Sequential Expression of Adhesion and Costimulatory Molecules in Graft-versus-Host Disease Target Organs after Murine Bone Marrow Transplantation across Minor Histocompatibility Antigen Barriers

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ABSTRACT
Graft-versus-host disease (GVHD) is a potentially fatal complication after allogeneic bone marrow transplantation. However, few data exist thus far on the molecular signals governing leukocyte trafficking during the disease. We therefore investigated the sequential pattern of distinct adhesion, costimulatory, and apoptosis-related molecules in GVHD organs (ileum, colon, skin, and liver) after transplantation across minor histocompatibility barriers (B10.D2 → BALB/c, both H-2d). To distinguish changes induced by the conditioning regimen from effects achieved by allogeneic cell transfer, syngeneic transplant recipients (BALB/c → BALB/c) and irradiated nontransplanted mice were added as controls. Irradiation upregulated the expression of vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and B7-2 in ileum, as well as VCAM-1 and B7-2 in colon, on day 3 in all animals. Whereas in syngeneic mice these effects were reversed from day 9 on, allogeneic recipients showed further upregulation of VCAM-1, ICAM-1, and B7-2 in these organs on day 22, when GVHD became clinically evident. Infiltration of CD4+ and CD8+ donor T cells was noted on day 9 in skin and liver and on day 22 in ileum and colon. Surprisingly, the expression of several other adhesion molecules, such as ICAM-2, platelet-endothelial cell adhesion molecule 1, E-selectin, and mucosal addressin cell adhesion molecule 1, did not change. Proapoptotic and antiapoptotic markers were balanced in GVHD organs with the exception of spleen, in which a preferential expression of the proapoptotic Bax could be noted. Our results indicate that irradiation-induced upregulation of VCAM-1, ICAM-1, and B7-2 provides early costimulatory signals to incoming donor T cells in the intestine, followed by a cascade of proinflammatory signals in other organs once the alloreponse is established.

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KEY WORDS
Minor histocompatibility antigens • GVHD • Murine model • Adhesion molecules • Costimulatory molecules • Apoptotic markers • Organ specificity

INTRODUCTION
Graft-versus-host disease (GVHD) is a pathologic condition in which allogeneic T cells react against recipient minor histocompatibility antigens (miHAgS) of the immunocompromised host [1]. Most frequently, this occurs after allogeneic bone marrow transplantation (BMT), when activated donor T cells are transplanted into a patient whose hematologic and immunologic compartments have recently been ablated by high-dose chemoradiotherapy. One of the most striking observations regarding GVHD is that it does not affect all host organs in a similar fashion but preserves a characteristic pattern of organ involvement regardless of the type of transplant used. The most frequently involved organs are the skin, liver, and intestine [2], as well as the hematopoietic system.
and thymus. The reasons for this characteristic organ tropism are not fully understood [3].

The circulation of leukocyte subsets from the blood into the different compartments of the body, where they can exert their specific functions, requires a complex interaction of selectins, integrins, and other members of the immunoglobulin superfamily with their respective ligands [4]. This system of leukocyte trafficking is highly flexible and allows redirection of leukocytes to new sites of inflammation within minutes. The process of leukocyte homing to their designated areas involves several consecutive steps [5]: (1) rolling of leukocytes on endothelial cell surfaces mediated by different types of selectins, (2) firm adhesion of target cells on endothelial cells mediated by integrins, and finally, (3) transendothelial migration of the cells into the tissue, which requires the platelet-endothelial cell adhesion molecule (PECAM) 1 [6] and a gradient of other chemotactic substances. Several of these cellular adhesion molecules (CAMs) have been shown to be expressed in a tissue-specific way. The mucosal addressin cell adhesion molecule (MAdCAM)–1 seems to be important for the homing of α4β7+ activated T cells to Peyer patches [7], whereas the interaction of intestinal intraepithelial lymphocytes with epithelial cells is mediated by E-cadherin and α6β4 integrin [8]. The lectin endothelial leukocyte adhesion molecule E-selectin directs cutaneous lymphocyte antigen–positive T cells into the skin [9]. Therefore, it has been postulated that the characteristic organ tropism of GVHD may be—at least in part—explained by a differential expression pattern of adhesion molecules in GVHD target organs [5].

