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_J Immunol_ 2014; 193:3355-3365; Prepublished online 3 September 2014;
doi: 10.4049/jimmunol.1400983
http://www.jimmunol.org/content/193/7/3355

Supplementary Material [http://www.jimmunol.org/content/suppl/2014/09/03/jimmunol.140098.3.DCSupplemental](http://www.jimmunol.org/content/suppl/2014/09/03/jimmunol.140098.3.DCSupplemental)

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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

Katharina Reinhardt,* Dirk Foell,† Thomas Vogl,‡ Markus Mezger,* Helmut Wittkowski,† Rupert Handgretinger,* Wolfgang Andreas Bethge,§ and Ursula Holzer*©

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 amplifiers 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.

Allogeneic 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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Received for publication April 16, 2014. Accepted for publication July 30, 2014.

This work was supported by Jürgen-Manchot-Stiftung grants and Deutsche Forschungsgemeinschaft Grant F0354/3-1.

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The online version of this article contains supplemental material.

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

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1400983

The Journal of Immunology
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-κB 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 of buffy 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 BL21(DE3) bacteria were transformed with pET11b expression vector containing S100A8 or S100A9 cDNA. After growing bacteria at 37°C in 2% yeast extract and tryptone for 24 h, bacteria were harvested and lysed, and the inclusion bodies were prepared. The inclusion body pellets were dissolved in 8 M 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 M 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 thiogalactopyranoside, the pellet was lysed by sonication and the insoluble material was removed by centrifugation. 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 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), 2 mM L-glutamine, 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 Fluor488 (eBioscience) Ab alone or additionally with anti-IFN-γ PTC (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 2–14 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 Axioscop connected to an AxioCam camera supplied with software Axiovision 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 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...
chronic GvHD (n = 7) using the RNeasy 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 (Peplab) 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^(-ΔΔCt(GAPDH)-ΔΔCt(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+, IFN-γ+, and IL-17/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 Th1/Th17 cells (Fig. 1C).

**S100 levels in the stool and serum are increased 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 Chiisolo et al. and Rodríguez-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...
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. Higher concentrations of S100 proteins could be detected in the serum, stool, and bowel tissue of patients with GvHD. Thus, it was investigated whether S100 proteins

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**FIGURE 1.** Percentage of IL-17+, IL-17+IFN-\(\gamma^+\), and IFN-\(\gamma^+\) 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 (\(\pm 10\) 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 \(\mu\)M) during the last 3 h. The percentage of (A) IL-17+ cells, (B) IL-17+IFN-\(\gamma^+\) cells, and (C) IFN-\(\gamma^+\) 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 S100 proteins (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-
stimulated with S100 proteins to CD4\(^+\) T cells induced increased percentages of IL-17\(^+\) cells compared with supernatant of unstimulated 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

**FIGURE 3.** Immunohistochemical S100A8, S100A9, and S100A12 staining of bowel tissue from patients with acute intestinal GvHD. S100A8, S100A9, and S100A12 were detected in bowel tissue from patients with acute intestinal GvHD stages I, II, and III and healthy controls with no pathological findings by specific Abs conjugated to HRP (1 \(\mu\)g/ml) and following reaction with the substrate Substrat 3-amino-9-ethyl-carbazole (AEC), leading to the characteristic brown coloring. 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 \(\times 200\) for all images). (A) Image sets represent stained sections of bowel tissue from individual healthy controls and patients with different stages of acute intestinal GvHD. 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 (\(n = 3\)), and stage II–III (\(n = 6\)). Data analysis was performed using ImageJ. Statistical significance was determined using unpaired, two-tailed Student \(t\) test: *\(p < 0.05\), **\(p < 0.01\).
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

FIGURE 5. Influence of stimulation of monocytes with S100 proteins on the induction of Th17 cells. CD4⁺ T cells from healthy donors were cocultured with anti-CD3 mAb (100 ng/ml) and monocytes from healthy donors at a monocyte:T cell ratio of 1:4 for 5 d. Monocytes remained unstimulated or were additionally stimulated with S100A8, S100A9, S100A8/S100A9, or S100A12 (5 μg/ml) for 4 h before coculturing with CD4⁺ T cells. After 5 d of coculture, the percentage of induced (A) IL-17⁺ cells, (B) IL-17⁺ IFN-γ⁺ cells, and (C) IFN-γ⁺ cells was determined by flow cytometry. Representative flow plots are shown in (D). Statistical significance was determined using one-way ANOVA, followed by Bonferroni’s post hoc test: *p < 0.05, ****p < 0.0001.
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 heterodimers, 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.
tively 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-
diated. In support of our observations, other studies demonstrated
that the induction of IL-17+ cells in the presence of in vitro ac-
tivated monocytes requires cell contact and is not solely attributed
to monocyte-derived proinflammatory cytokines (12, 49). In
contrast, in vivo activated monocytes appeared to rely on cell contact
with CD4+ T cells to promote Th17 responses, suggesting a more
important role for cell-to-cell signals such as costimulation or ad-
hesion (49).
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 pro-
gression of GvHD, it was investigated whether monocytes stimu-
lated with S100 proteins release increased levels of proinflammatory
cytokines. The data of the current 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 TNF-α consistent with the results of a previous study demonstrat-
ing that stimulation of monocytes with S100A8 results in signifi-
cantly increased secretion of IL-1β and TNF-α (32). Fur-
thermore, it could be shown that monocytes stimulated with
S100A12 release elevated levels of IL-1β, TNF-α, and IL-6 (31).
Therefore, our findings indicate that binding of S100 proteins to
TLR4 on monocytes activates the TLR4 signaling pathway, re-
sulting in the activation of the transcription factor NF-κB and the
expression and secretion of proinflammatory cytokines that pro-
mote the induction of Th17 cells (32). Thus, immune intervention
targeting proinflammatory S100 proteins might be an attractive
strategy to inhibit uncontrolled inflammatory processes during
GvHD.
In conclusion, we demonstrate that phagocyte-specific S100
proteins are released during GvHD and can be detected in the stool,
serum, and bowel tissue of patients with GvHD. S100 proteins act
as endogenous ligands of TLR4 and induce the activation of
monocytes, the expression and release of proinflammatory cyto-
kines, and the development of Th17 cells. Our findings highlight
that the induction of Th17 cells by activated monocytes and the
stimulatory effects of the proinflammatory S100 proteins on monocytes
might play a relevant role in the pathogenesis of GvHD.
Based on these data, S100 proteins might be novel sensitive fecal
and pathologic in situ biomarkers of intestinal GvHD.

Acknowledgments
We thank Melanie Saers and Susanne Schlenzeinbaum (University of
Muenster) for performing S100 measurements, Claudia Sole (University
of Muenster) for the immunohistochemical staining, Mirjam Breig (Medi-
cal Center, Tuebingen, Germany) for patient documentation, and
Mirjam Breig and Birgit Walter (University Children’s Hospital) for pro-
viding assistance with the patient characteristics.

Disclosures
The authors have no financial conflicts of interest.

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