.6). Apart from the different stimulus used, the ELISA data showed cytokine level that were accumulated over three days, whereas the flow cytometric data gave an exact picture of the frequency and expression level.

However, the observed increase of non-pathogenic T_H17 cell frequencies accompanied by a decrease of pathogenic T_H17 cells under IL-23 deprivation showed a clear shift of the balance towards a non-pathogenic T_H17 phenotype (

Figure 24B). In order to investigate if the shifted balance was caused by an actually increased differentiation of non-pathogenic T_H17 cells or rather a reduction of pathogenic T_H17 cells, absolute cell numbers were determined.

Analysis of absolute cell number in $CD4^+$ and effector T-cell cultures revealed a decrease of all IL-17 expressing cells regardless which cytokine was co-expressed. Also, total cell counts of cells expressing IL-22, TNF-α, IFN-γ and even IL-10 decreased after anti-IL-23 treatment. Only the IL-13^+ cell population remained unaffected, showing that the previous observed relative increase of frequencies in these cells are based on reduced numbers of other cell populations rather than an actual increase of the IL-13^+ cell population (Figure 20/ Figure 25). These data show that IL-23 specifically impacts T_H17 cells. Furthermore, they show that all T_H17 subtypes are affected no matter if they are pathogenic or non-pathogenic, indicating IL-23 to be essential for survival of all IL-17 producing effector T-cells.

Interestingly, we could show that IL-23 inhibition decreased IL-10^+ cell populations in a smaller extent than IFN-γ^+ and IL-17^+ cell populations likewise pathogenic T_H17 cells co-expressing IL-17/IFN-γ were reduced more extensively than non-pathogenic T_H17 cells co-expressing IL-17/IL-10 (Figure 21/ Figure 26). This emphasizes a certain resistance or rather a lower sensitivity of non-pathogenic T_H17 cells to IL-23 deprivation. The observed higher sensitivity of pathogenic T_H17 cells to anti-IL-23 could be based on differences regarding IL-23R expression among different T_H17 subsets. It has been shown that in context of IL-4 and IL-13 the respective receptor expression level correlated with the sensitivity of the cell for the respective cytokine (Junttila et al. 2008). In line with this, the observed higher sensitivity of pathogenic T_H17 cells to anti-IL-23 could be based on differences regarding IL-23R expression among the different T_H17 subsets, as well. In literature, IL-23R has been shown to be expressed especially on $CD45RO^+$ memory T-cells but almost not or barely at low level on the surface of $CD45RO^{neg}$ naïve T-cells. This was shown by flow cytometric data by staining IL-23R and by quantitative real-time polymerase chain reaction (qRT-PCR) data by detection of

IL-23R messenger RNA (mRNA)(Wilson et al. 2007; Chen et al. 2007). Although we tried different commercially available antibodies and even tried to amplify the signal by usage of a biotin-labeled antibody, we were unable to stain IL-23R in either $CD4^+$, effector or naïve T-cell cultures. This is likely due to an insufficient affinity of the commercially available antibody and lower expression of IL-23R in *in vitro* cultures.

Interestingly, we were capable of staining IL-23R in the pathogen mediated activation model, revealing no differences regarding IL-23R expression from untreated compared to anti-IL-23 treated samples (Figure 28). Potentially, pathogen mediated activation via coculturing induces a higher strength of expression of IL-23R. It is not clear why we could not stain IL-23R in the other cultures. But since we detected highest effects and yields of non-pathogenic T_H17 cells within effector T-cell culture, it would be important to check IL-23R expression within these cultures in the future, to evaluate differences among pathogenic and non-pathogenic T_H17 cells. But besides an altering expression of IL-23R there could be other alterations more downstream in signal transduction which could explain the observed effects and should be addressed in further studies, too.

Signaling pathway of IL-23R and of cytokine receptors in general is very complex and a lot of different molecules are engaged within signal transduction. The binding of the p40 and p19 subunit of the cytokine to the IL-12Rβ1 and IL-23R promotes receptor oligomerization and thereby leads to an activation of members of the Janus kinase (JAK) family. These kinases amplify the signal and activate members of the STAT family which represent transcription factors and guide the signal downstream towards gene expression (Pastor-Fernández et al. 2020). The importance of the signal amplification within this proximal signal cascade is underlined by the fact that cytokine receptors are only scarcely distributed on the cell surface. For example, only less than 1,000 Interferon-alpha/beta receptor alpha chain (IFNAR1) and IL-4Rα1 receptors are estimated to be on a cell surface (Moraga et al. 2014; Uzé et al. 1990; Lowenthal et al. 1988). So, the mediators downstream the receptor binding play a crucial role and alteration within their quantity could also be a reasonable explanation for the different sensitivity among T_H17 subsets against IL-23 deprivation.

Furthermore, the two different subsets could have different binding affinities of the IL-23R to IL-23 and thereby mediate the robustness of non-pathogenic T_H17 cells under IL-23 deprivation. For example, it could be shown that mutations of the Tyk2 protein, a member of the JAK family, decreased the affinity of IFNAR1 for binding IFN and similarly that binding protease USP18 leads to a decrease of the affinity of Interferon-alpha/beta receptor beta chain (IFNAR2) for binding IFN without altering the respective surface levels of the receptor (Moraga et al. 2014; Gauzzi et al. 1997; François-Newton et al.