To be able to exert effector functions, T cells not only have to enter the tissue where their antigen of interest is expressed, but also need further stimulation in context with antigen. Therefore, in addition to triggering through the T-cell receptor, T cells require a second, costimulatory signal, which is usually provided by the same antigen-presenting cell that presents the antigen. Although the importance of adhesion and costimulatory molecules for the induction of GVHD is well established, blocking studies against several candidate molecules have been only partially successful [10-13]. This failure could be explained by the lack of sequential data on the expression of these molecules during the different phases of murine GVHD.

In this study, we therefore decided to provide a comprehensive time- and organ-resolved analysis of distinct adhesion molecules (vascular cell adhesion molecule [VCAM]–1, intercellular adhesion molecule [ICAM]–1 and ICAM-2, E-selectin, PECAM-1, and MAdCAM-1) and their ligands (lymphocyte function-associated antigen [LFA]–1, α4 integrin, and β2 integrin), T-cell costimulatory signals (B7-1 and B7-2), and apoptotic markers (Bax, Bcl-2, Fas, and FAS ligand [Fas-L]) in a murine model of GVHD across miHAg barriers (B10.D2/nSnJ → BALB/c, both H-2b) [10,14]. The GVHD target organs skin, liver, ileum, and colon were examined on day 3 (aplasia), day 9 (hematopoietic reconstitution), day 22 (early phase of GVHD), and day 28 (advanced phase of GVHD) by immunohistochemistry or reverse transcription-polymerase chain reaction (RT-PCR). The results of the allogeneic transplanted group were compared with the results from untreated and irradiated control mice (BALB/c), as well as with results from a syngeneic transplanted group (BALB/c → BALB/c). These novel results provide insight into the sequential pattern of early effects induced by the conditioning regimen, as well as subsequent effects induced by the expanding allogeneic cells inside the host.

**MATERIALS AND METHODS**

**Mice**

Female B10.D2/nSnJ (H2d, Mtv-7−, Mtv-1−, Mtv-6−, Mtv-13−) donor and female BALB/c recipient (H-2b, Mtv-7+, Mtv-1+, Mtv-6+, Mtv-13+) mice differ in miHAgS [15], as well as endogenous superantigens [16]. Mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in high-efficiency particulate air–filtered cabins (Seantonier Type C110; Scanbur, Karlslunde, Denmark) under standard pathogen-free conditions at 22°C, with controlled air flow and a 12-hour day/night rhythm.

**Bone Marrow Transplantation**

**Allogeneic BMT.** BALB/c recipients (13-16 weeks old) were preconditioned for BMT by fractionated total body irradiation (10 Gy) from a linear accelerator (Linac; Electra, Hamburg, Germany). In initial titration studies, the lethal irradiation dose from the linear accelerator had been determined as 2 fractions of 5 Gy each with an interval of 8 hours. Bone marrow was obtained by flushing the femurs and tibiae from healthy B10.D2/nSnJ donors (13-15 weeks old). Splenocytes (as a source of T cells) were obtained by homogenizing spleens of B10.D2/nSnJ donors. Four to 6 hours after irradiation, recipient BALB/c hosts were reconstituted with a single intravenous injection of 10 × 10^6 B10.D2/nSnJ bone marrow cells and 100 × 10^6 spleen cells, as previously described [10].

**Syngeneic BMT.** As a control, bone marrow and spleen cells were obtained similarly from healthy BALB/c donors and given to irradiated BALB/c recipients. For an irradiation control, BALB/c mice were lethally irradiated (10 Gy) without receiving bone marrow or spleen cells. Organs of these mice were analyzed on day 3 after irradiation.
Because of the large number of animals (n = 60), transplantsations were performed on 2 separate days. The experimental protocol was approved by the Animal Care Committee of the University of Tuebingen.

