Role of monocyte-induced development of Th17 cells, the heat shock protein 90 and proinflammatory S100 proteins in the pathogenesis of graft-versus-host disease

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1 Summary

Allogeneic hematopoietic cell transplantation (HCT) is an effective treatment for patients with hematologic malignancies, aplastic anaemia, and congenital immunodeficiency disorders. One of the major serious morbidities associated with HCT is the development of acute or chronic graft-versus-host disease (GvHD). The pathophysiology of GvHD is complex and not fully understood. The role of Th17 cells during GvHD is discussed controversially and still remains unclear. In this study, the induction of Th17 cells by monocytes of patients with GvHD *in vitro* was analysed demonstrating that monocytes isolated from patients with acute skin and intestinal GvHD stage I-IV or chronic GvHD induce significantly increased levels of Th17 cells compared to patients without GvHD after HCT and healthy controls. Several studies suggest using the determined levels of regulatory (Treg) cells and the ratio of Th17 cells to Treg cells in the peripheral blood of patients as diagnostic markers for GvHD. However, the data of the present study have demonstrated that the determined percentages of Treg cells in peripheral blood mononuclear cells (PBMCs) isolated from patients with acute GvHD do not differ from the assessed percentages of Treg cells in PBMCs of healthy donors and patients without GvHD after HCT. By contrast, the percentages of Treg cells in PBMCs from patients with extensive chronic GvHD seem to be increased in comparison to the healthy controls and the non-GvHD group. The results of the present work further indicate that the calculated ratios of Th17 cells to Treg cells are not altered in patients with acute or chronic GvHD compared to patients without GvHD after HCT. Development and progression of GvHD is mediated by multiple cellular and inflammatory effectors. However, several of these molecules are still unknown. Previous studies have demonstrated that S100 proteins act as innate amplifier of inflammation and play an important role in many inflammatory diseases such as inflammatory bowel disease or rheumatoid arthritis. These proinflammatory S100 proteins belong to the group of Damage Associated Molecular Pattern (DAMP) molecules and are released by activated or damaged phagocytes under conditions of cell stress during infections and autoimmune diseases. Therefore, expression levels of S100 proteins in monocytes and the presence of S100 proteins in the stool, serum and bowel tissue were investigated in patients with acute or chronic GvHD and compared to healthy controls and patients

without GvHD after HCT. Additionally, the influence of S100 proteins on monocyte-mediated induction of Th17 cells was analysed. The data of this study demonstrate that the expression of S100 proteins is increased in monocytes from patients with GvHD compared to the controls. Overall, elevated levels of S100 proteins can be detected in the serum, stool and bowel tissue of patients with GvHD demonstrating the release of these phagocyte-specific proteins during GvHD. Furthermore, S100 proteins were found to bind to toll-like receptor 4 (TLR4) on monocytes resulting in the promotion of monocyte-induced Th17 development. These data emphasize the role of S100 proteins in Th17 triggered inflammation. Additionally, it was investigated if the induction of Th17 cells is mediated by proinflammatory cytokines released by monocytes or by cell contact between monocytes and $CD4^+$ T cells. The data of the present study have revealed that monocyte-mediated Th17 development occurs in a cell-cell-contact dependent manner with the involvement of proinflammatory cytokines secreted by *in vitro* or *in vivo* activated monocytes. A further part of this thesis examined the influence of heat shock protein 90 (Hsp90) in monocyte-mediated induction of Th17 cells. Hsp90 is a ubiquitously expressed molecular chaperon that is known to play an important role in signal transduction, transcription regulation and survival of the cell. The data of this work have demonstrated that Hsp90 inhibition in *in vivo* activated monocytes by the geldanamycin derivative 17-DMAG decreases Th17 responses. Further results have shown that the stimulatory effects of proinflammatory S100 proteins on monocyte-induced Th17 development can be blocked by chemical inhibition of Hsp90 using 17-DMAG or by specific siRNA-mediated knockdown of the stress-inducible Hsp90α in monocytes. In contrast to dexamethasone which is a potent synthetic member of the glucocorticoid class of steroids that are widely used drugs for the treatment of acute and chronic GvHD, the chemical Hsp90 inhibitor 17-DMAG does not seem to induce the development of proinflammatory Th17 cells expressing the multi-drug resistance protein 1 (MDR1). MDR1 is an ATP-dependent efflux pump that plays a crucial role in the bioavailability of a wide range of drugs and xenobiotics. Altogether, the results of the present work indicate that levels of proinflammatory S100 proteins are increased in the serum, stool and bowel tissue of patients with GvHD and might promote monocyte-induced development of Th17 cells during GvHD. Specific inhibition of Hsp90 might prevent the induction of inflammation-promoting Th17 cells. Therefore, Hsp90 could be a novel, critical target for the treatment of GvHD.

Zusammenfassung

Die allogene hämatopoetische Stammzelltransplantation stellt eine effektive Behandlungsmöglichkeit für Patienten mit hämatologischen Erkrankungen, aplastischen Anämien und angeborenen Immunerkrankungen dar. Eine der Hauptursachen für Morbidität und Mortalität nach allogener Stammzelltransplantation stellt die Spender-gegen-Empfänger Reaktion (Graft-versus-Host Disease (GvHD)) dar. Allerdings ist die Pathophysiologie der GvHD sehr komplex und noch nicht vollständig aufgeklärt. Die Rolle von Th17 Zellen während der GvHD wird derzeit kontrovers diskutiert und ist immer noch unklar. In dieser Studie wurde die Induktion von Th17 Zellen durch Monozyten von Patienten mit GvHD *in vitro* untersucht und es konnte gezeigt werden, dass Monozyten, die aus dem peripheren Blut von Patienten mit akuter Haut oder Darm GvHD Grad I-IV oder mit chronischer GvHD isoliert wurden, signifikant höhere Anteile an Th17 Zellen induzieren verglichen mit Monozyten von gesunden Spendern und Patienten nach Stammzelltransplantation ohne GvHD. Einige Studien schlagen vor, die Anteile an regulatorischen T Zellen oder das Verhältnis von Th17 Zellen zu regulatorischen Zellen im peripheren Blut von Patienten als diagnostische Marker für eine GvHD einzusetzen. Die Daten der vorliegenden Arbeit hingegen haben gezeigt, dass es keine Unterschiede in den Anteilen an regulatorischen T Zellen in den peripheren mononukleären Blutzellen (PBMCs) von Patienten mit einer akuter GvHD, von gesunden Spendern sowie Patienten ohne GvHD nach allogener Stammzelltransplantation gibt. Jedoch scheinen die Anteile an regulatorischen T Zellen in PBMCs von Patienten mit extensiver chronischer GvHD erhöht zu sein, verglichen mit gesunden Spendern und Patienten ohne GvHD nach Stammzelltransplantation. Die Ergebnisse dieser Arbeit zeigen außerdem, dass sich die Verhältnisse von Th17 Zellen zu regulatorischen T Zellen bei Patienten mit akuter oder chronischer GvHD und Patienten ohne GvHD nach Stammzelltransplantation nicht unterscheiden. Das Entstehen und Fortschreiten einer GvHD wird durch viele zelluläre und inflammatorische Effektoren vermittelt. Viele dieser Moleküle sind jedoch noch unbekannt. Bisherige Studien haben gezeigt, dass S100 Proteine als angeborene Verstärker einer Entzündung fungieren und eine wichtige Rolle bei vielen entzündlichen Erkrankungen wie bei inflammatorischen Darmerkrankungen oder bei der Rheumatoiden Arthritis spielen. Diese S100 Proteine gehören zur Gruppe der „Damage Associated Molecular Pattern“ (DAMP) Moleküle

und werden von aktivierten oder beschädigten Phagozyten bei Zellstress im Rahmen von Infektionen und Autoimmunerkrankungen freigesetzt. Aufgrund dieser Tatsachen wurden S100 Expressionslevel in Monozyten sowie das Vorkommen von S100 Proteinen im Stuhl, Serum und Darmgewebe bei Patienten mit akuter oder chronischer GvHD untersucht und mit gesunden Spendern sowie Patienten ohne GvHD nach allogener Stammzelltransplantation verglichen. Zusätzlich wurde der Einfluss von S100 Proteinen hinsichtlich der Monozyten-vermittelten Induktion von Th17 Zellen analysiert. Die Ergebnisse dieser Arbeit haben gezeigt, dass die Expression der S100 Proteine in Monozyten von Patienten mit einer GvHD im Vergleich zu den Kontrollen erhöht ist. Insgesamt ist das S100 Proteinlevel im Serum, Stuhl sowie im Darmgewebe von Patienten mit einer GvHD erhöht, was darauf hindeutet, dass diese Phagozyten-spezifischen Proteine während einer GvHD freigesetzt werden. Des Weiteren führt eine Stimulation von Monozyten mit S100 Proteinen, welche an den toll-like Rezeptor 4 (TLR4) auf Monozyten binden, zu einer verstärkten Induktion an Th17 Zellen, was die Rolle von S100 Proteinen bei einer durch Th17 Zellen ausgelösten Inflammation betont. Außerdem wurde untersucht, ob die Induktion von Th17 Zellen zytokinvermittelt oder über Zellkontakt zwischen Monozyten und $CD4^+$ T Zellen erfolgt. Die Daten dieser Studie haben gezeigt, dass die Monozyten-vermittelte Induktion von Th17 Zellen durch Zell-Zell-Kontakt, aber unter Beteiligung von proinflammatorischen Zytokinen, welche von *in vitro* und *in vivo* aktivierten Monozyten sezerniert werden, erfolgt. Ein weiterer Teil dieser Arbeit beschäftigte sich mit der Untersuchung des Einflusses des Hitzeschockproteins 90 (Hsp90) auf die Monozyten-induzierten Entstehung von Th17 Zellen. Hsp90 ist ein ubiquitär exprimiertes molekulares Chaperon, das bei der Signaltransduktion, Transkriptionsregulation und dem Überleben der Zelle eine wichtige Rolle spielt. Die Ergebnisse dieser Arbeit haben gezeigt, dass eine Hsp90 Inhibition in *in vivo* aktivierten Monozyten mittels Geldanamycin Derivat 17-DMAG dazu führt, dass die Th17 Antwort verringert wird. Weitere Daten legen dar, dass der stimulatorische Effekt der proinflammatorischen S100 Proteine hinsichtlich der Induktion von Th17 Zellen durch chemische Inhibition von Hsp90 mittels 17-DMAG sowie durch spezifisches siRNA-vermitteltes Ausschalten des Stress-induzierbaren Hsp90α in Monozyten gehemmt werden kann. Im Gegensatz zu Dexamethason, welches zur Gruppe der künstlichen Glucocorticoide gehört, welche sehr häufig zur Behandlung der akuten und chronischen GvHD eingesetzt werden, fördert der chemische Hsp90

Inhibitor 17-DMAG nicht die Entstehung von proinflammatorischen Th17 Zellen, welche das „multi-drug resistance protein 1“ (MDR1) exprimieren. Dieses MDR1 ist eine ATP-abhängige Effluxpumpe, welche eine wichtige Rolle bei der Bioverfügbarkeit vieler Medikamente und Xenobiotika spielt. Zusammenfassend deuten die Ergebnisse dieser Arbeit darauf hin, dass der Gehalt an proinflammatorischen S100 Proteinen im Serum, Stuhl und Darmgewebe von Patienten mit GvHD erhöht ist und somit die Monozyten-vermittelte Th17 Induktion während der GvHD fördern könnte. Eine spezifische Hemmung von Hsp90 könnte die Induktion von entzündungsfördernden Th17 Zellen verhindern. Daher könnte Hsp90 einen entscheidenden Angriffspunkt bei der Behandlung einer GvHD darstellen.

2 Introduction

2.1 Allogeneic hematopoietic stem cell transplantation

Allogeneic hematopoietic cell transplantation (HCT) is an effective treatment for patients with hematologic malignancies, aplastic anemia, and congenital immunodeficiency disorders.[1,2] The first successful allogeneic hematopoietic stem cell transplantation was done in 1968 using bone marrow as source of hematopoietic stem cells.[3] In the following years, bone marrow was used as source of stem cells for transplantation. In the 1960s, experiments have shown that peripheral blood contains a small number of stem cells[4,5], which can be enriched by treatment with chemotherapeutic drugs and hematopoietic growth factors like the granulocyte colony-stimulating factor prior to stem cell infusion.[6-8] Thus, mobilized peripheral blood stem cells became an alternative stem cell source for HCT. In 1978, cord blood was found to be a rich source of stem cells and was therefore used for allogeneic HCT.[9,10]

2.1.1 Types of allogeneic hematopoietic stem cell transplantation

The ability to perform an allogeneic HCT depends on the availability of a suitable donor. The best donor for HCT is a HLA-matched sibling or unrelated donor.[11] However, the presence of these donors is limited as only approximately 30% of the patients requiring transplantation have a HLA-matched sibling.[12] Additionally, the probability of identifying a HLA-matched unrelated donor in a worldwide donor registries is dependent on the diversity of HLA antigens within a population and on the patient`s race.[13] Furthermore, the search for a HLA-matched unrelated donor is time-consuming and the time from the initiation of the search for a donor to transplantation is four month or longer.[14] In this time period, the patient`s disease stage often deteriorates seriously so that allogeneic HCT will no longer be a therapeutic option.[15] Therefore, alternative donors such as a partially HLA-mismatched unrelated donor, a three-loci mismatched haploidentical family donor or an umbilical cord blood stem cell product are increasingly used as sources of

hematopoietic stem cells.[11,15] In the case of transplantation of stem cells from unrelated donors, HLA-matching at HLA-A, -B, -C and -DRB1 between donors and recipients is associated with best survival.[16] A single mismatch for HLA-A or -B or HLA-DR, determined by serologic typing, increases the risk of acute graft-versus-host disease (GvHD) and decreases overall survival.[17-19] Umbilical cord blood stem cell products are HLA mismatched at 1 to 6 antigens or alleles and the minimal number of T cells in the umbilical cord blood product allows its use across HLA barriers. However, using umbilical cord blood as stem cell source entail several disadvantages like the small stem cell number in the product which limits the stem cell dose in adults and often requires the use of a second umbilical cord blood product.[11] Additionally, HLA-mismatched cord blood transplants often induce a high transplant-related mortality.[20] Furthermore, umbilical cord blood products are usually not immediately available in comparison to haploidentical stem cell sources.[15] These haploidentical transplants have the advantage of speed as relatives are usually easy to contact for stem cell collection.[11] These transplants from parents, children, or other family members are matched for one of the patient`s two HLA haplotypes and are well tolerated.[21] Another advantage of haploidentical stem cell transplants over umbilical cord blood products is the large numbers of haploidentical stem cells can be collected from the donor by repeated collections and these large stem cell numbers allow the engraftment across the HLA-barrier even after reduced conditioning regimens.[22,23] Furthermore, haploidentical transplantation is associated with a low transplant-related toxicity and an acceptable rate of rejection.[22]

2.1.2 Conditioning regimens before allogeneic hematopoietic stem cell transplantation

The conditioning or preparative regimen to the recipient is essential for the success of a HCT. Effective conditioning protocols should provide sufficient immunoablation to prevent graft rejection, eliminate malignant cells, minimise the risk of GvHD development without reducing engraftment or graft-versus-tumour effects and to minimise tissue toxicity.[24,25] The selection of a conditioning regimen for any given patient depends on disease-related factors including the diagnosis or remission status and also patient-related factors such as age and availability of a stem cell

donor.[25] The intensity of conditioning regimens can be classified in myeloablative and reduced intensity conditioning as described by Bacigalupo et al.[26] High-dose myeloablative conditioning (MAC) consists of typically one or more alkylating agents and may contain whole-body irradiation.[24,25] The increase of the intensity of host conditioning results in the reduction of immunocompetent cells in the recipient and permits stem cell transplantation of even unrelated, mismatched donors.[27] However, high-dose conditioning also leads to increased tissue toxicity.[28] Reduced intensity conditioning (RIC) uses a dose of alkylating agents that is reduced by approximately 30%. This conditioning regimen incorporates more directed immunosuppressive agents including the purine analogue fludarabine to provide anti-tumour activity and to inhibit T-cell proliferation and mixed lymphocyte reaction.[29-31] RIC regimens may also include the use of total lymphoid irradiation and the application of T-cell-depleting antibodies.[32,33] Therefore, RIC regimen offers the possibility to transplant older patients with reduced toxicity and side effects and without a compromise in overall survival in comparison to high-dose conditioning regimen.[34,35]

2.2 Graft-versus-host disease

2.2.1 Pathophysiology of acute GvHD

The pathophysiology of acute GvHD can be divided into three phases (Figure 1).[36] In the first phase, conditioning regimen induces the damage to the intestinal mucosa and liver resulting in the activation of host cells and the release of proinflammatory cytokines such as TNFα and IL-1β and danger signals such as adenosine-5`-triphosphate (ATP), nicotine adenine dinucleotide and extracellular matrix proteins such as biglycan that promote the expression of adhesion molecules, MHC antigens and costimulatory molecules on host antigen presenting cells (APCs).[37-41] This activation and maturation of APCs enhances their recognition by donor T cells.[42-45] Damage of the gastrointestinal tract from the conditioning allows the translocation of lipopolysaccharide (LPS) which can activate innate immunity through toll-like receptors (TLRs) promoting the cytokine cascade.[37,38] In the second phase, donor T cell activation is induced by recipient antigens presented by host APCs and is intensified by donor APCs.[46,47] This is mediated by HLA proteins which are encoded by the MHC on chromosome 6. MHC compatibility determines the risk of developing

an acute GvHD and the frequency of an acute GvHD can be correlated with the mismatch at HLA-A, -B, -C, and -DRB1.[48] However, despite full 8 of 8 or even 12 of 12 match, 40% of stem cell recipients still develop an acute GvHD due to mismatches of minor histocompatibility antigens between HLA-identical donors and recipients.[36,49] The minor histocompatibility antigens are peptides derived from genetically polymorphic genes, whose difference between the donor and stem cell recipient results in donor T-lymphocyte-mediated immune responses towards the recipient.[50] In addition to the interaction between the T cell receptor and MHC molecules, activation of T cells requires a second costimulatory signal such as interaction between CD28 (present on the T cell) and CD80 or CD86 (present on APCs). The absence of these costimulatory signals leads to T cell anergy. Thus, blockade of these costimulatory interactions can prevent acute GvHD.[51] The third phase which also called the effector phase is a complex cascade of both cellular mediators such as cytotoxic T lymphocytes and natural killer cells and soluble inflammatory mediators such as TNFα, IFNγ and IL-1 leading to target tissue destruction.[24,52] In detail, activated T cells proliferate and differentiate into naïve, effector, memory, regulatory T cells, Th1, Th2, Th17 cells, and other subsets.[53-56] These activated T cells migrate from secondary lymphoid organs to target tissues (skin, liver, gut and lung) through chemokine-receptor, selectin-ligand and integrin-ligand interactions.[57] Once T cells have reached the target organs, T cells induce the destruction of the target tissue mediated by direct cytotoxic activity or by recruitment of other leukocytes.[58] Cytotoxic T cells that use the Fas/Fas ligand pathway for target lysis appear to be important for liver damage whereas cytotoxic T cells that use the perforin/granzyme pathways predominate in skin and intestinal GvHD.[24,59] Microbial products such as LPS that leak through the damaged intestinal mucosa or skin can promote the secretion of proinflammatory cytokines such as TNFα and IL-1 by monocytes. Released TNFα can damage tissue directly by inducing necrosis and apoptosis in the skin and gastrointestinal tract mediated either through TNF receptors or the Fas pathway. IL-1 also induces target cell apoptosis.[38,60,61] Tissue damage then leads to the increased inflammatory signals promoting the disease process by contributing to the cytokine storm.[24]

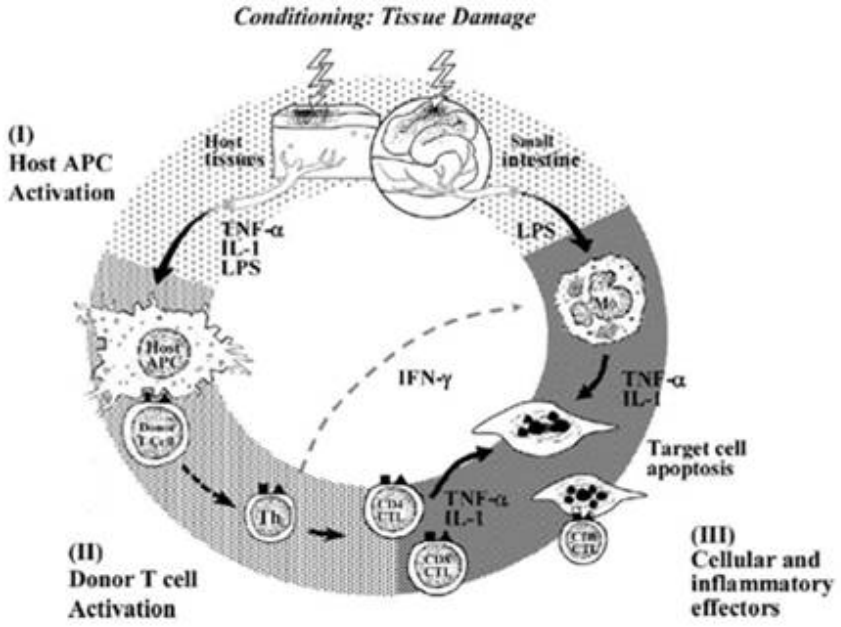

Figure 1: Pathophysiology of acute GvHD

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The pathophysiology of acute GvHD can be divided into three phases. In the first phase, the conditioning regimen leads to the damage of host tissue resulting in the release of proinflammatory cytokines that activate host APCs. In the second phase, host APCs activate mature donor T cells which proliferate, differentiate and produce additional effectors including cytotoxic T cells, natural killer cells, TNFα and IL-1 that mediate tissue damage. In the third phase, LPS leaks through the damaged intestinal mucosa and triggers the production of additional TNFα. TNFα leads to tissue damage in the skin and gastrointestinal tract.

2.2.2 Pathophysiology of chronic GvHD

The immune mechanisms leading to the development of a chronic GvHD are still not completely understood. However, it is known that a chronic GvHD develops due to a complex pathology involving donor B cells and T cells as well as other cells. Regarding B cells, it is known that patients with chronic GvHD have circulating antibodies that can react with the cells from the recipient.[62,63] It has been found that two classes of recipient antibodies are associated with chronic GvHD. The first class includes antibodies specific for antigens in the recipient that are not present in the donor. Examples for this class of alloantibodies are antibodies that are directed against Y-chromosome-encoded (HY) proteins (DBY, UTY, ZFY, RPS4Y, and EIF1AY). Alloantibodies specific for HY could be detected in more than 80% of male

patients with chronic GvHD who had female stem cell donors whereas anti-HY antibodies very seldom develop in male recipients who have male donors.[64-66] Additionally, the presence of HY antibodies seems to predict the subsequent development of chronic GvHD and is associated with maintenance of disease remission.[64] The second class of antibodies that is frequently present in patients with chronic GvHD includes antibodies that are directed against nonpolymorphic autoantigens such as the platelet-derived growth factor receptor (PDGFR). It has been demonstrated that higher levels of stimulatory autoantibodies directed against PDGFR could be detected in the serum of patients with extensive chronic GvHD with skin involvement and/or lung fibrosis in comparison to patients without chronic GvHD. These antibodies are known to induce tyrosine phosphorylation and accumulation of reactive oxygen species and stimulate type 1 collagen gene expression through the Ha-Ras-ERK1/2-ROS signalling pathway. Therefore, theses autoantibodies might play a causal role in the pathogenesis of chronic GvHD, ultimately leading to fibroblast activation.[67] In addition to antibody production, B cells contribute to the immune response by antibody-independent mechanisms including antigen presentation, production of cytokines and chemokines, as well as by acting as immunoregulatory cells.[63] It has been reported that low total B cell counts with high infection rate are associated with chronic GvHD.[68,69] Furthermore, it is known that chronic GvHD is associated with perturbed B-cell homeostasis. Patients with chronic GvHD have reduced levels of naïve B cells and high numbers of activated memory B cells.[70-73] Furthermore, increased levels of B cell activation factor (BAFF) can be correlated with the development and severity of GvHD. Thus, high levels of BAFF in the presence of low numbers of naïve B cells are supposed to foster the survival of activated alloreactive and autoreactive B cells.[73-75] Therefore, it was a logical step to introduce the treatment with rituximab in chronic GvHD which is a chimeric monoclonal antibody directed against CD20 expressed on the surface of B cells.[75] In addition to B cells, donor T cells play an important role in the immune pathology of chronic GvHD. A recent study has demonstrated that immune responses occurring in patients with chronic lichenoid GvHD show a mixed Th1/Th17 signature that is accompanied by upregulated Th1/Th17 cytokine/chemokine transcripts as well as increased numbers of IFNγ and IL-17 producing $CD8^+$ T cells.[76,77] Furthermore, patients with active chronic GvHD have a lower frequency of regulatory T cells

compared to patients without chronic GvHD and healthy individuals.[78] Analysis of reconstitution of regulatory T cells and $CD4^+$ conventional T cells in patients who underwent allogeneic HCT after MAC showed that thymic generation of naïve regulatory T cells is affected and that reconstituting regulatory T cells have mainly an activated/memory phenotype. In response to $CD4^+$ lymphopenia after stem cell transplantation, regulatory T cells exhibit higher proliferation rates compared to conventional T cells, but regulatory T cells undergoing homeostatic proliferation are more susceptible to Fas-mediated apoptosis.[79]

2.2.3 Epidemiology of GvHD

Despite prophylactic treatments with immunosuppressive agents, approximately 50% of transplantation recipients develop a GvHD.[80] Epidemiological investigations have shown that despite GvHD prophylaxis, matched sibling transplants have a 40% incidence of an acute GvHD grade II-IV and 50% risk of extensive chronic GvHD.[81] The risk is increased for matched unrelated donor transplants. The incidence for acute GvHD grade II-IV and extensive chronic GvHD is 75% and 55%, respectively.[82] Comparing the GvHD risks in patients receiving stem cells from matched or mismatched donors, the cumulative incidences of acute grades II-IV, grades III-IV and chronic GvHD in recipients of matched and mismatched stem cell transplants were 32 versus 40%, 11 versus 16% and 56 versus 55%. The work of another group could demonstrate that blood and marrow transplantation can achieve comparable outcomes with HLA-identical sibling and HLA-haploidentical transplantation as the cumulative incidences of grades II to IV acute GvHD in the HLA-matched and HLA-haploidentical cohorts were 32% versus 40%, respectively.[83] Other studies have shown that umbilical cord blood transplants are associated with a lower incidence of GvHD than marrow or mobilized peripheral blood stem cell transplants.[84,85]

2.2.4 Clinical features of GvHD

Acute GvHD initially affects the skin (81% of patients with GvHD) followed by gastrointestinal tract (54%) and liver (50%).[86] Lesions of the skin are usually the first manifestations and occur during white cell engraftment. Affected patients typically

have a maculopapular rash starting around neck and shoulders.[36] Severe forms of acute skin GvHD include the formation of dermal ulcerating blisters or bullae and toxic epidermal necrolysis.[87] Pathologic findings include dyskeratotic epidermal keratinocytes, lymphocytic exocytosis, perivascular lymphocytic infiltration and apoptosis at the crypts.[88] Gastrointestinal manifestations include abdominal cramping, diarrhoea, ileus, anorexia or nausea.[52] Severity of intestinal GvHD is determined by the volume of diarrhoea.[52,89] Histologic characteristics include apoptotic bodies in the base of crypts, crypt abscesses, loss and flattening of surface epithelium.[90] Acute GvHD that includes liver disease is induced by the damage to bile canaliculi leading to hyperbilirubinemia and increased alkaline phosphatase.[91] The severity of the disease is determined by measurement of serum bilirubin.[89] Histologic features of bile damage include the bile duct destruction, epithelial cell dropout and lymphocytic infiltration of bile ducts.[92]

2.2.5 Grading of GvHD

Grading of GvHD is based on dermal, gastrointestinal, and hepatic involvement and most centres use the Keystone criteria (table 1) for grading of acute GvHD. This grading is based on the extent of skin rash, level of bilirubin and volume of diarrhoea.[61,89] While acute GvHD grade I is defined as skin rash that may not require treatment, grade II disease is symptomatic and acute GvHD grade III and IV is severe with less than 25% survival.[93] Chronic GvHD can be divided into limited and extensive disease (table 2). While limited chronic GvHD is characterized by localised skin involvement and/or hepatic dysfunction, extensive disease includes generalised skin disease, or limited chronic GvHD with other organ involvement or unfavourable liver histology.[93-95]

Table 1 Clinical staging and grading of acute GvHD

Adapted from Glucksberg et al.[96]

Stage	Skin	Liver	Gut
1	Rash on <25% of skin	Billirubin 2-3 mg/dl	Diarrhoea >500 ml/day or persistent nausea with positive biopsy
2	Rash on 25-50% of skin	Billirubin 3-6 mg/dl	Diarrhoea >1000 ml/day
3	Rash on >50% of skin	Billirubin 6-15 mg/dl	Diarrhoea >1500 ml/day
4	Generalised erythroderma with bulla formation	Billirubin >15 mg/dl	severe abdominal pain with or without ileus
Grade			
1	Stage 1-2	None	None
2	Stage 3 or	Stage 1 or	Stage 1
3	-	Stage 2-3 or	Stage 2-4
4	Stage 4 or	Stage 4	-

Table 2 Clinical grading of chronic GvHD

Adapted from Shulman et al.[95]

Limited stage	localized skin involvement and/or hepatic dysfunction
Extensive stage	generalized skin involvement or limited skin involvement or hepatic involvement and any of the following a) Liver histology b) Eye involvement (Schirmer`s test with <5 mm wetting) c) Involvement of minor salivary glands or oral mucosa d) Involvement of any other organ

2.2.6 Prevention of GvHD

Acute GvHD prophylaxis developed during the 1970s used the folate antagonist methotrexate (MTX) due to its ability to delete proliferating donor lymphocytes through inhibition of dihydrofolate reductase and production of thymidylate and purines.[80,97] Initial MTX dosing regimens resulted in incidences of acute GvHD grade III-IV of approximately 25%.[98] Progression in prevention of acute GvHD could be achieved by combining MTX with the calcineurin inhibitors Cyclosporine A (CSA) and Tacrolimus in patients receiving bone marrow transplants from matched siblings.[98] However, these regimens did not induce any improvements in chronic GvHD incidence.[80] Additionally, the administration of these agents had numerous side effects including a delayed engraftment, an increased incidence of mucositis, renal impairment, thrombotic microangiopathy or demyelination.[93,99] Therefore, new drugs were introduced into clinical practice for a better control of GvHD. Post-transplant cyclophosphamide is another method of eliminating rapidly dividing T cells that has shown promising outcomes in recent clinical trials.[100,101] The inosine monophosphate dehydrogenase inhibitor mycophenolate mofetil (MMF) inhibits proliferation of lymphocytes via its metabolite mycophenolic acid and is synergistic with calcineurin inhibitors in preventing GvHD.[80] Rapamycin (Sirolimus™) binds to the protein mTOR

required for G1 to S phase transition and induces the prevention of cytokine-driven T-cell proliferation by causing cell cycle arrest.[93,102-104] In addition to effector T-cell inhibition, Rapamycin preserves regulatory T cells after stem cell transplantation, thereby adding to GvHD control.[80] Given the central role of T cells in GvHD, T cell depletion mediated by monoclonal antibodies has been explored as a preventative strategy of GvHD.[80] Randomized studies have shown that T cell depletion successfully decreases the risk of GvHD. Patients receiving anti-thymocyte globulin (ATG) showed significant reduction of grade II-IV and grade III-IV acute GvHD from 51%-33% and from 24.5%-11.7%, respectively. ATG recipients had also a reduced 3-year incidence of extensive chronic GvHD.[105]

2.2.7 Treatment of GvHD

Glucocorticoids are used as standard treatment of grade II-IV acute GvHD.[106] Glucocorticoids are potent inhibitors of the transcription factor NF-κB that activates many immunoregulatory genes in response to proinflammatory stimuli resulting in lymphocyte apoptosis, inhibition of the synthesis of lymphokines, and cell surface antigens required for immune functions.[107] However, the application of glucocorticoids has severe side effects including hyperglycemia, psychosis, osteoporosis or avascular necrosis of bone.[106] Additionally, only about half of patients respond to glucocorticoid treatment.[108] Besides ATG, Tacrolimus, Rapamycin and MMF (see 2.2.6), specific monoclonal antibodies are essential for GvHD therapy.[106] Alemtuzumab is a humanized antibody that binds to CD52 expressed on normal and malignant T-cells, B-cells and monocytes and results in cell death in the presence or absence of complement. Basiliximab is a chimeric antibody which binds to CD25 that is upregulated on activated T cells and induces the inhibition of T cell proliferation.[93] The murine monoclonal antibody Muromonab binds to CD3 expressed on T cells resulting in the silencing of T cell allo-reactivity by inducing apoptosis.[109] Additionally, Toclizumab, a humanized monoclonal antibody directed against the proinflammatory cytokine IL-6 shows promising effects in the treatment of refractory GvHD.[110] The chimeric monoclonal antibody Infliximab is directed against TNFα and blocks the interaction of the cytokine with the corresponding receptor causing the lysis of cells that produce TNFα. A study investigating the use of Infliximab to treat GvHD has

demonstrated that the antibody is well tolerated and active for the treatment of steroid-resistant acute GvHD.[111] Furthermore, many novel approaches have shown promising outcomes concerning GvHD incidence. Adoptive transfer of *ex vivo* expanded natural regulatory T cells has shown safety and decreased rates of GvHD compared to the controls.[112] Extracorporeal photophoresis (ECP) that involves the *ex vivo* incubation of patient leukocytes with 8-methyoxypsoralen and ultraviolet A (UVA) irradiation and the reinfusion into the patient resulting in immunomodulatory effects including lymphocyte apoptosis, increasing regulatory T cell production and a shift from a Th1 to Th2 phenotype.[113] Another strategy of GvHD treatment is the infusion of multipotent mesenchymal stem/stromal cells (MSCs) that have the potential of self-renewal and multi-lineage differentiation.[114] MSCs exert immunosuppressive effects on lymphocytes and APCs and have been used for GvHD treatment with promising response rates.[115,116]

2.3 S100 proteins

2.3.1 Protein structure of S100 proteins

To date, the S100 protein family comprises more than 20 members and represents the largest subgroup within the Ca^{2+}-binding EF-hand superfamily and are only present in vertebrates.[117,118] EF hand motifs consist of two α-helices flanking a central calcium-biding loop, resulting in the characteristic helix-loop-helix motif.[118] All S100 proteins have the same key structural motifs although their sequence homology does not exceed 65%.[119] Monomeric forms have a molecular weight between 10 and 13 kDa and two calcium-binding EF hands with different affinities for calcium connected by a central hinge region.

2.3.2 Characterization of phagocyte-specific S100 proteins

Three phagocyte-specific S100 proteins represent the group of calgranulins and express constitutively the genes S100A8 (also known as myeloid-related protein 8 [Mrp8], calgranulin A), S100A9 (Mrp14, calgranulin B) and S100A12 (calgranulin C). The expression of these S100 proteins is restricted to phagocytic myeloid cells, in particular granulocytes and monocytes.[120-122] Human S100A8 and S100A9 assemble

to heterodimers.[123] These heterocomplexes are also referred to as calprotectin.[124] S100A8 and S100A9 are the most abundant proteins in neutrophilic granulocytes representing approximately 40% of the soluble cytosolic protein content and majorly contribute to calcium-binding capacity in these cells.[125] S100A12 is expressed almost exclusively by granulocytes and comprises approximately 5% of cytosolic protein content in granulocytes.[126] No S100 expression can be detected in B or T cells pointing towards a role of these proteins in innate rather than adaptive immune response.[127]

2.3.3 Functions of phagocyte-specific S100 proteins

2.3.3.1 Intracellular functions

S100A8/S100A9 complexes interact with components of the cytoskeleton in a calcium-dependent manner.[123] Elevation of intracellular calcium concentrations induces interactions of S100A8/S100A9 complexes and S100A8/S100A9 tetramers promote tubulin polymerization and bundle microtubules leading to the stabilization of tubulin filaments.[128] Thereby, S100A9 represents the regulatory subunit in the S100A8/S100A9 heterocomplexes. S100A9 is phosphorylated by p38 mitogen-activated protein kinase (MAPK) resulting in the inhibition of S100A8/S100A9-induced tubulin polymerization. Phosphorylation of S100A9 is antagonistically regulated by binding of S100A8 and calcium.[128,129] Thus, S100A8/S100A9 heterocomplexes are critically involved in the tubulin-dependent cytoskeletal rearrangement and cell migration after activation of phagocytes.

