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Monocyte-Induced Development of Th17 Cells and the Release of S100 Proteins Are Involved in the Pathogenesis of Graft-versus-Host Disease

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Graft-versus-host disease (GvHD) is a major cause of morbidity and mortality after allogeneic hematopoietic cell transplantation. However, the pathophysiology of GvHD remains poorly understood. In this study, we analyzed the induction of Th17 cells by monocytes of patients with GvHD in vitro, demonstrating that monocytes isolated from patients with acute skin and intestinal GvHD stage I–IV and chronic GvHD induce significantly increased levels of Th17 cells compared with patients without GvHD. S100 proteins are known to act as innate amplifier of inflammation. We therefore investigated the presence of S100 proteins in the stool, serum, and bowel tissue of patients with GvHD and the influence of S100 proteins on the induction of Th17 cells. Elevated levels of S100 proteins could be detected in patients with acute GvHD, demonstrating the release of these phagocyte-specific proteins during GvHD. Furthermore, stimulation of monocytes with S100 proteins was found to promote Th17 development, emphasizing the role of S100 proteins in Th17-triggered inflammation. Altogether, our results indicate that induction of Th17 cells by activated monocytes and the stimulatory effects of proinflammatory S100 proteins might play a relevant role in the pathogenesis of acute GvHD. Regarding our data, S100 proteins might be novel markers for the diagnosis and follow-up of GvHD. The Journal of Immunology, 2014, 193: 3355–3365.

A
dollogeneic hematopoietic cell transplantation (HCT) is an effective treatment for patients with hematologic malignancies, aplastic anemia, congenital immunodeficiency disorders, and autoimmune diseases (1, 2). One of the major serious morbidities associated with HCT is the development of acute or chronic graft-versus-host disease (GvHD) (3). The pathophysiology of GvHD is complex and not fully understood. The role of Th17 cells during GvHD is discussed controversially and still remains unclear. High levels of IFN-γ-producing T cells detected in the skin of patients with cutaneous acute GvHD after allogeneic stem cell transplantation and increased IFN-γ expression in PBMCs have led to the conclusion that GvHD is mediated predominantly by proinflammatory Th1 cells (4, 5). However, it could be shown in mouse models that inhibition of Th1 cytokines resulted in exacerbation of acute GvHD (6, 7). Further investigations of T cell subsets involved in the pathophysiology of GvHD have been directed toward Th17 cells. Differentiation of IL-17–producing human Th cells is initiated by IL-23, IL-1β, and IL-6 (8–11). However, it has also been shown that the induction of Th17 cells is not solely cytokine dependent, but rather requires cell contact with activated monocytes (12). Full expression of the Th17 phenotype depends on the transcription factors retinoid-related orphan receptor γt, retinoid-related orphan receptor α, and STAT-3 (13–15). Th17 cells generate the proinflammatory cytokines IL-17A, IL-17F, IL-21, IL-22, and TNF (11, 15). IL-17A and IL-17F bind to the IL-17R expressed on leukocytes, epithelial cells, endothelial cells, keratinocytes, and fibroblasts. Binding of IL-17A and IL-17F to the receptor induces the production of other proinflammatory cytokines and chemokines and the recruitment of leukocytes and neutrophils (15–17). It is known that IL-17 plays a crucial role in the development of several autoimmune diseases such as multiple sclerosis, rheumatoid arthritis (RA), systemic lupus erythematosus, systemic sclerosis, and inflammatory bowel disorders (18–24). Several studies also indicate a contribution of Th17 cells to GvHD disorders. In mice, it could be demonstrated that i.v. injection of in vitro differentiated Th17 cells induced lethal GvHD with extensive pathologic lesions in the lung and skin (25). Numbers of Th17 cells in the skin of patients with acute GvHD and active chronic GvHD were increased compared with control samples (26). Development and progression of GvHD are mediated by numerous cellular and inflammatory effectors (27). However, several of these molecules are still unknown. Previous studies demonstrated that proinflammatory S100 proteins play an important role in many inflammatory diseases, such as inflammatory bowel disease (IBD) or RA (28–30). These S100 proteins belong to the group of damage-associated molecular pattern molecules and are released by activated or damaged phagocytes under conditions of cell stress during infections and autoimmune diseases (28, 29, 31). S100A8 (also

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Abbreviations used in this article: Ct, cycle threshold; GvHD, graft-versus-host disease; HCT, hematopoietic cell transplantation; IBD, inflammatory bowel disease; RA, rheumatoid arthritis.