**Flow Cytometry**

Spleens were homogenized, and cells were filtered through a 100-µm cell strainer (BD, Heidelberg, Germany) and washed. After density centrifugation (20 minutes, 1500g, 22°C) on a murine Ficoll gradient (Cedarline, Hornby, Ontario, Canada), mononuclear cells were harvested, washed, and resuspended at a concentration of 2 × 10^6/mL. Cells were stained with CD3-phycocerythrin (PE), CD4 (L3T4)–fluorescein isothiocyanate (FITC), CD8 (Ly2)–FITC, Vβ3 T-cell receptor–PE, DX5–PE (natural killer [NK] cells), CD45R/B220–FITC (B cells), and Ly9.1–FITC (directed against antigens expressed on lymphocytes of BALB/c, but not of B10.D2/nSnJ, mice; all obtained from Pharmingen, Hamburg, Germany) and washed. After density centrifugation through a 100-µm cell strainer (BD) primary antibodies were obtained from PharMingen: rat anti-mouse immunoglobulin (Ig)G—CD4 (H129.19), CD8 (53-6.7), CD11a (M17/4), CD29 (9EG7), CD31 (Mec13.3), CD49d (9C10), CD62E (10E9.6), CD80 (1G10), CD86 (GL1), CD102 (3C4), CD106 (429 MVCAM.A), MadCAM-1 (MECA-367), and β7 integrin (M293); hamster anti-mouse immunoglobulin—CD28 and CD54 (3E2). Biotinylated secondary antibodies were rabbit anti-rat IgG (Dako, Hamburg, Germany), goat anti-Armenian hamster IgG, and goat anti-Syrian hamster IgG (PharMingen).

**Preparation of Frozen Tissue for Sectioning**

Tissue expression of adhesion molecules was analyzed during the phases of aplasia (day 9) and engraftment (day 9) and during different stages of GVHD (days 21 and 28). Mice were killed with CO2, and target organs of GVHD (skin, liver, ileum, and colon) were prepared, embedded in frozen tissue matrix (OCT embedding compound; Leica, Nussloch, Germany), shock-frozen in liquid nitrogen, and stored at −80°C. Frozen sections were prepared with a Cryocut (CM 3000; Leica): 6 to 10 sections were placed on electrostatic pretreated glass slides, air-dried, fixated for 8 minutes in acetone, and stored at −20°C.

**Antibodies**

Primary antibodies were obtained from Pharmingen: rat anti-mouse immunoglobulin (Ig)G—CD4 (H129.19), CD8 (53-6.7), CD11a (M17/4), CD29 (9EG7), CD31 (Mec13.3), CD49d (9C10), CD62E (10E9.6), CD80 (1G10), CD86 (GL1), CD102 (3C4), CD106 (429 MVCAM.A), MadCAM-1 (MECA-367), and β7 integrin (M293); hamster anti-mouse immunoglobulin—CD28 and CD54 (3E2). Biotinylated secondary antibodies were rabbit anti-rat IgG (Dako, Hamburg, Germany), goat anti-Armenian hamster IgG, and goat anti-Syrian hamster IgG (PharMingen).

**Immunohistochemistry**

Frozen sections were stained with the avidin-biotin-peroxidase complex immunoperoxidase technique [17]. Sections were pretreated with 3% goat or rabbit serum in phosphate-buffered saline (PBS) to reduce nonspecific background. A washing step followed, after which the primary antibody (rat or hamster anti-mouse IgG, 1:100) was applied and incubated for 45 minutes at room temperature in a humidified chamber. The slides were washed again in PBS, and a biotin-labeled secondary antibody was applied for 1 hour (goat or rabbit anti-rat or anti-hamster antibody, 1:50, with 1% sterile bovine serum albumin). Slides were washed, and sections were covered with 1:200 streptavidin-peroxidase for 1 hour. After another washing step, the substrate solution (4 mg of 3-amino-9-ethylcarbazole in 0.5 mL of N,N-dimethylformamide and 9.5 mL of 0.1 mol/L sodium acetate buffer filtered through a 0.2-µm sterile filter) was placed on the sections and incubated for 8 to 15 minutes. The reaction was stopped with PBS, and a hematoxylin counterstain (Mayer’s) was performed. Stained sections were covered in glycerin/gelatin. Primary antibodies were replaced by PBS in negative controls. For immunofluorescence microscopy, a FITC-labeled streptavidin-peroxidase dilution was used. After 1 hour of incubation, slides were washed, covered with Citifluor (Plano, Wetzlar, Germany), and stored at 4°C in the dark until analysis.

Samples were visualized on an Olympus BX60 microscope (Olympus, Hamburg, Germany) and digitized with the Camera CF-15MC (MCU-II; Kappa GmbH, Germany). Each molecule was compared sequentially within 1 organ of allogeneic and syngeneic transplants, irradiation controls, and nontransplanted mice. The number of animals studied at each time point varied from 3 to 6 (Figures 1-4). From each organ, multiple sections of 2 slides were analyzed before the degree of expression was determined. The experiment was performed 2 times with 30 transplanted animals in each experiment. Because no variation was observed between the 2 experiments, data from the 2 experiments were pooled. Four hundred of the 7000 slides were re-evaluated by a second investigator (P.G.S.), and consistency with the primarily obtained results was confirmed.