2.3.3.2 Release from phagocytes

S100A8, A9 and A12 lack the leader sequence required for secretion via the endoplasmatic reticulum and Golgi complex.[120] There is evidence that these S100 proteins are released by activated cells via active non-classical secretion and by necrotic cells via passive release.[128,130] Secretion of S100 proteins by phagocytes is an energy-dependent process requiring the activation of protein kinase C in combination with a second calcium-dependent signal and interactions with microtubules.[128,130]

2.3.3.3 Extracellular functions

Secreted S100A8/S100A9 heterocomplexes can activate microvascular endothelium by interacting with heparan sulfate proteoglycans and carboxylated glycans expressed by endothelial cells.[131,132] Thus, a proinflammatory and thrombogenic response is induced in endothelial cells characterized by the induction of proinflammatory cytokines and adhesion molecules resulting in a loss of cell-cell contacts and an increased permeability of endothelial monolayers is induced.[133,134] Additionally, the heterodimer S100A8/S100A9 and the homodimer S100A12 promote the expression and affinity of the integrin receptor CD11b/CD18 (Mac-1) on neutrophil granulocytes resulting in the adhesion of these cells to the endothelium.[135] Thus, the release of S100A8/S100A9 and/or S100A12 at sites of inflammation results in the interaction of primed phagocytes with endothelial cells facilitating the further recruitment of even more leukocytes which promote the inflammatory process.[130,135,136] Furthermore, S100A8, S100A9 and S100A12 have antimicrobial properties providing evidence that these proteins participate in unspecific host defense mechanisms.[124] Thus, S100 proteins have a tissue specific role in intracellular homeostasis, whereas they become proinflammatory mediators in the extracellular space.[122]

2.3.3.4 Function as damage associated molecular patterns (DAMP)

The innate immune system can be activated by microbial products which are referred to as pathogen associated molecular patterns (PAMPs) such as LPS or flagellin. These exogenous ligands bind to pattern recognition receptors on cells of the innate immune system leading to the activation of host defense mechanisms. Additionally, endogenous molecules released in the context of tissue injury and inflammation also initiate innate immune responses such as damage associated molecular patterns (DAMPs).[122,137] The calcium-binding proteins S100A8, S100A9 and S100A12 belong to the group of DAMPs and are released by activated or damaged phagocytes under conditions of cell stress during infections and autoimmune diseases.[120,138,139] The heterodimer S100A8/S100A9 and the homodimer S100A12 are endogenous ligands of the toll-like receptor 4 (TLR4) expressed on phagocytes and induce the translocation of myeloid differentiation primary response protein 88 (MyD88) from the cytosol to the receptor complex at the plasma membrane and hyperphosphorylation

of interleukin-1 receptor associated kinase-1 (IRAK-1) resulting in the activation of the transcription factor NF-κB and in the expression and release of proinflammatory cytokines including TNFα, IL-1β, IL-12, IL-8 and IL-6.[138,140] S100A12 is also a ligand for the receptor for advanced glycation end products (RAGE) which is expressed on macrophages, endothelium and lymphocytes.[141,142] Binding of S100A12 to RAGE results in the activation of NF-κB, an increased expression of vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1) on the surface of endothalial cells and the attraction of leukocytes.[120,142]

2.3.3.5 Role of S100 proteins in inflammatory diseases

Several studies have shown that proinflammatory S100 proteins contribute to the pathogenesis of several inflammatory diseases and therefore represent promising novel therapeutic targets. In patients suffering from arthritis, activated phagocytes expressing S100 proteins infiltrate inflammatory lesions in the synovium.[143,144] Concentrations of the heterodimer S100A8/S100A9 are increased in the serum and synovial fluid reflecting the release of these proteins from activated phagocytes within the synovium and synovial fluid.[120,145,146] Serum levels of S100A8/S100A9 correlate better with disease activity and joint destruction in rheumatoid arthritis and psoriatic arthritis compared with classical markers of inflammation such as erythrocyte sedimentation rate and C-reactive protein (CRP).[147-149] Furthermore, histopathological analysis demonstrated that the infiltration of neutrophils and monocytes and the general activation of cutaneous epithelium reflected by the expression of proinflammatory S100 proteins appear to be typical for the transient rash in systemic-onset idiopathic juvenile arthritis (SOJIA).[150] Additionally, high local S100A12 expression levels are detectable in airway diseases like acute lung injury, respiratory distress syndrome and cystic fibrosis.[151,152] Other studies have shown that fecal S100A12 levels from patients with inflammatory bowel disease are elevated and correlate with the histology score.[139,151,153] Previous studies have demonstrated that levels of fecal S100A8/S100A9 are elevated in patients with severe intestinal GvHD in comparison to patients with acute GvHD without gastrointestinal symptoms and also patients with infective enteritis or diarrhoea after HCT indicating that calprotectin might be a new non-invasive marker of severe gastrointestinal GvHD.[154,155]

2.4 Heat shock proteins

Heat shock proteins are the most abundant and ubiquitous soluble intracellular proteins that are present in the cytosol of prokaryotes, and the cytosol, nuclei, endoplasmatic reticulum, mitochondria and chloroplasts of eukaryotes.[156] Genes encoding heat shock proteins were initially identified in 1962 in *Drosophila melanogaster* larvae that were advertently exposed to high temperatures resulting in a characteristic puffing pattern and gene activation profile in the polytene chromosomes of salivary glands.[157] The first products of these genes were identified and characterized as heat shock proteins in 1974.[158] Further investigations have demonstrated that the expression of heat shock proteins in mammalian cells is not only induced by hyperthermia but can also be induced by physical, biochemical or environmental influences including the exposure to heavy metals ultraviolet and gamma irradiation, amino acid analogues or cytotoxic drugs, glucose deprivation and virus infection.[159] Heat shock proteins normally constitute up to 5% of the total intracellular proteins, but their levels can rise to 15% or more under stress exposure.[160] While the expression of many heat shock proteins is induced by stress, some heat shock proteins are expressed constitutively.[161] Heat shock proteins are divided into different families according to their molecular weight (i.e. hsp100, hsp90, hsp70, hsp60, hsp40 and small hsp).[159] Since their discovery, an increasing number of diverse functions have been attributed to heat shock proteins.[160] Major functions of heat shock proteins are the chaperoning of misfolded or newly synthesized polypeptides, the assembly, stabilization and intracellular translocation of proteins, the protection of cells from proteotoxic stress, and the processing of immunogenic agents.[161-164]

2.5 Immunological properties of heat shock proteins

Heat shock proteins have essential housekeeping and cytoprotective functions and are also involved in the innate and adaptive immune response.[165-168] Heat shock proteins such as Hsp70, Hsp90, Gp96, Hsp110, Grp170 and Calreticulin associate with a broad range of peptides generated within the cells leading to the formation of HSP-peptide complexes that interact with APCs and promote the adaptive immune

response (Figure 2).[169-171] In detail, HSP-peptide complexes interact with CD91 expressed on macrophages and dendritic cells leading to the internalisation of these complexes into a non-acidic endosomal compartment, followed by the delivery of the complex or the peptide alone to the cytosol.[172-174] The peptides are processed by proteasomes, transported to the endoplasmatic reticulum and loaded onto MHC class I molecules which are presented to $CD8^+$ T cells.[172] Alternatively, peptides can be loaded onto MHC class I molecules in the endosome without transfer through the cytosol and endoplasmatic reticulum.[175] However, it has also been demonstrated that a small proportion of HSP-peptide complexes enters an acidic compartment after internalization via CD91 resulting in the interaction of peptides with MHC class II molecules that stimulate $CD4^+$ T cells.[176] Beside the involvement of heat shock proteins in the adaptive immune response, Gp96, Hsp70 and Hsp60 have been reported to participate in the innate immune response.[177] The interaction of heat shock proteins with APCs also leads to the induction of several peptide-independent activities, including the maturation of dendritic cells, the activation of the NF-κB pathway in macrophages and dendritic cells resulting in the secretion of inflammatory cytokines such as TNFα, IL-1β, IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF).[177-180] Additionally, heat shock proteins induce the production of nitric oxide by macrophages and dendritic cells and the secretion of chemokines such as monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2) and RANTES (regulated upon activation, normal T cells expressed and secreted) by T cells.[181-183]

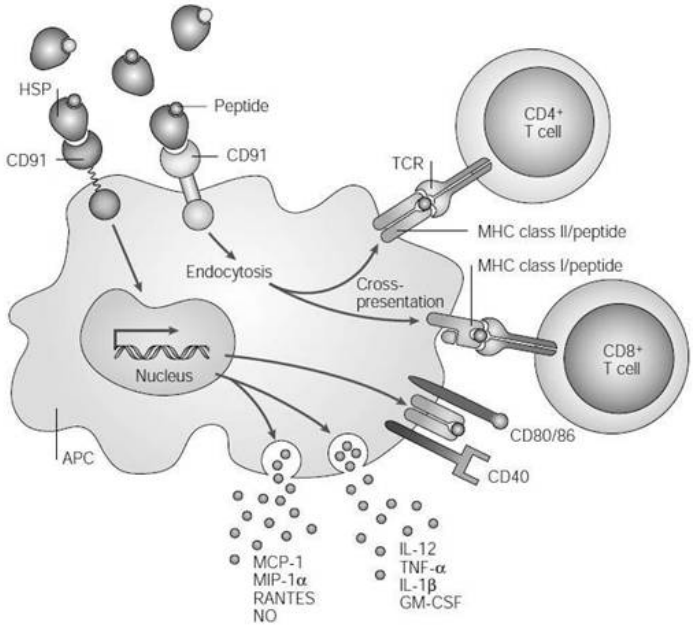

Figure 2: Role of heat shock proteins in the innate and adaptive immunity

Adapted from Srivastava[160]. Copyright by *Nature Publishing Group*

Heat shock proteins associate with a broad range of peptides generated within the cells leading to the formation of HSP-peptide complexes that interact with APCs and are internalized by receptor-mediated endocytosis. The peptides are processed loaded onto MHC class I and MHC class II molecules that stimulate $CD8^+$ and $CD4^+$ T cell responses (adaptive immunity). The interaction of heat shock proteins with APCs also induces peptide-independent activities, including the maturation and activation of APCs resulting in the secretion of inflammatory cytokines such as TNFα, IL-1β, IL-12 and GM-CSF (innate immunity).

2.6 Characterisation of heat shock protein 90

The 90 kDa heat shock protein Hsp90 was initially identified as one of the highly conserved heat shock proteins involved in the stress response.[157,184] Hsp90 is essential for the viability in eukaryotes by functioning as molecular chaperone that contributes to the folding, maintenance of structural integrity and proper regulation of cytosolic proteins.[185] Many of its substrates are proteins that are essential for cell cycle control and signal transduction.[185] Hsp90 acts with a multitude of Hsp90 co-chaperones that modulate its substrate recognition, ATPase cycle and chaperone function resulting in a large conformational flexibility of Hsp90 that allows Hsp90 to assist a wide range of substrates.[185] The chaperone cycle starts with the presentation of newly synthesized or misfolded client proteins to Hsp70 by its activator Hsp40 in

an ATP-dependent manner. The dimeric co-chaperone HOP binds to the Hsp40-Hsp70-client complex to Hsp90, thereby forming the Hsp70-HOP-Hsp90 complex.[186,187] HOP inhibits the ATPase activity and promotes the client transfer of from Hsp70 to Hsp90.[188-190] On ATP binding, Hsp90 forms a mature complex with p23 and other co-chaperones such as Cdc37 and immunophilins catalysing the conformational maturation of the client. Thus, the co-chaperone p23 and the immunophilins displace HOP and Hsp70 leading to the formation of the mature complex.[191] Over the past years, over 200 client proteins of Hsp90 have been identified, covering almost all cellular processes such as cell growth, signal transduction, cell-cycle control, transcriptional regulation and apoptosis.[192-198] For instance, these client proteins include transmembrane tyrosine kinases (Her-2, EGFR), metastable signalling proteins (Akt, Raf-1 and IKK), mutated signalling proteins (p53, v-Src), chimeric signalling proteins (Bcr-Abl), cell cycle regulators (Cdk4, Cdk6) and steroid receptors (androgen, estrogen, and progesterone receptors).[199] Many of these proteins are mutated and/or overexpressed in cancer cells.[187,200] Vertebrates express the two isoforms of cytosolic Hsp90 which are referred to as Hsp90α and Hsp90β.[201,202] In comparison to Hsp90α, Hsp90β is expressed constitutively to a higher level in most tissues and is essential for long-term cellular adaption, differentiation and evolution. The other isoform, Hsp90α is stress-inducible and may therefore be a more cytoprotective form of Hsp90.[198] Additionally, the expression of Hsp90α is upregulated in many cancers, as well as in the extracellular environment, where the induced effects on the metalloproteinase-2 activity may be important in cancer cell metastasis.[202-204] Several studies have demonstrated that Hsp90 is involved in multiple processes related to inflammation demonstrating that the inhibition of Hsp90 might be of benefit for treatment of inflammatory and autoimmune diseases. The severity and progression of these inflammatory and autoimmune disorders as well as cancer is associated with the activation of the NF-κB pathway.[205,206] A variety of stimuli including cytokines, chemokines, bacterial and viral products, UV radiation and free radicals activate the NF-κB pathway by inducing the phosphorylation of IκB proteins. The increased phosphorylation of IκB by the IκB kinase complex (IKK) results in the ubiquitination and proteasomal degradation of IκB. The NF-κB proteins are, thus, liberated from IκB and translocate to the nucleus where they bind to the promoter regions of NF-κB-

responsive genes resulting in increased gene expression.[206,207] Natural product inhibitors of Hsp90 induce the blockade of mitogen-activated protein (MAP) kinases and the degradation of the Hsp90 client IKK leading to the loss of cytokine production in macrophages and other cell types.[208-214] Additionally, the interleukin-1 receptor-associated kinase is also a client protein of Hsp90, and consequently, inhibition of Hsp90 diminishes innate immune responses via toll-like receptor signalling.[215] The relevance of Hsp90 inhibition *in vitro* could further be supported by preclinical models of rheumatoid arthritis, experimental allergic encephalomyelitis, uveitis and sepsis suggesting Hsp90 inhibitors as novel anti-inflammatory drugs.[216-220] Another study demonstrated that the blockade Hsp90 can specifically eliminate alloreactive T cells and might therefore be a potential approach to prevent and treat GvHD in hematopoietic stem cell transplantation recipients without impairing pathogen- and disease-specific T cell immunity.[221]

2.7 Hsp90 inhibitors for cancer treatment

2.7.1 Hsp90 inhibitors targeting the ATP binding site

2.7.1.1 Benzochinone ansamycins

The prototypical class of Hsp90 inhibitors is represented by the natural product ansamycin, including geldanamycin and its derivatives 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG).[222] These benzochinone ansamycins are competitive inhibitors of the ATP binding site of Hsp90 and their binding to the N-terminal ATP binding pocket restrains Hsp90 in the ADP-bound conformation and prevents binding of the client protein.[223-225] Thus, the client proteins are ubiquitinated and degraded by the proteasome.[226] Geldanamycin has shown potent anti-cancer activity in preclinical studies, but the high hepatoxicity observed in animal models diminishes its clinical potential.[227] The derivatives of geldanamycin 17-AAG and 17-DMAG with better toxicological properties have been synthesised and have progressed to phase I and phase II clinical trials and have demonstrated anti-cancer activity in human epidermal growth factor receptor 2 (HER2)–positive, trastuzumab-refractory breast cancer, melanoma and prostate cancer.[228-230] Although 17-AAG is a

potent inhibitor of Hsp90, several pharmacological deficiencies have been described including poor water solubility and complex organic formulations, with patient safety concerns.[231,232] Despite their clinical use, hepatotoxicity still remains a problem with the application of both 17-AAG and 17-DMAG.[228,230] The toxicity of quinones is due to their ability to redox cycle and/or arylate cellular nucleophiles. The redox cycling results in the production of reactive oxygen species and reaction with thiols leading to the formation of glutathione conjugates and adducts with cellular proteins.[233,234] The use of benzochinone ansamycins might be associated with additional drawbacks as their binding to Hsp90 does not only inhibit binding of ATP but also results in the induction of a stress response leading to the release, activation, nuclear localization and trimerization of heat shock factor-1 (HSF-1).[235] This transcription factor induces the upregulation of Hsp70 expression resulting in the inhibition of apoptosis signalling and a reduced Hsp90-targeted drug efficacy.[235-237] Thus, the clinical efficacy of ansamycins might be enhanced by a combination therapy using molecules that abrogate Hsp70 induction.[238] Additionally, ansamycins are substrates of the multidrug resistance protein 1 (MDR1; P-glycoprotein 1) (see 2.9) that is an ATP-dependent efflux pump with broad substrate specificity leading to decreased drug accumulation.[239]

2.7.1.2 Synthetic small molecules

In the recent years, synthetic small molecule inhibitors have been designed in order to achieve more specific targeting of Hsp90 and better pharmacological effects. The first group of these molecules was developed based on the purine scaffold.[240] These purine derivatives (PU-class) bind to the Hsp90-nucleotide binding pocket and have shown selective binding to Hsp90 in tumour cells.[241,242] Furthermore, other studies have demonstrated anticancer activity in multiple animal models.[243,244] 6-Chloro-9-(4-methoxy-3,5-dimethyl-pyridin-2-ylmethyl)-9*H*-purin-2-ylamine (BIIB021), is a synthetic HSP90 inhibitor that showed strong antitumor effects as a single agent and increased the efficacy of radiation in preclinical models.[245,246] Furthermore, it could be demonstrated that BIIB021 is not a substrate of MDR1 and showed potent antitumor activity against a multidrug resistance expression cell line.[247] A phase I study has been completed demonstrating that the drug was well-tolerated at doses that are pharmacodynamically active.[248] A recently carried out phase II clinical study has

revealed that BIIB021 leads to objective responses in patients with gastrointestinal stromal tumours and pharmacodynamics studies confirmed Hsp90 inhibition.[249]
Ganetespib (STA-9090) is a novel small-molecule inhibitor of Hsp90 with a unique triazolone-containing chemical structure that exhibits potent antitumor effects in a broad range of malignancies both *in vitro* and *in vivo* which is due to the rapid degradation of Hsp90 client proteins. Ganetespib has also shown strong antitumor activity in cell lines that confer drug resistance to agents currently in use in the clinic. In addition, ganetespib displays high tumour penetration and no evidence of cardiac or liver toxicity indicating a favourable drug safety profile.[222,250]

2.7.2 Hsp90 inhibitors targeting co-chaperone/Hsp90 interactions

Hsp90 requires multiple co-chaperones for its function. Thus, arresting the chaperone cycle by targeting co-chaperone-Hsp90 interactions offers a potential approach to inhibit Hsp90 activity.[187,251,252]

2.7.2.1 Targeting the Cdc37/Hsp90 interaction

The co-chaperone Cdc37 plays an important role in the maturation of several receptor tyrosine kinases that play a critical role in the development and progression in many types of cancer.[251,253] Cdc37 acts as adaptor loading these kinases onto the Hsp90 complex resulting in the maturation of these proteins.[251,254,255] Depletion of Cdc37 in human colon cancer cells diminishes the association of kinase clients with Hsp90 resulting in decreased levels of these clients and reduced cell proliferation.[255] In comparison to benzochinone ansamycins, silencing of Cdc37 does not induce an upregulation of Hsp70 expression.[255]

2.7.2.2 Targeting the Hsp70/Hsp90 interaction

Hsp90 can also be inhibited by targeting of the Hsp70/Hsp90 interaction. The assembly of Hsp70 and Hsp90 is achieved by association with the two tetratricopeptide repeat (TPR) domains (TPR1 and TPR2A) of Hop.[256] A study has previously demonstrated that a designed TPR module, CTPR390+, binds to the Hsp90 C-terminus with higher affinity and specificity compared to TPRA2 leading to

the prevention of formation of the Hsp70/Hsp90 complex. This specific Hsp90 inhibition results in decreased levels of the Hsp90-dependent client protein HER2 and the inhibition of breast cancer cell proliferation.[257] Another advantage of Hsp90 inhibition via targeting of Hsp70/Hsp90 interactions is that this method does not lead to the induction of Hsp70 which is undesirable because its anti-apoptotic function counteracts the effect of Hsp90 inhibition.[256,257]

2.7.3 Post-translational modifications of Hsp90

Post-translational modifications including hyperacetylation, hyperphosphorylation or thiol oxidation can affect co-chaperone association and/or ATP binding and thereby regulate the chaperone function of Hsp90. [258]

2.7.3.1 Hsp90 hyperacetylation

Histone deacetylases (HDACs) are promising targets in drug development for cancer therapy. Several studies have correlated HDACs with the function of the Hsp90 chaperone. HDACs and histone acetyltransferases (HATs) are responsible for the reversible acetylation of lysine residues on Hsp90 and thereby regulate Hsp90 activity.[259] A recent study has demonstrated that knockdown of the HDAC6 by RNA interference enhances the degree of Hsp90 acetylation leading to a reduced binding capacity of ATP and co-chaperones to Hsp90 promoting the degradation of Hsp90 client proteins.

2.7.3.2 Hsp90 thiol oxidation

Tubocapsenolide A is a withanolide-type steroid that inhibits the activity of Hsp90-Hsp70 chaperone complex by a direct thiol oxidation, leading to the destabilization and depletion of Hsp90 client proteins and thus causes cell cycle arrest and apoptosis several human cancer cell lines.[260]

2.7.3.3 Hsp90 phosphorylation

It has been demonstrated that hyperphosphorylation of Hsp90 leads to an apparent decrease in the efficiency of the Hsp90 chaperone system. However, the role of site-

specific phosphorylation in modulating Hsp90 function has not been fully elucidated yet.[261,262]

2.8 CD4$^+$ T cell subtypes

CD4$^+$ T cells are crucial components of an adaptive immune response. Upon antigenic stimulation and cytokine signalling, naïve CD4$^+$ T cells are activated and differentiate into various CD4$^+$ T cell subsets (Figure 3).[263,264]

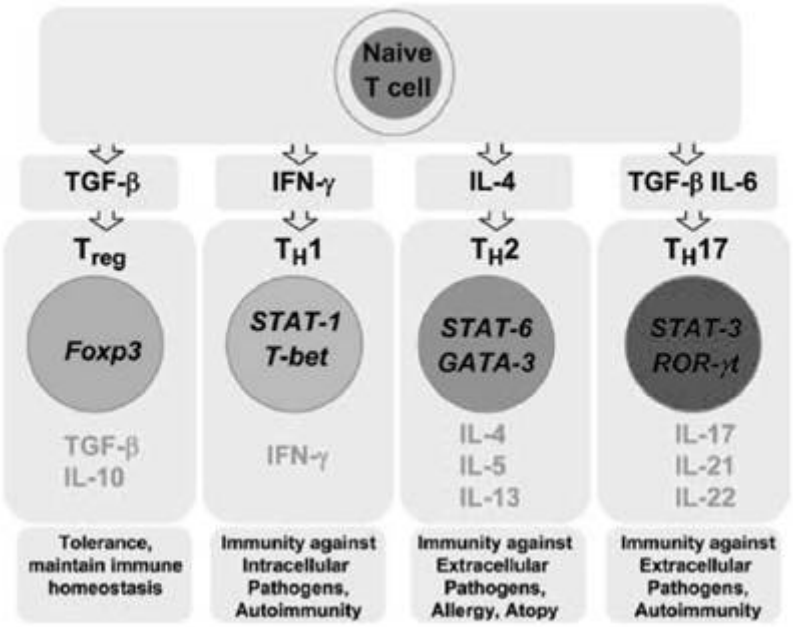

Figure 3: CD4$^+$ T cell subsets

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Antigenic stimulation and specific cytokine signalling induce the differentiation of naïve T cells into various CD4$^+$ T cell subsets including Th1, Th2, Th17 cells and regulatory T cells.[263]

2.8.1 The Th1-Th2 paradigm

The CD4$^+$ T cell subsets were initially classified in 1986 by Mossman and Coffman proposing a Th1-Th2 paradigm of T cells.[265] This hypothesis is based on the observation that CD4$^+$ T cell subsets produce different cytokine pattern and thereby induce different effector functions.[266] Thus, CD4$^+$ T cells were categorized into two main subsets named type 1 helper T cells (Th1 cells) and type 2 helper T cells (Th2 cells) due to their different cytokine production profiles.[265,266] Differentiation of naïve

$CD4^+$ T cells into effector cells is induced by antigenic stimulation and cytokine signalling.[263,264] Signal transducer and activator of transcription (STAT) proteins are the key signalling transcription elements in the differentiation pathway of $CD4^+$ T cells.[263] The cytokines IFNγ and IL-12 induce the activation of STAT4 and STAT1 resulting in the activation of T-bet (T-box expressed in T cells) which is the master transcription factor for Th1 cell differentiation.[267,268] Differentiation of Th2 cells is induced by IL-4 which activates the transcription factors STAT-6 and GATA-binding protein 3 (GATA-3).[269,270] Th1 cells secrete high levels of IFNγ and IL-2 that stimulate macrophages, induce delayed type hypersensitivity and the maturation of cytotoxic T cells, and are thus essential for cell-mediated immunity against intracellular pathogens.[266,268] Additionally, Th1-dominant immune responses have been considered pathologic in organ-specific autoimmune and other chronic inflammatory diseases including Crohn's disease, autoimmune thyroid disease or multiple sclerosis.[268,271] Th2 cells secrete IL-4, IL-5, IL-10 and IL-13 which induce differentiation, activation and the in situ survival of eosinophils, promote the production of high amounts of antibodies, including IgE by B lymphocytes and the growth of mast cells and basophils, and are therefore required for the mediation of humoral immunity to clear extracellular pathogens.[266,271-274] Furthermore, cytokines and chemokines produced by Th2 cells and those produced by other cell types in response to the Th2 specific cytokines or as a reaction to the tissue damage mediated by Th2 cells account for multiple pathophysiological aspects of allergic disorders such as the production of IgE antibodies or the recruitment or activation of mast cells.[274]

2.8.2 Characterisation of Th17 cells

The Th1-Th2 paradigm was subsequently extended to encompass a number of additional $CD4^+$ T cell subsets including Th17 cells and regulatory T cells. In 2006 three publications demonstrated that the immunoregulatory cytokine transforming growth factor β (TGF-β) in combination with IL-6 induce the differentiation of naïve $CD4^+$ T cells into Th17 cells.[275-277] The cytokine IL-23 is required for Th17 cell expansion and maintenance.[277] Moreover, IL-1β and TNFα were found to amplify the Th17 response induced by TGF-β and IL-6.[278] The cytokines contributing to the development of human Th17 cells were controversially discussed since several

studies in 2007 claimed that TGF-β is not required for the differentiation of human Th17 cells.[279,280] However, recent studies demonstrated that low concentrations of the anti-inflammatory cytokine TGF-β in combination with IL-1β and IL-6, IL-21 or IL-23 promote human Th17 differentiation from naïve $CD4^+$ T cells.[281-283] Although the differentiation of human and mouse Th17 cells is not identical, they express similar cytokines, such as IL-17A, IL-17F, IL-22 and IL-21, and cell surface receptors such as IL-23R and the C-C chemokine receptor type 6 (CCR6).[284,285] Several studies identified Th17 cells as an independent lineage of T helper cells because they do not express any Th1 cell- (STAT-1, STAT-4 and T-bet) and Th2 cell-associated transcription factors (STAT-6 and GATA-3).[286,287] IL-6, IL-21 and IL-23 induce the activation of STAT-3, regulating the expression of IL-23R and IL-21 which are both required for amplifying the generation of Th17 cells.[288-290] Full expression of the Th17 phenotype depends on the orphan nuclear receptor, retinoid-related orphan receptor γt (RORγt) which is upregulated in a STAT-3-dependent manner and induces the expression of IL-17A, IL-17F, contributes to the generation of IL-23R and mediates the production of IL-22. Furthermore, high levels of another related nuclear receptor, RORα, was found in Th17 cells. RORα is induced by a combination of TGFβ and IL-6 in a STAT-3-dependent manner and synergizes with RORγt to promote differentiation and function of Th17 cells.[291-294] Other data suggest that Runt-related transcription factor 1 (Runx1) induces RORγt expression by binding to and acting together with RORγt during IL-17 transcription.[295]

Th17 cells secrete the cytokines IL-17A, IL-17F, IL-21 and IL-22. IL-17A and IL-17F can both bind to the IL-17 receptor broadly expressed on a variety of cells such as B cells, T cells, natural killer cells, monocytes, granulocytes, fibroblasts, epithelial cells endothelial cells, stromal cells and osteoblasts.[296-301] This binding of the ligand to its receptor induces the activation of NF-κB and MAPK pathways resulting in the induction of the expression of proinflammatory cytokines including IL-1, IL-6 and TNFα as well as chemokines such as C-X-C motif chemokine (CXCL) 8 and matrix metalloproteinases, thereby leading to the recruitment, activation and migration of neutrophil granulocytes.[298,302-304] The cytokine IL-21 seems to play an autocrine-amplifying role on Th17 response as it is produced by Th17 cells and also acts as an activator of STAT-3 inducing the generation of Th17 cells.[298] Upon binding to the IL-22 receptor complex, IL-22 induces the activation of STAT-3 and, to a lesser extent,

STAT-1 and STAT-5. Additionally, IL-22 leads to the activation of MAPK pathways which plays an important role in complex cellular programs including proliferation, differentiation, development, transformation, and apoptosis.[298,305] Thus, Th17 cells and Th17-asssociated cytokines are potent inducer of tissue inflammation and are known to play an important role in the development of several autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis and inflammatory bowel disorders.[306-312]

Furthermore, it could be demonstrated that Th17 cells show a great degree of context-dependent plasticity.[313] Several groups have reported that a remarkable number of Th17 cells express IFNγ in addition to IL-17A both *in vivo* and *in vitro*. Additionally, it has been shown that both Th17 cells and the IL-17- and IFNγ-producing Th17/Th1 clones express the transcription factors RORγt and the Th1-related transcription factor T-bet. Incubation of Th17 clones with IL-12 results in the production of IFNγ besides IL-17, and this effect is associated with a reduced RORγt and increased T-bet expression.[285,314,315]

2.8.3 Characterisation of regulatory T cells

Regulatory T (Treg) cells are characterized as a T cell subpopulation that modulates the immune system, retains tolerance to self-antigens and eliminates autoimmunity. These Treg cells comprise approximately 5-10% of the mature $CD4^+$ T cells in mice and also in humans, and approximately 1-2% of Treg cells can be detected in the peripheral blood. Treg cells can be characterized by the expression of CD25 (alpha chain of the IL-2 receptor), CTLA-4 (CD152, cytotoxic T-lymphocyte antigen 4), and glucocorticoid-induced tumor-necrosis-factor-related protein (GITR). Furthermore, Treg cells express the transcription factor forkhead box P3 (FoxP3) which is essential for the development and function of Treg cells.[316] The expression of FoxP3 is controlled by DNA-methylation and can be increased by TGF-β, IL-2, or T cell receptor stimulation of T cells.[317] Treg cells can be divided into two main classes: naturally occurring Treg (nTreg) cells and induced or adaptive Treg (iTreg) cells.[316] The nTreg cells develop as a distinct lineage in the thymus, from where they are exported and are responsible for the maintenance of self-tolerance.[318] It is known that nTreg cells derived in the thymus are anergic *in vitro*, but show proliferation at steady state *in vivo*.[319,320] The iTreg cells, however, are not formed in the thymus but

differentiate from mature naïve $CD4^+$ T cells in peripheral lymphoid organs and other tissues upon cellular activation in the presence of TGF-β.[321,322] Both nTreg and iTreg cells are essential for the maintenance of immune self-tolerance and homeostasis by suppressing various effector lymphocytes, especially the $CD4^+$ T cell subsets such as Th1, Th2, Th17 and follicular T helper cells.[316] Thereby, Treg cells produce cytokines including IL-10 and IL-35, sequester cytokines crucial for cell growth such as IL-2, and utilize the perforin-granzyme pathway to kill activated targets or tumour cells.[323-328] It could also be demonstrated that the cell numbers and suppressive activity of circulating Treg cells are significantly reduced in patients with autoimmune disorders such as juvenile idiopathic arthritis, psoriatic arthritis, multiple sclerosis, systemic lupus erythomatosus, autoimmune hepatitis, and type-1 diabetes.[277]

2.8.4 Plasticity in the development of Th17 cells and regulatory T cells

At early differentiation stages, each lineage can be reprogrammed to other directions. In contrast to Th1 and Th2 cells, which have been thought to represent terminal products of their respective developmental programs, Th17 cells and Tregs demonstrate considerable plasticity throughout the entire differentiation process.[329,330] Several studies demonstrated that the induction of the key transcription factors of Th17 cell and Tregs, RORγt and FoxP3, share a dependence on TGFβ-signalling determining Th17 versus Treg lineage specification.[329] It is known that nTreg cells develop during thymic selection through a mechanism that is independent of TGF-β, whereas the extrathymic development of iTreg cells is TGF-β-dependent. Recent data have shown that TGF-β alone induces both FoxP3 and RORγt.[329] The presence of IL-6, produced by dendritic cells that are activated by microbial products, or IL-21, produced by IL-6-stimulated T cells inhibits the induction of FoxP3 or eliminates the suppression that further potentiates the generation of Th17 cells thereby establishing an autocrine loop. While low dose TGF-β synergize with IL-6 and IL-21 to induce Th17 development, higher doses of TGF-β inhibit the expression of IL-23R and increases the expression of FoxP3, favouring the generation of iTreg. Thus, in the absence of proinflammatory signals from the innate immune system, priming of naïve CD4 T cells by antigen in a TGF-β rich environment induces the development of iTregs, whereas activation in an environment where both active TGF-β and IL-6 are available promotes the induction of Th17 cells.[329,331]

2.8.5 Role of T cells in the pathophysiology of GvHD

The pathophysiology of GvHD is complex and not fully understood yet. The role of Th17 cells during GvHD is discussed controversially and still remains unclear. High levels of IFNγ producing T cells detected in the skin of patients with cutaneous acute GvHD after allogeneic stem cell transplantation and increased IFNγ expression in PBMCs gave rise to conclude that GvHD is mediated predominantly by proinflammatory Th1 cells.[76,332] However, it could conversely be shown in mouse models that inhibition of Th1 cytokines results in the exacerbation of acute GvHD.[333,334] Further investigations of T cell subsets involved in the pathomechanism of GvHD have been directed towards Th17 cells as several studies indicated a contribution of Th17 cells to GvHD disorders. In mice it could be demonstrated that infusion of *in vitro*-differentiated Th17 cells induces lethal GvHD with extensive pathologic lesions in the lung and skin.[335] Another study showed that the development of GvHD is significantly delayed in an allogeneic bone marrow transplantation model when murine IL-17$^{-/-}$ CD4^{+} T cells are transferred compared to recipients of wild-type CD4^{+} T cells.[336] In a haploindentical murine transplantation model it could be demonstrated that infusion of CD4^{+} T cells lacking the transcription factor RORγt diminishes the severity and lethality of acute GvHD.[337] In patients receiving HCT the dose of IL-17-producing T cells in allografts was shown to be associated with an increased risk for acute GvHD.[338] Additionally, Th17 cells and IL-21 plasma levels were significantly elevated in patients at the onset of chronic GvHD and correlate with the clinical course.[339] Another study could show increased numbers of Th17 cells in the skin of patients with acute GvHD and active chronic GvHD compared to control samples.[340] Regarding the role of Treg cells during GvHD, several studies could demonstrate in different allogeneic hematopoietic cell transplantation animal models that addition of Treg cells results in the suppression of GvHD.[341-344] Recently, clinical studies have been published reporting that adoptive transfer of Treg cells might prevent GvHD. By infusion of freshly isolated donor Treg cells after myeloablative conditioning and before infusion of a megadose of CD34^{+} cells and conventional CD4^{+} and CD8^{+} T cells, haploidentical transplantation was possible in the absence of any posttransplantation immunosuppression and occurred with a very low rate of acute and chronic GvHD. Furthermore, the adoptive transfer of Treg cells promotes lymphoid reconstitution, improves immunity to opportunistic

pathogens, and does not weaken the graft-versus-leukemia (GvL) effect.[345] Additionally, a second study demonstrated that umbilical cord third-party Treg cells can be expanded *ex vivo* and confirmed that the adoptive transfer of Treg cells can be performed without apparent toxicity and seems to reduce the risk of acute GvHD.[112] A study published recently suggests that the ratio of Th17 to Treg cells (Th17/Treg) might be a novel, sensitive and specific pathologic in situ biomarker of GvHD as low Th17/Treg ratios seem to correlate with severe clinical and pathologic GvHD, apoptosis intensity, and TNFα expression.[346]