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known as myeloid-related protein 8, calgranulin A) and S100A9 (myeloid-related protein 14, calgranulin B) exist mainly as S100A8/S100A9 heterodimers. It has been demonstrated that S100A8 and S100A12 are endogenous ligands of TLR4 that induce the translocation of MyD88 from the cytosol to the receptor complex at the plasma membrane and hyperphosphorylation of IL-1R-associated kinase-1, which results in the activation of the transcription factor NF-kB and the expression of proinflammatory cytokines, such as TNF-α, IL-1β, IL-12, IL-8, and IL-6 (31, 32). S100 proteins represent promising novel therapeutic targets, as it is known that the expression of S100A8 and S100A9 is upregulated in several inflammatory disorders, such as sepsis, RA, IBD, vasculitis, and cancer (28, 30, 32). Additionally, fecal S100A12 levels from patients with IBD are elevated and correlate with the histology score (29, 33–35). In the current study, the role of Th17 cells and S100 proteins in the context of acute and chronic GVHD and their use as novel markers for diagnosis, follow-up, and therapy were investigated.

Materials and Methods

Donors

Blood from 32 healthy donors was obtained from the blood bank Tuebingen in the form ofuffy coats. Fifty-two 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%), as follows: HLA-matched unrelated donors in 19 cases (40%), HLA-haploidentical donors in 20 cases (43%), and HLA-mismatched unrelated donors in 2 cases (4%). Intensity of conditioning regimens was classified, as described by Bacigalupo (36). In this study, samples of 17 children and 6 adults undergoing HCT were examined consecutively (before conditioning days 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 diarrhea caused by infectious gastroenteritis were additionally included. Characteristics of these patients are shown in Supplemental Tables 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.

Expression and purification of S100 proteins

Human S100A8, S100A9, and S100A12 were recombinantly expressed in Escherichia coli and purified, as described earlier (31, 32, 37, 38). Briefly, E. coli B212(DE3) bacteria were transformed with pET11/20 expression vector containing S100A8 or S100A9 cDNA. After growing bacteria at 37°C 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-thiogalactopyranoside, the pellet was lysed by sonication and the insoluble material was removed by centrifugation. The supernatant was adjusted up to 10 mM CaCl2 and applied onto a phenyl-Sepharose column. After elution, material was removed by centrifugation. The supernatant was adjusted up to thiogalactopyranoside, the pellet was lysed by sonication and the insoluble (Biochrom) density gradient centrifugation. CD4+ T cells were isolated PBMCs were isolated from heparinized peripheral blood by Ficoll-Hypaque (Biochrom) density gradient centrifugation. CD4+ T cells were isolated from PBMCs by negative selection using CD4+ T cell isolation kit II (Miltenyi Biotec). CD4+ cells were isolated by positive selection via CD14 Microbeads (Miltenyi Biotec). The purity of these isolated cells, confirmed by flow cytometry using anti-CD3, anti-CD4, and anti-CD14 Abs, was >90%.

Cell culture

CD4+ T cells from healthy donors were cocultured with 100 ng/ml anti-CD3 mAb Orthoclone OKT3 (Janssen-Cilag) and monocytes from healthy donors, patients with acute or chronic GVHD, or patients before conditioning and at days 30, 60, and 100 post-HCT (≤ 10 d) without GVHD at a monocyte:T cell ratio of 1:4 for 5 d. Optionally, monocytes from healthy donors were additionally stimulated with 5 µg/ml S100A8, S100A9, S100A8/S100A9, or S100A12 for 4 h before coculturing with CD4+ T cells. Binding of S100 proteins to monocytes was inhibited by pre-incubation of monocytes with TLR4 antagonist (0.1 µg/ml; InvivoGen) for 30 min, followed by 4-h stimulation with S100 proteins. To investigate whether Th17 induction occurred, cell-contact–dependent or cytokine-mediated monocytes were cultured in RPMI 1640 media (Biochrom) containing 10% pooled human serum (University Children’s Hospital), penicillin/streptomycin (100 U/ml and 100 µg/ml), and 10 mM HEPES buffer (all from Biochrom). 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 d in the presence of Orthoclone OKT3 (100 ng/ml) and anti-CD28 mAb (1 µg/ml; BD Biosciences).