To determine the relative degree of expression of the respective molecules, a semiquantitative scoring system was developed, according to previously published studies [18,19]. Low expression (grade 1) was defined as the occasional detection of isolated positive cells (2–5 per section); formation of cellular aggregates was interpreted as intermediate expression (grade 2), and confluence of aggregates or, in the intestine, aggregates in each villus was interpreted as strong expression (grade 3). Representative examples of all degrees of expression can be seen in Figure 3. Different types of cells (lymphocytes, macrophages, endothelial cells, epithelial cells, and so on) were identified by their distinct location and morphology.
Reverse Transcription-Polymerase Chain Reaction

In allogeneic transplanted animals, RNA was extracted from skin, liver, ileum, and colon after homogenizing samples in GuaSCN buffer by using a homogenizing device (Heidolph, Schwabach, Germany) and loading the lysate onto a CsCl gradient. Complementary DNA was synthesized by reverse transcription by using standard procedures. Complementary DNA was amplified in a 50-μL standard PCR reaction. Before analysis, PCR conditions were optimized for each primer pair. PCR primers were as follows: Bax sense, 5′-TACAGGGTTTCATCCAGGATCG-3′; Bax antisense, 5′-GTGTCCACGTCAGCAATCATCC-3′ (fragment size, 175 base pairs [bp]; annealing temperature, 60°C; optimal PCR performance with 28 cycles); Bcl-2 sense, 5′-ATG-GTACATACCCAGTCAGAGG-3′; Bcl-2 antisense, 5′-ACAGAGTGAGTATTGGAGGAGG-3′ (248 bp; 60°C; 28 cycles); Fas sense, 5′-CTCAAGGTACTAATAGCATCTCCGAGAG-3′; Fas antisense, 5′-TTGCACITTGCACTTGATATG-3′ (316 bp; 60°C; 35 cycles); Fas-L sense, 5′-GAAGGAACCTGCCGAACACTCCGTG-3′; and Fas-L antisense, 5′-ATTGTACAGAGAGATTGAGATATGGTGACA-3′ (481 bp; 60°C; 35 cycles). PCR conditions were 94°C at 5 min for denaturation, cycling (denaturation at 94°C for 30 seconds, primer-specific annealing temperature for 30 seconds, and elongation at 72°C for 30 seconds; the number of cycles was optimized for each primer), and a final elongation step at 72°C for 7 minutes. After amplification, PCR products were denatured and visualized on an 8% polyacrylamide gel.

Each PCR included amplification of β-actin from the same organ as an internal standard, and both PCR products were loaded onto the same lane. In a semi-quantitative analysis, the optical density of the molecule of interest was divided by the optical density of the internal β-actin standard by using AIDA software (Raytest, Straubenhardt, Germany).

Statistics

For comparison of different grades of tissue expression (ordinal scale) among naive BALB/c mice, irradiation controls, and syngeneic and allogeneic transplants (nominal scale), the χ² test was used at selected time points to test for significant differences between groups. If both variables had only 2 levels, the Fisher exact test was applied. P < .05 was considered statistically significant.

RESULTS

Transplantation

After lethal irradiation and subsequent BMT, there was a profound loss of weight in the syngeneic group, as well as in the allogeneic group. Weight
that (middle) and allogeneic (right) transplanted animals. This indicates cells could predominantly be found in the epidermis of syngeneic skin after transplantation. The significant increase on day 9 are shown. This increase of expression was statistically significant (\( P = .0146 \)). B, Immunofluorescence staining confirming the significant increase (\( P = .0005 \)) of \( \alpha_4 \) integrin–positive cells in ileum of allogeneically transplanted animals from day 3 (left) to day 22 (right). Whereas in liver and ileum, the expression of \( \alpha_4 \) and \( \beta_7 \) integrin increased in parallel and lymphocytes positive for both markers were colococalized, in skin these 2 molecules were expressed on distinct cell populations. C, Different location of cutaneous \( \alpha_4 \) integrin–positive and \( \beta_7 \) integrin–positive cells. \( \alpha_4 \) Integrin–positive cells were localized in the dermis (left), whereas \( \beta_7 \) integrin–positive cells could predominantly be found in the epidermis of syngeneic (middle) and allogeneic (right) transplanted animals. This indicates that \( \alpha_4 \) and \( \beta_7 \) integrin are expressed on different cell populations in skin after transplantation.