2.9 The multi-drug resistance protein type 1

The multi-drug resistance protein type (MDR1), which is also referred to as P-glycoprotein plays an important role in bioavailability and cell-toxicity limitation of a wide range of drugs and xenobiotics.[347] MDR1 is an ATP-dependent efflux pump that belongs to the family of ATP-binding cassette (ABC) transporters. The ATPase activity of MDR1 is stimulated by binding of hydrophobic drug substrates to transmembrane regions resulting in a conformational change that releases the substrate to either the outer leaflet of the membrane or the extracellular space. Thereby, two ATP hydrolysis events are needed to transport one drug molecule. MDR1 is expressed in many human cancers including leukaemias and solid tumours and promote tumour resistance to chemotherapy.[348] In non-malignant cells, MDR1 is expressed on intestinal epithelium, endothelial cells of the blood-brain-barrier, and hepatocytes, where it is known to control the accumulation of xenobiotics and exogenous pharmacologic molecules.[349] Furthermore, MDR1 is also expressed in progenitor cell types and might be involved in the survival and longevity of these cells.[350,351] In a recent study, it could also be demonstrated that glucocorticoids promote the development of a subset of Th17 cells that stably expresses MDR1. These proinflammatory Th17 cells are further characterized by the expression of the chemokine receptors CCR6, CXCR3 and CCR4 on the cell surface. These $MDR1^{+}$Th17 cells are $CCR6^{+}$ and express CXCR3 at high levels and CCR4 at low levels. $MDR1^{+}$ $CCR6^{+}CXCR3^{hi}CCR4^{lo}$ Th17.1 cells differ from the $MDR1^{-}$Th17 cells which are $CCR6^{+}CXCR3^{lo}CCR4^{hi}$ and $MDR1^{-}$Th1 cells which are $CCR6^{-}CXCR3^{hi}CCR4^{lo}$. Proinflammatory $MDR1^{+}$Th17.1 cells produce Th17-associated (IL-

17A, IL-17F, IL-22) as well as Th1-associated cytokines (IFNγ) upon T cell receptor stimulation and do not express any anti-inflammatory molecules like IL-10. *In vivo*, these proinflammatory MDR1^{+} CCR6^{+}CXCR3^{hi}CCR4^{lo} Th17 cells are enriched and activated in the gut of patients with Crohn`s disease.[352] Glucocorticoids are usually administered to the patients with autoimmune disorders such as Crohn`s disease resulting in the modulation of the expression of several genes involved in the innate and adaptive immune response and the immunosuppression of peripheral T cells.[352-354] However, Ramesh et al. could also demonstrate that these proinflammatory MDR1^{+} CCR6^{+}CXCR3^{hi}CCR4^{lo} Th17 cells are induced by and refractory to glucocorticoids.[352]

3 Materials

3.1 Equipment

equipment	manufacturer
Assistent Rotating Mixer RM 5	Karl Hecht GmbH & Co. KG
autoMACS® Pro Separator	Miltenyi Biotec
Axioskop20	Carl Zeiss AG
AxioCam MR	Carl Zeiss AG
Bag sealer	Petra-electric
Bench-top centrifuge Micro 22R	Andreas Hettich GmbH & Co. KG
Bench-top centrifuge Rotina 420R	Andreas Hettich GmbH & Co. KG
Bio-Plex Protein Array	Bio-Rad Laboratories
Clean bench HERAsafe	Heraeus Holding GmbH
CFX96 Real-Time PCR Detection System	Bio-Rad Laboratories
ELISA Reader ELx800	BioTek
FACSCalibur™	BD Biosciences
Floor-standing centrifuge Rotixa 50 RS	Andreas Hettich GmbH & Co. KG
GeneAmp PCR System 9700	Perkin Elmer
Heating block Thermomixer 5436	Eppendorf AG
Incubation/Inactivation Bath Model 1002	Gesellschaft für Labortechnik
Incubator Hera cell	Heraeus Holding GmbH
Inverted Microscope IX50	Olympus
Magnetic stirrer	Thermo Fisher Scientific
Multichannel pipettes LABMATE™	Abimed GmbH
NanoDrop1000 Spectrophometer	Thermo Fisher Scientific
Neubauer counting chamber	La Fontaine International GmbH
Pipetboy acu 2	Integra Biosciences GmbH
Pipettes LABMATE™	Abimed GmbH
Pipettes	Eppendorf AG
Pipettes	Gilson
Power supply power Pac 1000	Bio-Rad Laboratories
Precision balance MC1 Analytic AC 210 S	Sartorius AG

Rotating tube mixer	Hecht-Assistent
Shaker Polymax1040	Heidolph Instruments Gmbh & Co. KG
Shaker Titramax1000	Heidolph Instruments Gmbh & Co. KG
Trans-Blot® SD Semi-Dry Apparatus	Bio-Rad Laboratories
Vortex shaker MS1	IKA-Werke-GmbH & Co. KG
XCell *Sure Lock™* Mini-Cell	Invitrogen

3.2 Consumables

consumable	manufacturer
4-20% precise protein gels (10-well, 12-well)	Thermo Fisher Scientific
Cell culture plates (flat bottom, 6-well, 96-well)	BD Biosciences
Cell culture plates (U-bottom, 96-well)	Greiner Bio-One GmbH
Cell scraper	Corning Incorporated
CL-XPosure film (clear blue X-ray film)	Thermo Fisher Scientific
Disposable pipettes (5 ml, 10 ml, 25 ml)	Corning Incorporated
Filter Paper sandwich	Thermo Fisher Scientific
Individual PCR Tubes™	Bio-Rad Laboratories
Invitrolon™ PVDF membranes (0.45 µm pore size)	Invitrogen
MACS Separation Columns (LS and MS Colums)	Miltenyi Biotec
Microplates (flat-bottom, 96-well)	Greiner Bio-One GmbH
MaxiSorp™ plates (96-well)	Thermo Fisher Scientific
PCR SingleCap 8er-SoftStrips	Biozym
PCR Tube Strips	Biorad
Pipette tips (10 µl)	Abimed GmbH
Pipette tips (200 µl)	Sarstedt AG & Co.
Pipette tips (1 ml)	Sarstedt AG & Co.
Polystyrene tubes (5 ml)	Sarstedt AG & Co.
Polypropylene tubes (15 ml)	Greiner Bio-One GmbH
Polypropylene tubes (50 ml)	Greiner Bio-One GmbH

Primaria™ 6-well flat bottom tissue culture plate	BD Biosciences
Reagent reservoir (100 ml)	Corning Incorporated
Safe-Lock tubes (0.5 ml, 1.5 ml, 2 ml)	Eppendorf
SafeSeal Tips® premium (10 µl, 20 µl, 100 µl, 200 µl, 1 ml)	Biozym
Sample Bags	Perkin Elmer
Sterile filter (0.2 µM)	Sartorius AG

3.3 Chemicals, reagents and solutions

chemicals	manufacturer
17-DMAGH$_2$	InvivoGen
Albumin Fraction V	Carl Roth GmbH & Co. KG
Ampuwa® Water	Fresenius Kabi Deutschland GmbH
Biocoll Separating Solution	Biochrom AG
Dexamethasone	Sigma-Aldrich
Dimethylsufoxid (DMSO)	Carl Roth GmbH & Co. KG
Ethanol	Merck KGaA
Ethylendiamintetraacetat (EDTA)	Sigma-Aldrich
FACS Clean®	BD Biosciences
FACS Flow®	BD Biosciences
FACS Rinse®	BD Biosciences
Human Serum Albumin (HSA; 20% solution for infusion)	CSL Behring GmbH
Ionomycin	Sigma-Aldrich
Lipofectamine™ RNAiMAX Transfection Reagent	Invitrogen
Lipopolysaccharides (LPS)	Sigma-Aldrich
Methanol	VWR
Monensin	eBioscience
Nonfat Dry Milk	Cell Signaling
Phorbol myristate acetat (PMA)	Sigma-Aldrich

Phosphate buffered saline (PBS)	Biochrom AG
Propidium iodide	BD Biosciences
Protease Inhibitor Cocktail Tablets Complete, MINI, EDTA-free	Roche
Rhodamine123	Sigma-Aldrich
TrackIT™ 1 kb DNA Ladder	Invitrogen
Trypan blue	Sigma-Aldrich
Tween® 20	Carl Roth GmbH & Co. KG

3.4 Reagent Kits

kit	manufacturer
BCA Protein Assay Kit	Thermo Fisher Scientific
Bio-Plex Human Cytokine Group I 5-plex Assay	Bio-Rad Laboratories
KAPA™ SYBR® FAST (QPCR mastermix)	PEQLAB Biotechnologie GmbH
QuantiTect® Reverse Transcription Kit	Qiagen
RNeasy Mini Kit	Qiagen
Super Signal® West Pico Chemiluminescent Substrate	Thermo Fisher Scientific

3.5 Ready-to-use buffers

buffer	manufacturer
10x eBioscience Permeabilization Buffer	eBioscience
autoMACS™ Pro Running Buffer	Miltenyi Biotec
autoMACS™ Pro Washing Buffer	Miltenyi Biotec
BupH Tris-Glycine Buffer Packs	Thermo Fisher Scientific
BupH Tris-HEPES-SDS Running buffer	Thermo Fisher Scientific
eBioscience IC Fixation Buffer	eBiosciece
FOXP3 Fix/Perm Buffer Set	Biolegend
NuPAGE® LDS Sample Buffer (4x)	Invitrogen
RIPA Buffer	Sigma-Aldrich

RLT Buffer	Qiagen
RPE Buffer with β-mercaptoethanol	Qiagen

3.6 Composition of used buffers

buffer	composition
Blotting buffer (pH8) **(BupH Tris-Glycine Buffer Packs)**	25 nM Tris 192 mM Glycine 20% Methanol
ELISA block buffer	PBS 0.1% Tween® 20 0.25% bovine serum albumin
ELISA/western blot wash buffer	PBS 0.1% Tween® 20
FACS buffer	PBS 2% fetal bovine serum
MACS buffer	PBS 0.5% human serum albumin 2 mM EDTA
RIPA Buffer	50 mM NaCl 1.0% IGEPAL® CA-630 0.5% sodium deoxycholate 0.1% SDS 50 mM Tris pH 8
SDS PAGE Reducing Sample Buffer	NuPAGE® LDS Sample Buffer 200 mM DTT
SDS PAGE Running Buffer (pH8±0.5) **(BupH Tris-HEPES-SDS Running buffer)**	100 mM Tris 100 mM HEPES 3 mM SDS

3.7 MACS® cell separating reagents

separation kit	manufacturer
CD14 MicroBeads, human	Miltenyi Biotec
CD4+ T cell Isolation Kit II, human	Miltenyi Biotec

3.8 Cell culture media, sera, supplements

Media/sera/supplements for cell culture	manufacturer
Fetal bovine serum (FCS)	Biochrom AG
HEPES buffer (1M)	Biochrom AG
L-glutamine (200 mM)	Biochrom AG
Opti-MEM® I Reduced Serum Medium	Invitrogen
Penicillin/Streptomycin	Biochrom AG
Pooled human serum (PHS)	Children's Hospital Tuebingen
VLE-RPMI 1640	Biochrom AG

3.9 Composition of cell culture media

media	composition
Antibiotic-free medium	VLE-RPMI 1640 2 mM Glutamine 10 mM HEPES buffer 10% heat-inactivated PHS
Freezing medium	90% fetal bovine serum 10% DMSO
VLE-RPMI complete medium	VLE-RPMI 1640 2 mM Glutamine 10 mM HEPES buffer 10% heat-inactivated PHS penicillin/streptomycin (100 units/ml and 100 µg/ml)

3.10 Antibodies

3.10.1 Antibodies for flow cytometry

specificity	isotype	clone	format	manufacturer
anti-human CD3	mouse IgG2a,κ	HIT3a	FITC	Biolegend
anti-human CD4	mouse IgG1,κ	RPA-T4	PE	Biolegend
Anti-human CD4	mouse IgG1,κ	SK3	PerCP	BD Biosciences
Anti-human CD8a	mouse IgG1,κ	HIT8a	APC	Biolegend
Anti-human CD25	mouse IgG1,κ	M-A251	PE	BD Biosciences
Anti-human CD194 (CCR4)	mouse IgG1,κ	L291H4	PE	Biolegend
Anti-human CD196 (CCR6)	mouse IgG2b,κ	G034E3	APC	Biolegend
Anti-human CD183 (CXCR3)	mouse IgG1,κ	G025H7	PerCP/Cy5.5	Biolegend
Anti-human CD80	mouse IgG1,κ	L307.4	PE	BD Biosciences
Anti-human CD80	mouse IgG1,κ	2D10	APC	Biolegend
Anti-human CD86	mouse IgG1,κ	2331 (FUN-1)	FITC	BD Biosciences
Anti-human CD86	mouse IgG2b,κ	IT2.2	PE	Biolegend
Anti-human CD14	mouse IgG2a,κ	M5E2	FITC	Biolegend
Anti-human CD11b	mouse IgG1,κ	ICRF44	FITC	Biolegend
Anti-human CD11c	mouse IgG1,κ	3.9	PerCP/Cy5.5	Biolegend

Anti-human CD16	mouse IgG1,κ	3G8	PE	BD Biosciences
Anti-human CX3CR1	rat IgG2b,κ	2A9-1	APC	Biolegend
Anti-human CD54	mouse IgG1,κ	HA58	APC	Biolegend
Anti-human CD45	mouse IgG1,κ	HI30	PE	Biolegend
Anti-human HLA-DR	mouse IgG2a,κ	L243	PerCP	Biolegend
Anti-human IL-17A	mouse IgG1,κ	eBio64DEC17	Alexa Fluor 647	eBioscience
Anti-human IL-17A	mouse IgG1,κ	eBio64DEC17	eFluor660	eBioscience
Anti- human IFNγ	mouse IgG1,κ	4S.B3	FITC	eBioscience
Anti-human Hsp90α	mouse IgG2a	K41009	PE	Enzo Life Sciences
Anti-human FOXP3	mouse IgG1,κ	259D	Alexa Fluor 647	Biolegend
Isotype control	mouse IgG1,κ	MOPC-21	FITC	Biolegend
Isotype control	mouse IgG2a,κ	MOPC-173	FITC	Biolegend
Isotype control	mouse IgG1,κ	MOPC-21	PE	Biolegend
Isotype control	mouse IgG2a,κ	MOPC-173	PE	Biolegend
Isotype control	mouse IgG2b,κ	MPC-11	PE	Biolegend
Isotype control	mouse IgG1,κ	MOPC-21	PerCP	Biolegend
Isotype control	mouse IgG2a,κ	MOPC-173	PerCP	Biolegend
Isotype control	mouse IgG1,κ	MOPC-21	PerCP/Cy5.5	Biolegend
Isotype control	mouse IgG2b,κ	MG2b-57	APC	Biolegend
Isotype control	mouse IgG1,κ	MOPC-21	APC	Biolegend
Isotype control	rat IgG2b,κ	RTK4530	APC	Biolegend
Isotype control	mouse IgG1,κ	MOPC-21	Alexa Fluor 647	Biolegend
Isotype control	mouse IgG1,κ	MOPC-21	eFluor660	Biolegend

3.10.2 Antibodies for western blot

specificity	isotype	clone	format	manufacturer
Anti-human Hsp90α	mouse IgG2a	K41009	purified	Enzo Life Sciences
Anti-human Hsp90β	mouse IgG1	MBH90B	purified	Abcam
Anti-human GAPDH	mouse IgG1	1D4		Enzo Life Sciences
Anti-mouse IgG	goat IgG		HRP	Enzo Life Sciences

3.10.3 Antibodies for ELISA

specificity	isotype	format	manufacturer
Anti-human S100A8/S100A9	rabbit IgG	Polyclonal affinity-purified rabbit antisera	Institute of Immunology, University of Muenster
Anti-human S100A12	rabbit IgG	Polyclonal affinity-purified rabbit antisera	Institute of Immunology, University of Muenster

3.10.4 Antibodies for cell culture

specificity	isotype	clone	format	manufacturer
Anti-human CD3 (ORTHOCLONE OKT3, muromonab-CD3)	mouse IgG2a	OKT3	purified	Janssen-Cilag
Anti-human CD28	mouse IgG1,κ	CD28.2	purified	BD Biosciences

Anti-human IL-17	mouse IgG2b	#41809	purified	R&D Systems
Anti-human TNFα	mouse IgG1	#28401	purified	R&D Systems
Anti-human IL-12/IL-23 p40 Antibody	mouse IgG1	#24901	purified	R&D Systems
Anti-human IL-1β	mouse IgG1	#8516	purified	R&D Systems
Anti-human IL-6	mouse IgG2b	#1936	purified	R&D Systems
Isotype Control	mouse IgG1	#11711	purified	R&D Systems
Isotype Control	mouse IgG2b	#20116	purified	R&D Systems
Anti-human TLR4	mouse IgG2a	HTA125	purified	Abcam
Isotype Control	mouse IgG2a	MOPC-173	purified	Abcam

3.11 siRNA

3.11.1 Applied siRNAs

siRNA	manufacturer
Hsp90α siRNA (ON-TARGETplus SMART pool, 5 nmol, L-005186-00-0005)	GE Healthcare Dharmacon
siRNA Control (ON-TARGETplus Non-targeting Pool, 5 nmol, D-001810-10-05)	GE Healthcare Dharmacon

3.11.2 Sequence of Hsp90α targeting siRNA

siRNA	sense/antisense strand
Duplex 1	Sense 5’ GAAGUAGACUAAUCUCUGGUU 3’ Antisense 3’ UUCUUCAUCUGAUUAGAGACC 5'
Duplex 2	Sense 5' GACCAAAUCUUGUUAUUGAUU 3' Antisense 3' UUCUGGUUUAGAACAAUAACU 5'

Duplex 3	Sense	5' GUUCAGUACUCUACAAUUCUU 3'
	Antisense	3' UUCAAGUCAUGAGAUGUUAAG 5'
Duplex 4	Sense	5' ACUAAGUGAUGCUGUGAUAUU 3'
	Antisense	3' UUUGAUUCACUACGACACUAU 5'

3.11.3 Sequence of non-targeting siRNA

siRNA	sense/antisense strand	
Duplex 1	Sense	5' UGGUUUACAUGUCGACUAAUU 3'
	Antisense	3' UUACCAAAUGUACAGCUGAUU 5'
Duplex 2	Sense	5' UGGUUUACAUGUUGUGUGAUU 3'
	Antisense	3' UUACCAAAUGUACAACACACU 5'
Duplex 3	Sense	5' UGGUUUACAUGUUUUCUGAUU 3'
	Antisense	3' UUACCAAAUGUACAAAAGACU 5'
Duplex 4	Sense	5' UGGUUUACAUGUUUUCCUAUU 3'
	Antisense	3' UUACCAAAUGUACAAAAGGAU 5'

3.12 Proteins

protein	expression organism	expression vector	Manufacturer
S100A8	*E.coli*	pET11/20 expression vector containing S100A8 cDNA	Institute of Immunology, University of Muenster
S100A9	*E.coli*	pET11/20 expression vector containing S100A9 cDNA	Institute of Immunology, University of Muenster
S100A12	*E.coli*	pET11b vector encoding tag-free S100A12 cDNA	Institute of Immunology, University of Muenster

3.13 Software

software	provider
BD CellQuest™ Pro Version 4.0.2	BD Biosciences
Bio-Plex Manager™	Bio-Rad Laboratories
CFX Manager™ Software	Bio-Rad Laboratories
Graph Pad Prism 5.0	GraphPad Software
ImageJ	Wayne Rasband
Microsoft Word, Excel, Power Point	Microsoft

3.14 List of manufacturers

manufacturer	city, country
Abcam	Abcam, Cambridge, UK
ABIMED GmbH	Langenfeld, Germany
Adobe Systems	San Jose, CA, USA
Andreas Hettich GmbH & Co.KG	Tuttlingen, Germany
BD Biosciences	San Jose, CA, USA
Biochrom AG	Berlin, Germany
Biolegend	San Diego, CA, USA
Bio-Rad Laboratories	Munich, Germany
Biozym	Hessisch Oldendorf, Germany
Carl Roth GmbH & Co. KG	Karlsruhe, Germany
Cell Signaling Technology	Danvers, MA, USA
Corning Incorporated	Corning, NY, USA
eBioscience	San Diego, CA, USA
Eppendorf	Hamburg, Germany
Enzo Life Sciences	Farmingdale, NY, USA
Fresenius Kabi Deutschland GmbH	Bad Homburg, Germany
GE Healthcare	Garching, Germany
Gesellschaft für Labortechnik	Burgwedel, Germany
Gilson	Middleton, WI, USA
GraphPad Software	La Jolla, CA, USA
Greiner Bio-One GmbH	Frickenhausen, Germany

Heidolph Instruments GmbH & Co. KG	Schwabach, Germany
Heraeus Holding GmbH	Hanau, Germany
Hettich AG	Bäch, Switzerland
Invitrogen	Carlsbad, CA, USA
InvivoGen	San Diego, CA, USA
Integra Biosciences	Fernwald, Germany
Janssen-Cilag	Neuss, Germany
Karl Hecht GmbH & Co. KG	Sondheim, Germany
La Fontaine International GmbH	Waghäusel, Germany
Merck KGaA	Darmstadt, Germany
Microsoft	Redmond, WA, USA
Miltenyi Biotec	Bergisch Gladbach, Germany
Olympus	Hamburg, Germany
PEQLAB Biotechnologie GmbH	Erlangen, Germany
PerkinElmer	Waltham, MA, USA
Petra-electric	Burgau, Germany
R&D Systems	Minneapolis, MN, USA
Roche	Basel, Switzerland
Sarstedt AG & Co.	Nümbrecht, Germany
Sartorius AG	Göttingen, Germany
Sigma-Aldrich	Saint Louis, MO, USA
VWR International	Darmstadt, Germany
Thermo Fisher Scientific	Rockford, IL, USA

4 Methods

4.1 Preparation of pooled human serum

50-60 ml of peripheral venous blood was taken from healthy donors without addition of any anticoagulants and immediately transferred into 50 ml tubes. After incubating the tubes for 1 h at 37°C to allow coagulation, they were centrifuged at 2716 g for 10 min at room temperature (RT). The supernatant serum was taken off, pooled and heat inactivated for 30 min at 56°C. The obtained pooled human serum (PHS) was aliquoted and frozen at -80°C.

4.2 Isolation of mononuclear cells from human peripheral blood

PBMCs were isolated from heparinized peripheral blood by Ficoll-Hypaque density gradient centrifugation. Therefore, two volume fractions of heparinized blood were slowly layered above one volume fraction of Ficoll-Hypaque in a 15 ml or 50 ml conical tube and centrifuged at 958 g for 20 min at 20°C without brake. The plasma was aspirated and the mononuclear cell layer was transferred into a new 15 ml or 50 ml tube. The isolated cells were resuspended in PBS and centrifuged at 400 g for 5 min. After removing the supernatant, two additional wash steps with PBS were carried out. Finally, cells were counted and stored in PBS on ice until further use.

4.3 Immunomagnetic cell separation

Subpopulations of PBMCs were isolated using magnetic-activated cell sorting (MACS). MACS technology is based on nanosized and superparamagnetic particles (MACS MicroBeads), which are conjugated to highly specific antibodies against a particular antigen on the cell surface. Functionality of isolated cells is preserved as MACS MircroBeads are non-toxic, biodegradable and do not saturate cell surface epitopes due to their small size. Cells magnetically labelled with MACS MicroBeads were applied to MACS Columns (composed of a spherical steel matrix) and placed in

a high-gradient magnetic field of a MACS separator. Unlabelled cells pass through while magnetically labelled cells are retained within the column. By removing the column from the magnetic field, the magnetically labelled cells can be flushed out.

In the present study, $CD4^+$ T cells were isolated from PBMC by negative selection using $CD4^+$ T cell Isolation Kit II. Therefore, non-target cells were indirectly labelled with a cocktail consisting of several biotinylated monoclonal antibodies directed against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ, and Glycophorin A) and anti-biotin monoclonal antibodies conjugated to MicroBeads according to the manufacturer`s protocol. Cells were isolated by the autoMACS separator using the program “Depletes”. The purity of isolated $CD4^+$ T cells, confirmed by flow cytometry using fluorochrome-conjugated antibodies directed against CD3 and CD4 and anti-CD14 antibodies, was >90%.

Additionally, $CD14^+$ monocytes were isolated from PBMC by positive selection using CD14 MicroBeads. Therefore, $CD14^+$ cells were magnetically labelled with CD14 MicroBeads and isolated according to the manufacturer`s protocol using the program “Possel”. The purity of isolated $CD14^+$ cells (>95%) was determined by flow cytometric analysis of CD14 surface expression.

4.4 Determination of cell number

Cell numbers and vitality were determined using a Neubauer chamber. Therefore, cell suspension was diluted with trypan blue and transferred into the chamber. Trypan blue is a diazo dye that is not absorbed by viable cells. However, it traverses the membrane of dead cells leading to the distinctive blue colouring under the microscope. Uncoloured living cells were counted in the four main squares. The cell concentration in the present cell suspension was calculated as indicated in the following formula:

$$\frac{\text{number of cells}}{\text{ml}} = \frac{\text{number of counted cells}}{\text{number of main squares}} \times \text{dilution factor} \times 10.000$$

4.5 Expression and purification of S100 proteins

Human S100A8, S100A9 and S100A12 were recombinantly expressed in *E.coli* and purified as described earlier.[126,129,138,140] Briefly, *E.coli BL21(DE3)* bacteria were transformed with pET11/20 expression vector containing S100A8 or S100A9 cDNA. After growing bacteria at 37°C in 2x yeast extract and tryptone for 24 h, bacteria were harvested, lysed and the inclusion bodies were prepared. The inclusion body pellets were dissolved in 8M urea buffer and samples were adjusted to pH 2.0-2.5 first by adding hydrochloric acid. Samples were dialyzed to get adapted to pH 7.4 for proper refolding in the presence of 2 mM DTT. After centrifugation to pellet aggregated material, samples were further dialyzed and applied to anion exchange column and gel filtration chromatography. Recombinant human S100A12 was expressed in *E.coli* from the pET11b vector encoding tag-free S100A12. After inducing the protein expression with isopropyl-β-D-thiogalactopyranosid (IPTG), the pellet was lysed by sonication and the insoluble material was removed by centrifugation. The supernatant was adjusted up to 10 mM $CaCl_2$ and applied onto a phenyl-sepharose column. After elution, S100A12 containing fractions were concentrated by ultrafiltration and loaded onto a ResQ column (Pharmacia). The protein was eluted using a 0-1M NaCl gradient using an AKTA purifier chromatography system (Amersham). All recombinant S100A12 preparations were purged over LPS-removal columns (Endotrap-Hyglos). Possible endotoxin contaminations in S100A8, S100A9 and S100A12 preparations were determined by Limulus amoebocyte lysate (LAL) assay (Lonza) and confirmed to be lower than 1 pg LPS/µg S100 protein.

4.6 Monocyte-T-cell co-culture experiments for analysis of induction of Th1, Th17/Th1, Th17 and Th17.1 cells

Monocytes from healthy donors, from patients with or without GvHD after HCT and $CD4^+$ T cells were isolated by magnetic cell separation as described in 4.3. $CD4^+$ T cells from healthy donors were co-cultured with 100 ng/ml anti-CD3 mAb Orthoclone OKT3 and monocytes from healthy donors, patients with acute or chronic GvHD or patients before conditioning and at day 30, 60 and 100 post HCT (+/- 10 days) without GvHD at a monocyte:T cell ratio of 1:4 for 5 days in 96-well flat bottom plates

in VLE-RPMI medium containing 10% PHS, penicillin/streptomycin (100 units/ml and 100 µg/ml), 2 mM L-glutamine, and 10 mM HEPES buffer. Where indicated, monocytes from healthy donors were additionally stimulated with 5 µg/ml S100A8, S100A9, S100A8/S100A9 or S100A12 for 4 hours before co-culturing with $CD4^+$ T cells. Binding of S100 proteins to monocytes was inhibited by pre-incubation of monocytes with TLR4 antagonist (0.1 µg/ml) for 30 min followed by 4 h stimulation with S100 proteins. To investigate if Th17 induction occurred cell-contact dependent or cytokine mediated, monocytes were cultured in VLE-RPMI1640 complete medium for 24 h. The supernatant was added to the culture medium of freshly isolated $CD4^+$ T cells at a ratio of 1:1. $CD4^+$ T cells were cultured for 5 days in the presence of Orthoclone OKT3 (100 ng/ml) and anti-CD28 mAb (1 µg/ml). To analyse the effect of cytokine neutralisation on the induction of Th17 cells, monocytes from healthy donors were stimulated for 4 h with S100 proteins prior to co-culture with $CD4^+$ T cells isolated from healthy donors in the presence of 100 ng/ml anti-CD3 mAb Orthoclone OKT3 and the neutralizing antibodies specific for the cytokines IL-17, TNFα, IL-1β, IL-6, IL-12/IL-23p40 (5 µg/ml) and the corresponding isotype controls (mouse IgG1 and mouse IgG2B; 5 µg/ml). For inhibition of Hsp90 in monocytes, cells were treated with 17-DMAG for 16 h and washed before culturing with $CD4^+$ T-cells. Optionally, physiological concentrations of dexamethasone (0.1 µM) were added to the co-culture of monocytes and $CD4^+$ T cells. Furthermore, monocytes were transfected optionally with siRNA targeting Hsp90α or non-targeting siRNA prior to the co-culture with $CD4^+$ T cells.

4.7 Immunofluorescent staining for flow cytometry

Flow cytometry is used for immunophenotyping of a variety of specimens, including whole blood, bone marrow, serous cavity fluids, cerebrospinal fluid, urine, and solid tissues. This technique allows the measurement of multiple characteristics of individual microscopic particles such as cells by suspending them in a stream of fluid and passing them by an electronic detection apparatus. A beam of light (usually laser light) of a single wavelength is directed onto a hydrodynamically-focused stream of fluid. Cells passing through the beam scatter light, which is detected as forward scatter (FSC; proportional to the cell size) and side scatter (SSC; proportional to cell

granularity). Cells are labelled with fluorochromes emitting light when excited by the laser with corresponding excitation wavelength. The use of multiple fluorochromes allows several cell properties to be measured simultaneously.[355] In this work, flow cytometric analysis was carried out using a 4-colour FACSCalibur™ flow cytometer, equipped with an air-cooled 488 nm argon laser and a 635 nm red diode laser. Following fluorochromes were used: FITC (detected in the FL-1 channel); PE (detected in the FL-2 channel); PerCP (detected in the FL-3 channel); APC and AlexaFluor 647 (detected in the FL-4 channel). Data acquisition and analysis were performed using BD CellQuest™ Pro software.

4.7.1 Immunofluorescent staining of cell surface antigens

Cells to be analyzed were aliquoted to polystyrene tubes (0.1-1 x 10^6 cells/tube) and washed by adding 2 ml PBS containing 2% FBS to each tubes. The tubes were centrifuged at 400 g for 5 min, the supernatant was discarded, and the cell pellet was resupended in the remaining liquid (50-100 µl). Fluorochrome labelled antibodies were added to final concentration of 1-5 µg/ml and incubated in the dark at 4°C for at least 15 min.

4.7.2 Intracellular staining of cytokines

For intracellular cytokine staining (ICC) of IL-17 and IFNγ in $CD4^+$ T lymphocytes, cells were treated for 5 h with PMA (50 ng/ml) and ionomycin (750 ng/ml) to stimulate intracellular cytokine production. The calcium ionophore ionomycin and the phorbol ester synergistically enhance activation of protein kinase C inducing the phosphorylation of a number of cellular proteins and the initiation of the transcription of several genes resulting in the production of cytokines.[356] Secretion of cytokines was blocked by adding the protein transport inhibitor monensin at a final concentration of 2 µM during the last 3 h of activation. Monensin is a carboxyl ionophore which interrupts the intracellular transport process leading to the accumulation of cytokines in the Golgi complex.[357] After 5 h $CD4^+$ T cells were harvested and transferred to polysterene tubes. Cells were initially stained for cell surface markers (4.7.1). For intracellular cytokine staining, cells were fixed by adding

100 µl of eBioscience IC Fixation Buffer to each tube. The Fixation Buffer containing 4% paraformaldehyde (PFA) cross-links proteins preserving their native 3D structure.[358] For fixation, cells were incubated in the dark at room temperature for 20 min after vortexing the tubes. Cells were permeabilized by washing twice with 500 µl 1x eBioscience Permeabilization Buffer (10x eBioscience Permeabilization Buffer was diluted 1:10 in MilliQ water). The Permeabilization Buffer contains saponin, an organic detergent which interacts with cholesterol of the cell membrane, making it permeable to antibodies.[359] For intracellular cytokine staining, cells were resuspended in 100 µl Permeablization Buffer and incubated with the fluorochrome-conjugated antibodies directed against IL-17A and IFNγ in the dark at room temperature for 20 min. Subsequently, cells were washed once with 500 µl Permeabilization Buffer. After another washing step with 2 ml PBS containing 2% FBS, the supernatant was discarded and cells were analyzed using the FACSCalibur™ flow cytometer.

4.7.3 Intracellular staining of heat shock protein 90α

Heat shock protein 90α was detected in monocytes by intracellular staining. After isolating PBMCs by Ficoll-Hypaque density gradient centrifugation, monocytes were obtained by immunomagnetic cell separation as described in 4.3. Monocytes were transferred to polysterene tubes and initially stained for cell surface markers (4.7.1). For intracellular staining of Hsp90α, monocytes were fixed by adding 100 µl of eBioscience IC Fixation Buffer to each tube and incubated in the dark at room temperature for 20 min after vortexing the tubes. Cells were permeabilized by washing twice with 500 µl 1x eBioscience Permeabilization Buffer (10x eBioscience Permeabilization Buffer was diluted 1:10 in MilliQ water). For intracellular staining of Hsp90α, monocytes were resuspended in 100 µl Permeablization Buffer and incubated with the fluorochrome-conjugated antibodies directed against Hsp90α in the dark at room temperature for 20 min. After washing the cells once with 500 µl Permeabilization Buffer, another washing step with 2 ml PBS containing 2% FBS was carried out. The supernatant was discarded and monocytes were analyzed using the FACSCalibur™ flow cytometer.

4.7.4 Intracellular staining of transcription factors

Detection of $CD4^+CD25^+FoxP3^+$ regulatory T cells in total $CD4^+$ T cells was carried out according to the manufacturer`s protocol. Therefore, $CD4^+$ T cells were transferred to polystyrene tubes and staining of surface markers (CD4, CD25) was performed (4.5.1). For fixation, cells were incubated with 1 ml of 1x FoxP3 Fix/Perm buffer (4x FoxP3 Fix/Perm buffer was diluted 1:4 in PBS) in the dark at room temperature for 20 min after vortexing. Subsequently, cells were centrifuged at 400g for 5 min and the supernatant was discarded. Thereafter, the cells were washed twice by adding 2 ml PBS containing 2% FBS and 1 ml 1x FoxP3 Perm buffer (10x FoxP3 Perm buffer was diluted 1:10 in PBS) to each tube. For permeabilization, cells were resuspended in 1 ml 1x FoxP3 Perm buffer and incubated in the dark at room temperature for 15 min. Tubes were centrifuged at 400g for 5 min and the supernatant was discarded. After resuspending the cells in 100 µl 1x FoxP3 Perm buffer, 2 µl fluorochrome-conjugated antibody directed against FoxP3 were added. After an incubation time of 30 min, cells were washed twice with PBS containing 2% FBS and analyzed on the FACSCalibur™ flow cytometer.

4.7.5 Detection of multi-drug protein type1 (MDR1)

A 1 mg/ml Rhodamine123 stock solution was prepared in DMSO. The aliquots were stored at -20°C until usage. For flow cytometric analysis, $CD4^+$ T cells were resuspended in complete medium and loaded with Rhodamine123 at a final concentration of 1 µg/ml for 30 min on ice in the dark. Then, cells were washed in complete medium and moved to a 37°C incubator for 2 h. After this efflux period, cells were washed once in PBS, stained with the surface markers and washed again in PBS. Stained cells were kept on ice before flow cytometric analysis.

4.7.6 Detection of necrosis

Detection of necrosis was performed using propidium iodide (PI), a dye which penetrates damaged cellular membranes and forms intercalation complexes with double-stranded DNA inducing an amplification of the fluorescence. Thus, the

number of non-vital cells in the total cell population can be assessed. Initially, staining of surface markers was performed. Afterwards, 10 µl PI staining solution (composed of 50 µg PI/ml in PBS) were added to the cells right before flow cytometric analysis on the FACSCalibur^{TM} flow cytometer.

4.8 Cytokine measurements

Secretion of proinflammatory cytokines by monocytes was analyzed using Human Cytokine Group 1 5-plex Assay from Bio-Rad. The Bio-Plex suspension array system is based on the covalent binding of fluorescently dyed beads, each with a distinct colour code, to specific monoclonal antibodies directed against the desired biomarkers. These coupled beads react with the sample containing the biomarker of interest. After several wash steps to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed by adding the streptavidin-phycoerythrin conjugate. Flow cytometric quantification was performed using the Bio-Plex Protein Array System. A red (635 nm) laser illuminates the fluorescent dyes within each bead to provide bead classification and thus identification of the particular cytokines. At the same time, a green (532 nm) laser excites phycoerythrin to generate a reporter signal, which is detected by a photomultiplier tube and enables the quantification of the cytokine amount.