Intracellular cytokine staining

After 5 d of coculture of monocytes and CD4+ T cells, as described above, cells were stimulated for 5 h with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (750 ng/ml; Sigma-Aldrich) in the presence of monensin (2 µM; eBioscience) during the last 3 h. For flow cytometric analysis, cells were harvested, stained for the cell surface marker CD4 (anti-CD4-PE; BioLegend), fixed, permeabilized, and labeled with anti–IL-17 Alexa Flour647 (eBioscience) Ab alone or additionally with anti–IFN-γ FITC (eBioscience), according to the manufacturer’s protocol.

S100 ELISA

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 days 7, 14, 21, 30, 60, and 100 (± 5 d) post-HCT (stool sample size = 24; serum sample size = 62). Furthermore, stool samples of 7 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 2–4 d. S100A8/ S100A9 concentrations in the stool were determined using calprotectin assay (Buehlmann). For S100A12 detection, ∼100 mg stool samples were suspended in extraction buffer at 1:50 dilution for homogenization, as described and validated previously (34, 39, 40). Concentrations of S100A8/ S100A9 and S100A12 in the serum were determined by double-sandwich ELISA, as described previously (34, 41).

Immunohistochemistry

S100A8, S100A9, and S100A12 were detected on paraffin-embedded bowel specimens of healthy controls (n = 2) and acute intestinal GVHD stages I, II, and III (n = 3 in each group) by staining with specific Abs, as described previously (34, 37, 42). Secondary Abs and substrates for color reaction were used, as described before (42). Cell nuclei were stained using hematoxylin. Images were captured using a Zeiss Axioskop connected to an Axioscam camera supplied with software Axiosvision 3.0 for Windows (Zeiss). Percentage of S100A8-, S100A9-, or S100A12-stained areas in the individual sections was determined using ImageJ.

Cytokine assay

Cytokine levels of IL-1β, IL-6, IL-8, IL-10, and TNF-α in the supernatant of monocytes were measured by Bio-Plex Human Cytokine Group I 5-plex Assay (Bio-Rad). For further investigation of the pathogenesis of GVHD, monocytes isolated from healthy donors were optionally stimulated with S100A8, S100A9, S100A8/S100A9, or S100A12 (5 µg/ml) for 4 h before incubating for 24 h to assess cytokine release. Analysis of data was performed using Bio-Plex Manager software.

Real-time PCR

RNA was extracted from isolated monocytes from healthy donors (n = 5), patients with diarrhea caused by infectious gastroenteritis (n = 5), patients after HCT without GVHD (n = 6), and patients after HCT with acute or...
chonic GvHD (n = 7) using the RNaseq mini kit (Qiagen). First-strand cDNA was synthesized from 500 ng total RNA using QuantiTect Reverse Transcription Kit (Qiagen), according to manufacturer’s instructions. 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. (43). A total of 5 μl SYBR mix was added to 2 μl RNase-free water (Qiagen), 1 μl cDNA, and 1 μl 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 3 s at 95°C and 25 s at 59°C were run on the CFX96 Real-Time PCR Detection System (Bio-Rad). For analysis, cycle threshold (Ct) values normalized to the housekeeping gene GAPDH were calculated as 2\(^{(Ct_{[GAPDH]} - Ct_{[S100]})}\), as described previously (44).

**Results**

**Monocytes from patients with GvHD induce increased levels of Th17 cells**

Like RA, GvHD is an inflammatory process that is driven by the release of TNF-α, IL-1β, and IL-6 and the activation of APCs leading to the damage of tissue (45–48). It is known that in vivo activated monocytes from the site of inflammation in humans specifically promote Th17 responses during RA (49). Thus, the influence of monocytes on the induction of Th17, Th1, and Th17/Th1 cells in patients with GvHD was investigated. Therefore, 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 days 30, 60, and 100-post HCT (±10 d) without GvHD, and 32 healthy donors. These monocytes were cocultured with CD4+ T cells from healthy donors, and the percentage of induced IL-17+, IL-17*IFN-γ*, and IFN-γ* cells was determined. The data demonstrate that monocytes from patients with acute GvHD and patients with chronic GvHD induced significantly higher levels of IL-17* cells and IL-17*IFN-γ* cells compared with monocytes isolated from patients without GvHD and healthy donors (Fig. 1A, 1B). Monocytes from patients with GvHD also induced elevated levels of Th1 cells. However, the increase was lower compared with Th17 and Th17/Th1 cells (Fig. 1C).