Figure 2. Expression of \( \alpha_4 \) and \( \beta_7 \) integrin in GVHD target organs. Allogeneic bone marrow transplantation resulted in accumulation of activated donor T cells in GVHD target organs. This tissue infiltration could be detected from day 9 on in skin and liver and later from day 22 on in the ileum and colon. A, Representative examples of immunohistochemical \( \alpha_4 \) integrin staining in skin of untreated controls (left) and allogeneic transplanted mice (right) on day 9 are shown. This increase of expression was statistically significant (\( P = .0146 \)). B, Immunofluorescence staining confirming the significant increase (\( P = .0005 \)) of \( \alpha_4 \) integrin–positive cells in ileum of allogeneically transplanted animals from day 3 (left) to day 22 (right). Whereas in liver and ileum, the expression of \( \alpha_4 \) and \( \beta_7 \) integrin increased in parallel and lymphocytes positive for both markers were colococalized, in skin these 2 molecules were expressed on distinct cell populations. C, Different location of cutaneous \( \alpha_4 \) integrin–positive and \( \beta_7 \) integrin–positive cells. \( \alpha_4 \) Integrin–positive cells were localized in the dermis (left), whereas \( \beta_7 \) integrin–positive cells could predominantly be found in the epidermis of syngeneic (middle) and allogeneic (right) transplanted animals. This indicates that \( \alpha_4 \) and \( \beta_7 \) integrin are expressed on different cell populations in skin after transplantation.

returned to baseline after approximately 2 weeks. However, in contrast to syngeneic transplanted mice, which maintained their body weights throughout the entire observation period thereafter, allogeneic transplanted mice exhibited a second loss of weight after day 21 (Figure 5A), which coincided precisely with the onset of clinical symptoms compatible with GVHD. On day 22, 10 of 16 mice in the allogeneic group presented with erythema of the skin and loss of fur. On day 24, all mice in the allogeneic group (16/16) showed these symptoms of murine GVHD, with a further aggravation toward the end of the observation period.

Flow cytometric assessment of splenic lymphocyte subsets on days 9 and 22 revealed immune reconstitution of T, B, and NK cells in both groups, which were almost exclusively Ly9.1\(^+\) in the autologous group and Ly9.1\(^-\) in recipients of allogeneic grafts, indicating full donor engraftment in the latter group (data not shown). It is interesting to note that there was a marked expansion of V\( \beta_3\)^CD4\(^+\) and V\( \beta_3\)^CD8\(^+\) T cells on day 9 and day 22 in the allogeneic group, but not in the syngeneic group (Figure 5B).

Expression of VCAM-1, \( \alpha_4 \) Integrin, and \( \beta_7 \) Integrin

VCAM-1 was weakly expressed in lymphoid aggregates of Peyer patches, the lamina propria, and the submucosa of normal murine ileum and colon. On day 3, VCAM-1 was upregulated from a weak expression in untreated controls to an intermediate expression in ileum and colon in all treated groups (syngeneic, allogeneic, and irradiation controls). In the ileum and colon of syngeneic controls, VCAM-1 was downregulated back to pretransplantation levels on day 9, whereas it remained increased in allogeneic transplanted mice until day 22 (ileum, \( P = .0337 \); colon, \( P = .0476 \); Figure 1A and B). In contrast to the intestinal organs, expression of VCAM-1 in the skin and liver of the syngeneic group remained weak, but an upregulation was observed on day 9 in the allogeneic group. In skin, VCAM-1 was constitutively expressed on dermal and subcutaneous cells. In the course of GVHD, upregulation of VCAM-1 was seen in the dermis, and single positive cells also appeared in the epidermis (Figure 1C). In the liver, VCAM-1 was constitutively expressed in close proximity to liver veins, but with upregulation from weak to moderate expression on day 9 and 22 after allogeneic BMT, VCAM-1\(^+\) cells could also be found in the liver parenchyma (Figure 1D).