Therefore, 0.3 x 10^6 monocytes additionally pre-stimulated with 5 µg/ml S100A8, S100A9, S100A8/S100A9 or S100A12 for 4 hours were seeded in 180 µl VLE-RPMI complete medium in a 96-well-flat bottom plate. After an incubation time of 24 h the supernatant was removed and frozen at -80°C until analysis. Furthermore, serum samples of patients with GvHD and healthy donors were collected and stored until analysis at -80°C. The Bio-Plex assay was performed according to the manufacturer`s protocol. Therefore, the filter plate was pre-wetted with 100 µl assay buffer per well. After adding 50 µl of coupled beads the wells were washed twice with 100 µl wash buffer using a vacuum manifold. Subsequently, 50 µl samples (serum samples 1:4 diluted in sample diluent; undiluted supernatants), standards and blank were added and incubated for 30 min in the dark while shaking. After three wash steps 25 µl biotinylated detection antibody was added to each well and incubated for

30 min in the dark on the shaker. The plate was washed three times and 50 µl streptavidin-PE was added to each well and incubated for 10 min while shaking in the dark. Unbound streptavidin-PE was removed by three wash steps and the beads were resuspended in 125 µl assay buffer. The plate was read and the cytokine profile of each sample was determined by referring to the standard curve using the Bio-Plex Manager™ Software.

4.9 siRNA transfection

siRNA transfection was performed using Lipofectamine™ RNAiMAX, a lipid-based transfection reagent developed for the delivery of siRNA into eukaryotic cells. This transfection method is based on the mechanism of lipofection. Lipid-based transfection reagents consist of specific cationic lipids that form micelles or liposomes that interact with negatively charged nucleic acids, fuse with the cell membrane and facilitate the delivery of nucleic acids into the cell.
The main advantages of lipofection are its high efficiency, the ease of use, reproducibility, and low toxicity. In addition, this method is suitable for all transfection applications (transient, stable, co-transfection, reverse, sequential or multiple transfections).[360]

Human monocytes were isolated by magnetic cell separation as described in 4.3. Subsequently, 1 x 10^6 monocytes were seeded in 2 ml antibiotic-free VLE-RPMI medium containing 10% PHS, 2 mM L-glutamine, and 10 mM HEPES buffer per well in a Primaria™ 6-well flat bottom tissue culture plate. Monocytes were transfected with 20 nM, 50 nM or 120 nM Hsp90α targeting siRNA or non-targeting control siRNA using 5 µl Lipofectamine™ RNAiMAX transfection reagent per well. Briefly, both siRNA duplexes and Lipofectamine™ RNAiMAX transfection reagent were diluted with 200 µl Opti-MEM® I Reduced Serum Medium per test. To allow the formation of siRNA-transfection reagent complexes, predilutions were mixed and incubated for 15 min at room temperature. After adding 400 µl of formed RNAi duplex-Lipofectamine™ RNAiMAX complexes to each well monocytes were incubated at 37°C and 5% CO_2 until they were analysed for gene knockdown at various time points (24, 48, 72, 96, 140 or 168 h) by flow cytometry or immunoblotting.

4.10 Preparation of cell lysates

For lysis of monocytes, cells were washed once with PBS to remove non-adherent cells. Adherent cells were detached from the tissue culture dish using a cell scraper and transferred to 1.5 ml tubes. Freshly isolated monocytes were directly transferred to 1.5 ml tubes and lysed. After resuspending $1*10^6$ monocytes in 100 µl RIPA Lysis Buffer containing protease inhibitors, cells were incubated on ice for 15 min while vortexing several times. Tubes were centrifuged at 10.000 g for 15 min at 4°C and the cell lysates were stored at -80°C.

4.11 Determination of protein concentration

Cell lysates were obtained as described in 4.10. Total protein concentration in these cell lysates were determined using Pierce™ BCA Protein Assay Kit which is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (biuret reaction) with the sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) using a reagent containing bicinchoninic acid.[361] Chelation of two molecules of BCA with one cuprous ion results in the purple-colored reaction product of this assay. This complex exhibits absorbance at 562 nm that is nearly linear with increasing protein concentrations over a wide working range (20-2000 µg/ml). The macromolecular structure of the protein, the number of peptide bonds and the presence of the amino acids cysteine, cysteine, tryptophan and tyrosine are reported to be responsible for the color formation with BCA.[362]

Initially, diluted albumin standards at a concentration range of 0-2000 µg/ml were prepared. The cell lysates to be measured were diluted in RIPA Lysis Buffer at a ratio of 1:10. 25 µl of each calibration standard and of the diluted samples were pipetted into a 96-well flat-bottom microplate. After preparing the BCA working reagent by mixing BCA reagent A with BCA reagent B at a ratio of 50:1, 200 µl of this working solution were added to each well and mixed with the samples by placing the plate on the shaker for 30 sec. The plate was covered and incubated at 37°C for 30 min. After cooling the plate to RT, the absorbance was measured at 570 nm on the plate

reader. The assay was performed in duplicates or triplicates for each of the standards and samples. The 570 nm absorbance measurement of the Blank standard replicates was subtracted from the 570 nm measurements of the standard and sample replicates. A standard curve was prepared by plotting the average Blank-corrected 570 nm measurement for each BSA standard against the protein concentration. The protein concentrations of the cell lysates were deduced from this calibration curve.

4.12 SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is a technique to separate proteins according to their electrophoretic mobility. It allows the estimation of the relative molecular mass of proteins, the identification of major proteins in a sample and the determination of the distribution of proteins among fractions.[363] The anionic detergent SDS, the reducing agent dithiothreitol (DTT) and heat are widely used to denature the native structure of proteins. Thereby, SDS breaks up secondary and non-disulfide-linked tertiary structures, and additionally applies a negative charge to each protein in proportion to its mass.
Heating the samples to at least 60°C further allows SDS to bind to the hydrophobic regions of the proteins and further promotes the denaturation of the proteins. The reducing agent DTT denatures the protein by reducing disulfide linkages leading to the complete break-up of the tertiary and quartenary structure of the proteins.[364-366]

Samples for SDS-PAGE were prepared by mixing 3 volumes of the cell lysates containing 5-20 µg of total protein with 1 volume of NuPAGE® LDS Sample Buffer containing 200 mM DTT. The samples were denatured at 95°C for 10 min and loaded on Tris-HEPES-SDS 4-20% precast protein gels. For estimation of the protein size, PageRuler™ Prestained Protein Ladder was used. The gels were run at constant voltage of 120V for 50 min using a XCell *SureLock™* Mini-Cell Gel Running apparatus and BupH Tris-HEPES-SDS Running buffer.

4.13 Western Blot and immunodetection

Western blot or immunoblot is an analytical technique that allows the detection of specific proteins. Thereby, proteins are transferred from polyacrylamide gels to a membrane so that they are accessible for antibody detection.[367,368]
After gel electrophoresis, the separated proteins were transferred to Invitrolon™ PVDF membranes (0.45 µm pore size) at constant amperage of 60 mA for 45 min using Trans-Blot® SD Semi-Dry Apparatus and BupH Tris-Glycine Buffer Packs. Membranes were blocked using 5% skim milk in PBS-T (PBS with 0.1% Tween® 20) for at least 2 h at RT to prevent unspecific antibody binding to the membrane. The membranes were then incubated with the primary monoclonal antibodies directed against Hsp90α and the housekeeper glyceraldehyde 3-phosphate dehydrogenase (GAPDH) overnight at 4°C. These primary antibodies were diluted 1:1000 in 5% skim milk in PBS-T. The next day, the blots were washed three times in PBS-T before incubating with an anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (diluted 1:3500 in 5% skim milk). After three additional washing steps with PBS-T, bound HRP was detected utilizing SuperSignal® West Pico Chemiluminescent Substrate. Light emission was visualized using X-ray films (CL-XPosure Films).

4.14 S100 ELISA

S100 levels in stool and serum samples were detected by double sandwich enzyme-linked immunosorbent assay (ELISA). This method allows the quantification of antigens between two layers of antibodies (i.e. capture and detection antibody). Quantification of this assay occurs by detecting the amount of labelled detection antibody using a chromogenic substrate.
For determination of S100 concentrations, stool samples of 9 patients after HCT without GvHD and serum samples of 14 patients after HCT without GvHD were collected consecutively on day 7, 14, 21, 30, 60 and 100 (+/- 5 days) post HCT (stool sample size=24; serum sample size=62). Furthermore, stool samples of 9 patients and serum samples of 11 patients (stool sample size=10; serum sample size=34) were collected at the onset of GvHD and in the further progression of GvHD every 2 to 14 days. Stool and serum samples were stored at -80°C.

S100A8/S100A9 concentrations in the stool were determined using Calprotectin-Assay (Buehlmann). Thereby, a capture antibody specific to the S100A8/S100A9 heterodimeric and polymeric complexes, respectively, was coated onto the microtiter plate. A second monoclonal detection antibody conjugated to horseradish peroxidase HRP was used to detect fecal S100A8/S100A9 molecules bound to the monoclonal antibody coated onto the plate after a washing step. After incubation and a further washing step, tetramethylbenzidine (TMB) was added (blue colour formation) followed by a stopping reaction (change to yellow color). The absorption is measured at 450 nm using an ELISA reader.

Concentrations of S100A8/S100A9 in the serum and concentrations of S100A12 in the serum and in the stool were determined by sandwich ELISA as described in several studies.[130,369-374] For detection of fecal S100A12 levels, approximately 100 mg of stool samples were suspended in extraction buffer at 1:50 dilution for homogenisation as described and validated previously.[371-373] Briefly, flat bottom 96-well maxisorb microtitre plates were coated with 10 µg of monoclonal antibodies directed against human S100A12 or human S100A8/S100A9 per well (in 50 µl) and incubated for 16 h at 4°C. The plates were washed three times with PBS-T (wash buffer) and blocked with wash buffer containing 0.25% bovine serum albumin (blocking buffer) for one hour at 37°C. After washing the plates once with wash buffer, 50 µl of samples in three dilutions using block buffer were added and incubated for two hours at room temperature. The assay was calibrated using purified S100A12 in concentrations ranging from 0.016 to 125 ng/ml or different amounts (0.25-250 ng/ml) of the native complex of S100A8 and S100A9. After three washings biotinylated rabbit anti human-S100A12 or biotinylated rabbit anti human-S100A8/S100A9 (10 µg/well) was added and incubated for 30 min at 37°C. After an additional washing step, the plates were washed and incubated with streptavidin-horseradish peroxidase conjugate (1:5000 dilution) for 30 min at 37°C. The plates were washed three times and incubated with ABTS (2,2′-azinobis (3-ethylbenzthiazoline sulphonic acid) and H_2O_2 in 0.05 M citrate buffer, pH 4.0, for 20 min at room temperature. Absorbency at 405 nm was measured after 20 min using an ELISA reader. The assay has a linear range between 0.5 and 30 ng/ml and a sensitivity of <0.5 ng/ml.

4.15 Immunohistochemistry

Immunochemistry refers to the process of binding of specific antibodies to antigens in cells of a tissue section. This method is widely used in the diagnosis and prognosis of several diseases.[375] S100A8, S100A9 and S100A12 were detected on paraffin-embedded bowel specimens of healthy controls (n=2) and patients with acute intestinal GvHD stage I, II and III (n=3 in each group) as described previously.[126,371,376] In brief, paraffin sections were prepared from bowel tissue of patients with intestinal GvHD and as controls from patients with no pathological findings. After inhibiting endogenous peroxidase using 1% NaN_3 (1%) (w/v) and 0.1% H_2O_2 (v/v) in PBS, sections were blocked with 1% BSA in PBS and incubated with specific antibodies targeting S100A8, S100A9 or S100A12 (1 µg/ml). Following binding of the peroxidase-bound secondary antibody and reaction with the substrate 3-Amino-9-ethyl-carbazol (AEC) resulted in a characteristic brown colouring. Cell nuclei were stained using haematoxylin. Images were captured using a Zeiss Axioskop connected to an Axiocam camera supplied with software Axiovision 3.0 (original magnification x200 for all images). Percentage of S100A8, S100A9 or S100A12 stained areas in the individual sections were determined using ImageJ.

4.16 Isolation of RNA from monocytes

The extraction of RNA from human monocytes was carried out using the RNeasy® Mini Kit (Qiagen). This kit allows the efficient purification of RNA from small amounts of starting material by selective binding of RNA to the silica membranes in the spin columns. Therefore, monocytes from healthy donors (n=5), patients with diarrhoea caused by infectious gastroenteritis (n=5), patients after HCT without GvHD (n=6) and patients after HCT with acute or chronic GvHD (n=7) were isolated by magnetic cell separation (4.3). 0.1-5*10^6 monocytes were resuspended in 350 µl RLT buffer mixed with the reducing agent β-mercaptoethanol according to the manufacturer`s instructions. RLT buffer contains guanidine-thiocyanate which inactivates RNases to ensure purification of intact RNA. The lysate was directly pipetted into a QIAshredder spin column and centrifuged for 2 min at maximum speed (10.000 g) at RT to homogenize. The homogenized lysate was mixed with one volume fraction of 70%

ethanol. After applying the sample to an RNeasy Mini spin column where the total RNA binds to the membrane, it was centrifuged for 15 sec at 8000 g at RT. The flow-through was discarded and two wash steps with 700 µl RW1 buffer and 500 µl RPE buffer for 15 sec at 8000 g were carried out to remove any contaminants. To eliminate any remaining ethanol, 500 µl RPE buffer were added to the RNeasy spin column and another centrifugation step for 2 min at 8000 g was carried out. RNA was eluted by addition of 30 µl RNase-free water and subsequent centrifugation for 1 min at 8000 g.

4.17 Determination of RNA concentration

The concentration of RNA samples was determined using NanoDrop 1000 Spectrophotometer. The spectrometer determines the absorbance at 260 nm and calculates the RNA concentration using the Beer-Lambert equation: c = (A * e)/b.
In this equation c stands for the nucleic acid concentration in ng/microliter, the absorbance in AU, e is the wavelength-dependent extinction coefficient in ng-cm/microliter (for RNA: 40 ng-cm/µl) and b is the path length in cm. For the NanoDrop 1000 Spectrophotometer path lengths of 1.0 mm and 0.2 mm are used. RNase free water was used to blank the spectrophotometer before measuring the samples.
Additionally, the spectrophotometer measures the absorbance at 280 nm to calculate the ratio of sample absorbance at 260 nm and 280 nm. This ratio is used to assess the purity of DNA and RNA. For RNA, the ratio should be ideally 2.0.

4.18 Reverse Transcription (cDNA synthesis)

Reverse transcription means the conversion of template RNA into a complementary DNA (cDNA) using a reverse transcriptase. This enzyme is an RNA-dependent DNA-polymerase that generates cDNA from an RNA template. Thereby, cDNA was synthesized from 500 ng RNA using the QuantiTect® Reverse Transcription Kit (Qiagen). As a first step contaminations with genomic DNA in the RNA sample were removed by adding 2 µl Wipeout Buffer to the template RNA. RNAse-free water was

added to the sample to obtain a total volume of 14 µl. After incubating the sample for 5 min at 42°C, the sample was placed on ice and a mastermix consisting of 4 µl Quantiscript RT Buffer, 1 µl Quantiscript Reverse Transcriptase and 1 µl RT Primer Mix was added. The synthesis of cDNA was carried out by incubating the sample for 30 min at 42°C using an GeneAmp PCR System 9700 (Perkin Elmer) The reverse transcriptase was then inactivated by heating up the sample to 95°C for 3 min. The obtained cDNA was stored at -20°C.

4.19 Quantitative Real Time PCR

The quantitative real-time polymerase chain reaction (RT-PCR) is a technique that is used to amplify and simultaneously quantify a targeted DNA molecule.[377] This method is based on the binding of the cyanine dye SYBR® Green I to double-stranded DNA allowing the detection of the accumulation of the PCR product. An increase in DNA product during PCR therefore leads to an increased fluorescence intensity and is measured at each cycle, thus allowing DNA concentrations to be quantified in “real time”. The DNA-dye-complex absorbs blue light (λ_{max} = 497 nm) and emits green light (λ_{max} = 520 nm).[378] The quantitative RT-PCR was carried out using the thermal cycler CFX96 Real-Time PCR Detection System (Biorad) that illuminates and detects fluorescence from each sample with high sensitivity and no cross talk. The cycle number at the threshold level of log-based fluorescence is defined as ct-value, which is the observed value in most RT-PCR experiments.[379] The gene expression can be quantified by normalization to one or more reference genes, the so-called housekeeping genes, which are stably expressed throughout the given experiment.[380]

In this work, RNA expression levels of S100A8, S100A9 and S100A12 in monocytes isolated from different donors was further investigated. Therefore, RNA was extracted from isolated monocytes from healthy donors (n=5), patients with diarrhoea caused by infectious gastroenteritis (n=5), patients after HCT without GvHD (n=6) and patients after HCT with acute or chronic GvHD (n=7) using the RNeasy mini kit (Qiagen). First-strand cDNA was synthesized from 500 ng of total RNA using QuantiTect Reverse Transcription Kit as described in 4.18. Amplification of S100 genes was performed using KAPA Sybr Fast QPCR MasterMix for Bio-Rad iCycler

(Peqlab) and specific primers for human S100A8, S100A9 and S100A12 as proposed by Yamaoka et al.[381] 5 µl of Sybr Mix was added to 2 µl of RNase-free water, 1 µl of cDNA and 1 µl of S100 specific primer (each 5 pmol, Eurofins MWG Operon), respectively. After an initial denaturation step for 1 min at 95°C, 40 PCR cycles with 3s at 95°C and 25s at 59°C were run. For analysis, ct-values normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were calculated as $2^{(ct(GAPDH)-ct(S100))}$ as described by previously.[382] Analysis of the obtained PCR-fragments was carried out by size fractionation using 2% agarose gels.

4.20 Statistical analysis

Data were tested for statistical significance with GraphPad Prism 5. For comparing more than two samples that were independent, or not related, a one-way analysis of variance (ANOVA) was used. In the case of a normal distribution of the residuals, the One-way ANOVA was followed by Bonferroni's post-hoc test. The Kruskal-Wallis test (with Dunn's post hoc test) was used if the residuals were not normally distributed. For comparison of two independent samples, the two-tailed Student`s *t*-test was used. Values of $p<0.05$ were considered significant.

4.21 Patients and samples

Blood from 32 healthy donors was obtained from the blood bank Tuebingen in the form of buffy coats. 52 patients were recruited at the University Children´s Hospital Tuebingen and the Medical Center Tuebingen of which 35 children (67%) and 12 adults (23%) were treated with a HCT. Stem cells of HLA-matched related donors were used in 6 cases (13%), HLA-matched-unrelated donors in 19 cases (40%), HLA-haploidentical donors in 20 cases (43%) and HLA-mismatched unrelated donors in 2 cases (4%). Intensitiy of conditioning regimens were classified as described by Bacigalupo et al.[26] In this study, samples of 17 children and 6 adults undergoing HCT were examined consecutively (before conditioning, day 7, 14, 21, 30, 60 and 100 post HCT and on onset of GvHD) of which 8 children (47%) and 5 adults (83%) developed an acute GvHD. Among 2 children (12%) and 2 adults (33%) this acute GvHD was followed by a chronic GvHD. Furthermore, 2 children after HCT without

GvHD, 16 children with GvHD and 6 adults with GvHD were additionally included at the onset of GvHD. In total, 24 patients with acute GvHD, 5 patients with chronic GvHD and 6 patients with acute GvHD followed by chronic GvHD were included in this study. As controls, 5 patients with diarrhoea caused by infectious gastroenteritis were additionally included. Characteristics of these patients are shown in table 1 and 2. Approval for this study was obtained from the independent ethics committee of the University of Tuebingen [336/2011BO1]. All donors have given informed consent to participate in this study.

Table 3 Patient characteristics

Characteristic	Patients without GvHD after HCT (control group) n=12 (number in %)	Patients with GvHD after HCT n=35 (number in %)	*p*-value
Sex			
male	10 (83)	15 (43)	
female	2 (17)	20 (57)	*0.1350*
Age group in years			
<5	2 (17)	3 (9)	
5-18	9 (75)	21 (60)	
>18	1 (8)	11 (31)	*0.2556*
Disease			
Leukemia/MDS	8 (66)	23 (66)	
Lymphoma	0	4 (11)	
Solid tumors	2 (17)	3 (9)	
Non-malignant	2 (17)	5 (14)	*0.3002*
Transplantation type			
haploidentical	5 (42)	15 (43)	
matched unrelated donor	5 (41)	14 (40)	
matched related donor	2 (17)	4 (11)	
mismatched unrelated donor	0	2 (6)	*0.3141*
Conditioning regimen			
reduced intensity regimen	2 (17)	9 (26)	
myeloablative regimen	10 (83)	26 (74)	*0.3455*
GvHD prophylaxis			
ATG	9 (75)	25 (71)	
MMF	5 (42)	19 (54)	
MTX	6 (50)	11 (31)	
Ciclosporin	5 (41)	9 (26)	
Tacrolimus	2 (17)	8 (23)	
Sirolimus	0	1 (8)	
MPA	0	1 (8)	*0.0853*

MDS indicates myelodysplastic syndrome; ATG, anti-thymocyte globulin; MMF, mycophenolate mofetil; MTX, methotrexate; and MPA, mycophenolic acid

Table 4 GvHD overview

GvHD characteristic	Patients with GvHD after HCT n=35* (number in %)
Acute GvHD	
Skin	
stage I	11 (31)
stage II	9 (26)
stage III	4 (11)
stage IV	1 (8)
Gut	
stage I	5 (14)
stage II	4 (11)
stage III	3 (9)
stage IV	1 (8)
Liver	
stage I	0
stage II	1 (8)
stage III	0
stage IV	0
Acute GvHD	
grade I	14 (40)
grade II	7 (20)
grade III	8 (23)
grade IV	1 (8)
Chronic GvHD	
limited	3 (9)
extensive	8 (23)
GvHD therapy	
Steroids (Prednisolon, Urbason)	28 (80)
MMF	26 (74)
Calcineurin inhibitors (Ciclosporin, Tacrolimus)	21 (60)
Protopic, Soderm	15 (43)
TNF-blocker (Etanercept, Infliximab,	10 (28)
Phototherapy (ECP, PUVA)	7 (20)
Sirolimus	5 (14)
mAb (Basiliximab, Alemtuzumab, Toclizumab, Muromonab)	5 (14)
MSC	3 (9)
ATG	1 (8)
Azathioprin	1 (8)

MMF indicates mycophenolate mofetil; ECP, extracorporeal photopheresis; PUVA, psoralen and ultraviolet A irradiation; MSC, mesenchymal stem cells; and ATG, anti-thymocyte globulin

***** 35 patients with GvHD include 24 patients with acute GvHD, 5 patients with chronic GvHD and 6 patients with acute GvHD followed by chronic GvHD

5 Results

5.1 Monocyte-induced Th17 development in healthy donors, patients with and without GvHD after HCT

Like rheumatoid arthritis, GvHD is an inflammatory process which is driven by the release of TNFα, IL-1 and IL-6 and the activation of APCs leading to tissue damage.[36,383-385] It is known that *in vivo* activated monocytes from the site of inflammation in humans specifically promote Th17 responses during rheumatoid arthritis.[386] Thus, the influence of monocytes on the induction of Th17-, Th1- and Th17/Th1 cells in patients with acute or chronic GvHD, patients without GvHD and healthy donors was investigated. Monocytes were isolated from the peripheral blood of 9 patients with acute GvHD grade I-IV, 4 patients with chronic GvHD (2 patients with extensive chronic GvHD, 2 patients with limited chronic GvHD), 20 patients before conditioning, 21 patients at day 30, 60 and 100 post HCT (+/- 10 days) without GvHD and 32 healthy donors. These monocytes were co-cultured with anti-CD3 mAb Orthoclone OKT3 (100 ng/ml) and $CD4^+$ T cells isolated from the peripheral blood of healthy donors at a monocyte:T cell ratio of 1:4 for 5 days. Non-adherent $CD4^+$ T cells were harvested and the percentage of induced IL-17^+, IL-17^+IFNγ^+ and IFNγ^+ cells was assessed by flow cytometry. For analysis, total proliferating $CD4^+$ T cells were gated (gate R1) and the percentages of induced Th17, Th17/Th1 and Th1 cells were determined (Figure 4A). Representative density plots are shown in figure 4A. The data represented in figure 4A-C demonstrate that monocytes isolated from the peripheral blood from patients with acute GvHD and patients with chronic GvHD induced significant higher levels of IL-17^+ cells and IL-17^+IFNγ^+ cells compared to monocytes isolated from patients without GvHD after HCT and healthy donors (**$p<0.01$) (Figure 4A,B). Monocytes from patients with GvHD induced also elevated levels of Th1 cells (**$p<0.01$). However, the increase was lower compared to Th17- and Th17/Th1 cells (Figure 4A, D). Comparing the induced percentages of IL-17^+-, IL-17^+/IFNγ^+-, IFNγ^+-cells, it could be demonstrated that monocytes isolated from patients with GvHD have the strongest effects *in vitro* on the induction of Th17 cells.

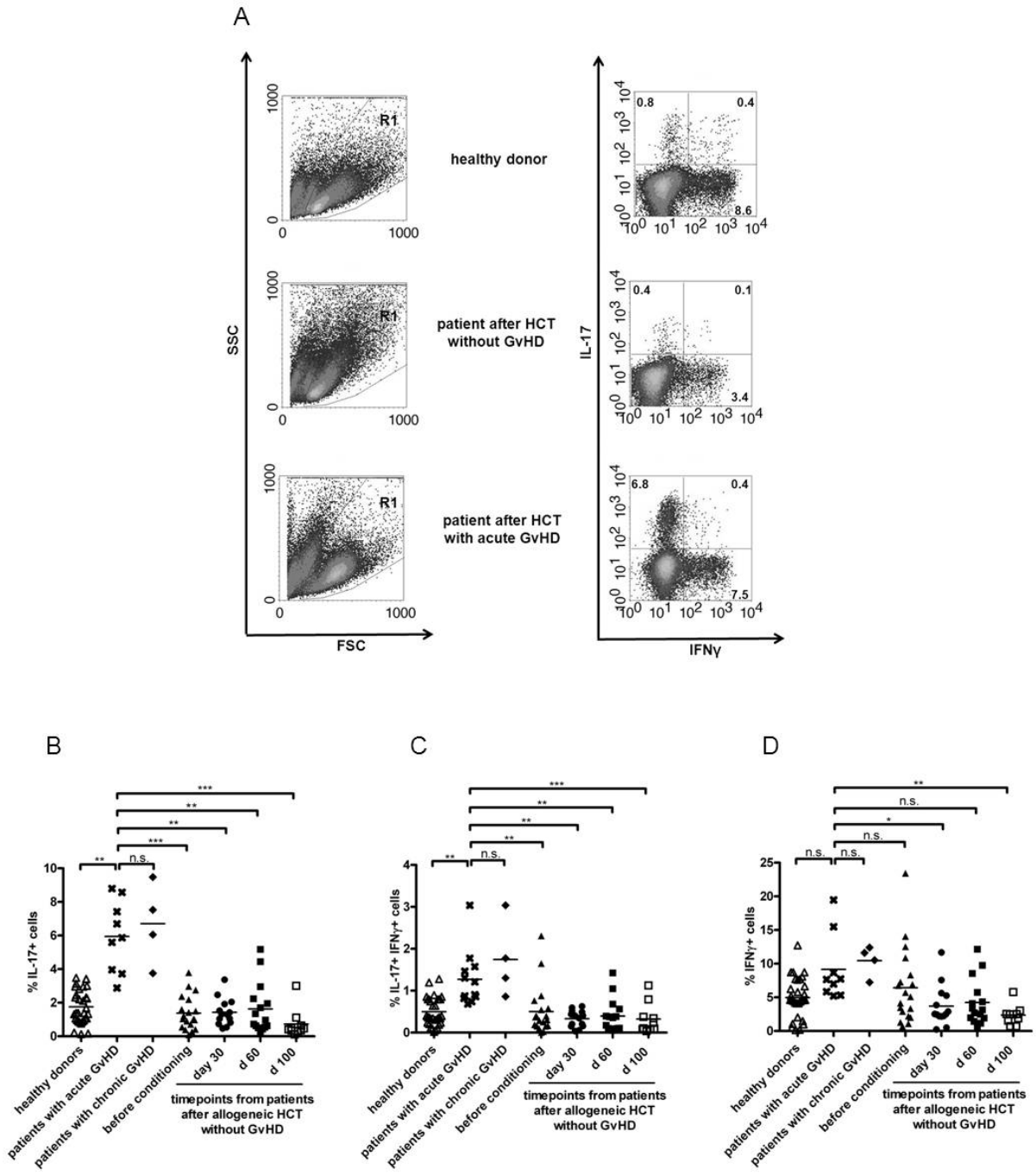


Figure 4: Percentage of IL-17$^+$, IL17$^+$INFγ$^+$ and IFNγ$^+$ cells induced by monocytes isolated from patients with acute or chronic GvHD, without GvHD after HCT and healthy donors

CD4$^+$ T cells from healthy donors were co-cultured with anti-CD3 mAb Orthoclone OKT3 (100 ng/ml) and monocytes from healthy donors, from patients before conditioning, patients without GvHD on day 30, 60 and 100 (+/- 10 days) post HCT and patients with acute and chronic GvHD at a monocyte:T cell ratio of 1:4 for 5 days. After 5 days of co-culture, cells were stimulated for 5 h with PMA (50 ng/ml) and ionomycin (750 ng/ml) in the presence of monensin (2 µM) during the last 3 h and flow cytometric analysis was performed. (A) Density plots representing Th17, Th17/Th1 and Th1 cells induced by monocytes from healthy donors, patients with GvHD and patients after HCT without GvHD. The dot plots represent the determined percentages of monocyte-induced (B) IL-17$^+$ cells, (C) IL-17$^+$IFNγ$^+$

cells and (D) IFNγ$^+$ cells in the included healthy donors, patients without GvHD and patients with acute or chronic GvHD. Statistical significance was determined using Kruskal-Wallis Test (with Dunn's post hoc test): *p<0.05; **p<0.01; ***p<0.001. Reinhardt et al.[387]

5.2 Progression of Th17 induction in patients with and without GvHD after HCT

It could be demonstrated that monocytes from patients with GvHD induced significantly elevated levels of Th17 cells compared to healthy donors and patients without GvHD (Figure 4A). To emphasize these data, the progression of monocyte-mediated Th17 induction after HCT was analysed in patients with and without GvHD. The results of one patient with acute and chronic GvHD and one patient with no GvHD after HCT are exemplary represented in figure 5A and 5B. Monocytes isolated from these patients before conditioning induced low percentages of IL-17$^+$ cells. At the onset of acute GvHD increased levels of Th17 cells were induced. After abatement of acute GvHD, monocyte-induced Th17 levels decreased and increased after clinical onset of the limited chronic GvHD (Figure 5A). On the contrary, monocytes isolated from the patient without GvHD at day 30, 60 and 100 (+/- 10 days) after HCT induced almost consistent low levels of IL-17$^+$ cells (Figure 5B).

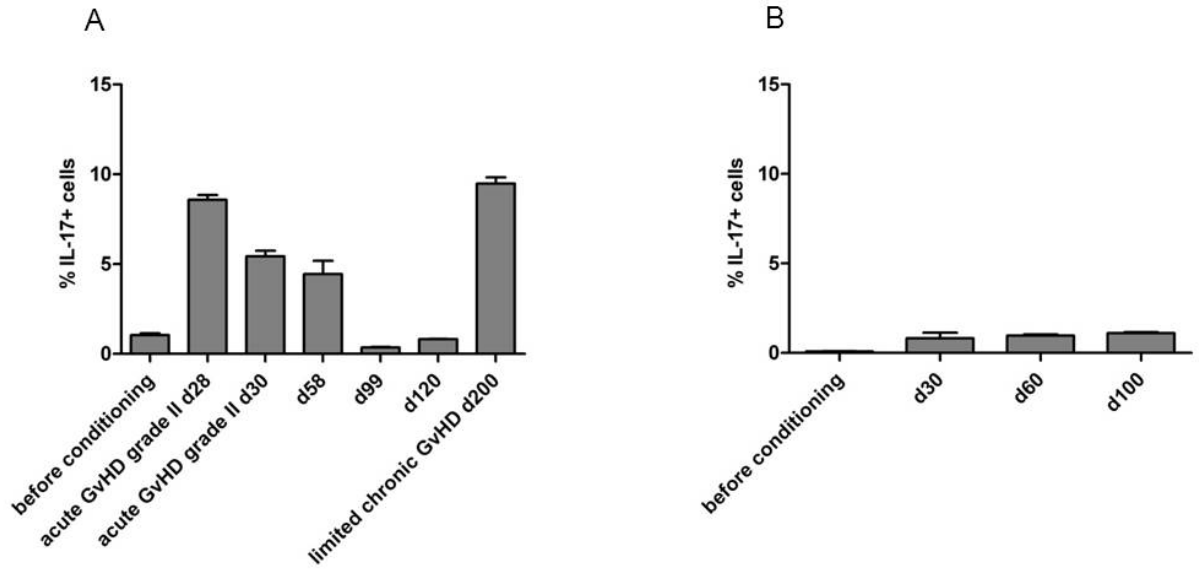


Figure 5: Progression of monocyte-induced Th17 induction in one patient with acute and chronic GvHD and one patient with no GvHD after HCT

CD4$^+$ T cells were isolated from a healthy donor were co-cultured with monocytes isolated from (A) one patient who developed an acute and chronic GvHD after HCT and (B) from another patient without GvHD after HCT at the indicated time points after HCT in the presence of anti-CD3 mAb Orthoclone

OKT3 (100 ng/ml) at a monocyte:T cell ratio of 1:4 for 5 days. After 5 days of co-culture, cells were restimulated with PMA/ionomycin, stained for IL-17 expression and analysed by flow cytometry. Bars represent the mean values of duplicates +SEM.

5.3 Level of Treg cells and Th17/Treg ratios in the peripheral blood of healthy donors, patients with and without GvHD after HCT

Several studies suggest using the assessed percentage of regulatory T cells and the ratio of Th17 cells to regulatory T cells in the peripheral blood of patients as markers for the development of GvHD.[78,346,388-390] Thus, beside the monocyte-induced percentage of Th17 cells (Figure 6A) the percentage of regulatory T (Treg) cells within the PBMCs isolated from 20 healthy donors, 3 patients with acute GvHD grade I and II, 2 patients with extensive chronic GvHD and 21 patients before conditioning and at day 30, 60 and 100 post HCT (+/- 10 days) was determined by flow cytometry using the characteristic markers CD3, CD4, CD25 and FoxP3 (Figure 6B). As the percentage of $CD3^+CD4^+$ T cells in the peripheral blood of patients after HCT is relatively low a cut-off point of 1000 gated $CD3^+CD4^+$ T cells was defined for the determination of the $CD25^+FoxP3^+$ subpopulation. The determined percentages of $CD3^+CD4^+CD25^+FoxP3^+$ T cells in PBMCs isolated from patients with acute GvHD (n=3) did not differ from the determined percentages of Treg cells assessed in PBMCs of healthy donors (n=20), patients before conditioning (n=18) and patients without GvHD on day 30 (n=7), day 60 (n=8) and day 100 (n=6) (+/- 10 days) after HCT. On the contrary, the percentages of Treg cells in PBMCs from patients with chronic extensive GvHD (n=2) were increased compared to healthy donors, patients with acute GvHD, patients before conditioning and patients without GvHD after HCT on the indicated time points. However, the sample size of patients with chronic GvHD is too low to perform a statistical evaluation. Additionally, the ratio of Th17 cells to Treg cells was determined. The data in figure 6C demonstrate that there was no statistically significant difference between the calculated Th17/Treg ratios of healthy donors, patients with acute or chronic GvHD, patients before conditioning and patients without GvHD at day 30, 60 and 100 (+/- 10 days) after HCT.