**S100 levels in the stool and serum are elevated in patients with GvHD**

Several studies have demonstrated that S100A12 and S100A8/ S100A9 are overexpressed during chronic active IBD and act as markers of inflammation in serum and stool (29, 33, 34, 50). Like IBD, GvHD is also promoted by a number of complex immune responses consisting of several inflammatory mediators leading to tissue necrosis (46, 51). Furthermore, previous results from Chiusolo et al. and Rodriguez-Otero et al. (52, 53) indicated that fecal S100A8/S100A9 levels were elevated in patients with intestinal GvHD. Therefore, S100 levels were determined in the stool and serum of patients, as described above. For analysis, stool and serum samples were collected consecutively from patients on days 7, 14, 21, 30, 60, and 100 (±5 d) post-HCT. Stool and serum samples were collected immediately at the onset of GvHD and also in the further progression of GvHD every 2–14 d, depending on the duration of GvHD. The data points from the same patients are highlighted in one color in Fig. 2. S100 levels in stool samples of 7 patients with intestinal GvHD (2 patients with acute intestinal GvHD stage II, 1 patient with acute intestinal GvHD stage IV, and 4 patients with extensive chronic GvHD) and 9 patients without GvHD were compared. The results represent that S100A8/S100A9 and S100A12 levels were significantly increased in samples of the stool of patients with acute intestinal GvHD (n = 6) and also in the stool of patients with extensive chronic GvHD (n = 4) compared with samples of patients without GvHD (n = 24) (Fig. 2A, 2B).

Furthermore, S100A8/S100A9 and S100A12 concentrations in the serum of 11 patients with acute or chronic GvHD (5 patients with acute GvHD grades I–IV, 2 patients with acute GvHD grade II/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 without GvHD were determined at the stated time points after HCT. S100A8/S100A9 serum concentrations of samples of patients with acute GvHD (n = 34) were significantly elevated compared with serum concentrations of samples of patients without GvHD (n = 62) (Fig. 2C). In patients with chronic GvHD, serum levels of S100A8/S100A9 were not increased in eight samples compared with patients without GvHD post-HCT. However, the sample size of patients with chronic GvHD was lower compared with the one of the patients with acute GvHD. Similar results were obtained for S100A12 levels in the serum. Samples of patients with acute GvHD (n = 33) showed significantly increased S100A12 levels in the serum compared with patients without GvHD post-HCT (n = 53). Serum concentrations of S100A12 did not differ in samples of patients with chronic GvHD (n = 8) and patients without GvHD (Fig. 2D). S100 levels in the stool seemed to increase with severity of intestinal acute GvHD, although no statistical significance could be obtained, whereas S100 concentrations in the serum did not correlate with the grade of GvHD (Supplemental Fig. 1A–D).

**Release of S100A8, S100A9, and S100A12 into bowel tissue is increased in patients with acute intestinal GvHD**

As patients with acute GvHD showed significantly increased S100 levels in the serum and stool compared with patients without GvHD after HCT, it was further investigated whether proinflammatory S100 proteins are also present in inflamed bowel tissue from patients with acute intestinal GvHD. Therefore, S100A8, S100A9, and S100A12 were stained in intestinal biopsies of healthy controls with no pathological findings (n = 2) and patients with acute intestinal GvHD stage I (n = 3) and stage II–III (n = 6). 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 Fig. 3A. In bowel tissue from patients with acute intestinal GvHD stages I, II, and III, S100A8, S100A9, or S100A12 was 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, percentages of S100A8-, S100A9-, and S100A12-stained areas were higher in sections of bowel tissue from patients with acute intestinal GvHD stages II and III compared with the patients with acute intestinal GvHD stage I. Percentages of S100A8- and S100A12-stained areas in bowel specimens of patients with intestinal GvHD stage II–III were significantly increased in comparison with the healthy controls, whereas no statistical significance could be determined in S100A9-stained areas. In intestinal tissue of healthy controls, hardly any S100 staining was detected (Fig. 3B–D).