The \( \alpha_4 \) integrin, which is expressed on activated lymphocytes, is known to serve as a ligand for VCAM-1. It was constitutively expressed on lymphocytes in all organs at weak to moderate levels. Only in skin, \( \alpha_4 \) integrin was also expressed on cells with a dendritic morphology. In all treated animals, expression remained constant or decreased because of irradiation-induced aplasia on day 3 and returned to pretransplantation levels in syngeneic controls on day 9. Afterward, no significant changes in expression of \( \alpha_4 \) integrin could be observed in syngeneic controls. However, in the allogeneic group there was a significant increase of \( \alpha_4 \) integrin–positive cells between day 3 and day 9 in skin (\( P = .0076 \)) and liver (\( P = .0112 \)) and between day 3 and day 22 in ileum (\( P = .0005 \)) and colon (\( P = .0421 \); Figure 2A). In liver, clusters of \( \alpha_4 \) integrin–positive lymphocytes appeared after day 9, not only in the surrounding of liver veins, but also in...
the liver parenchyma. The increased expression of α₄ integrin in intestinal organs was confirmed by immunofluorescence staining (Figure 2B). Expression remained increased in the ileum, skin, and liver but decreased in the colon on day 28 (data not shown).

α₄ Integrin interacts with VCAM-1 as a het-

Figure 3. Expression of ICAM-1 and its ligand LFA-1 in ileum. Expression of ICAM-1 and LFA-1 is shown in untreated and irradiated controls (♦) and in syngeneic (○) and allogeneic (◇) transplanted mice. Expression was assessed as absent (0), weak (1), moderate (2), or strong (3). Each symbol represents 1 animal, analyzed at the respective time points; bars represent median values. Data were pooled from 2 separate experiments. In immunohistochemistry slides, positive, AEC-stained cells appear in red. A, Expression of ICAM-1 in all animals (upper panel) and representative examples of immunohistochemistry in an untreated BALB/c mouse (left) and an allogeneic transplanted animal on day 22 (right; lower panel). B, Expression of LFA-1 in ileum of all animals (upper panel) and representative examples of immunohistochemistry in an untreated BALB/c mouse (left) and an allogeneic transplanted animal on day 22 (right, lower panel). Of note, in allogeneic mice, increased expression of ICAM-1 on day 3 preceded infiltration of LFA-1⁺ cells on day 22.

Figure 4. Expression of B7-1 and B7-2 in ileum. Expression of B7-1 and B7-2 is shown in untreated and irradiated controls (♦) and syngeneic (○), and allogeneic (◇) transplanted mice. Expression was assessed as absent (0), weak (1), moderate (2), or strong (3). Each symbol represents 1 animal, analyzed at the respective time points; bars represent median values. Data were pooled from 2 separate experiments. A, Expression of B7-1 in ileum. B, B7-1 expression in skin. In ileum and skin of allogeneic mice, there was a late upregulation of B7-1 on day 22 (P < .05), which could be observed neither in syngeneic mice nor in colon and liver of allogeneic transplant recipients. C, B7-2 expression in ileum. D, B7-2 expression in skin of all animals. B7-2 expression in ileum was significantly increased by irradiation in all treated animals (P < .05) and remained upregulated in the allogeneic group. In skin, B7-2 expression increased in the allogeneic group from day 9 on, but not in irradiation or syngeneic controls.
Expression of ICAM-1, ICAM-2, and LFA-1

ICAM-1 was weakly expressed in the ileum and colon, but not in the liver, of untreated mice on a variety of cell types in the lamina propria (endothelial cells, fibroblasts, macrophages, and lymphocytes). In skin, ICAM-1+ cells occurred only occasionally in the surrounding of blood vessels. Irradiation and syngenic controls showed only a very mild and transient increase of ICAM-1 expression on day 3 in ileum, colon, and liver, whereas in skin, no alterations could be observed (data not shown). In allogeneic transplanted mice, however, ICAM-1 was upregulated in the ileum on day 3 (P < .0293; Figure 3A), as well as in the colon, liver, and skin on day 9 (P < .0293, .0043, and .0017, respectively), and remained increased throughout the entire observation period. In the skin of allogeneic mice, dermal endothelial cells and keratinocytes became ICAM-1 positive (data not shown).

ICAM-2 was constitutively expressed in all organs on macrophages and endothelium at weak to intermediate levels, and its expression was not altered by irradiation. Only in the skin of the allogeneic group was there a mild—but not significant—increase in ICAM-2 expression on days 9, 22, and 28 (data not shown).