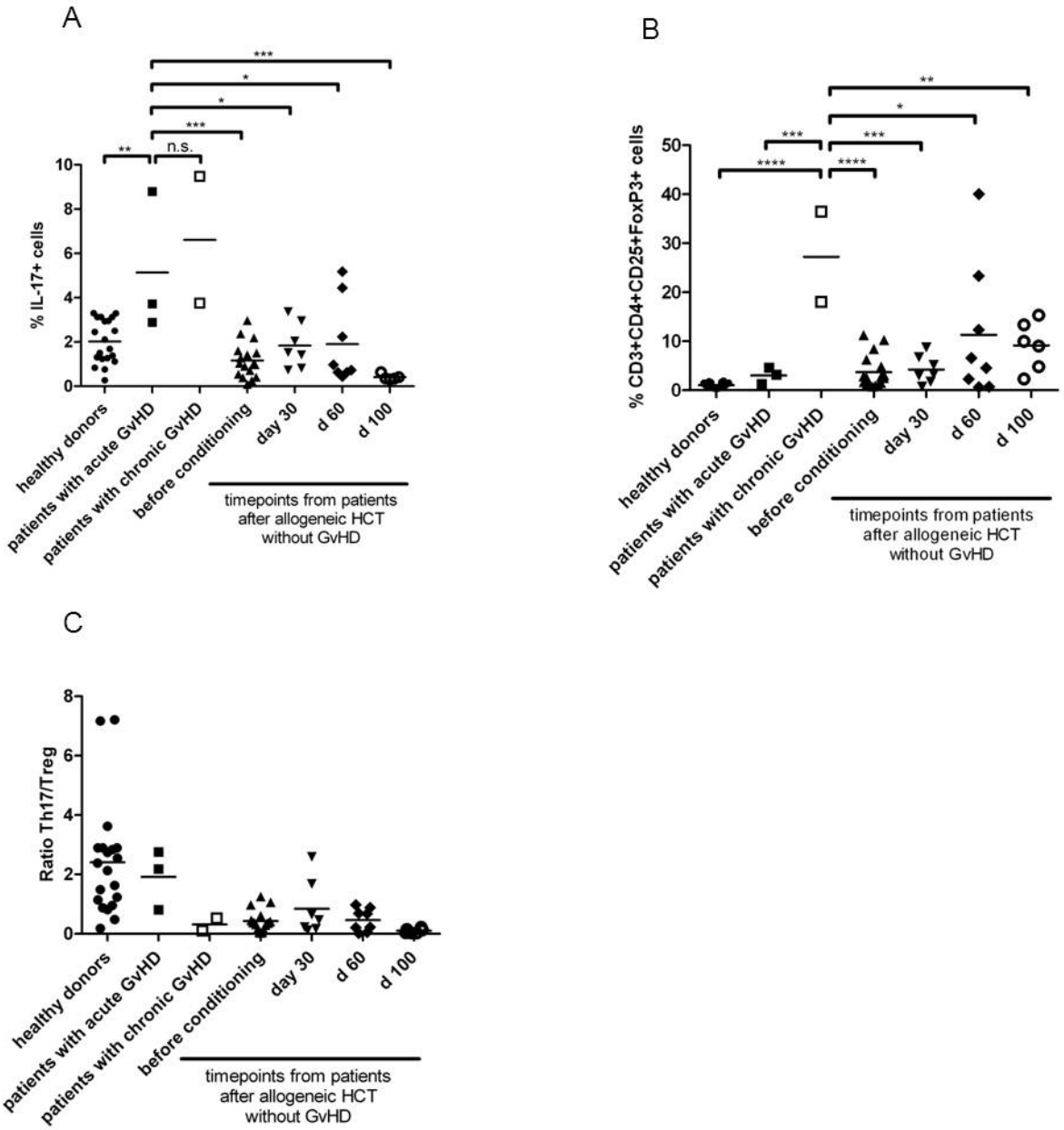


Figure 6: Determined percentages of monocyte-induced Th17 cells, Treg cells in total PBMCs and Th17/Treg ratio in patients with acute or chronic GvHD, without GvHD after HCT and healthy donors

(A) $CD4^+$ T cells from healthy donors were co-cultured with anti-CD3 mAb Orthoclone OKT3 (100 ng/ml) and monocytes from healthy donors, from patients before conditioning, patients without GvHD on day 30, 60 and 100 (+/- 10 days) post HCT and patients with acute and chronic GvHD at a monocyte:T cell ratio of 1:4 for 5 days. After 5 days of co-culture, cells were restimulated with PMA/ionomycin and stained for IL-17 expression. (B) The percentage of regulatory T cells in total PBMCs was determined by flow cytometry using antibodies specific for CD3, CD4, CD25 and FoxP3. (C) The ratios of Th17 cells to regulatory T cells of healthy donors, patients with acute or chronic GvHD, patients before conditioning and patients without GvHD at day 30, 60 and 100 (+/- 10 days) after HCT were calculated.

5.4 Phenotypical analysis of monocytes at the onset of acute GvHD

As monocytes isolated from patients with acute or chronic GvHD induced elevated levels of Th17 cells, the expression pattern of several activation markers on monocytes was analysed. After isolating PBMCs from patients before conditioning, patients at the onset of acute GvHD and patients without GvHD at day 30, 60 and 100 post HCT (+/- 10 days), monocytes were isolated by magnetic cell separation. Monocytes from 11 patients without GvHD and from 10 patients at the onset of acute GvHD were investigated by flow cytometry regarding the expression of CD86, CD80, HLA-DR, CD16, CX3CR1, CD11b, CD11c, CD54. Expression levels of the activation markers CD86, CD80, HLA-DR, CD16, CD11b, CD11c and CD54 in monocytes isolated from patients at the onset of acute GvHD did not differ from the detected expression levels in patients without GvHD (Figure 7A-G). By contrast, the percentage of $CD14^{+}CX3CR1^{+}$ cells was decreased in monocytes isolated from patients at the onset of acute GvHD compared to monocytes isolated from patients before conditioning and patients without GvHD after HCT (Figure 7H).

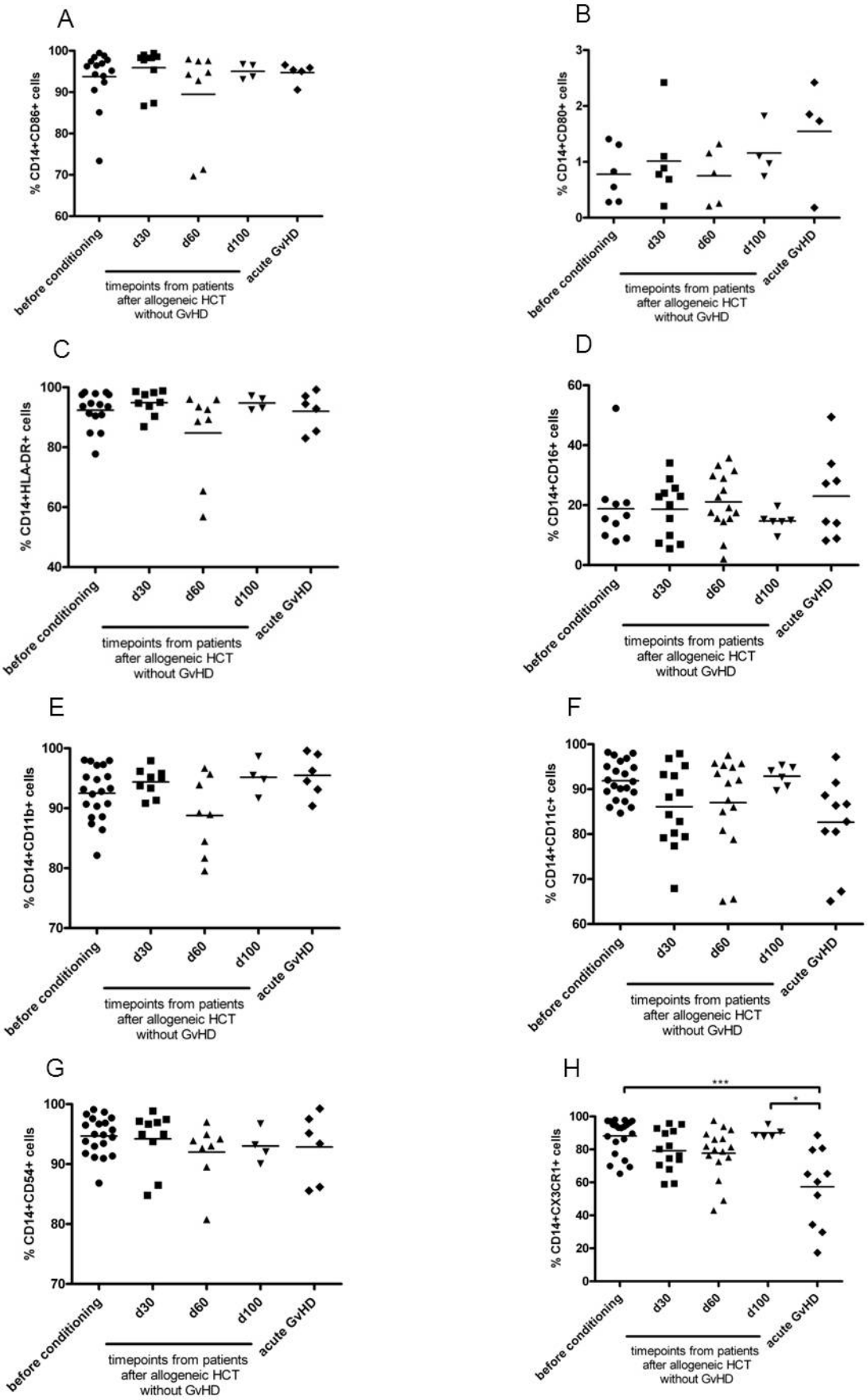

A
% CD14+CD86+ cells
100
90
80
70
60
before conditioning
d30
d60
d100
acute GvHD
timepoints from patients after allogeneic HCT without GvHD
B
% CD14+CD80+ cells
3
2
1
0
C
% CD14+HLA-DR+ cells
40
D
% CD14+CD16+ cells
E
% CD14+CD11b+ cells
F
% CD14+CD11c+ cells
G
% CD14+CD54+ cells
H
% CD14+CX3CR1+ cells
***
*

Figure 7: Expression levels of activation markers on monocytes isolated from patients before conditioning, at the onset of acute GvHD and without GvHD at day 30, 60 and 100 post HCT (+/- 10 days)

Monocytes were isolated from total PBMCs from patients before conditioning, at the onset of acute GvHD and without GvHD at day 30, 60 and 100 post HCT (+/- 10 days) by magnetic cell sorting. Monocytes were analysed by flow cytometry using antibodies specific for (A) CD86, (B) CD80, (C) HLA-DR, (D) CD16, (E) CD11b, (F) CD11c, (G) CD54 and (H) CX3CR1. Statistical significance was determined using Kruskal-Wallis Test (with Dunn's post hoc test): *p<0.05; ***p<0.001.

5.5 S100 expression in monocytes from healthy controls, patients with diarrhoea, and patients with or without GvHD after HCT

A recent study has demonstrated that peripheral blood mRNA levels of proinflammatory S100 proteins are closely associated with inflammation.[381] Additionally, several works have shown that S100A8/S100A9 and S100A12 are over-expressed during chronic active inflammatory bowel disease and act as inflammation markers.[139,153,371] Like inflammatory bowel disease, GvHD is induced and promoted by several complex immune responses consisting of several inflammatory mediators leading to tissue necrosis.[36,391] Therefore, RT-PCR analysis was carried out investigating expression levels of S100A8, S100A9 and S100A12 in monocytes isolated from patients without GvHD and patients with acute or chronic GvHD. As further controls healthy donors and patients with diarrhoea of other causes were included. Initially, amplification products of five healthy donors were loaded onto a 2% agarose gel following RT-PCR. The estimated size of the amplification product in the gel corresponded with the expected calculated sizes (Figure 8A). The formation of primerdimers during RT-PCR could be excluded as only a single band for the appropriate amplification product could be detected. For RT-PCR analysis, ct-values of S100A8, S100A9 and S100A12 were normalized to the housekeeping gene GAPDH. As shown in figure 8B-D, normalized ct-values of S100A8, S100A9 and S100A12 from patients with GvHD (4 patients with acute GvHD grade I, 1 patient with acute GvHD grade II, 1 patient with acute GvHD grade III and 1 patient with extensive chronic GvHD) (n=7) were increased compared to healthy donors (n=5), patients with diarrhoea caused by infectious gastroenteritis (n=5) and patients after HCT without GvHD (n=6). Highest expression levels of S100A8, S100A9 and S100A12 could be detected in monocytes of patients with GvHD. However, S100

expression levels were also elevated in monocytes of patients without GvHD after HCT and patients with diarrhoea compared to healthy donors. While ct-values of S100A8 from patients with GvHD only differed significantly from the ct-values of healthy donors ($p<0.01$), S100A9 ct-values from patients with GvHD were significantly increased compared to the corresponding ct-values of healthy donors, patients without GvHD after HCT and patients with diarrhoea ($p<0.001$). Ct-values of S100A12 from patients with GvHD were significantly elevated compared to healthy donors and patients with diarrhoea ($p<0.05$). Averaged ct-values for S100A8, S100A9 and S100A12 were increased 13.8-, 5- and 3.2-fold higher in patients with GvHD compared to healthy donors. Equally, averaged ct-values for S100A8, S100A9 and S100A12 were 2.9-, 2.8- and 2-fold higher in patients with GvHD compared to patients after HCT without GvHD. A 2.6-, 2- and 1.9-fold increase could be determined comparing ct-values for S100A8, S100A9 and S100A12 in patients with GvHD with corresponding ct-values in patients with diarrhoea (Figure 8B-D).

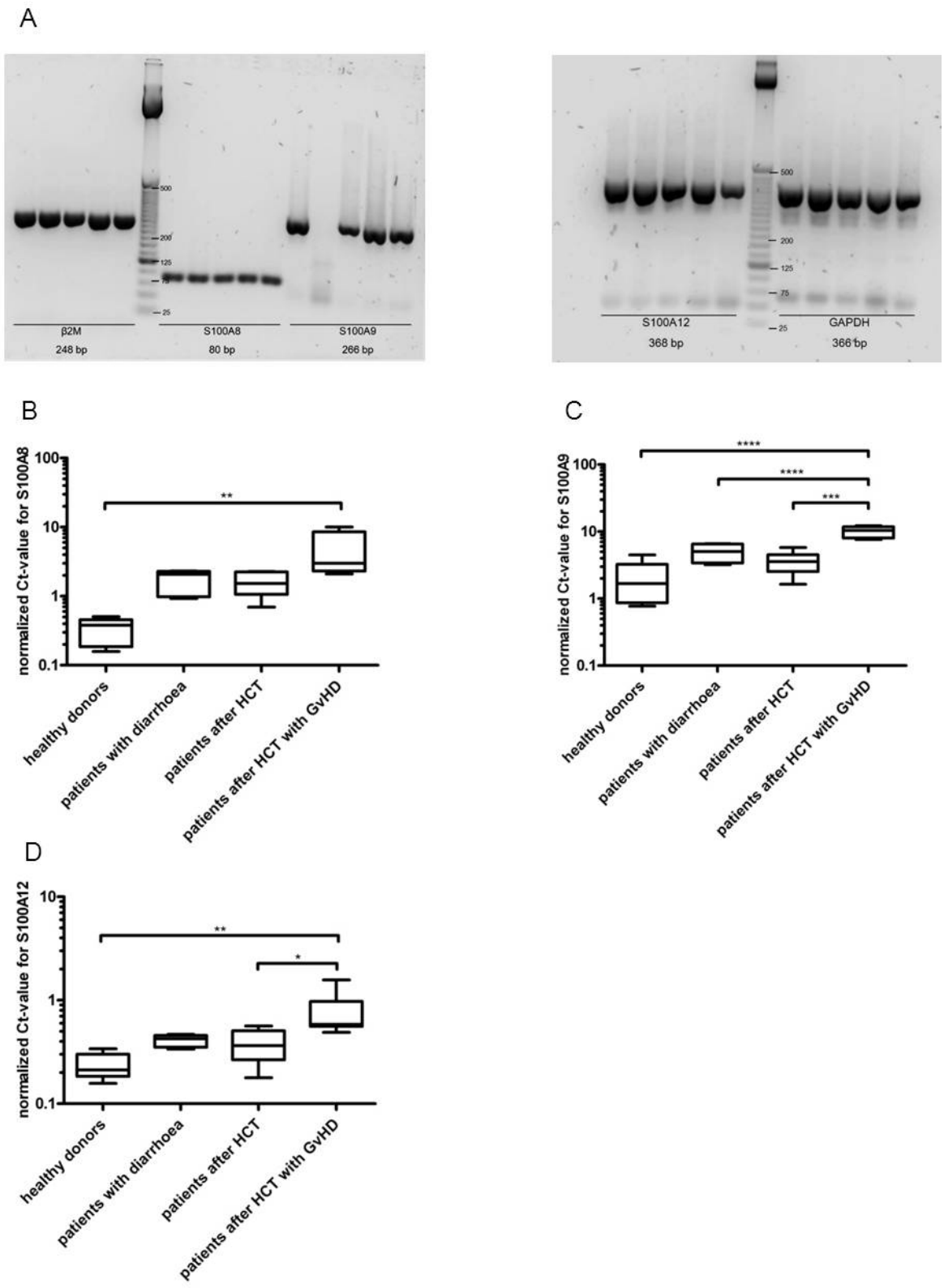


Figure 8: Normalized ct-values for S100A8, S100A9 and S100A12 from monocytes isolated from healthy donors, patients with diarrhoea, patients after HCT without GvHD and patients with acute or chronic GvHD

Real-time PCR analysis was carried out using genomic DNA generated from monocytes isolated from healthy donors (n=5), patients with diarrhoea caused by infectious gastroenteritis (n=5), patients after HCT without GvHD and no diarrhoea (n=6) and patients with acute or extensive chronic GvHD (n=7).

(A) Amplification products of five healthy donors were loaded onto a 2% agarose gel following RT-PCR using the primers for S100A8, S100A9, S100A12 and the housekeeping gene β2 microglobulin (β2M) and GAPDH. Ct-values for (B) S100A8, (C) S100A9 and (D) S100A12 were normalized to the housekeeping gene GAPDH. Statistical significance was determined using One-way ANOVA followed by Bonferroni's post-hoc test: *p<0.05; **p<0.01;***p<0.001; ****p<0.0001. Reinhardt et al.[387]

5.6 Comparison of S100 levels in the stool and serum in patients with or without GvHD

Several studies have demonstrated that S100A8/S100A9 and S100A12 can act as inflammatory markers in the serum and stool for diseases like inflammatory bowel disease or rheumatoid arthritis.[120,139,153,371,392] Furthermore, it has been shown previously that fecal S100A8/S100A9 levels are elevated in patients with intestinal GvHD.[154,155] Thus, S100 levels were determined in the stool and serum of patients by sandwich ELISA as described in 4.14. For analysis, stool and serum samples were collected consecutively from patients on day 7, 14, 21, 30, 60 and 100 (+/- 5 days) post HCT. Stool and serum samples were collected immediately at the onset of GvHD and in the further progression of GvHD every 2 to 14 days depending on the duration of GvHD. Data points from the same patients are highlighted in one colour in figure 9. S100 concentrations in stool samples of 9 patients with intestinal GvHD (1 patient with acute intestinal GvHD stage I, 1 patient with acute intestinal GvHD stage II, 3 patients with acute intestinal GvHD stage III and 4 patients with extensive chronic GvHD) and 9 patients without GvHD were compared. The data in figure 9A and 9B represent that S100A8/S100A9 and S100A12 levels were significantly increased in fecal samples of patients with acute intestinal GvHD (n=8) and also in the stool of patients with extensive chronic GvHD (n=4) compared to stool samples of patients without GvHD (n=24) ($p<0.01$). Furthermore, determined S100 concentrations in the stool were correlated with the severity of intestinal GvHD. As shown in figure 9C and 9D, fecal S100 levels seem to increase with the severity of intestinal GvHD.

In addition to the determined S100 levels in the stool, S100A8/S100A9 and S100A12 concentrations were detected in the serum of 11 patients with acute or chronic GvHD (5 patients with acute GvHD grade I-IV, 2 patients with acute GvHD grade I/III and limited chronic GvHD, 2 patients with acute GvHD grade II/III and extensive chronic

GvHD, 2 patients with extensive chronic disease) and 14 patients after HCT without GvHD at the indicated time points after HCT. S100A8/S100A9 concentrations in serum samples of patients with acute GvHD (n=34) were significantly elevated compared to serum concentrations of samples of patients without GvHD (n=62) ($p<0.01$) (Figure 9E). In patients with chronic GvHD serum levels of S100A8/S100A9 were not increased in 8 samples compared to patients without GvHD post HCT. However, the sample size of patients with chronic GvHD in this experiment was lower compared to the one of patients with acute GvHD. Similar results were obtained for S100A12 levels in the serum. S100A12 concentrations in serum samples of patients with acute GvHD (n=33) were significantly increased compared to patients without GvHD post HCT (n=53) ($p<0.05$). Serum levels of S100A12 did not differ in samples of patients with chronic GvHD (n=8) and patients without GvHD (Figure 9F). S100 concentrations in the serum were also correlated with the grade of GvHD. On the contrary to fecal S100 levels, levels in the serum did not correlate with the severity of GvHD (Figure 9G,H).

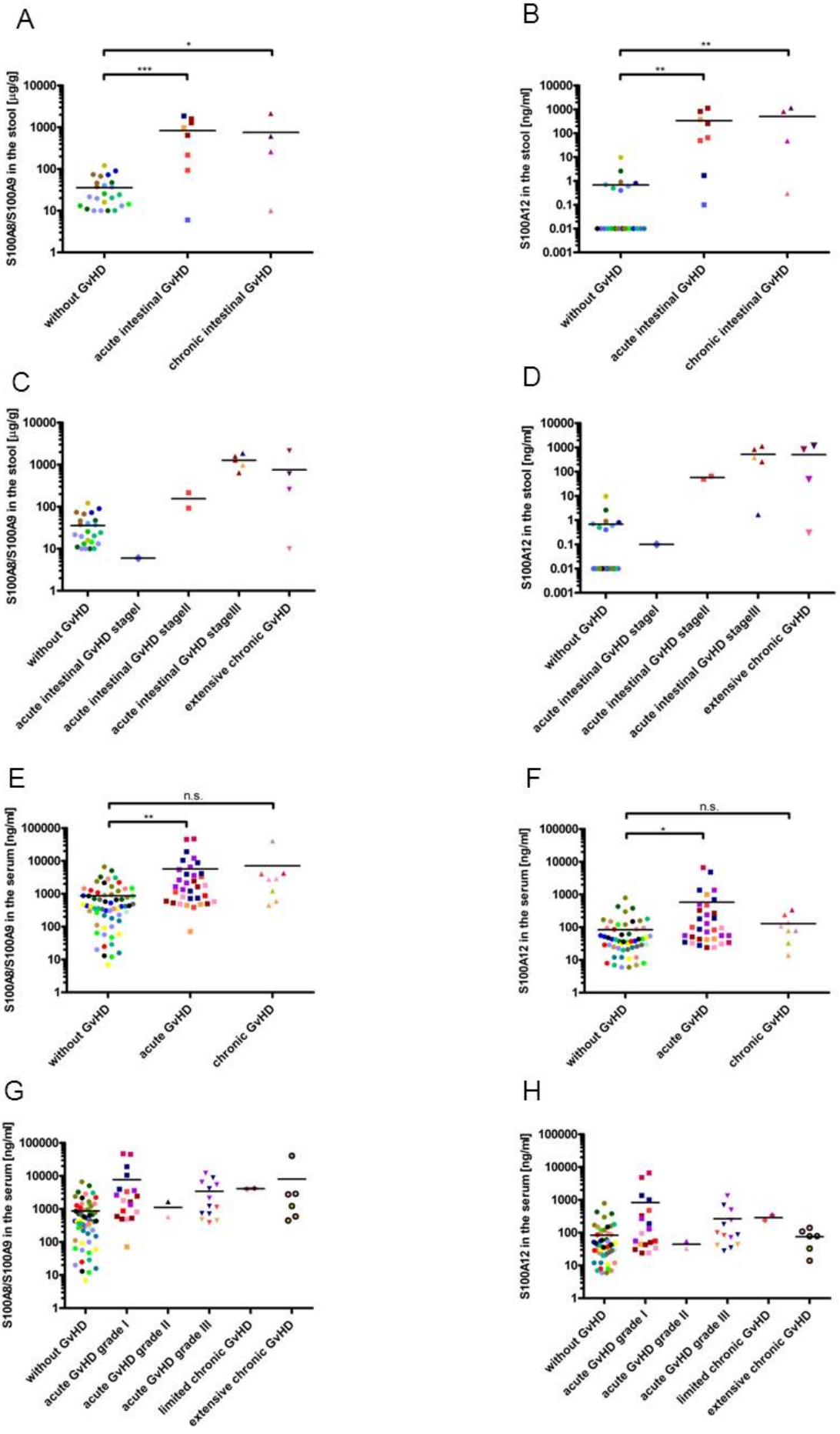

A
S100A8/S100A9 in the stool [µg/g]
without GvHD
acute intestinal GvHD
chronic intestinal GvHD
B
S100A12 in the stool [ng/ml]
C
without GvHD
acute intestinal GvHD stageI
acute intestinal GvHD stageII
acute intestinal GvHD stageIII
extensive chronic GvHD
D
E
S100A8/S100A9 in the serum [ng/ml]
n.s.
acute GvHD
chronic GvHD
F
S100A12 in the serum [ng/ml]
G
acute GvHD grade I
acute GvHD grade II
acute GvHD grade III
limited chronic GvHD
H

Figure 9: S100 concentrations in the stool and serum of patients with acute or chronic GvHD and patients without GvHD after HCT

Stool and serum samples were collected from the same patients on day 7, 14, 21, 30, 60 and 100 (+/- 5 days) post HCT. Data points from the same patient are highlighted in the same colour. At the onset of GvHD, stool and serum samples were collected immediately and in the further progression of GvHD every 2 to 14 days. (A) Fecal S100A8/S100A9 and (B) S100A12 were determined in the stool of patients with acute intestinal and extensive chronic GvHD and patients without GvHD by Calprotectin-Assay or double sandwich ELISA. (C) S100A8/S100A9 and (D) S100A12 levels in the stool were correlated with the severity intestinal GvHD. (E) S100A8/S100A9 and (F) S100A12 concentrations were determined in the serum of patients with acute skin or intestinal GvHD and patients with limited and extensive chronic GvHD and patients without GvHD by double-sandwich ELISA. Serum concentrations of (G) S100A8/S100A9 and (H) S100A12 were correlated with GvHD grade. Statistical significance was determined using student`s unpaired t-test: *p<0.05; **p<0.01; ***p<0.001. Reinhardt et al.[387]

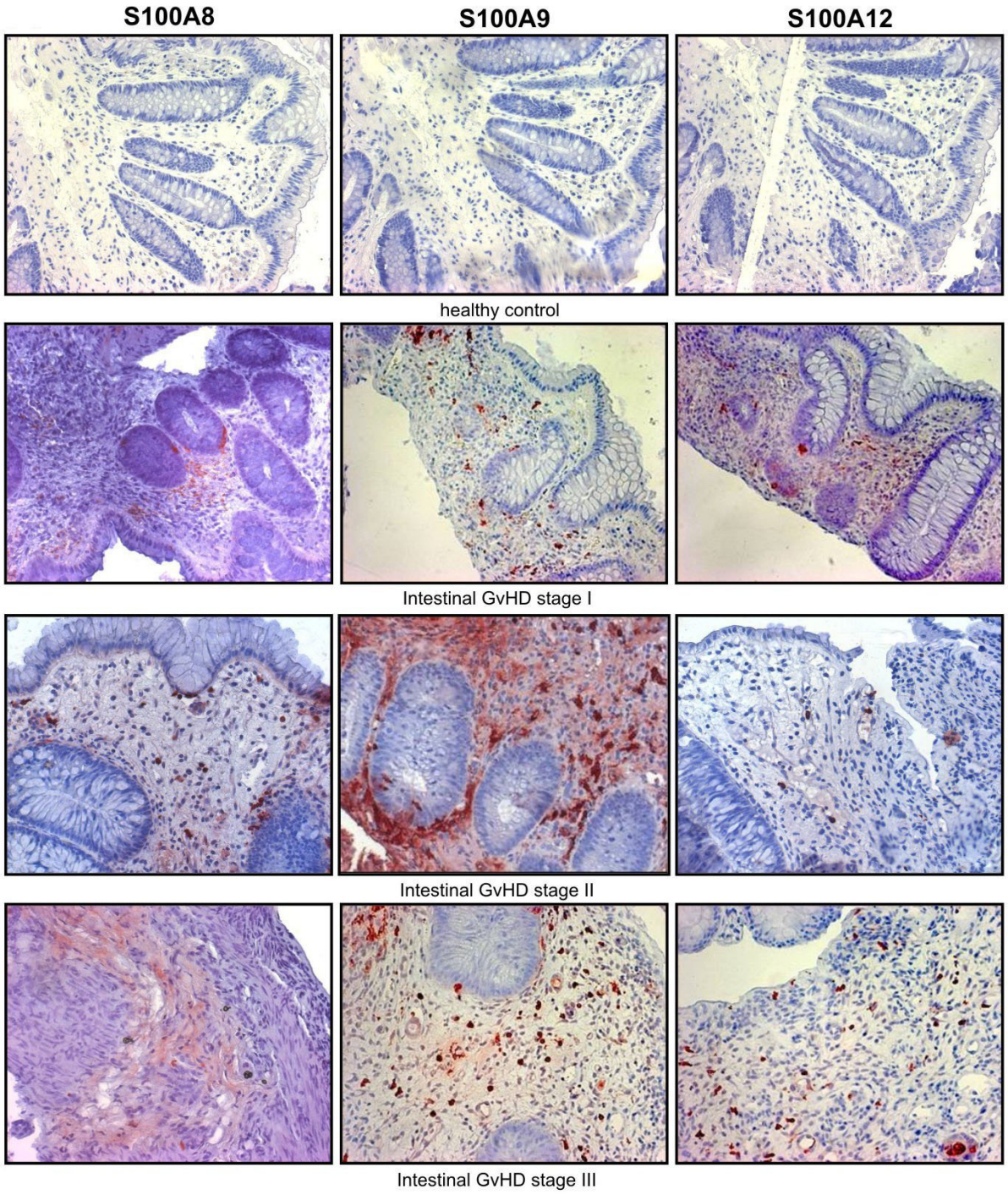
5.7 Immunohistochemical S100-staining in bowel tissue from patients with acute intestinal GvHD and healthy controls

Previous experiments have shown that S100 levels in the serum and stool of patients with acute GvHD were elevated compared to patients without GvHD past HCT. Thus, the question was addressed whether proinflammatory S100 proteins are also present in inflamed bowel tissue of patients with acute intestinal GvHD. Therefore, immunohistochemical staining of S100A8, S100A9 and S100A12 in intestinal biopsies of healthy controls with no pathological findings (n=2), patients with acute intestinal GvHD stage I (n=3), stage II (n=3) and stage III (n=3) was performed.

Representative image sets of stained sections of bowel tissue from individual healthy donors and patients with different stages of acute intestinal GvHD are shown in figure 10A. In bowel tissue from patients with acute intestinal GvHD stage I, II and III, S100A8, S100A9 or S100A12 were present in an extracellular distribution surrounding S100A8-, S100A9- or S100A12-positive cells. Percentages of S100A8, S100A9 or S100A12 stained areas in individual sections were determined using ImageJ. Overall, determined percentages of S100A8, S100A9, and S100A12 stained areas were higher in sections of bowel tissue from patients with acute intestinal GvHD stage II-III compared to the patients with acute intestinal GvHD stage I (Figure 10B-D). Significant differences could be detected comparing the S100A8 stained areas in bowel tissue of patients with acute intestinal GvHD stage II-III with the

stained areas in bowel specimens of patients with acute intestinal GvHD stage I or healthy controls ($p<0.01$) (Figure 10B). Furthermore, the percentages of S100A12 stained areas in bowel specimens of patients with intestinal GvHD stage II-III were significantly increased ($p<0.05$) in comparison to the healthy controls (Figure 10D) whereas no statistical significance could be determined in S100A9 stained areas (Figure 10C). In intestinal tissue of healthy controls hardly any S100 staining was detected (Figure 10A-D).

A


S100A8
S100A9
S100A12
healthy control
Intestinal GvHD stage I
Intestinal GvHD stage II
Intestinal GvHD stage III

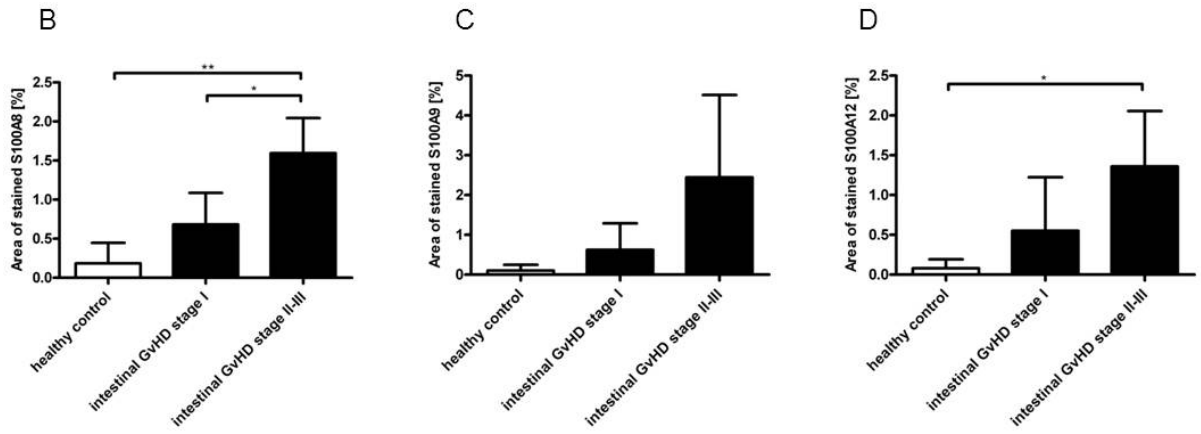


Figure 10: Immunohistochemical staining of S100A8, S100A9 and S100A12 in bowel tissue from patients with acute intestinal GvHD

S100A8, S100A9 and S100A12 were detected in bowel specimens from patients with acute intestinal GvHD stage I, II and III and healthy controls with no pathological findings by specific antibodies conjugated to horseradish peroxidase (1 µg/ml) and following reaction with the substrate Substrat AEC (3-Amino-9-ethyl-carbazole) leading to the characteristic brown colouring. Cell nuclei were stained with haematoxylin (blue). Images were captured using a Zeiss Axioskop connected to an Axiocam camera supplied with software Axiovision 3.0 (original magnification x200 for all images). (A) Image sets represent stained sections of bowel tissue from individual healthy controls and patients with acute intestinal GvHD stage I, II or III. Data bars represent the determined percentages of (B) S100A8, (C) S100A9 or (D) S100A12 stained areas + SD in sections of bowel tissue from healthy controls (n=2), patients with acute intestinal GvHD stage I (n=3), stage II (n=3) and stage III (n=3). Data analysis was performed using ImageJ. Statistical significance was determined using unpaired, two-tailed student`s t-test: *$p<0.05$; **$p<0.01$. Reinhardt et al.[387]

5.8 Influence of S100 proteins on monocyte-induced Th17 development

So far, the data of this work have demonstrated that monocytes isolated from the peripheral blood of patients with GvHD induce increased levels of IL-17$^+$ cells and express elevated levels of the proinflammatory proteins S100A8, S100A9 and S100A12. Additionally, increased concentrations of S100 proteins could be detected in the serum, stool and bowel tissue of patients with GvHD. As a previous study has demonstrated that the TLR4 ligand LPS induces the activation of monocytes followed by the induction of elevated levels of IL-17$^+$ cells[393], it was investigated if the TLR4 ligands S100A8, S100A9 and S100A12 have similar effects on monocyte-induced development of Th17 cells. Therefore, monocytes isolated from healthy donors were pre-stimulated with S100 proteins for 4 h before co-culturing with CD4$^+$ T cells from

healthy donors. The results in figure 11A and 11B demonstrate that stimulation of monocytes with the TLR4 ligands S100A8, S100A9, the heterodimer S100A8/S100A9 or S100A12 induced significant higher percentages of IL-17$^+$ cells compared to unstimulated monocytes ($p<0.05$). Stimulation of monocytes with S100 proteins resulted in induced Th17 levels similar to the ones induced by monocytes from patients with GvHD (S100A8: 7.5±1.5; S100A9: 7.2±1.8; S100A8/S100A9: 6.5±1.7; S100A12: 4.7±1.2; GvHD: 6.2±2.2) (Figure 4A,B and 11A,B). Furthermore, monocytes stimulated with S100A8 or S100A9 induced significantly increased percentages of Th17/Th1 cells ($p<0.05$). The heterodimer and S100A12 showed weaker stimulatory effects on monocyte-induced development of IL-17$^+$IFNγ$^+$ cells compared to S100A8 and S100A9. Overall, the stimulatory effects of S100 proteins on the induction of Th17/Th1 cells were less pronounced compared to Th17 cells (Figure 11A,C). Stimulation of monocytes with S100 proteins did not show any effect on Th1 cells (Figure 11A,D).