**Monocytes from patients with GvHD express elevated levels of S100A8, S100A9, and S100A12**

As S100 proteins could be detected in increased levels in the serum, stool, and bowel tissue of patients with GvHD, it was investigated whether expression levels of S100A8, S100A9, and S100A12 are different in monocytes from healthy donors, patients with diarrhea of other causes, patients without GvHD, and patients with acute or chronic GvHD. RT-PCR analysis showed that normalized Ct values for S100A8, S100A9, and S100A12 proteins 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 without GvHD (n = 34) were significantly increased compared with serum concentrations of samples of patients without GvHD (n = 62) (Fig. 2C). In patients with chronic GvHD, serum levels of S100A8/S100A9 were not increased in eight samples compared with patients without GvHD post-HCT. However, the sample size of patients with chronic GvHD was lower compared with the one of the patients with acute GvHD. Similar results were obtained for S100A12 levels in the serum. Samples of patients with acute GvHD (n = 33) showed significantly increased S100A12 levels in the serum compared with patients without GvHD post-HCT (n = 53). Serum concentrations of S100A12 did not differ in samples of patients with chronic GvHD (n = 8) and patients without GvHD (Fig. 2D). S100 levels in the stool seemed to increase with severity of intestinal acute GvHD, although no statistical significance could be obtained, whereas S100 concentrations in the serum did not correlate with the grade of GvHD (Supplemental Fig. 1A–D).
with extensive chronic GvHD) \((n = 7)\) were increased compared with healthy donors \((n = 5)\), patients with diarrhea caused by infectious gastroenteritis \((n = 5)\), and patients after HCT without GvHD \((n = 6)\), demonstrating that mRNA expression levels of S100 proteins are elevated in monocytes of patients with acute or chronic GvHD. Although highest expression levels of S100A8, S100A9, and S100A12 could be detected in monocytes of patients with GvHD, S100 expression levels were also elevated in monocytes of patients after HCT and patients with diarrhea compared with healthy donors. Averaged Ct values for S100A8, S100A9, and S100A12 were 2.9-, 2.8-, and 2-fold higher in patients with GvHD compared with patients after HCT without GvHD. Equally, averaged Ct values for S100A8, S100A9, and S100A12 were elevated 2.6-, 2-, and 1.9-fold in patients with GvHD compared with patients with diarrhea (Fig. 4).

Stimulation of monocytes with S100 proteins results in a significant increase in the percentage of induced Th17 cells

It could be shown that monocytes from patients with GvHD induce increased levels of IL-17+ cells and express elevated levels of S100 proteins could be detected in the serum, stool, and bowel tissue of patients with GvHD. Thus, it was investigated whether S100 proteins

**FIGURE 1.** Percentage of IL-17+, IL-17+IFN-γ+, and IFN-γ+ cells induced by monocytes from patients with acute and chronic GvHD, without GvHD after HCT, and healthy donors. CD4+ T cells from healthy donors were cocultured with anti-CD3 mAb (100 ng/ml) and monocytes from healthy donors; from patients before conditioning; patients without GvHD on days 30, 60, and 100 (±10 d) post-HCT; and patients with acute and chronic GvHD at a monocyte:T cell ratio of 1:4 for 5 d. After 5 d of coculture, 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. The percentage of (A) IL-17+ cells, (B) IL-17+IFN-γ+ cells, and (C) IFN-γ+ cells in CD4+ T cells was assessed by flow cytometry. Statistical significance was determined using the Kruskal–Wallis test (with Dunn’s post hoc test): *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\).
influence monocyte-induced development of Th17 cells. Therefore, monocytes from healthy donors were prestimulated with S100 proteins for 4 h before coculturing with CD4+ T cells. Stimulation of monocytes with S100A8, S100A9, the heterodimer S100A8/S100A9, or S100A12 induced significantly higher percentages of Th17 cells compared with unstimulated monocytes (Fig. 5A). 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) (Figs. 1A, 5A). The percentage of Th17/Th1 cells could significantly be increased by stimulation of monocytes with S100A8 and S100A9. However, the stimulatory effects of S100 proteins on the induction of Th17/Th1 cells were less pronounced compared with Th17 cells (Fig. 5B). Stimulation of monocytes with S100 proteins had no effects on Th1 cells (Fig. 5C). Representative flow plots are shown in Fig. 5D.