The ligand for ICAM-1 and ICAM-2 on lymphocytes is LFA-1, the constitutive expression of which could be detected at weak to intermediate levels in all organs on lymphocytes and macrophages. Similar to the results of α4 integrin, expression of LFA-1 remained constant or decreased because of irradiation-induced aplasia on day 3 and returned to pretransplantation levels in syngenic controls on day 9. In allogeneic, but not in syngenic transplanted mice, there was a significant increase in LFA-1+ cells in skin and liver on day 9 (P < .005) and in ileum and colon on day 22 (P < .002). The increase remained until the end of the observation period (Figure 3B). The increased staining of α4 integrin and LFA-1 in the respective tissues of the allogeneic group was closely mirrored by an increased expression of CD4 and CD8, with the exception of the liver, where staining of CD4 and CD8 was less intense than in the other 3 organs (data not shown). Starting day 9 in the allogeneic group, there was a spreading of LFA-1+ cells from the dermis to the epidermis in the skin and from the veins to the parenchyma in the liver.

Expression of PECAM-1, E-Selectin, and MAdCAM-1

PECAM-1 was constitutively detectable in all tissues at an intermediate degree of expression on endothelium and macrophages, the level of which was not altered by irradiation or syngenic BMT. Only in the colon of allogeneic transplanted animals there was a mild, but not significant, increase of expression on days 22 and 28 (data not shown).

E-selectin was not constitutively expressed in any of the analyzed organs. Furthermore, we could not observe an early upregulation of E-selectin due to irradiation or transplantation. At later time points (days 9 and 22), E-selectin was upregulated to a cer-
tain degree in all organs; however, this effect could partially also be observed in the syngeneic group (data not shown). Therefore, we could not find clear evidence for the involvement of E-selectin in the process of GVHD in this model.

MAdCAM-1 was weakly expressed on high endothelial venules of Peyer patches, lamina propria, and tunica muscularis in the ileum and colon of untreated mice. This pattern of expression remained unchanged in irradiation and syngeneic controls, as well as in the ileum of the allogeneic group. Only in the colon of allogeneic transplanted mice did a transient upregulation of MAdCAM-1 occur, on day 9 ($P = .0476$); it waned until day 28 (data not shown).

Expression of B7-1 and B7-2

In untreated controls, B7-1 was expressed on macrophages in Peyer patches, lamina propria, and submucosa of the ileum and colon. In skin, B7-1 was only sporadically expressed in the dermis of controls and syngeneic transplanted mice. B7-1 was not expressed in the liver of controls or of syngeneic or allogeneic transplanted animals. On day 3, B7-1 expression in ileum became almost undetectable in all groups but recovered on day 9 (Figure 4A). Only in allogeneic transplanted mice did B7-1 expression temporarily increase in ileum and colon (data not shown) during GVHD at similar levels. However, in the spleen of allogeneic transplanted mice, there was a striking dysbalance of these molecules in favor of the proapoptotic Bax. C. Expression of Fas/Fas-L in colon of allogeneic transplanted mice during GVHD. Like Bax/Bcl-2, Fas/Fas-L expression temporarily increased during GVHD. A similar pattern was noted in ileum and spleen (data not shown). Expression of Fas/Fas-L was balanced in all GVHD organs and in spleen.

Expression of apoptotic markers Bax, Bcl-2, Fas, and Fas-L

Expression of Bax and Bcl-2, as measured by RT-PCR, increased in the ileum of allogeneic transplanted mice on days 3, 9, and 14 but returned to baseline levels at later time points (Figure 6A). A similar pattern was noted in colon (data not shown). In skin, no clear change in the pattern of Bax/Bcl-2 expression could be observed, whereas in liver, the expression of both molecules was decreased after transplantation (data not shown). In general, the expression of Bax and Bcl-2 was balanced in all GVHD organs and in spleen.

B7-2 was weakly expressed on macrophages in all organs of untreated control mice. In contrast to B7-1, B7-2 expression was inducible by irradiation in ileum (Figure 4C) and colon. On day 3, B7-2 expression in the ileum and colon was increased in irradiation controls, as well as in syngeneic and allogeneic transplanted animals. In the syngeneic group, B7-2 expression returned to normal levels on day 9 but remained increased in allogeneic transplanted mice in intestinal organs. In skin and liver, there was no effect of irradiation on B7-2 expression. In skin, there was a delayed upregulation of B7-2 from day 9 to 28 in the allogeneic group, but not in syngeneic controls (Figure 4D), whereas in liver, no modulation in any group could be observed throughout the observation period.