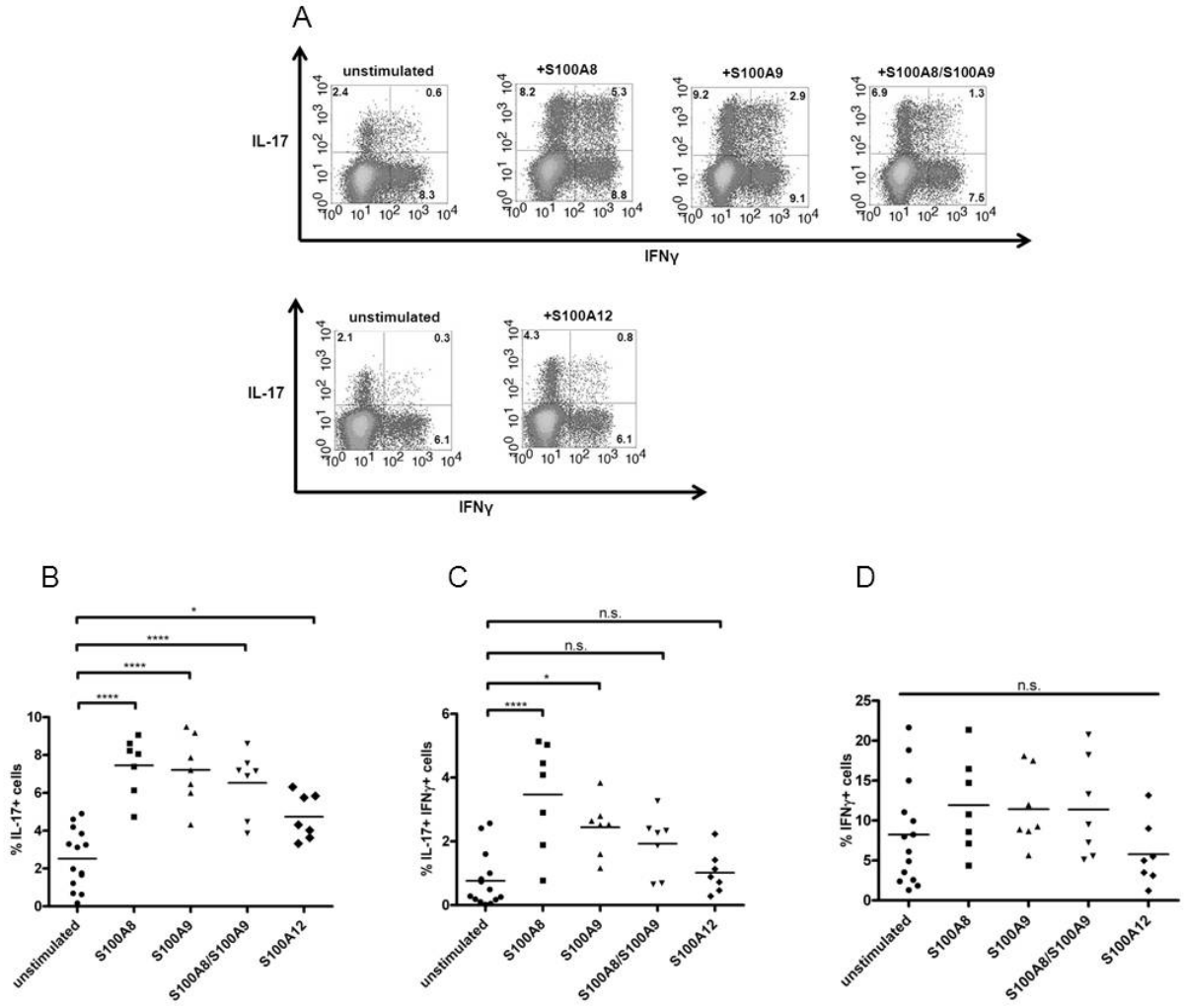


Figure 11: Influence of stimulation of monocytes with S100 proteins on the induction of Th17 cells

$CD4^+$ T cells from healthy donors were co-cultured with anti-CD3 mAb (100 ng/ml) and monocytes from healthy donors at a monocyte:T cell ratio of 1:4 for 5 days. Monocytes remained unstimulated or were additionally stimulated with S100A8, S100A9, S100A8/S100A9 or S100A12 (5 µg/ml) for 4 h before co-culturing with $CD4^+$ T cells. After 5 days of co-culture, cells were restimulated with PMA/ionomycin and flow cytometric analysis was performed. (A) Representative density plots demonstrating the effect of S100 proteins on the induction of Th17, Th17/Th1 and Th1 cells. Percentages of monocyte-induced (B) $IL\text{-}17^+$ cells, (C) $IL\text{-}17^+IFN\gamma^+$ cells and (D) $IFN\gamma^+$ cells after stimulation with S100 proteins in healthy donors were determined by flow cytometry. Statistical significance was determined using One-way ANOVA followed by Bonferroni's post-hoc test: *p<0.05; ****p<0.0001. Reinhardt et al.[387]

5.9 Effect of TLR4 blockade prior to stimulation of monocytes with S100 proteins on Th17 development

Several studies have demonstrated that S100 proteins are specific ligands of TLR4 and induce the translocation of MyD88 from the cytosol to the receptor complex at the plasma membrane and the hyperphosphorylation of IRAK-1 leading to NF-κB-dependent gene expression.[138,140] The data shown above have demonstrated that stimulation of monocytes with S100 proteins induces increased levels of Th17 cells. To further investigate if these stimulatory effects occur in a TLR4-dependent manner, the receptor was blocked on monocytes using a TLR4 antagonist prior to incubation with S100 proteins. This specific blockade of S100-binding to TLR4 on monocytes resulted in significantly reduced levels of induced IL-17$^+$ cells ($p<0.05$) (Figure 12A, B). Furthermore, levels of induced IL-17$^+$IFNγ$^+$ cells were also significantly reduced by incubation of monocytes with TLR4 antagonist ($p<0.001$) (Figure 12A, C). Any unspecific effects of TLR4 antagonist could be excluded as monocytes solely treated with the antagonist induced approximately the same levels of Th17 cells and Th17/Th1 cells compared to untreated monocytes. The TLR4 antagonist did not show any effects on Th1 cells (Figure 12A, D).

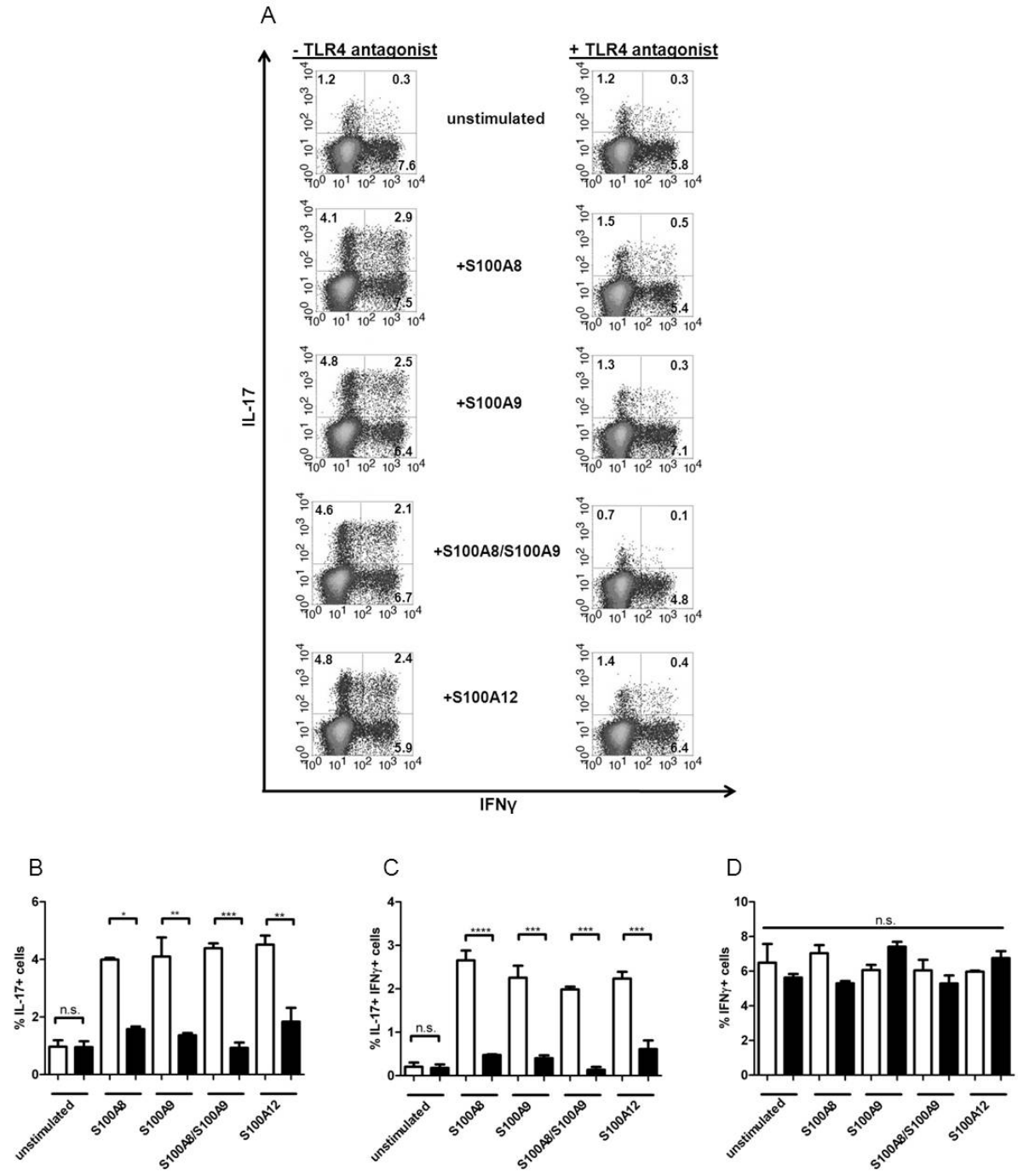


Figure 12: Influence of TLR4 blocking followed by stimulation of monocytes with S100 proteins on the induction of Th17, Th17/Th1 and Th1 cells

Monocytes from healthy donors were pre-incubated for 30 min with 0.1 µg/ml TLR4 antagonist, followed by 4 h stimulation with S100 proteins (5 µg/ml) as indicated. Monocytes were co-cultured with $CD4^+$ T cells isolated from healthy donors with anti-CD3 mAb (100 ng/ml) at a monocyte:T cell ratio of 1:4 for 5 days. After 5 days of co-culture, cells were stimulated for 5 h with PMA (50 ng/ml) and ionomycin (750 ng/ml) in the presence of monensin (2 µM) during the last 3 h and flow cytometric analysis was performed. (A) Representative density plots demonstrating the effect of TLR4 blockade on the induced percentages of Th17, Th17/Th1 and Th1 cells. Density plots exemplary illustrate cell populations analysed in the corresponding graphs below. Bars represent the percentage of induced

(B) IL-17$^+$ cells, (C) IL-17$^+$IFNγ$^+$ cells and (D) IFNγ$^+$ cells from one of three independent experiments with similar outcomes. Data represent mean values of duplicates + SEM. Statistical significance was determined using One-way ANOVA followed by Bonferroni's post-hoc test: *p<0.05; **p<0.01;***p<0.001. White bars: without TLR4 antagonist; black bars: with TLR4 antagonist. Reinhardt et al.[387]

5.10 Impact of cytokine-neutralizing antibodies on the Th17 response

Next, the question was addressed whether proinflammatory cytokines are involved in monocyte-induced development of Th17 cells. Therefore, monocytes from healthy donors were pre-stimulated with S100 proteins for 4 h. These monocytes were co-cultured with CD4$^+$ T cells isolated from healthy donors in the presence of 100 ng/ml anti-CD3 mAb Orthoclone OKT3 and the neutralizing antibodies specific for the cytokines IL-17, TNFα, IL-1β, IL-6, IL-12/IL-23p40 or the corresponding isotype controls. The antibodies were added to the culture either separately or together. As already shown in figure 11A and 11B, S100A8 and S100A9 had strong stimulatory effects on monocyte-induced development of Th17 cells. In this experiment, the heterodimer S100A8/S100A9 showed only weak effects on monocyte-mediated induction of IL-17$^+$ cells (Figure 13A-G). Addition of one single antibody to the culture did not seem to have any neutralizing effect on the level of induced Th17 cells except for the anti-IL-1β monoclonal antibody (Figure 13A-E). This antibody showed weak neutralizing effects resulting in decreased percentages of induced IL-17$^+$ cells in cultures with monocytes pre-stimulated with S100A9 (Figure 13E). The data also indicate that levels of induced Th17 cells could be decreased in co-cultures of monocytes pre-stimulated with S100A8 or S100A9 and CD4$^+$ T cells by adding all five neutralizing antibodies (Figure 13F). The isotype controls mouse IgG1 or mouse IgG2b did not influence the induction of Th17 cells (Figure 13G,H).

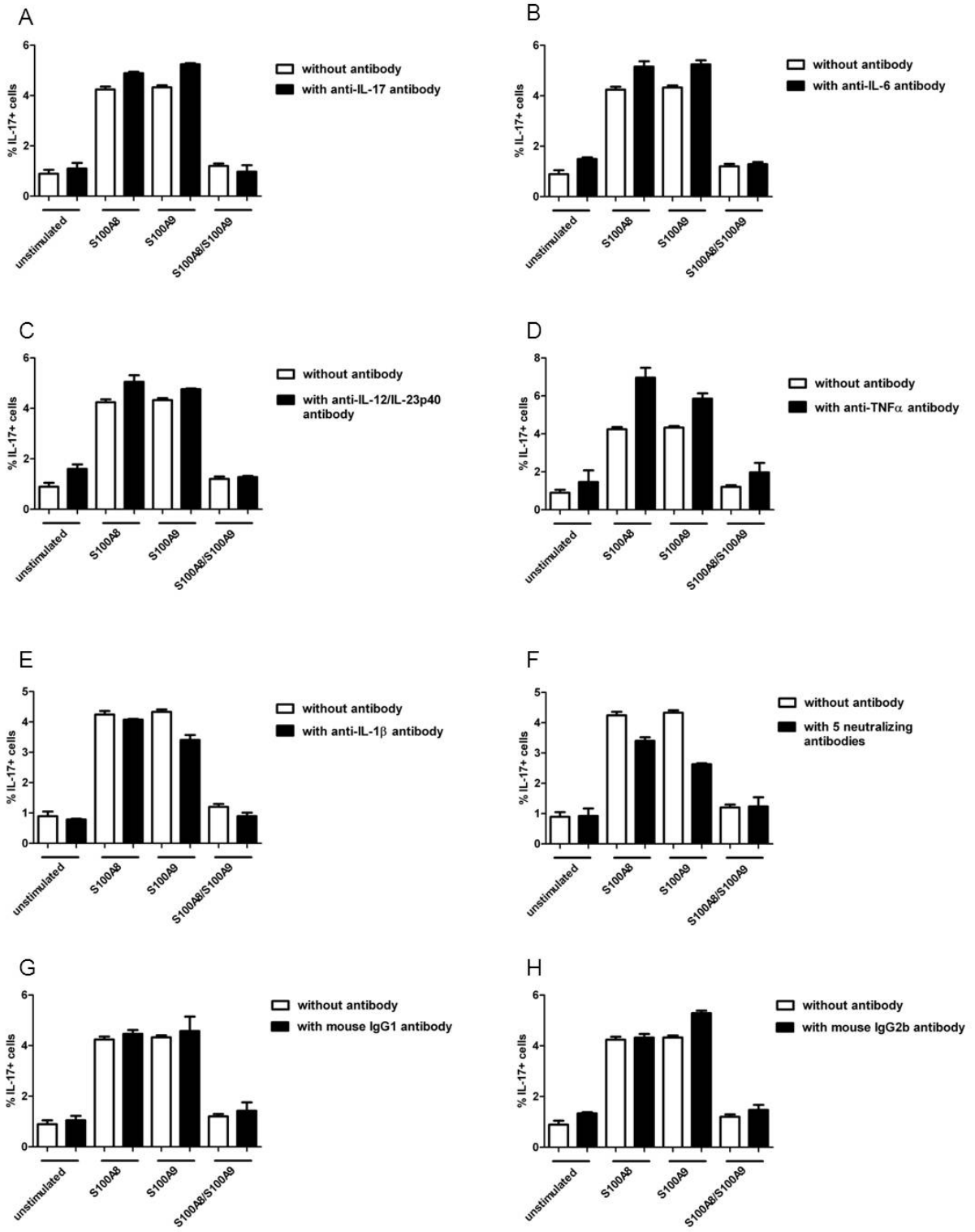


Figure 13: Influence of neutralizing antibodies on the induction of Th17 cells

$CD4^+$ T cells isolated from a healthy donor were co-cultured with anti-CD3 mAb (100 ng/ml) and monocytes from a healthy donor at a monocyte:T cell ratio of 1:4 for 5 days. Monocytes remained unstimulated or were additionally stimulated with S100A8, S100A9 or S100A8/S100A9 (5 µg/ml) for 4h before co-culturing with $CD4^+$ T cells. Neutralizing antibodies specific for (A) IL-17, (B) IL-6, (C) IL-12/IL-23p40, (D) TNFα, (E) IL-1β, (F) all 5 neutralizing antibodies together or the corresponding isotype controls (G) mouse IgG1, (H) mouse IgG2b were added to the co-cultures. After 5 days, cells

were restimulated with PMA/ionomycin and flow cytometric analysis was performed determining the percentage of induced IL-17$^+$ cells. Data represent mean values of duplicates + SEM of one experiment.

5.11 Influence of cytokines on monocyte-mediated induction of Th17 cells

As the data shown in figure 13 indicate that cytokines might be involved in the induction of Th17 cells, further experiments were performed to assess whether the induction of Th17 cells is mediated mainly via cytokines or whether cell-cell contact is essential for the efficient induction of IL-17$^+$ cells. Therefore, monocytes from healthy donors, optionally pre-stimulated with S100A8, S100A9, the heterodimer or S100A12, were seeded and incubated for 24 h. The supernatant was then added to the culture medium of CD4$^+$ T cells freshly isolated from healthy donors at a ratio of 1:1. CD4$^+$ T cells were cultured as described above. Addition of supernatant of monocytes pre-stimulated with S100 proteins to CD4$^+$ T cells induced significantly increased levels of IL-17$^+$ cells compared to supernatant of unstimulated monocytes ($p<0.05$). S100A8, S100A9 and S100A12 showed stronger stimulatory effects on monocytes in comparison with the heterodimer S100A8/S100A9 (Figure 14A). Overall, percentages of induced IL-17$^+$ cells in CD4$^+$ cells were notably lower when only soluble factors were present compared to Th17 induction mediated via both cell-contact and soluble factors (Figure 4B,11B,14A). Next, it was investigated which proinflammatory cytokines are released by monocytes and therefore promote the development of Th17 cells. Proinflammatory cytokine levels in the supernatant of monocytes were measured by Human Cytokine Group I 5-plex Assay as described in 4.8. Therefore, monocytes isolated from healthy donors were optionally pre-stimulated with S100A8, S100A9, the heterodimer S100A8/S100A9 or S100A12 for 4 h before incubating for 24 h to determine cytokine release. Results are shown in figure 14B as n-fold increase relative to the detected cytokine levels in the supernatant of unstimulated monocytes. In case of cytokine levels in the supernatant of unstimulated monocytes below detection levels, results were expressed as n-fold of the quantification limit of the lowest standard. The data demonstrate that monocytes stimulated with S100A8, S100A9, S100A8/S100A9 or S100A12 released 2-7200-fold increased levels of IL-1β, IL-6, IL-8, IL-10 and TNFα compared to unstimulated monocytes. Consistent with the data shown in figure 14A, stimulation of

monocytes with S100A8, S100A9 or S100A12 had stronger stimulatory effects on monocytes compared to the heterodimer S100A8/S100A9 resulting in increased levels of released proinflammatory cytokines (Figure 14B).

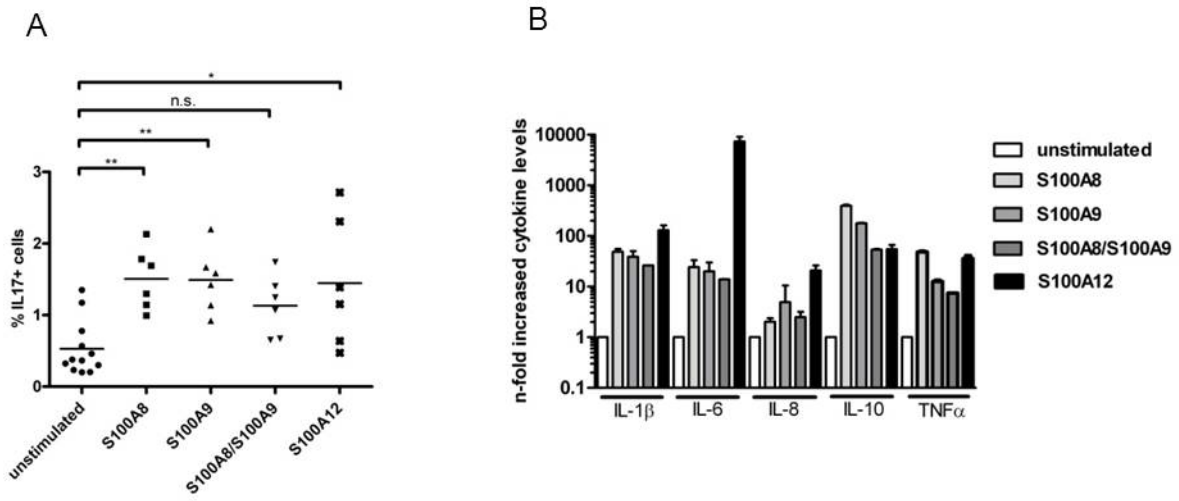


Figure 14: Influence of cytokines on monocyte-induced development of Th17 cells and determination of cytokine levels released by monocytes

Monocytes from healthy donors optionally pre-stimulated with S100A8, S100A9, S100A8/S100A9 or S100A12 (5 µg/ml) proteins for 4 h were seeded and incubated for 24 h. (A) The next day, the supernatant was added to the culture medium of freshly isolated $CD4^+$ T cells at a ratio of 1:1. $CD4^+$ T cells were cultured for 5 days in the presence of OKT3 (100 ng/ml) and a monoclonal antibody directed against CD28 (1 µg/ml). After 5 days, cells were restimulated for 5 h with PMA (50 ng/ml) and ionomycin (750 ng/ml) in the presence of monensin (2 µM) during the last 3 h. The percentage of $IL\text{-}17^+$ cells in $CD4^+$ T cells was assessed by flow cytometry. Statistical significance was determined using One-way ANOVA followed by Bonferroni's post-hoc test: *$p<0.05$. (B) Levels of proinflammatory cytokines (IL-1β, IL-6, IL-8, IL-10 and TNFα) in the supernatant of monocytes optionally pre-stimulated with S100A8, S100A9, S100A8/S100A9 or S100A12 (5 µg/ml) were determined in duplicates by Bio-Plex Human Cytokine Group I 5-plex Assay. Results are shown as n-fold increase relative to cytokine levels in the supernatant of unstimulated monocytes. Reinhardt et al.[387]

5.12 Levels of proinflammatory cytokines in the serum of patients with GvHD

In addition to the measurement of proinflammatory cytokine levels in the supernatant of monocytes, concentrations of IL-1β, TNFα, IL-8, IL-10 and IL-6 were determined in the serum of healthy donors (n=5), patients with acute GvHD (n=5) and patients with extensive chronic GvHD (n=3) using Human Cytokine Group I 5-plex Assay. In the event that serum cytokine concentrations were below detection levels, they were set to zero. Overall, proinflammatory cytokines were not detectable or negligibly low in

the serum of healthy donors. Regarding the measurement of IL-1β concentrations, only one patient with acute GvHD showed weakly elevated IL-1β levels in the serum compared to the healthy donors (Figure 15A). Similar results were obtained for the determination of TNFα levels in the serum. One patient with extensive chronic GvHD showed increased TNFα serum levels in comparison to healthy donors whereas TNFα concentrations were very low in the serum collected from the other patients with GvHD (Figure 15B). Overall, IL-8 levels in the serum of patients with acute or chronic GvHD were increased compared to the healthy donors except for one patient with acute GvHD (Figure 15C). IL-10 levels in the serum were elevated in patients with acute GvHD in comparison to healthy donors. Weakly elevated levels of IL-10 could also be detected in the serum of patients with chronic GvHD (Figure 15D). Serum concentrations of IL-6 were elevated in individual patients with acute or chronic GvHD compared to the healthy donors (Figure 15E).

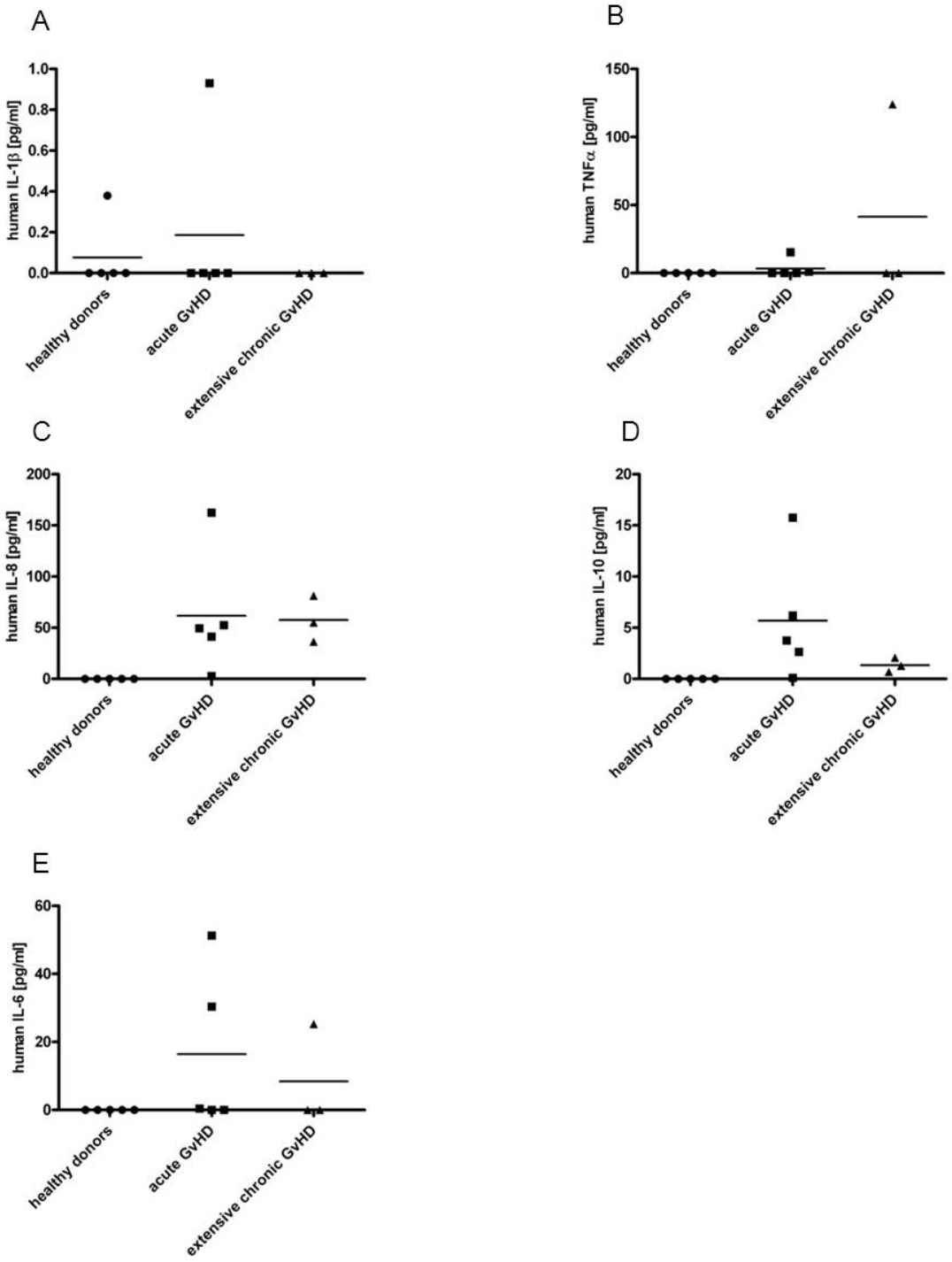


Figure 15: Proinflammatory cytokine levels in the serum of healthy donors and patients with acute or chronic GvHD

Levels of (A) IL-1β, (B) TNFα, (C) IL-8, (D) IL-10 and (E) IL-6 were determined in the serum of healthy donors and patients with GvHD using Bio-Plex Human Cytokine Group I 5-plex Assay.

5.13 Impact of chemical inhibition of Hsp90 in monocytes on the induction of Th17 cells

The heat shock protein 90 (Hsp90) is an ubiquitously expressed molecular chaperon which plays an important role in the folding, maturation and stabilization of lots of proteins that are involved in the signal transduction, transcription regulation and survival of the cell.[394] It is also known that Hsp90 is involved in monocyte activation via the NF-κB pathway. Compounds targeting Hsp90 were found to inhibit the production of proinflammatory cytokines like IL-6 and IL-1β from activated macrophages, which are known to induce the development and expansion of IL-17$^+$ cells.[213,279,395] Therefore, it was investigated if Hsp90 is also involved in the induction of Th17 cells and thus in the pathogenesis of GvHD. Therefore, monocytes isolated from the peripheral blood of 3 healthy donors and 3 patients with acute GvHD grade II or III were incubated with the geldanamycin derivative 17-DMAG for 16 h to inhibit the function of Hsp90. Subsequently, monocytes were washed and co-cultured with CD4$^+$ T cells from healthy donors and the percentage of induced Th17 cells was determined. Monocytes from healthy donors which were incubated with 17-DMAG induced lower percentages of IL-17$^+$ cells compared to untreated monocytes. As already shown in figure 4A, the percentage of monocyte-induced Th17 cells was significantly elevated in patients with acute GvHD. Inhibition of Hsp90 in monocytes from patients with GvHD resulted in significantly decreased levels of induced IL-17$^+$ cells which were approximately the same as in healthy donors ($p<0.001$) (Figure 16A). Treatment of monocytes from healthy donors and from patients with acute GvHD with the Hsp90 inhibitor resulted in diminished percentages of induced IL-17$^+$IFNγ$^+$ cells compared to untreated cells. However, this decrease in Th17/Th1 cells was not significant (Figure 16B). Incubation of monocytes with 17-DMAG did not show any significant effects on the percentages of IFNγ$^+$ cells (Figure 16C). Representative density plots demonstrating the effect of monocyte treatment with 17-DMAG on the induced percentages of Th17, Th17/Th1 and Th1 cells are shown in figure 16D.

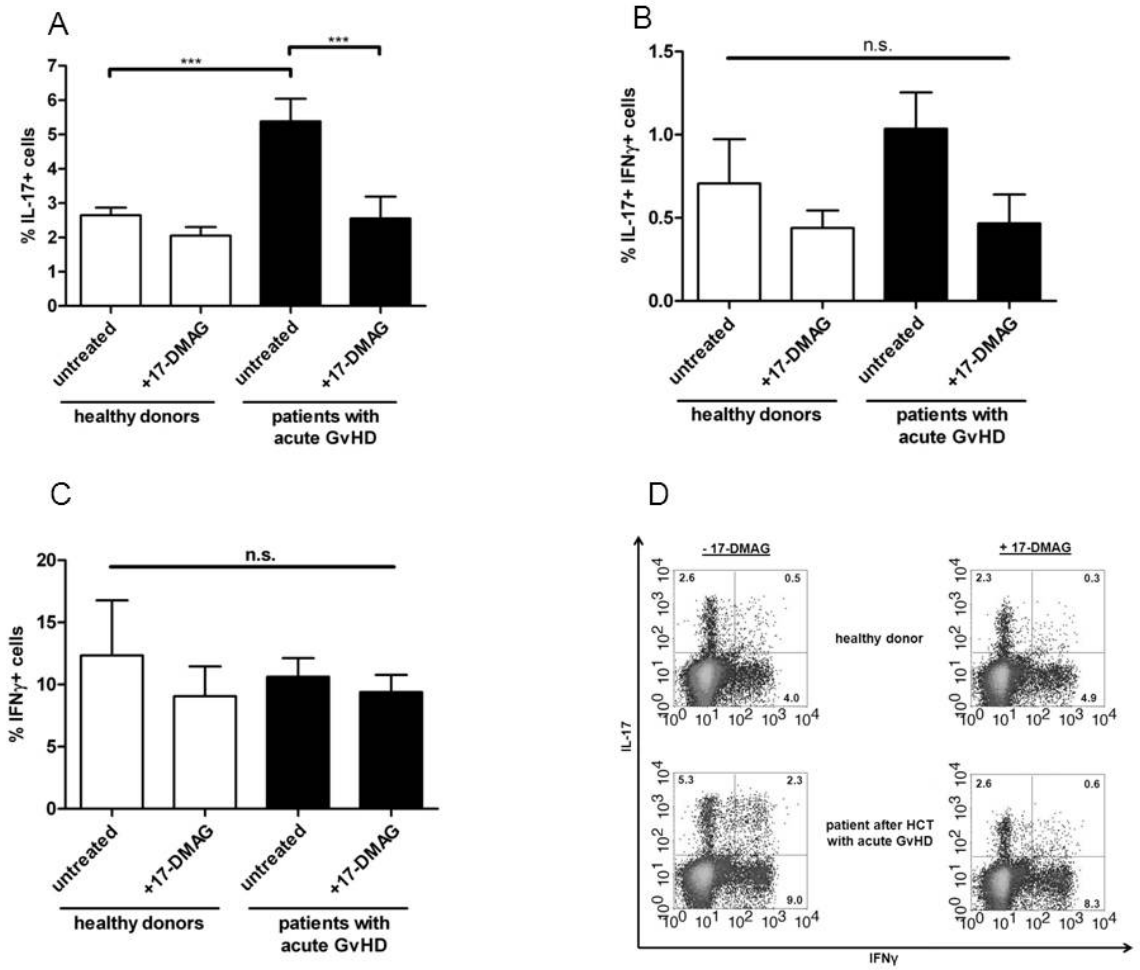


Figure 16: Influence of Hsp90 inhibition in monocytes from healthy donors and patients with acute GvHD by 17-DMAG on the induction of Th17, Th17/Th1 and Th1 cells

(A) Monocytes from 3 healthy donors, 3 patients with acute GvHD grade II or III were co-cultured with $CD4^+$ T cells isolated from healthy donors in the presence of anti-CD3 mAb (100 ng/ml) at a monocyte:T cell ratio of 1:4 for 5 days. Monocytes remained untreated or were incubated with 17-DMAG (5 µM) for 16 h before co-culturing with $CD4^+$ T cells. After 5 days, the percentage of induced (A) $IL\text{-}17^+$ (B) $IL\text{-}17^+IFN\gamma^+$ and (C) $IFN\gamma^+$ cells was assessed by flow cytometry. Statistical significance was determined using One-way ANOVA followed by Bonferroni's post-hoc test: ***p<0.001 (*n=3*; mean +SD). (D) Representative density plots demonstrating the effect of Hsp90 inhibition with 17-DMAG in monocytes on the induction of Th17, Th17/Th1 and Th1 cells.

5.14 Influence of chemical inhibition of Hsp90 in monocytes on the stimulatory effect of S100 proteins on monocyte-induced Th17 development

Previous results have shown that monocytes express elevated levels of S100A8, S100A9 and S100A12. Furthermore, increased concentrations of S100 proteins could be detected in the serum, stool and bowel tissue of patients with GvHD and it could be demonstrated that S100 proteins promote the induction of Th17 cells by binding to TLR4 on monocytes. Recently published studies indicate that Hsp90 plays an important role in the TLR4-mediated signalling pathway. De Nardo et al. could show that the inhibition of Hsp90 in murine macrophages with geldanamycin induced the degradation of IRAK-1 and the inhibition of the TLR4-induced expression of IL-1β.[215] This proinflammatory cytokine is involved in the initiation of the differentiation of IL-17-producing human T helper cells.[279] To assess the question if the signalling pathways of Hsp90 and S100 proteins overlap, monocytes isolated from healthy donors were treated for 16 h with the Hsp90 inhibitor 17-DMAG before stimulating with S100A8, S100A9 or S100A8/S100A9. As already shown in figure 11, stimulation of monocytes with S100A8, S100A9, S100A8/S100A9 or S100A12 induced significantly increased percentages of Th17 and Th17/Th1 cells whereas levels of Th1 cells were not affected by stimulation of monocytes with S100 proteins via TLR4 (Figure 17A-C). Treatment of monocytes with the Hsp90 inhibitor 17-DMAG prior to stimulation with S100 proteins resulted in significantly decreased levels of IL-17$^+$ cells compared to monocytes that were not incubated with 17-DMAG before S100 stimulation ($p<0.01$). Thus, S100 proteins did not show any stimulatory effect on monocyte-induced development of Th17 cells when monocytes were treated with the Hsp90 inhibitor before (Figure 17A). The data further indicate that monocytes incubated with 17-DMAG prior to S100 stimulation induced significantly decreased percentages of Th17/Th1 cells in comparison to monocytes with active Hsp90 that were stimulated via TLR4 ($p<0.05$) (Figure 17B). Regarding the determined levels of Th1 cells, monocytes with inactivated Hsp90 induced diminished levels of IFNγ$^+$ cells compared to monocytes that were not treated with the Hsp90 inhibitor ($p<0.001$) (Figure 17C). Representative density plots demonstrating the effect of Hsp90 inhibition in monocytes with 17-DMAG prior to S100 stimulation on the induced percentages of IL-17$^+$, IL-17$^+$IFNγ$^+$ and IFNγ$^+$ cells are shown in figure 17D.