S100 proteins induce elevated levels of Th17 cells by binding to TLR4 on monocytes

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 hyperphosphorylation of IL-1R–associated kinase-1, which induces NF-κB–dependent gene expression (31, 32). The data shown above demonstrate that stimulation of monocytes with S100 proteins induces increased levels of Th17 cells. To further investigate whether this stimulatory effect occurs in a TLR4-dependent manner, the receptor was blocked using a TLR4 antagonist. The blockade of S100 binding to TLR4 on monocytes led to significantly reduced levels of IL-17+ cells. Any unspecific effects of TLR4 antagonist could be excluded as monocytes solely treated with the antagonist induced approximately the same levels of Th17 cells compared with untreated monocytes (Fig. 6).

Induction of Th17 cells occurs cell–cell contact dependent and cytokine mediated

To assess whether the induction of Th17 cells is mediated via cytokines or cell-contact dependent, monocytes from healthy donors, optionally prestimulated with S100A8, S100A9, the heterodimer, or S100A12, were seeded and incubated for 24 h. The supernatant was then added to the culture medium of freshly isolated CD4+ T cells at a ratio of 1:1. CD4+ T cells were cultured,
as described above. Addition of supernatant of monocytes pre-
timulated with S100 proteins to CD4+ T cells induced increased
percentages of IL-17+ cells compared with supernatant of unsti-
mulated monocytes. However, S100A8, S100A9, and S100A12 showed
stronger stimulatory effects on monocytes compared with the
heterodimer S100A8/S100A9 (Fig. 7A). Overall, percentages of
induced IL-17+ cells in CD4+ cells were notably lower when
only soluble factors were present compared with Th17 induction
mediated via both cell-contact and soluble factors (Figs. 1A, 5A,
7A). Next, it was investigated which proinflammatory cytokines
are released by monocytes and therefore promote the induction of
Th17 cells. Proinflammatory cytokine levels in the supernatant of
monocytes were measured by Human Cytokine Group I 5-plex Assay. Therefore, monocytes isolated from healthy donors were
optionally stimulated with S100A8, S100A9, S100A8/S100A9, or S100A12 for 4 h before incubating for 24 h to determine cytokine release. Results are shown as n-fold increase relative to 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. It could be demonstrated 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 TNF-α compared with unstimulated monocytes. Consistent with the data shown in Fig. 7A, stimulation of monocytes with S100A8, S100A9, or S100A12 had stronger stimulatory effects on monocytes compared with the heterodimer S100A8/S100A9, resulting in increased levels of released proinflammatory cytokines (Fig. 7B).

Discussion

Our data demonstrate that monocyte-induced development of Th17 cells and the release of proinflammatory S100 proteins might play an important role during GvHD. It is known that GvHD is mediated by several cellular and inflammatory factors and the release of proinflammatory cytokines such as TNF-α, IL-1, and IL-6 (27). Although the role of Th17 cells in GvHD is still discussed controversially, several studies have demonstrated an involvement of Th17 cells in the pathogenesis of GvHD (6, 7, 25, 26). Consistent with previous results, the data of the current study show that monocytes isolated from patients with acute or chronic GvHD induce significantly increased levels of Th17 cells in vitro compared with monocytes from patients without GvHD and healthy donors, pointing toward a crucial role of activated monocytes in the initiation and progression of GvHD. Monocytes from patients with GvHD also induce elevated levels of Th1 cells. However, the increase is notably lower compared with Th17 cells. In support of our observations, it has been demonstrated recently that in vivo activated monocytes from inflamed joints of patients with active RA specifically induce a Th17 response in blood-derived CD4+ T cells (49).