2011). So, the existence of yet unknown molecules that affect binding affinity of IL-23R could explain the varying sensitivity towards IL-23, as well.

Comparison of the population size of pathogenic T_H17 cells expressing IL-17 and IFN-γ and non-pathogenic T_H17 expressing IL-17 and IL-10 showed that pathogenic T_H17 are much more abundant with 10 times of the population size of non-pathogenic T_H17 cells (Figure 25B). Many publications provide information about T_H17 and T_{reg} balance in diverse diseases and animal models, proposing that a disruption of this equilibrium leads to a disease manifestation (Noack and Miossec 2014). Similarly, this hypothesis could be transferred to T_H17 subtypes keeping a balance of pathogenic and non-pathogenic T_H17 phenotypes in sense of an immune homeostasis, as well. It could be doubted that 10 times less non-pathogenic cells represent a plausible balanced ratio of inflammatory and anti-inflammatory T_H17 subtypes, since analysis of T_H17/T_{reg} ratio in different diseases showed far higher values for the anti-inflammatory side (Alvarez-Rodriguez et al. 2019; Yu et al. 2015; Hao et al. 2019). However, immune homeostasis and the underlying pathogenesis of different diseases are just barely comparable. In addition, our results are obtained from an *in vitro* model that potentially does not mirror proportions of T_H17 subtypes *in vivo*, but it still shows the existence of these phenotypes *in vitro* and that the balance of them can be altered by IL-23.

6. Conclusion and outlook

6.1. IL-23 as a key cytokine in the context of T_H17 plasticity

Within this thesis we confirmed the role of IL-23 as a key cytokine regarding survival and pathogenicity of T_H17 cells. We investigated the effect of IL-23 deprivation on T_H17 phenotype in different stages of the T_H17 cell life cycle and showed that IL-23 inhibition affects T_H17 cells in an effector state, as well as during their differentiation process.

Moreover, we showed that both T_H17 subtypes depend to IL-23 exposure, but pathogenic T_H17 cells are more sensitive to IL-23 deprivation than non-pathogenic cells. In addition, we revealed the need for IL-23 for specific differentiation of pathogenic T_H17 cells and could transfer the essential role of IL-23 into a more physiological model of pathogen induced T-cell activation.

Interestingly, non-pathogenic T_H17 cells could not be efficiently induced by either cytokine mediated T-cell differentiation or in a model of pathogen mediated activation and differentiation of T-cells. Together with the observed larger non-pathogenic T_H17 populations within *in vitro* effector T-cell cultures, these data hint to non-pathogenic T_H17 cells originating as a result of T_H17 plasticity from T_H17 effector cells rather than direct differentiation from naïve T-cells.

Regardless of whether non-pathogenic T_H17 cells represent a discrete subtype of T_H17 cells or are just a transient phenotype during a process of T-cell plasticity, we could clearly show that they differ regarding their sensitivity to IL-23 deprivation showing that IL-23 neutralization significantly affects pathogenic T_H17 cells more severely compared to non-pathogenic T_H17 cells.

6.2. Modification of immune system in treatment of psoriasis: a potential for disease modification

Our *in vitro* results give hints that anti-IL-23 therapy has the potential to modify balance of pathogenic and non-pathogenic T_H17 subsets, thereby modifying the pathomechanism of psoriasis by preferentially inhibiting pathogenic T_H17 cells. Long lasting effects after termination of treatment were shown in a small number of patients.

This could be due to a predisposition of these patients to a non-pathogenic T_H17 phenotype. Treating these patients with an anti-IL-23 agent efficiently depletes pathogenic subsets, while maintaining non-pathogenic T_H17 cells, which are then able to suppress further inflammatory processes and sustain clear skin appearance. This hypothesis and whether our *in vitro* results are transferable to an *in vivo* model needs to be investigated. A currently running double-blind, randomized trial named the Guide study, evaluating guselkumab therapy in participants with moderate-to-severe plaque-type psoriasis can maybe deliver answers to these questions in the near future. Among other things this study is aimed to identify super responders (PASI=0 at weeks 20 and 28) after 4 injections of guselkumab (weeks 0; 4; 12 and 20), which in turn will receive guselkumab in 8- or 16-week intervals. On the other hand, participants with PASI<3 after week 68 will be withdrawn from guselkumab and followed up until week 116. Analysis of these results could elucidate whether super responders have a predisposition for a non-pathogenic T_H17 answer and whether they need less injections of anti -IL-23 agents. Despite their specific intervention in orchestrating an autoimmune response in pathogenesis of psoriasis, anti-IL-23 agents do not aim at curing the disease. This is why sooner or later all patients, including super responders, suffer a relapse after termination of treatment with anti-IL-23 agents. In general, finding a cure for psoriasis might be challenging due to its multifactorial genesis. Even so anti-IL-23 agents may not provide a cure, the results of my doctoral thesis reveal clearly that there is a disease modifying effect by anti-IL23 treatment, that does not deplete T_H17 cells in general and to the same extent. In contrast, it has selective effects on T_H17 phenotypes and thereby precisely effectuates the equilibrium of inflammation in an anti-inflammatory manner. Beyond that, along with the other biologics like anti-IL-17 and anti-TNF-α agents, anti-IL-23 agents represent a highly effective therapy for psoriasis without comparable alternatives.

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Publications resulting from this thesis

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