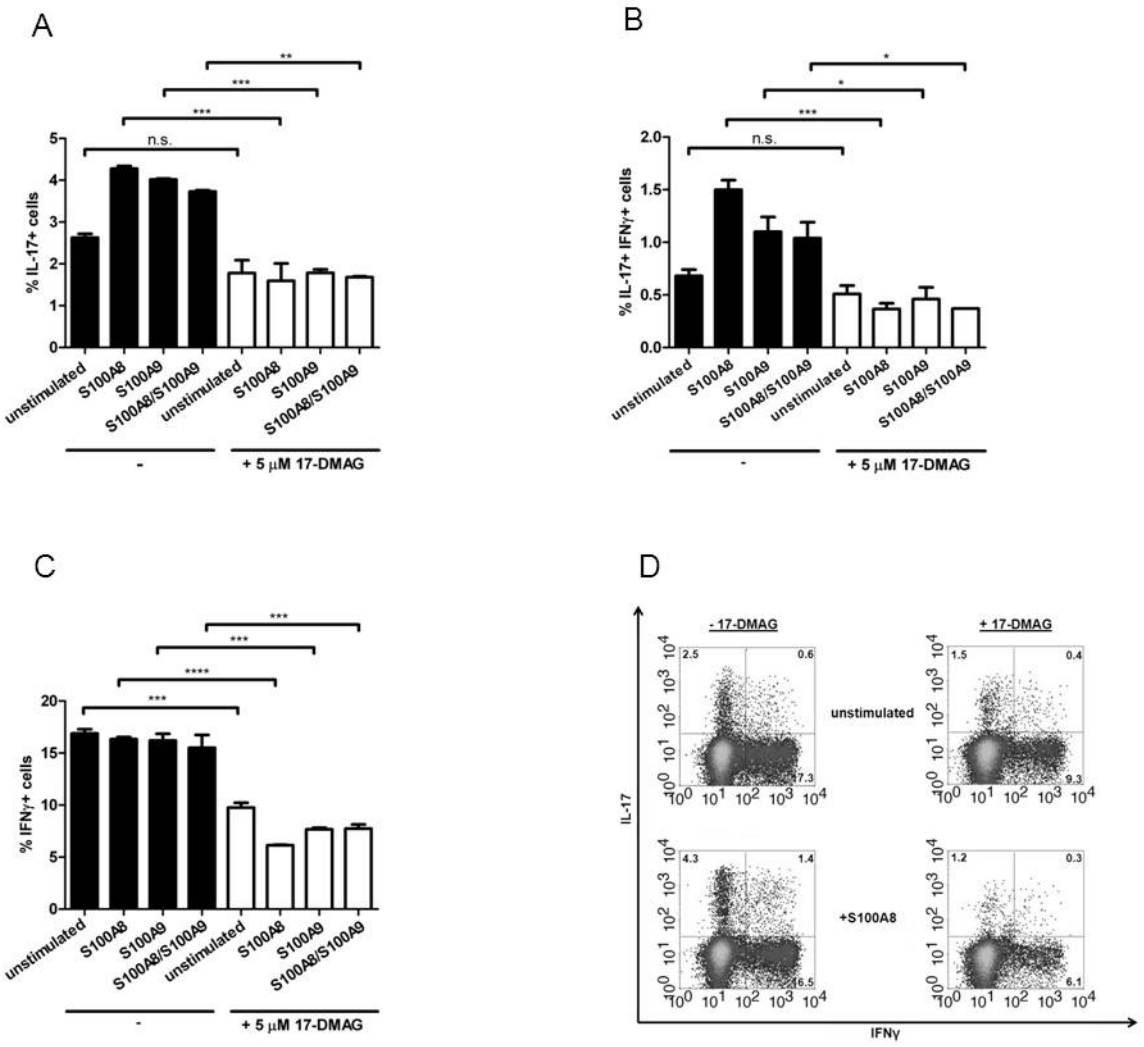


Figure 17: Influence of Hsp90 inhibition in monocytes on the stimulatory effect of S100 proteins on monocyte-induced development of Th17 cells.

Monocytes from 3 healthy donors, 3 patients with acute GvHD grade II or III were co-cultured with $CD4^+$ T cells isolated from healthy donors in the presence of anti-CD3 mAb (100 ng/ml) at a monocyte:T cell ratio of 1:4 for 5 days. Monocytes remained untreated or were incubated with 17-DMAG for 16 h prior to stimulation with S100 proteins for 4 h. Monocytes were co-cultured with $CD4^+$ T cells and anti-CD3 mAb (100 ng/ml) at a monocyte:T cell ratio of 1:4 for 5 days. The percentages of induced (A) Th17, (B) Th17/Th1 and (C) Th1 cells were determined by flow cytometry. Graph shows data from one of three independent experiments with similar outcomes. Data represent mean values of duplicates + SEM. Statistical significance was determined using One-way ANOVA followed by Bonferroni's post-hoc test: *p<0.05;**p<0.01;***p<0.001;****p<0.0001. (D) Representative density plots demonstrating the effect of monocyte treatment with the Hsp90 inhibitor 17-DMAG prior to S100 stimulation on the induced percentages of $IL\text{-}17^+$, $IL\text{-}17^+IFN\gamma^+$ and $IFN\gamma^+$ cells.

5.15 Depletion of HSP90α protein in monocytes by siRNA

Previous results shown in figure 16 indicate that Hsp90 is involved in monocyte-mediated development of Th17 cells during GvHD and in the TLR4-mediated signalling pathway. In order to confirm these findings, siRNA experiments were performed. Vertebrates express two isoforms of Hsp90, Hsp90α and Hsp90β. While Hsp90β is expressed constitutively to a high level in most tissues, Hsp90α is stress-inducible.[396] As the inflammatory processes during GvHD leading to tissue damage display enormous cellular stress for the human body, siRNA targeting the stress-inducible isoform Hsp90α in monocytes was used in the experiments. Monocytes from healthy donors were transfected with Hsp90α-targeting siRNA using a liposome-mediated transfection method. As negative control, non-targeting siRNA was used. In order to determine the silencing efficacy of the siRNA targeting Hsp90α, cells were assayed for knockdown at various time points over a period of 168 h after transfection. In immunoblots, a decrease of Hsp90α expression was detectable 96 h after transfection. No Hsp90α expression could be detected in the cells transfected with Hsp90α targeting siRNA 168 h after transfection. Overall, in monocytes treated with non-targeting siRNA and transfection reagent only, no decrease in Hsp90α expression could be detected in this period. However, freshly isolated monocytes from healthy donors did show any Hsp90α expression (Figure 18A). Time course analysis of Hsp90α silencing effect by flow cytometry displays a decrease in the expression of Hsp90α 72 h after transfection. Maximum silencing effect could be detected 168 h after transfection (Figure 18B and C). As already shown, flow cytometry is a more sensitive method for the detection of heat shock proteins compared to western blotting.[397] 72 h after transfection of human monocytes, Hsp90α expression was reduced to approximately 32% of the negative control. The effect was strongest 168 h after transfection with a more than 80% reduction of Hsp90α expression (Figure 18C).

A time point of 140 h after transfection was chosen and the expression of Hsp90α was analysed by immunoblot and flow cytometry. As already shown in figure 18A, freshly isolated monocytes from healthy donors did not express Hsp90α. Monocytes that remained untreated and were cultured for 140 h showed marked Hsp90α expression levels as well as monocytes treated with transfection reagent alone or in combination with non-targeting siRNA. Monocytes transfected with Hsp90α targeting

siRNA before culturing for 140 h Hsp90α did not show any Hsp90α expression in the immunoblot (Figure 18D). Similar results were obtained by flow cytometric analysis shown in figure 18E. In this experiment, transfection of human monocytes with Hsp90α targeting siRNA resulted in a 70% reduction of the Hsp90α expression in comparison to the negative control (Figure 18E).

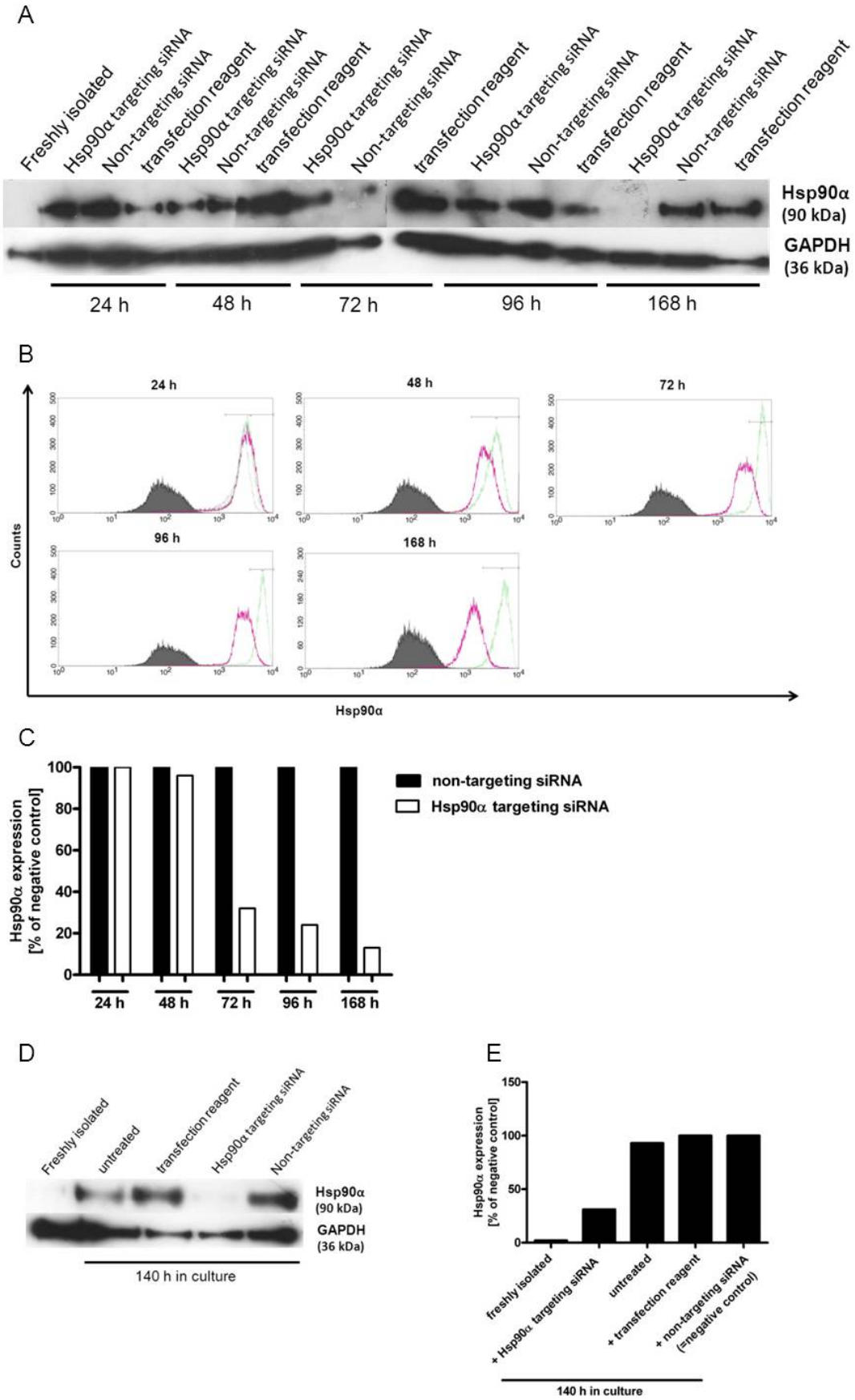

A
Freshly isolated
Hsp90α targeting siRNA
Non-targeting siRNA
transfection reagent
Hsp90α targeting siRNA
Non-targeting siRNA
transfection reagent
Hsp90α targeting siRNA
Non-targeting siRNA
transfection reagent
Hsp90α targeting siRNA
Non-targeting siRNA
transfection reagent
Hsp90α targeting siRNA
Non-targeting siRNA
transfection reagent
Hsp90α (90 kDa)
GAPDH (36 kDa)
24 h
48 h
72 h
96 h
168 h
B
24 h
48 h
72 h
96 h
168 h
Counts
Hsp90α
C
Hsp90α expression [% of negative control]
non-targeting siRNA
Hsp90α targeting siRNA
24 h
48 h
72 h
96 h
168 h
D
Freshly isolated
untreated
transfection reagent
Hsp90α targeting siRNA
Non-targeting siRNA
Hsp90α (90 kDa)
GAPDH (36 kDa)
140 h in culture
E
Hsp90α expression [% of negative control]
freshly isolated
+ Hsp90α targeting siRNA
untreated
+ transfection reagent
+ non-targeting siRNA (=negative control)
140 h in culture

Figure 18: Depletion of Hsp90α protein expression in monocytes by siRNA
(A) Immunoblot of lysed human lysates (5 µg per lane) were resolved by SDS-PAGE and immunoblot for Hsp90α expression (upper row) and GAPDH (as loading control; lower row). (B) Flow cytometric analysis of Hsp90α expression in human monocytes at the indicated time points after treatment with Hsp90α targeting siRNA (pink) or non-targeting siRNA (green). The gray shaded histograms represent staining of monocytes treated with Hsp90α targeting siRNA with isotype-matched negative control antibodies. M1 defines monocytes expressing Hsp90α. (C) Relative Hsp90α expression in monocytes after treatment with Hsp90α targeting siRNA or non-targeting siRNA was determined at the indicated time points. For analysis, the number of Hsp90α expressing monocytes treated with non-targeting siRNA (negative control) in M1 at the respective time points was set as 100%. (D) Immunoblot of lysed human monocytes that remained untreated or were optionally treated with 20 nM Hsp90α targeting siRNA, 20 nM non-targeting siRNA or transfection reagent alone. Lysates were prepared 140 h after transfection. Equal protein amounts of whole cell lysates (5 µg per lane) were analysed by SDS-PAGE and immunoblot for Hsp90α expression (upper row) and GAPDH (as loading control; lower row). (E) Relative Hsp90α expression levels in monocytes optionally treated with Hsp90α targeting siRNA, non-targeting siRNA or transfection reagent alone were determined. Therefore, the number of Hsp90α expressing monocytes treated with non-targeting siRNA (negative control) in M1 at the respective time points was set as 100%.

5.16 Impact of HSP90α knockdown in monocytes on Th17 development

Previous results have shown that monocytes incubated with the chemical Hsp90 inhibitor 17-DMAG prior to stimulation with S100 proteins induced significantly decreased levels of IL-17$^+$ cells compared to untreated monocytes (Figure 17A and D). However, chemical inhibition of Hsp90 is known to have unspecific effects like the induction of other heat shock proteins, including Hsp40 and Hsp70, via activation of heat shock factor 1 (HSF-1).[195,398-400] Therefore, the expression of stress-inducible Hsp90α was specifically silenced in monocytes from healthy donors (n=3) using Hsp90α targeting siRNA. As negative control, monocytes were treated with non-targeting siRNA. Subsequently, monocytes were stimulated with S100 proteins, co-cultured with CD4$^+$ T cells and analysed regarding their potential to induce Th17 cells by flow cytometry. Overall, S100 proteins showed stimulatory effects on monocyte-induced development of IL-17$^+$ cells and IL-17$^+$IFNγ$^+$ cells as already demonstrated in figure 11 and 17. As shown in figure 19A, monocytes transfected with Hsp90α targeting siRNA prior to S100 stimulation induced decreased levels of IL-17$^+$ cells in comparison to monocytes that were treated with non-targeting siRNA prior to TLR4-mediated stimulation of monocytes. Furthermore, treatment of monocytes with

Hsp90α targeting siRNA before stimulation with S100 proteins resulted in weakly decreased levels of induced IL-17$^+$IFNγ$^+$ cells in comparison to monocytes transfected with non-targeting siRNA prior to monocytes stimulated via TLR4 (Figure 19B). Overall, monocytes transfected with Hsp90α targeting siRNA induced similar levels of Th1 cells compared to monocytes treated with non-targeting siRNA (Figure 19C).

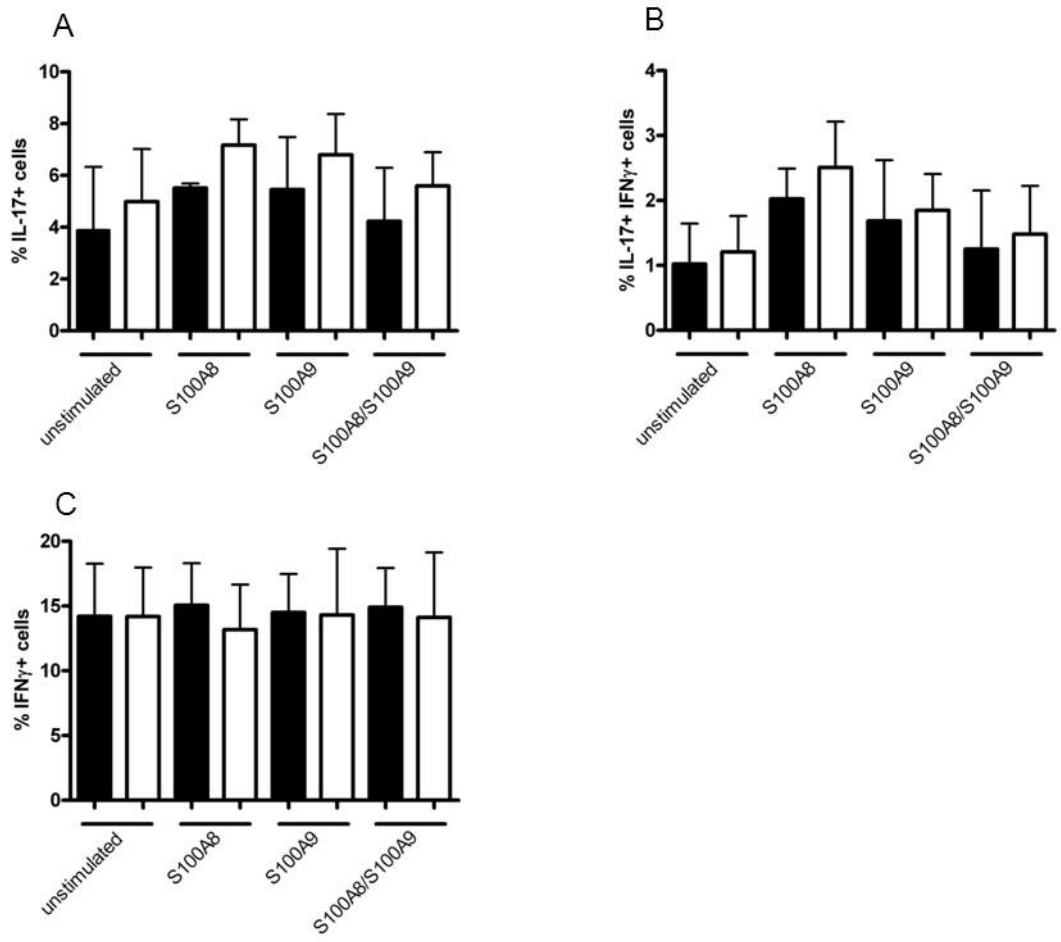


Figure 19: Influence of depletion of HSP90α protein in monocytes on the induction of IL-17$^+$ cells

Monocytes were isolated from healthy donors and transfected with 20 nM Hsp90α targeting siRNA or 20 nM non-targeting siRNA. 140 h after transfection, monocytes were optionally stimulated with S100A8, S100A9 and S100A8/S100A9 for 4 h. Monocytes were washed and co-cultured with CD4$^+$ T cells isolated from healthy donors in the presence of anti-CD3 mAb (100 ng/ml) at a monocyte:T cell ratio of 1:4 for 5 days. After 5 days of co-culture, cells were stimulated for 5 h with PMA (50 ng/ml) and ionomycin (750 ng/ml) in the presence of monensin (2 µM) during the last 3 h and flow cytometric analysis was performed to determine the percentages of (A) IL-17$^+$ cells, (B) IL-17$^+$IFNγ$^+$ and (C) IFNγ$^+$ cells within CD4$^+$ T cells (*n=3*; mean +SD). Black bars: Hsp90α targeting siRNA; White bars: non-targeting siRNA

5.17 Effect of dexamethasone and 17-DMAG on the induction of proinflammatory Th17 cells expressing MDR1

Acute or chronic GvHD is usually treated with glucocorticoids.[401] However, the use of glucocorticoids is associated with an increased susceptibility for infections, the toxicity of the drugs and an increased risk of relapse due to a diminished graft-versus-tumour effect.[402-404] Additionally, glucocorticoids induce the development of proinflammatory $CCR6^{+}CCR4^{lo}CXCR3^{hi}$Th17.1 cells expressing the multi-drug resistance protein type 1. These proinflammatory $MDR1^{+}$Th17.1 cells are known to be resistant to immunosuppression mediated by glucocorticoids.[352] We therefore investigated the influence of dexamethasone and the Hsp90 inhibitor 17-DMAG on the induction of $MDR1^{+}$Th17.1 cells. Thus, monocytes isolated from healthy donors were optionally treated with 17-DMAG for 16 h. These monocytes were co-cultured with $CD4^{+}$ T cells and anti-CD3 antibody at a monocyte:T cell ratio of 1:4. Additionally, 0.1 µM dexamethasone was added to the culture as indicated. After 5 days of co-culture, the percentage of $CCR6^{+}MDR1^{+}$ cells within $CD4^{+}$ T cells and the percentage of $MDR1^{+}CCR6^{+}CCR4^{lo}CXCR3^{hi}$Th17.1 cells within these $CCR6^{+}MDR1^{+}$ cells were determined by flow cytometry as indicated in the density plots in figure 20A. The results shown in figure 20B and 20C demonstrate that monocytes treated with dexamethasone induced increased levels of $CCR6^{+}MDR1^{+}$ cells and $MDR1^{+}CCR6^{+}CCR4^{lo}CXCR3^{hi}$Th17.1 cells whereas monocytes that remained untreated or were incubated with the Hsp90 inhibitor 17-DMAG in monocytes induced similar low levels of $MDR1^{+}$Th17.1 cells.

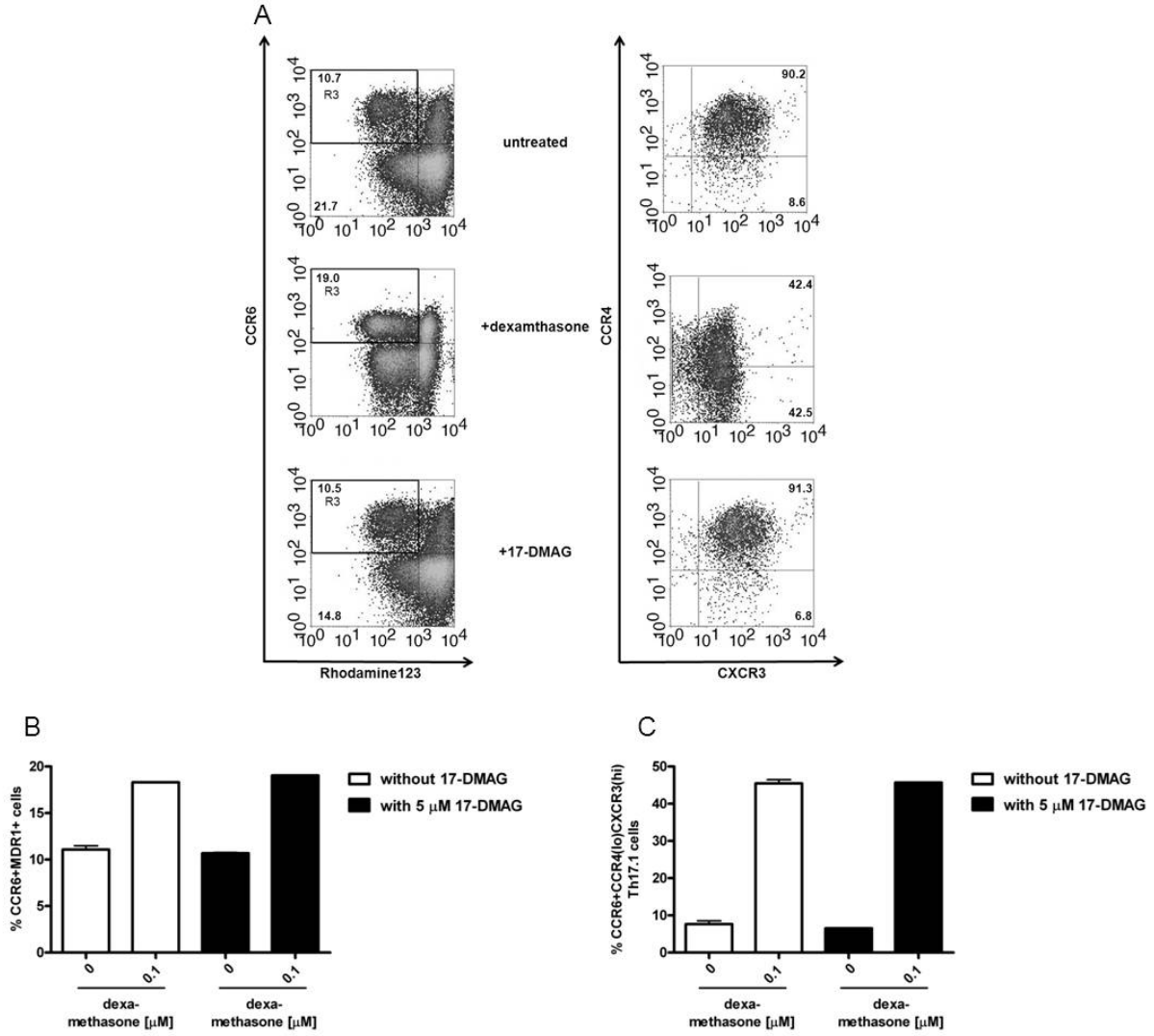


Figure 20: Influence of dexamethasone and 17-DMAG on the induction of MDR1$^+$Th17.1 cells

Monocytes from healthy donors were optionally treated with 5 µM 17-DMAG for 16 h. These monocytes were co-cultured with CD4$^+$ T cells and anti-CD3 mAb (100 ng/ml) at a monocyte:T cell ratio of 1:4. Additionally, 0.1 µM dexamethasone was added to the culture as indicated. After 5 days of co-culture, the percentage of CCR6$^+$MDR1$^+$ cells and the percentage of MDR1$^+$CCR6$^+$CCR4^{lo}CXCR3^{hi} cells within these CCR6$^+$MDR1$^+$ cells were determined by flow cytometry as exemplary indicated in the density plots in (A). Bars represent the determined percentages of (B) MDR1$^+$CCR6$^+$ cells within CD4$^+$ T cells and (C) MDR1$^+$CCR6$^+$CCR4^{lo}CXCR3^{hi} Th17.1 cells within MDR1$^+$CCR6$^+$ cells and are representative for two independent experiments. Data represent the mean values of duplicates +SEM.

6 Discussion

6.1 Monocyte-induced Th17 cells play an important role in the pathophysiology of GvHD

Various studies have demonstrated that Th17 cells are involved in several inflammatory autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, and experimental autoimmune encephalomyelitis.[405-408] However, the role of Th17 cells in the pathomechanism of GvHD is still discussed controversially. A previous study has concluded that the progression of GvHD is predominantly promoted by proinflammatory Th1 cells as the results demonstrate high levels of IFNγ producing T cells in the skin of patients with acute cutaneous GvHD.[76] In a murine acute GvHD model, administration of IL-17$^{-/-}$ donor T cells to lethally irradiated MHC mismatched recipients induced enhanced GvHD as a result of robust Th1 expansion.[409] However, inhibition of Th1 cytokines in mice led to the exacerbation of acute GvHD.[333,334] Furthermore, it could be demonstrated in mice that infusion of highly purified Th17 cells was capable to elicit lethal GvHD, hallmarked by extensive pathologic cutaneous and pulmonary lesions.[335] Additionally, enhanced numbers of Th17 cells could be detected in the skin of patients with acute and chronic GvHD compared to controls and a strong correlation between Th17 levels and clinical status of patients with GvHD could be demonstrated.[340] Consistent with these results representing the involvement of Th17 cells in the pathogenesis of GvHD, the data of the present study have shown that monocytes isolated from the peripheral blood of patients with acute or chronic GvHD induce significantly increased levels of IL-17^{+} cells *in vitro* compared to monocytes from patients without GvHD and healthy donors pointing towards a crucial role of activated monocytes in the initiation and progression of GvHD (Figure 4A and B). Furthermore, it could be demonstrated that levels of induced Th17 cells increase at the onset of acute GvHD and diminish after alleviation of GvHD indicating that induced Th17 levels might correlate with GvHD progression whereas the percentages of induced IL-17^{+} cells remain broadly constant in patients without GvHD after HCT (Figure 5A and B). In support to our observations, it has been demonstrated recently that *in vivo* activated monocytes derived from inflamed joints of patients with active rheumatoid arthritis specifically

induce a Th17 response in blood-derived $CD4^+$ T cells.[386] Th17 cells secrete the proinflammatory cytokines IL-17A, IL-17F, IL-21 and IL-22. The cytokines IL-17A and IL-17F are known to bind to the IL-17 receptor resulting in the activation of NF-κB and the MAPK pathways resulting in the production of proinflammatoy cytokines and chemokines and in the recruitment of leukocytes and neutrophils.[298,302-304,337,410] IL-21 plays an autocrine-amplifying role on the Th17 response as it activates the transcription factor STAT3 in naïve $CD4^+$ T cells and promotes thereby the generation of further Th17 cells.[298] Additionally, IL-22 induces the activation of the MAPK pathways which play an important role in cell proliferation, differentiation, development, transformation, and apoptosis.[298,305] As our results demonstrate that levels of induced Th17 cells are elevated during GvHD, these Th17-associated cytokines might be further promote GvHD by the induction of tissue inflammation. In this study, it was also investigated if monocytes from patients induce a general increase in T cell activation. Therefore, the extent of Th1 responses was examined by analysing the percentage of $IFN\gamma^+$ cells. It could be demonstrated that monocytes from patients with GvHD induce elevated levels of Th1 cells. However, the increase is notably lower compared to Th17 cells (Figure 4A and D). The results of the present study also reveal that monocytes from patients with active acute or chronic GvHD induce increased levels of $IL\text{-}17^+IFN\gamma^+$ cells (Figure 4A and C). These Th17/Th1 cells can also be found in increased levels in the gut of patients with Crohn's disease. Th17 cells as well as these Th17/Th1 cells express the transcription factors RORγt and the Th1-transcription factor T-bet, and stimulation in the presence of IL-12 down-regulates the expression of RORγt and the production of IL-17, but induces IFNγ expression suggesting that a functional relationship between Th17 and Th1 cells may exist.[285]

6.2 Th17/Treg ratios are not altered in the peripheral blood of patients with GvHD compared to patients without GvHD and healthy donors

In animal models, it could be demonstrated that the application of *ex vivo*-expanded Treg cells delay or prevent lethal GvHD by suppressing the early expansion of alloreactive donor T cells, their interleukin-2-receptor-chain expression without abrogating the graft-versus-tumour effect of these donor T cells.[341,342,344] Another study has demonstrated that the transfer of Treg cells that originate from the donor but not host, protect from lethal GvHD by secretion of IL-10. Additionally, it was stated that the balance of donor-type $CD4^+CD25^+$ Treg cells and conventional $CD4^+CD25^-$ T cells can determine the outcome of an acute GVHD.[343] Recently, the data of clinical studies have been published demonstrating that the application of freshly isolated donor Treg cells after myeloablative conditioning and before infusion of a megadose of $CD34^+$ cells and conventional $CD4^+$ and $CD8^+$ T cells results in a very low rate of acute and chronic GvHD in the absence of any posttransplantation immunosuppression. Additionally, the adoptive transfer of Treg cells leads to a better lymphoid reconstitution, improves immunity to opportunistic pathogens and does not weaken the graft-versus-leukemia effect.[345] Another clinical trial has shown that the adoptive transfer of *ex vivo* expanded Treg cells isolated from the umbilical cord blood seems to reduce the risk of acute GvHD and can be carried out without apparent toxicity.[112] Altogether, these data of the murine models and the clinical studies indicate that Treg cells might play an important role in inducing and maintaining allogeneic tolerance and might inhibit GvHD after allogeneic HCT. Thus, it seems to be important to clarify the kinetics of Treg cell recovery and its correlation with the occurrence of GvHD in humans. The work of Li et al. has demonstrated that the frequency of Treg cells is reduced in patients with acute GvHD grade II-IV and extensive chronic GvHD in comparison to the healthy controls. Furthermore, it could be shown that the levels of Treg cells in the peripheral blood of these patients can be correlated with the severity of GvHD. However, levels of Treg cells in the peripheral blood of patients without GvHD are significantly higher compared to the healthy controls. Altogether, this study suggests that the measurement of Treg cell levels in the peripheral blood together with the determination of TGF-β and TNFα serum levels at the early reconstitution after allogeneic HCT might indicate the onset and severity of both acute and chronic GvHD.[389] On the contrary, Arimoto et al. revealed that the

expression level of FoxP3 messenger RNA does not correlate with the occurrence of acute and chronic GvHD. The data of this study indicate that the level of $FoxP3^+$ cells is normal relative to other cell types and their frequency in the peripheral blood relative to total leukocytes or T cells does not indicate the occurrence of an acute or chronic GvHD.[411] The results of Arimoto et al. are consistent with the data of the present study demonstrating that the determined percentages of Treg cells in PBMCs isolated from the peripheral blood of patients with acute GvHD does not differ from the Treg levels determined in PBMCs isolated from healthy controls and the non-GvHD group. However, the percentages of Treg cells in PBMCs from patients with chronic extensive GvHD are increased compared to the determined Treg cell levels in PBMCs from healthy donors, patients with acute GvHD, patients before conditioning and patients without GvHD after HCT, but the sample size of patients with chronic GvHD is too low to perform a statistical evaluation (Figure 6B). Thus, more samples need to be analysed to confirm these results. Furthermore, Ratajczak et al. have demonstrated recently that low Th17/Treg ratios seem to correlate with severe clinical and pathological GvHD, apoptosis intensity of epithelial cells, Fas expression in the cellular infiltrate, and the expression of TNF and the TNF receptor. These data indicate that Th17/Treg ratio could be a sensitive and specific pathologic in situ biomarker of GVHD.[346] On the contrary, Malard et al. evaluated the presence of Th17, Th1 and Treg cells in human liver biopsies and could show that levels of Th17 cells are elevated in the liver of patients with chronic GvHD whereas the numbers of Th1 cells and Treg cells are low. Furthermore, Th17/Teg ratios are significantly increased in the liver of patients with chronic GvHD suggesting a defect in the regulatory mechanism driven by Treg cells or an enhanced activation of effector cells, especially Th17 cells, or both mechanisms, in chronic liver GvHD in humans.[390] Therefore, the Th17/Treg ratios were determined in the present study. However, the data of the present study confirms neither the results of the work of Ratajczak et al. nor the study of Malard et al. as Th17/Treg ratios from patients with acute or chronic GvHD do not differ from the ratios of healthy controls and patients without GvHD after HCT (Figure 6C). However, the sample size needs to be increased to validate these data.