Several studies have demonstrated that expression of phagocyte-derived S100 proteins is strongly upregulated in several inflammatory diseases (28–30, 32, 33). A previous study revealed that levels of fecal S100A8/S100A9 were elevated in patients with intestinal GvHD stage II–III compared with 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 diarrhea such as infectious gastroenteritis (53). On the contrary, Chiusolo et al. (52) could show that fecal S100A8/S100A9 is increased in patients with intestinal GvHD not only compared with patients with acute GvHD without gastrointestinal involvement, but also compared with patients with infective enteritis and patients with diarrhea after HCT. 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 with patients after HCT without GvHD. Furthermore, our results point out that serum concentrations of S100A8/S100A9 and S100A12 are significantly elevated in patients with acute GvHD compared with patients without GvHD, whereas S100 levels are not increased in the serum of patients with chronic GvHD. However, it must be taken into account that sample size of patients with chronic GvHD was lower compared with patients with acute GvHD. Altogether, these data indicate the release of S100 proteins by activated phagocytes during GvHD. Fecal S100A8/S100A9 and S100A12 might display attractive biomarkers as they provide a noninvasive examination and could be used for follow-up progression of GvHD.

Further results demonstrate that S100A8, S100A9, and S100A12 can be detected in extracellular distribution surrounding S100-
positive cells in bowel tissue of patients with acute intestinal GvHD stages I, II, and III, promoting the hypothesis that these proinflammatory molecules are secreted by phagocytes during GvHD. Staining of S100 proteins was more pronounced in bowel tissue of patients with acute intestinal GvHD stages II and III compared with bowel tissue of patients with acute intestinal GvHD stage I. This release of phagocyte-specific S100 proteins has already been demonstrated during IBD (29, 33). Our findings support the notion that S100 proteins could be novel pathological markers for the diagnosis of acute intestinal GvHD. However, a large multicenter study should
be carried out to further verify the obtained results and to investigate whether severity of intestinal GvHD can be correlated with the area of stained S100A8, S100A9, and S100A12. Furthermore, the present data reveal that monocytes isolated from patients with acute or chronic GvHD express elevated mRNA levels of S100A8, S100A9, and S100A12 compared with monocytes isolated from healthy donors, patients with diarrhea caused by infectious gastroenteritis, and patients after HCT without GvHD. However, S100 expression levels in monocytes of patients with diarrhea and patients after HCT are higher compared with healthy donors. This is not surprising as S100 proteins are damage-associated molecular pattern molecules released by activated or damaged phagocytes under conditions of cell stress, for example, during infections. A recent study demonstrating that peripheral blood mRNA levels of S100 family are closely associated with inflammation confirms our finding (43). 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 (37, 54, 55). In contrast to S100A12, S100A8 and S100A9 are expressed and secreted by both granulocytes and monocytes (37, 42).

According to the presented results, 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. Therefore, we investigated whether S100A8, S100A9, S100A8/S100A9, and S100A12 influence monocyte-induced development of Th17 cells. It is known that S100 proteins are endogenous TLR4 ligands that activate monocytes (31, 32). We could demonstrate that binding of S100A8, S100A9, S100A8/S100A9, or S100A12 to TLR4 on monocytes induces significantly increased levels of Th17 cells compared with unstimulated monocytes. However, S100A8 and S100A9 showed stronger stimulatory effects concerning the induction of Th17 cells compared with the heterodimer S100A8/S100A9 and S100A12. S100A8 and S100A9 mainly exist under physiological conditions in the form of homodimers, and the amount of S100A8 and S100A9 homodimers is unknown, but can be classified as rela-

FIGURE 6. Influence of TLR4 blocking, followed by stimulation of monocytes with S100 proteins on the induction of Th17 cells. Monocytes from healthy donors were preincubated 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 cocultured with CD4+ T cells from healthy donors with anti-CD3 mAb (100 ng/ml) at a monocyte:T cell ratio of 1:4 for 5 d. After 5 d of coculture, the percentage of induced IL-17+ cells was determined by flow cytometry. Graph shows data from one of three independent experiments with similar outcomes. Data represent mean values of duplicates ± SD. Statistical significance was determined using one-way ANOVA, followed by Bonferroni’s post hoc test: *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 7. Induction of Th17 cells mediated by cytokines. Monocytes from healthy donors optionally prestimulated 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 d in the presence of OKT3 (100 ng/ml) and a mAb directed against CD28 (1 µg/ml). After 5 d, 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. The percentage of IL-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, **p < 0.01. (B) Levels of the proinflammatory cytokines (IL-1β, IL-6, IL-8, IL-10, and TNF-α) in the supernatant of monocytes optionally prestimulated 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.
私立low. On the contrary, S100A12 is only present as homo-
dimer and seems to induce a weaker immune activation due to any
pathophysiological reasons. Additionally, it has to be kept in mind
that the activity of the S100 protein preparations varies from batch
to batch. Nevertheless, our findings point out that proinflammatory
S100 proteins can promote the induction of Th17 cells by binding
to TLR4 on monocytes and 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 LPS
in vitro (12).