6.3 The onset of an acute GvHD is not accompanied by an upregulation of the expression of activation markers on monocytes

It has recently been reported by Arpinati et al. that monocytes of patients with chronic GvHD patients have greater CD86 mean fluorescence intensity in the marrow and peripheral blood indicating an increased activation of donor-derived marrow and blood monocytes in patients with chronic GvHD and suggesting the targeting of monocytes for the treatment of chronic GvHD.[412] Additionally, Evans et al. have demonstrated that CD86, CD40, and CD54 is highly expressed on monocytes from the synovial fluid but blockade of a single costimulatory or adhesion pathway (CD80/CD86, CD54, or CD40) in co-cultures with either *in vitro* or *in vivo* activated monocytes does not lead to a decrease in the percentage of Th17 cells indicating that a certain level of redundancy exists in the cell membrane-derived signals required for Th17 responses or additional factors are involved that remain to be identified.[386] Consistent with the data of Evans et al., the data of the present study demonstrate that monocytes isolated from both patients with acute or chronic GvHD, which is like rheumatoid arthritis an inflammatory disorder, induce increased levels of Th17 cells in a cell-contact dependent manner with the involvement of proinflammatory cytokines secreted by activated monocytes (Figure 14A). However, in contrast to the data of Arpinati et al.[412] and Evans et al.[386], the results of the present study reveal that the expression levels of the activation markers CD86, CD80, HLA-DR, CD16, CD11b, CD11c and CD54 on monocytes isolated from patients at the onset of acute GvHD do not differ from the assessed expression levels in the non-GvHD group indicating that costimulatory or adhesion pathways (CD80/CD86, CD54, or CD40) might play a minor role in the development of an acute GvHD (Figure 7A-G). These data further promote the hypothesis of Evans et al. suggesting the existence of a certain level of redundancy in the cell membrane-derived signals required for monocyte-mediated induction of Th17 cells and the involvement of additional factors that still need to be identified.[386] Furthermore, Namba et al. have demonstrated that the absolute number of $CX3CR1^+$ monocytes in the peripheral blood is decreased in patients with severe chronic GvHD.[413] These data are similar to the results of the present study revealing that the percentage of $CD14^+CX3CR1^+$ cells is significantly decreased in patients with acute GvHD compared to patients without GvHD after allogeneic HCT (Figure 7H). However,

more samples need to be analysed regarding the expression of the activation markers on monocytes in the progress of both acute and chronic GvHD and to further analyse the involvement of costimulatory or adhesion pathways in acute and chronic GvHD.

6.4 S100 proteins seem to be promising novel biomarkers for the diagnosis of GvHD

Development and progression of GvHD is mediated by numerous cellular and inflammatory effectors.[414] Several studies have demonstrated that the expression of phagocyte-derived S100 proteins is strongly upregulated in several inflammatory diseases such as sepsis, rheumatoid arthritis, cryopyrin-associated periodic syndromes, inflammatory bowel disease, vasculitis and cancer.[120,139,140,153,415] Previous works have revealed that S100A12 and S100A8/S100A9 are over-expressed during chronic active inflammatory bowel disease and serve as markers of inflammation in serum and stool.[139,153,371,392] Furthermore, a study published recently has demonstrated for the first time that levels of fecal S100A8/S100A9 are elevated in patients with intestinal GvHD stage II-III compared to patients with acute GvHD without gastrointestinal symptoms. However, the sensitivity for the diagnosis of intestinal GvHD stage I was quite low and intestinal GvHD could not be discriminated from other causes of diarrhoea such as infectious gastroenteritis.[155] On the contrary, Chiusolo et al. could show that fecal S100A8/S100A9 is elevated in patients with intestinal GvHD not only compared to patients with acute GvHD without gastrointestinal involvement but also compared to patients with infective enteritis and patients with diarrhoea after HCT.[154] These results agree with our data demonstrating that S100A12 and S100A8/S100A9 levels are significantly increased in the stool of patients with acute intestinal GvHD and also in patients with extensive chronic GvHD compared to patients without GvHD after HCT (Figure 9A and B). Furthermore, our results point out that serum concentration of both S100A8/S100A9 and S100A12 are significantly increased in patients with acute GvHD compared to patients without GvHD whereas S100 levels are not elevated in the serum of patients with chronic GvHD (Figure 9E and F). However, it must be taken into account that the sample size of patients with chronic GvHD was lower compared to patients with acute GvHD. Furthermore, correlation of S100 levels in the serum and stool with the severity of

GvHD shows that determined S100 concentrations in the serum do not correlate with GvHD grade whereas S100 levels in the stool seem to increase with the stage of intestinal GvHD (Figure 9C-D and G-H). However, stool sample size needs to be increased to confirm this assumption. Altogether, these data indicate the release of S100 proteins by activated phagocytes during GvHD. Especially fecal S100A8/S100A9 and S100A12 might display an attractive biomarker as it provides a non-invasive examination and could be used for follow up progression of GvHD.

Further results of this study demonstrate that S100A8, S100A9 and S100A12 can be detected in the extracellular distribution surrounding S100-positive cells in bowel tissue of patients with acute intestinal GvHD stage I, II and III promoting the hypothesis that these proinflammatory molecules are secreted by phagocytes during GvHD (Figure 10A-D). Staining of S100 proteins was more pronounced in bowel tissue of patients with acute intestinal GvHD stage II and III compared to bowel tissue of patients with acute intestinal GvHD stage I indicating that phagocyte-specific S100 proteins are released at sites of intestinal inflammation during acute GvHD with gastrointestinal involvement. A large multicentre study should be carried out to further verify the obtained results and to investigate if the severity of intestinal GvHD can be correlated with the area of stained S100A8, S100A9 and S100A12. However, overall our findings give first evidence that S100 proteins could be novel pathological markers for the diagnosis of acute intestinal GvHD. Similar results were obtained in studies investigating the release of S100A12 and S100A8/S100A9 in inflammatory bowel disease. These works have demonstrated the direct release of phagocyte-derived S100 proteins from inflamed tissues reflecting the secretion from infiltrating neutrophils (S100A12) and also monocytes or epithelial cells (S100A8/S100A9). These released proteins are found to promote inflammation in intestinal tissue via activation of pattern recognition receptors.[139,153]

Furthermore, the present data have revealed that monocytes isolated from patients with acute or chronic GvHD express elevated mRNA levels of S100A8, S100A9 and S100A12 compared to monocytes isolated from healthy donors, patients with diarrhoea caused by infectious gastroenteritis and patients after HCT without GvHD (Figure 8B-D). However, S100 expression levels in monocytes of patients with diarrhoea and patients after HCT are higher compared to healthy donors. These results are not surprising as diarrhoea and HCT represent enormous cell stress for the human body and S100 proteins are DAMP molecules released by activated or

damaged phagocytes under conditions of cell stress during infections.[120,139] Therefore, a cut-off point for S100 expression levels in monocytes of patients with GvHD must be determined so that S100A8, S100A9 and S100A12 expression levels in monocytes can be used as another tool for the diagnosis and follow up of acute or chronic GvHD and to discriminate intestinal GvHD from other causes of diarrhoea like infectious gastroenteritis. A recent study demonstrating that peripheral blood mRNA levels of S100 family are closely associated with inflammation confirms our finding.[381] Furthermore, our data are consistent with the findings of other studies demonstrating that S100A12 shows an enriched expression in PBMC and monocytes on mRNA level whereas S100A12 expression on protein level is restricted to granulocytes.[126,416,417] In the contrast to S100A12, S100A8 and S100A9 are expressed and secreted by both granulocytes and monocytes.[126,376] Overall, the data of this study indicate that determination of S100 levels in the serum, stool or gastrointestinal tissue as well as the analysis of S100 expression levels in monocytes might be novel tools for the diagnosis and follow-up of GvHD.

6.5 S100 proteins can promote monocyte-induced development of Th17 cells

According to the results of this study, the release of S100 proteins by activated phagocytes and the induction of Th17 cells solely mediated by monocytes seem to play an important role in the pathomechanism of GvHD. It is known that S100 proteins are endogenous TLR4 ligands which activate phagocytes by binding to TLR4 resulting in the translocation of MyD88 from the cytosol to the receptor complex at the plasma membrane and the hyperphosphorylation of IRAK-1 leading finally to the activation of NF-κB and the following expression and release of proinflammatory cytokines such as TNFα, IL-1β, IL-12, IL-6 and IL-8.[138,140] As it is known that the cytokine IL-6 is involved in the induction of Th17 differentiation and that TGF-β and IL-1β amplify the Th17 response[276-278], it was investigated in this study if stimulation of monocytes with phagocyte-specific S100 proteins promotes the induction of Th17 cells. It could be demonstrated that in comparison to unstimulated monocytes, stimulation of monocytes with S100A8, S100A9, S100A8/S100A9 or S100A12 results in significantly increased levels of Th17 cells which were similar to the ones induced by monocytes isolated from patients with GvHD (Figure 4A,B and 11A,B). Furthermore, stimulation of monocytes with S100 proteins also promotes the

induction of IL-17^{+}IFNγ^{+} cells (Figure 11A,C). However, S100A8 and S100A9 showed stronger stimulatory effects concerning the induction of Th17 and Th17/Th1 cells in comparison to the heterodimer S100A8/S100A9 and S100A12 (Figure 11A-D). S100A8 and S100A9 mainly exist under physiological conditions in the form of heterodimers and the amount of S100A8 and S100A9 homodimers is unknown but can be classified as relatively low. On the contrary, S100A12 is only present as homodimer and seems to induce a weaker immune activation due to any pathophysiological reasons. Additionally, it has to be kept in mind that the preparation method of S100A12 and S100A8/S100A9 differs as described in 4.5. Furthermore, the activity of the S100 protein preparations varies from batch to batch. In this study, it was further investigated if S100 proteins promote the induction of Th17 and Th17/Th1 cells in a TLR4-dependent manner. Therefore, TLR4 on monocytes was blocked prior to stimulation with S100 proteins by using a TLR4 antagonist. The data have shown that the specific blockade of S100-binding to TLR4 on monocytes results in significantly decreased levels of Th17 and Th17/Th1 cells (Figure 12A-C). These results point out that S100 proteins induce elevated levels of IL-17^{+} and IL-17^{+}IFNγ^{+} cells by binding to TLR4 on monocytes and are conform with the data of a previous study revealing that levels of induced Th17 cells could further be enhanced by stimulation of monocytes with the TLR4 ligand lipopolysaccharide (LPS) *in vitro*.[393] The data of the present study also demonstrate that stimulation of monocytes with S100 proteins does not have any effect on Th1 cells (Figure 11 A,D and 12 A,D). These findings may be attributed to the fact that monocytes isolated from human PBMC produce large amounts of IL-1, IL-6 but not IL-12, which is essential for the activation of the transcription factor T-bet, in response to various TLR stimulants including LPS, and are therefore potent inducers of Th17 but not Th1 cells.[279] In mice it is reported that IFNγ has down-regulating effects on the development of autoimmune diseases such as experimental autoimmune encephalomyelitis and experimental autoimmune uveitis.[418,419] Thus, the results of the present study indicate that Th17 and Th17/Th1 cells and not Th1 cells might promote the development and progression of GvHD.

6.6 Induction of Th17 cells occurs in a cell-contact dependent manner with the involvement of monocyte-associated proinflammatory cytokines

In this study, it was also examined if the induction of Th17 cells is mediated by proinflammatory cytokines released by monocytes or by cell contact between monocytes and $CD4^+$ T cells. It is known that S100 proteins can induce the activation of the transcription factor NF-κB by binding on TLR4 on phagocytes resulting in the expression and release of proinflammatory cytokines such as TNFα, IL-1β, IL-12, IL-6 and IL-8.[138,140] Thus, monocytes were stimulated with S100 proteins before incubating for 24 h. The supernatant containing the released cytokines was added to $CD4^+$ T cells and seems to promote the induction of Th17 cells (Figure 14A). However, the percentages of induced IL-17^+ cells in $CD4^+$ T cells are lower when only soluble factors are present in comparison to the Th17 levels induced by both cell-contact and soluble factors (Figure 4B,11B, 14A). These results could further be confirmed by the data of another experiment demonstrating that the addition of five cytokine-neutralizing antibodies (anti IL-17, anti-TNFα, anti-IL-1β, anti-IL-6, anti-IL-12/IL-23p40) to the co-culture consisting of monocytes pre-stimulated with S100 proteins and $CD4^+$ T cells results in diminished levels of induced Th17 cells in comparison to co-cultures set up without cytokine-neutralizing antibodies. However, addition of a single neutralizing antibody to the culture does not show any neutralizing effects (Figure 13). These results are similar to the data of Evans et al. demonstrating that blocking of either IL-1β or TNFα by neutralizing antibodies at the start of the co-culture does not prevent the increase in the percentage of IL-17^+ T cells induced by LPS-stimulated monocytes *in vitro*, whereas blocking both IL-1β and TNFα leads to the reduction of the percentage of IL-17^+ T cells.[386] In support to our observations, other studies have demonstrated that the induction of IL-17^+ cells in the presence of *in vitro* activated monocytes requires both cell contact and APC-derived proinflammatory cytokines. Kryczek et al. could demonstrate that myeloid APCs potently support the induction of IL-17^+ T cells during psoriasis which secrete IL-1β, IL-23 and CCL20.[420] Another study showed that *in vivo* activated monocytes from patients with type 1 diabetes secrete the proinflammatory cytokines IL-1β and IL-6 and induce more IL-17-secreting cells from memory T cells compared to monocytes from healthy controls. This induction of IL-17-secreting T cells by monocytes isolated from patients with type 1 diabetes can be reduced *in vitro* with a combination of an IL-

6-blocking antibody and IL-1 receptor antagonist.[395] Additionally, Evans et al. have reported recently that *in vivo* activated monocytes derived from the inflamed joints of patients with active rheumatoid arthritis promote Th17, but not Th1 or Th2 responses. Furthermore, it could be shown that *in vitro* activated monocytes induce Th17 responses in co-cultured $CD4^+$ T cells in an IL-1β/TNF-α-dependent manner whereas *in vivo* activated monocytes from the site of inflammation in rheumatoid arthritis induce increased Th17 responses in a cell-contact dependent.[386] Altogether, these multiple reports and the results of the present study indicate that the development of Th17 cells is induced by activated APC in a manner that relies on direct cell-contact and soluble factors in the form of proinflammatory cytokines especially IL-6, IL-23 and IL-1β.

As our results demonstrate that the release of S100 proteins and their stimulatory effect on monocyte-mediated induction of Th17 cells might play an important role in the development and progression of GvHD, it was investigated if monocytes stimulated via TLR4 with S100 proteins release increased levels of proinflammatory cytokines which might be involved in the Th17 cell development. The data of the present study demonstrate that *in vitro* stimulation of monocytes with S100A8, S100A9, S100A8/A9 or S100A12 results in increased release of IL-1β, IL-6, IL-8, IL-10 or TNFα (Figure 14B) and are therefore consistent with the results of a previous study demonstrating that stimulation of monocytes with S100A8 results in significantly increased secretion of IL-1β and TNFα.[140] Furthermore, it could be shown that monocytes stimulated with S100A12 release elevated levels of IL-1β, TNFα and IL-6.[138] Thus, our findings indicate that binding of S100 proteins to TLR4 on monocytes may activate the TLR4 signalling pathway resulting in the activation of the transcription factor NF-κB and the expression and secretion of proinflammatory cytokines including IL-6 promoting the differentiation of Th17 cells and IL-1β and TNFα which are involved in the amplification of the Th17 response.[140,276-278] Taken together, immune intervention targeting proinflammatory S100 proteins might be an attractive strategy to inhibit uncontrolled inflammatory processes during GvHD.

6.7 Serum levels of proinflammatory cytokines are elevated in patients with GvHD

It is known that many cytokines are involved in the development of acute GvHD. Inflammatory cytokines released during conditioning regimen play a primary role in the activation of T cells, e.g. TNFα, IL-1 and IL-6 and there is strong evidence that these cytokines and the cell-mediated cytotoxicity induce target tissue destruction during acute GvHD.[421] Several studies have demonstrated that the serum level of TNFα can be correlated with acute and chronic GvHD and might therefore be a sensitive and specific parameter for GvHD.[421-423] The data of another work indicates that IL-1 may be a critical mediator of GvHD as the administration of IL-1 receptor to patients with steroid-resistant acute GvHD results in an improvement of GvHD.[424] However, the results of the present study show that TNFα and IL-1β serum levels are only elevated in one single patient with GvHD in comparison to the appropriate serum levels in healthy donors (Figure 15A, B). These results might be due to the high sample dilution according to the manufacturer's advices resulting in the fact that many samples were below detection levels and were therefore set to zero so that no differences could be detected in these serum samples. Another study could show that IL-8 concentrations in the serum of patients with GvHD are higher in comparison to patients with no complications after HCT and patients with graft rejection.[425] These published results are consistent with the data of the present study revealing that serum levels of IL-8 are increased in patients with acute or chronic GvHD (Figure 15C). Furthermore, anti-inflammatory cytokines such as IL-10 have also been associated to GvHD in several studies. Min et al. studied the relationship between the serum concentrations of the pro- and anti-inflammatory cytokines IL-6, TNFα, IL-8 and IL-10 and transplantation-related complications in patients undergoing allogeneic HCT and could demonstrate that IL-6 and IL-10 correlate with early complications including fever, severe stomatitis and acute GvHD.[426] Consistent with these results, the data of the present study have also shown that IL-10 levels are increased in the serum of patients with acute GvHD in comparison to the determined IL-10 serum levels of healthy donors. Weakly elevated levels of IL-10 can also be detected in the serum of patients with chronic GvHD (Figure 15D). Additionally, IL-6 serum levels are elevated in individual patients with acute GvHD (Figure 15E). However, more serum samples should be collected and examined to further confirm the results of the

present study. It should be investigated if the measurement of cytokine levels in the serum of patients with acute or chronic GvHD is a sensitive and specific method for the prognosis, diagnosis and follow-up of GvHD.

6.8 Hsp90 plays a critical role in monocyte-mediated induction of Th17 cells during GvHD

The binding of S100 proteins or LPS to TLR4 on phagocytes leads to the activation of NF-κB and MAPK pathways resulting in the expression and release of proinflammatory cytokines such as TNFα, IL-1β and IL-6 which play a major role in the differentiation and expansion of Th17 cells.[138,140] TLR4 signalling and the constitutive activation of the NF-κB pathway result in the persistent increase of multiple inflammatory effector molecules and is associated with several inflammatory and autoimmune diseases such as rheumatoid arthritis, respiratory distress syndrome or inflammatory bowel disease.[139,147,148,152,153,206] The molecular chaperone Hsp90 is critically involved in the activation of monocytes via the NF-κB and MAPK pathways by maintaining the conformational stability of several key signalling proteins.[208,211,213,214,427] Several studies have demonstrated that targeting of Hsp90 with specific inhibitors results in the degradation of its client proteins such as IκB kinase complex or the interleukin-1 receptor-associated kinase diminishing innate immune responses via TLR signalling.[208-211,213,215] In the present study, the geldanamycin derivative 17-DMAG was used to inhibit the function of Hsp90. Geldanamyin is a naturally occurring benzochinone ansamycin with anti-proliferative anti-inflammatory effects which is produced by *Streptomyces hygroscopicus*.[225,428] Geldanamycin has demonstrated potent anti-tumor activity in preclinical studies, but the high hepatoxicity observed in animal models and the poor aqueous solubility diminished its clinical potential.[227] These findings resulted in the generation of geldanamycin derivatives differing only in the 17-substituent. 17-allylamino-17-demethoxygeldanamycin (17-AAG) and the water-soluble 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) with better toxicological properties have been generated and progressed to phase I and phase II clinical trials demonstrating potent anti-cancer activity in breast cancer, melanoma and prostate cancer.[228-230] In the present study, it could be demonstrated that treatment of *in vivo* activated monocytes isolated from patients with acute GvHD with

the Hsp90 inhibitor 17-DMAG results in significantly decreased levels of induced Th17 cells which are similar to those achieved with monocytes from healthy donors (Figure 16A). Furthermore, treatment of monocytes from healthy donors and from patients with acute GvHD with 17-DAMG leads to diminished percentages of Th17/Th1 cells in comparison to untreated cells (Figure 16B) whereas incubation of monocytes with the Hsp90 inhibitor does not seem to influence Th1 development (Figure 16C). Additionally, it could be demonstrated in this work that treatment of monocytes with the Hsp90 inhibitor 17-DMAG prior to stimulation with proinflammatory S100 proteins results in reduced levels of IL-17$^+$ and IL-17$^+$IFNγ$^+$ cells compared to monocytes that are not treated with 17-DMAG (Figure 17A, B). However, in this experimental set-up incubation of monocytes with the Hsp90 inhibitor prior to TLR4-mediated stimulation also results in decreased levels of IFNγ$^+$ cells in comparison to monocytes with functional Hsp90 stimulated with S100 proteins (Figure 17C). As it is known that chemical inhibition of Hsp90 can induce unspecific effects like the induction of other heat shock proteins such as Hsp40 and Hsp70[195,398-400], the expression of stress-inducible Hsp90α was silenced in monocytes from healthy donors using Hsp90α targeting siRNA. The data demonstrate that treatment of monocytes with Hsp90α targeting siRNA prior to S100 stimulation leads to diminished levels of Th17 (Figure 19A) and Th17/Th1 (Figure 19B) cells in comparison to monocytes treated with non-targeting control siRNA prior to monocyte stimulation via TLR4. Monocytes transfected with Hsp90α targeting siRNA induce similar percentages of Th1 cells (Figure 19C) in comparison to monocytes treated with the control siRNA. Overall, these experiments reveal that the inhibition of Hsp90 in monocytes induces decreased levels of both Th17 and Th17/Th1 cells whereas Th1 cells seem not be influenced. Altogether, these results indicate that targeting Hsp90, and thereby NF-κB and MAPK pathways[429], might dampen the induction of Th17 responses mediated by *in vivo* activated monocytes. Additionally, Hsp90 inhibition involves that these pathways cannot be activated any more by IL-17 and other proinflammatory cytokines including IL-1β, IL-6 and TNFα released during GvHD[36,52,429-431]. Additionally, it could be demonstrated that the application of Hsp90 inhibitors leads to the selective depletion of alloreactive T cells without impairing antiviral T cell immunity.[221] Thus, targeting Hsp90 might be an attractive novel therapy for the treatment of acute or chronic GvHD. However, hepatoxicity still remains a problem with the application of the geldanamycin

derivatives 17-AAG and 17-DMAG.[228,230] Therefore, novel synthetic small molecule inhibitors have been generated in the recent years in order to achieve a more specific targeting of Hsp90, better pharmacological effects and better tolerance. Ganetespib (STA-9090) is a novel small molecule inhibitor of Hsp90 which induces the rapid degradation of Hsp90 client proteins resulting in potent anti-tumour effects in a broad range of malignancies both *in vitro* and *in vivo*. Additionally, Ganetespib overcomes drug resistance in multiple tumor types and does not show any cardiac or liver toxicity.[222,250] Additionally, targeting the interactions between Hsp90 and its co-chaperones might be alternative approaches for the inhibition of Hsp90. In *in vitro* and *in vivo* studies, it could be demonstrated that celastrol, a quinone methide triterpene isolated from the Chinese medicinal plant *Tripterygium wilfordii*, blocks the binding of the adaptor Cdc37 to Hsp90 resulting in the degradation of Hsp90 client proteins and the exhibition of potent anticancer activity against pancreatic cancer cells.[252] Another study could show that the prevention of the Hsp70/Hsp90 complex formation by the peptide CTPR390+ induces the decrease of the Hsp90 client protein HER2 and the inhibition of breast cancer cell proliferation *in vitro*.[256,257] Additionally, in comparison to benzochinone ansamycins, targeting of Cdc37/Hsp90 and Hsp70/Hsp90 interactions does not induce an upregulation of the expression of Hsp70 which is undesirable as its anti-apoptotic effects counter the effects mediated by the inhibition of Hsp90.[255-257] Thus, targeting the co-chaperone-Hsp90 interactions are very promising tools for the inhibition of Hsp90 but their further investigation and validation will be required.

6.9 Monocytes induce elevated levels of proinflammatory Th17 cells expressing MDR1 in the presence of glucocorticoids

Glucocorticoids are usually used for the treatment of acute or chronic GvHD.[401] Glucocorticoids are small lipophilic compounds that mediate their biological effects by binding to the intracellular glucocorticoid receptor that translocates to the nucleus and directly or indirectly regulates the transcription of several genes involved in the innate and adaptive immune response. Glucocorticoids have immunosuppressive effects on peripheral T cells which are due to inhibition of expression of a wide variety of activation induced gene products including a great number of cytokines (IL-1, TNF, IL-6, IL-8 etc.) and multiple chemokines. Additionally, these compounds suppress the

cellular (Th1) immunity and promote the humoral (Th2) immunity. Additionally, glucocorticoids induce apoptosis in $CD4^+CD8^+$ thymocytes whereas resting peripheral T cells are resistant to glucocorticoid-induced death.[353,354] However, the application of glucocorticoids is accompanied by an increased susceptibility for infections, the toxicity of the drugs and an enhanced risk of relapse due to a diminished graft-versus-tumour effect which is mainly mediated by donor T cells.[402-404] Recently, it could also be demonstrated that glucocorticoids promote the development of a subset of Th17 cells that stably expresses the ATP-dependent efflux pump MDR1 which transports a wide range of drugs and xenobiotics to either the outer leaflet of the membrane or the extracellular space.[347,348,352] These so-called proinflammatory Th17.1 cells can be further characterized by the expression of the chemokine receptors CCR6, CXCR3 and CCR4 on their cell surface. These $MDR1^+$Th17.1 cells are $CCR6^+$ and express CXCR3 at high levels and CCR4 at low levels. In comparison to these $MDR1^+$ $CCR6^+CXCR3^{hi}CCR4^{lo}$ Th17.1 cells, $MDR1^-$ Th17 cells are $CCR6^+CXCR3^{lo}CCR4^{hi}$ and $MDR1^-$Th1 cells are $CCR6^-$ $CXCR3^{hi}CCR4^{lo}$. Additionally, Ramesh et al. could show that these proinflammatory $MDR1^+$Th17.1 cells are refractory to glucocorticoids.[352] Based on these data, the influence of the glucocorticoid dexamethasone on the induction of $MDR1^+$Th17.1 cells was compared with the effect of the Hsp90 inhibitor 17-DMAG in the present study. Consistent with the data of Ramesh et al. it could be demonstrated that treatment of monocytes with dexamethasone results in the induction of increased levels of $CCR6^+MDR1^+$ cells and $MDR1^+$ $CCR6^+CXCR3^{hi}CCR4^{lo}$ Th17.1 cells whereas monocytes that remained untreated or were alternatively incubated with the Hsp90 inhibitor 17-DMAG induce low levels of $MDR1^+$ Th17.1 cells (Figure 20A, B). The data of several studies demonstrating the potent antitumor activity of 17-DMAG[228-230] together with the results of the present study indicating that 17-DMAG does not promote the development of proinflammatoy Th17.1 cells expressing MDR1 suggest that Hsp90 might be a novel target for the treatment of GvHD.

6.10 Hypothesis for the pathomechanism of GvHD

Based on the results of the present study the following hypothesis can be proposed for the pathomechanism of GvHD. Cell stress induces the expression and release of the heterodimer S100A8/S100A9 and the homodimer S100A12 by activated

monocytes and granulocytes during GvHD. These S100 proteins act as endogenous ligands of TLR4 and induce the activation of monocytes, the expression and release of proinflammatory cytokines including TNFα, IL-1β, IL-12, IL-8 and IL-6 and thereby promote the development of Th17 cells. These Th17 cells release proinflammatory cytokines such as IL-17A, IL-17F, IL-21 and IL-22 leading to the activation of the NF-κB and MAPK pathways which further promote GvHD (Figure 21).

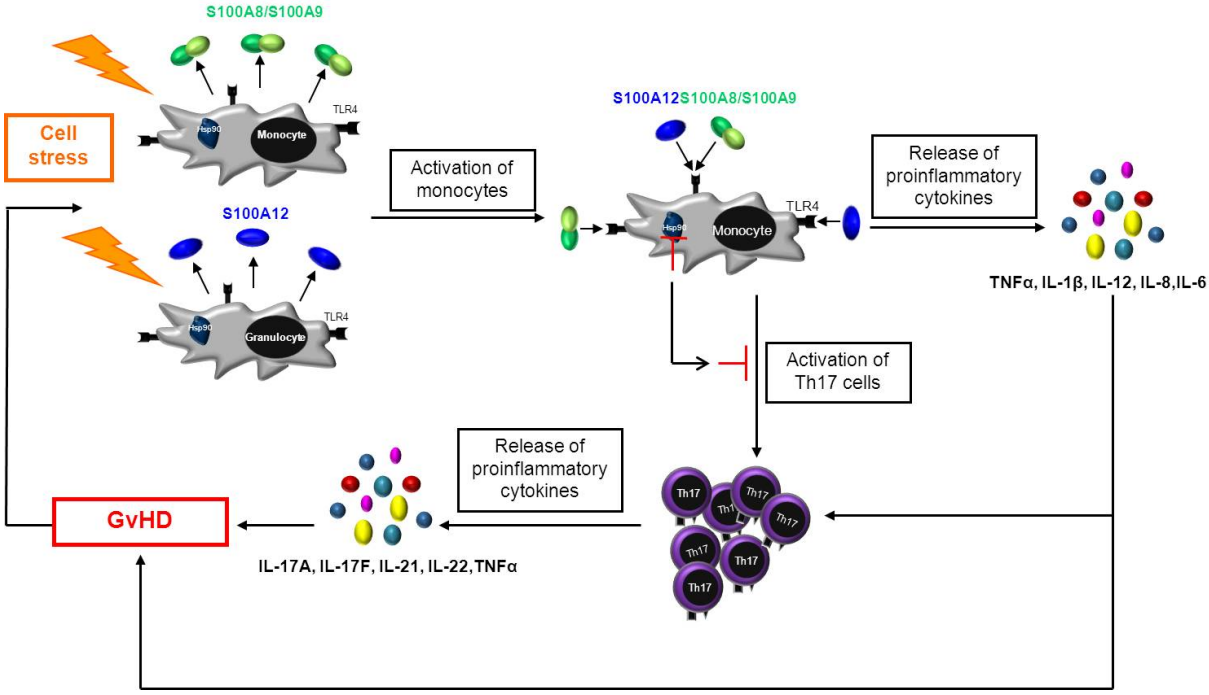


Figure 21: Hypothesis for the pathomechanism of GvHD

At the onset of GvHD, S100A8/S100A9 and S100A12 are released by activated phagocytes and act as endogenous ligands of TLR4. Subsequent activation of monocytes induces the expression and release of proinflammatory cytokines and the development of Th17 cells. Th17 cells release proinflammatory cytokines which further promote GvHD.

7 References

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8 Supplement

8.1 Abbreviations

°C	degree Celsius
17-AAG	17-allylamino-17-demethoxygeldanamycin
17-DMAG	17-dimethylaminoethylamino-17-demethoxygeldanamycin
APC	antigen presenting cell
APC	allophycocyanin
ATG	anti-thymocyte globulin
ATP	adenosine-5'-triphosphate
BAFF	B cell activation factor
BCA	bicinchoninic acid
CCR	CC chemokine receptor
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CRP	C-reactive protein
CSA	cyclosporine A
CXC	chemokine with cysteine separated by an amino acid defined as X
CXCR	CXC chemokine receptor
DAMP	damage associated molecular pattern
DC	dendritic cell
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECP	extracorporeal photophoresis
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated protein kinase
EU	endotoxin units
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
Fox	forkhead box
FSC	forward scatter
g	g-force
GAPDH	glycerinaldehyde 3-phosphate dehydrogenase
GATA-3	GATA-binding protein 3

GITR	glucocorticoid-induced tumour-necrosis-factor-related protein
Gp96	96-kilodalton glycoprotein
Grp170	170-kilodalton glucose-regulated protein
GvHD	graft-versus-host disease
GvL	graft-versus-leukemia
h	hour(s)
HAT	histone acetyltransferase
HCT	hematopoietic cell transplantation
HDAC	histone deacetylase
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HSF-1	heat shock factor-1
Hsp	heat shock protein
Hsp70	70-kilodalton heat shock protein
Hsp90	90-kilodalton heat shock protein
IκB	inhibitor of κB
ICAM-1	intracellular adhesion molecule 1
ICC	intracellular cytokine staining
IFN	interferon
Ig	immunoglobulin
IKK	IκB kinase complex
IL	interleukin
IPTG	Isopropyl-β-D-thiogalactopyranosid
IRAK-1	interleukin-1 receptor associated kinase-1
iTreg	induced regulatory T cell
JIA	juvenile idiopathic arthritis
kDa	kilodalton
LPS	lipopolysaccharides
MAC	myeloablative conditioning
MACS	magnetic-activated cell sorting
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase(s)
MCP-1	monocyte-chemoattractant protein-1
MDR1	multi-drug resistance protein 1

MDS	myelodysplastic syndrome
MHC	major histocompatibility complex
min	minute(s)
MIP-2	macrophage inflammatory protein-2
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
MMF	mycophenolate mofetil
MPA	mycophenolic acid
MSC	mesenchymal stem cells
MTX	methotrexate
MyD88	myeloid differentiation primary response protein 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cell
nm	nanometre(s)
nM	nanomolar
nTreg	naturally occurring regulatory T cell
OD	optical density
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell(s)
PBS	phosphate-buffered saline
PDGRF	platelet-derived growth factor receptor
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin-chlorophyll-protein
PHS	pooled human serum
PI	propidium iodide
PMA	phorbol-12-myristate-13-acetate
PRR	pattern recognition receptor
PUVA	psoralen and ultraviolet A irradiation
RA	rheumatoid arthritis
RAGE	receptor for advanced glycation end products
RANTES	regulated upon activation, normal T cells expressed and secreted
RIC	reduced intensity conditioning

RNA	ribonucleic acid
RNAi	RNA interference
ROR	retinoic acid receptor-related orphan nuclear receptor
RT	room temperature
RT-PCR	real-time polymerase chain reaction
Runx1	Runt-related transcription factor
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphatepolyacrylamide gel electrophoresis
siRNA	small interfering RNA
SSC	side scatter
STAT	signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TLR	toll like receptor
TMB	tetramethylbenzidine
TNF	tumour necrosis factor
TPR	tetratricopeptide
Treg cell	regulatory T cell
U	units
UVA	ultraviolet A
V	volts
VCAM-1	vascular cell adhesion molecule 1
VLE	very low endotoxin
µg	microgram(s)
µl	microliter(s)
µM	micromolar

8.2 Publications

8.2.1 Original contributions

Reinhardt K, Foell D, Vogl T, Mezger M, Wittkowski H, Fend F, Gille C, Feuchtinger T, Lang P, Handgretinger R, Bethge W, Holzer U. Monocyte-induced development of Th17 cells and the release of S100 proteins are involved in the pathogenesis of graft-versus-host disease *J Immunol* 2014 Oct 1;193(7):3355-65. doi: 10.4049/jimmunol.1400983.

Holzer U, **Reinhardt K**, Lang P, Handgretinger R, Fischer N. Influence of a mutation in IFN-gamma receptor 2 (IFNGR2) in human cells on the generation of Th17 cells in memory T cells. *Hum Immunol*. 2013;74(6):693-700.

8.2.2 Conference articles

Reinhardt K, Foell D, Vogl T, Fend F, Gille C, Feuchtinger T, Lang P, Handgretinger R, Bethge W, Holzer U. Involvement Of S100 Proteins and Hsp90 In The Pathogenesis Of Graft-Versus-Host Disease After Allogeneic Hematopoietic Cell Transplantation. *American Society of Hematology (ASH) - New Orleans – 2013*

Reinhardt K, Foell D, Vogl T, Fend F, Gille C, Feuchtinger T, Lang P, Handgretinger R, Bethge W, Holzer U. Role of monocytes, S100 proteins and Hsp90 in pathogenesis of graft-versus-host disease. *European Bone Marrow Transplantation (EBMT) - London - 2013*

8.2.3 Awards

Abstract Achievement Award

Reinhardt K, Foell D, Vogl T, Fend F, Gille C, Feuchtinger T, Lang P, Handgretinger R, Bethge W, Holzer U. Involvement Of S100 Proteins and Hsp90 In The Pathogenesis Of Graft-Versus-Host Disease After Allogeneic Hematopoietic Cell Transplantation. *American Society of Hematology (ASH) - New Orleans – 2013*

8.3 Danksagung

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8.4 Lebenslauf

Persönliche Daten

Name	Katharina Reinhardt
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Schulbildung

1996-2005 Mörike-Gymnasium Ludwigsburg
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1992 – 1996 Grundschule Benningen

Studium

10/2005-08/2011 Technische Biologie - Universität Stuttgart
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07/2010-05/2011 Diplomarbeit - Universität Stuttgart - Institut für Zellbiologie und Immunologie - Bereich Biomedical Engineering
- Thema: „Mouse and human 4-1BBL antibody fusion proteins for targeted cancer immunotherapy“

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Studienarbeit - Proimmune Ltd. in Oxford
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Promotion

seit 09/2011 Doktorandin - Universitätsklinik für Kinder- und Jugendmedizin in Tübingen - Abteilung Hämatologie/Onkologie -
- Thema: „Role of monocyte-induced development of Th17 cells, pro-inflammatory S100 proteins and heat-shock protein 90 in the pathogenesis of graft-versus-host disease“

9 Declaration

I hereby declare that this study was carried out and written entirely by myself without any non-permitted help and without the usage of any means except the mentioned ones.

Benningen, November 11th 2014

Katharina Reinhardt