We also examined whether the induction of Th17 cells is me-
diated by proinflammatory cytokines released by monocytes or
by cell contact between monocytes and CD4+ T cells. Our data
represent that efficient induction of Th17 cells by in vitro stimu-
lated monocytes occurs cell-contact dependent and cytokine me-
Diagrams could be added here.

Our findings highlight the importance of the induction of
IL-17+ cells in the presence of in vitro ac-

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topoietic and mesenchymal stem cell transplantation for severe and refractory


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Disclosures

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Table I. Patient characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients without GvHD after HCT (control group) n=12 (number in %)</th>
<th>Patients with GvHD after HCT n=35 (number in %)</th>
<th>p-value</th>
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<td><strong>Age group in years</strong></td>
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<td>11 (31)</td>
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<td>23 (66)</td>
<td></td>
</tr>
<tr>
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<tr>
<td>Solid tumors</td>
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<td>matched related donor</td>
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<tr>
<td>ATG</td>
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<tr>
<td>MMF</td>
<td>5 (42)</td>
<td>19 (54)</td>
<td></td>
</tr>
<tr>
<td>MTX</td>
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<tr>
<td>Ciclosporin</td>
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<td>Tacrolimus</td>
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</tr>
<tr>
<td>MPA</td>
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MDS indicates myelodisplastic syndrome; ATG, anti-thymocyte globulin; MMF, mycophenolate mofetil; MTX, methotrexate; and MPA, mycophenolic acid
<table>
<thead>
<tr>
<th>GvHD characteristic</th>
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<tr>
<td></td>
<td>n=35* (number in %)</td>
</tr>
<tr>
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<td>Skin</td>
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</tr>
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</tr>
<tr>
<td>stage II</td>
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<tr>
<td>stage III</td>
<td>4 (11)</td>
</tr>
<tr>
<td>stage IV</td>
<td>1 (8)</td>
</tr>
<tr>
<td>Gut</td>
<td></td>
</tr>
<tr>
<td>stage I</td>
<td>5 (14)</td>
</tr>
<tr>
<td>stage II</td>
<td>4 (11)</td>
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<tr>
<td>stage III</td>
<td>3 (9)</td>
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<tr>
<td>stage IV</td>
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<tr>
<td>Liver</td>
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</tr>
<tr>
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<tr>
<td>stage II</td>
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<td>stage III</td>
<td>0</td>
</tr>
<tr>
<td>stage IV</td>
<td>0</td>
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<tr>
<td><strong>Acute GvHD</strong></td>
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</tr>
<tr>
<td>grade II</td>
<td>7 (20)</td>
</tr>
<tr>
<td>grade III</td>
<td>8 (23)</td>
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<tr>
<td>grade IV</td>
<td>1 (8)</td>
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<tr>
<td><strong>Chronic GvHD</strong></td>
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<td>8 (23)</td>
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<tr>
<td>mAb (Basiliximab, Alemtuzumab, Tocilizumab, Muromonab)</td>
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<td>MSC</td>
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<tr>
<td>ATG</td>
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<tr>
<td>Azathioprin</td>
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</tbody>
</table>

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 an acute GvHD followed by a chronic GvHD
Figure S1: S100 concentrations in the stool and serum correlated with the clinical severity of GvHD.

(A) Fecal S100A8/S100A9 and (B) S100A12 were determined in the stool of patients with intestinal GvHD and patients without GvHD by Calprotectin-Assay or double sandwich ELISA and were correlated with the severity intestinal GvHD. Furthermore, (C) S100A8/S100A9 and (D) S100A12 concentrations were determined in the serum of patients with and without GvHD by double-sandwich ELISA and were correlated with GvHD grade.