Figure 6. Expression of apoptotic markers in intestine and spleen of allogeneic transplanted mice. Results of semiquantitative RT-PCR are given as ΔOD, which is the optical density (OD) of the respective PCR product divided by the OD of an internal β-actin standard from the same organ. A, Expression of Bax/Bcl-2 in ileum. B, Expression of Bax/Bcl-2 in spleen of untreated and allogeneic transplanted mice. Bax/Bcl-2 temporarily increased in ileum and colon (data not shown) during GVHD at similar levels. However, in the spleen of allogeneic transplanted mice, there was a striking dysbalance of these molecules in favor of the proapoptotic Bax. C, Expression of Fas/Fas-L in colon of allogeneic transplanted mice during GVHD. Like Bax/Bcl-2, Fas/Fas-L expression temporarily increased during GVHD. A similar pattern was noted in ileum and spleen (data not shown). Expression of Fas/Fas-L was balanced in all GVHD organs and in spleen.
DISCUSSION

The H-2d identical mice strains B10.D2 and BALB/c used in this study are known to differ in their expression of miHAgS [15] and endogenous proviruses [16]. In our model, GVHD induced by BMT and cotransplantation of $1 \times 10^8$ spleen cells became evident on day 22 by clinical symptoms, infiltration of tissues with donor lymphocytes, and subsequent destruction of tissue architecture. Without any treatment, GVHD in this model is reported to be rapidly lethal, with a median survival of 68 days and a mortality of >90% on day 100 after transplantation [10]. We could show that between day 9 and 22, successful immune reconstitution of T, B, and NK lineages occurred in syngeneic and allogeneic transplanted mice which were of donor type in the latter group. The dramatic expansion of Vβ3+ T cells is another hallmark of this GVHD model [21] and indicates initiation of an allogeneic response to miHAgS of BALB/c recipient mice. miHAg-driven T-cell responses usually decline rapidly [22,23]. The fact that Vβ3+ expansion is still detectable on day 22 in our model can be interpreted as a delayed regression of the cytotoxic T-lymphocyte response or a redistribution of T cells from GVHD organs into the circulation. However, the preferential expression of Bax in spleen on day 22 indicates that activation-induced cell death is also operational in our model.

Numerous studies have shown that adhesion molecules are involved in the pathophysiology of GVHD [7,18,24-33]. The interaction between ICAM-1 and LFA-1 seems to be involved in the development of cutaneous [24-26], hepatic [18,27], and intestinal [28,29] GVHD. Also, VCAM-1 is upregulated in these 3 major GVHD target organs after transplantation [28,30,31], whereas PECAM-1 is reported to be upregulated in skin [25], but not in intestinal organs [29] affected by GVHD. Furthermore, E-selectin and ICAM-1, but not VCAM-1 or P-selectin, can be upregulated on vascular endothelium by the irradiation used in the conditioning regimen [32,33]. A recent study could demonstrate that adhesion molecules play an essential role in the initiation of an antihost cytotoxic T-lymphocyte response in intestinal GVHD [7]. However, none of these studies has considered the dynamics of CAM expression during GVHD. Therefore, in this study, we provide the first systematic analysis of CAMs and costimulatory molecules and their ligands in murine GVHD in a time- and organ-resolved fashion.

As a direct consequence of lethal irradiation with 10 Gy, VCAM-1 was upregulated on day 3 in all treated groups in the ileum and colon, and ICAM-1 was upregulated in the ileum of allogeneic mice. All other adhesion molecules, in particular E-selectin, were not affected by irradiation in the early phase after transplantation. However, it cannot be completely excluded that some of these molecules might have been upregulated very early but had already declined to baseline expression by day 3 [33].

Whereas the number of B7-1+ macrophages and α4 integrin–positive and LFA-1+ lymphocytes temporarily decreased because of irradiation-induced aplasia, there was an early upregulation of B7-2 on day 3 in the ileum and colon of all irradiated animals. This probably reflects the increased capacity of B7-2 to become upregulated on macrophages and B cells in response to lipopolysaccharide [34] or granulocyte-macrophage colony-stimulating factor [35] compared with B7-1 and could be an indicator of intestinal mucosal damage. Therefore, in our model, irradiation caused early upregulation of VCAM-1, ICAM-1, and B7-2 only in intestinal organs, but not in skin or liver.

In syngeneic transplanted mice, these irradiation-induced phenomena waned by day 9 or 22. In contrast, allogeneic transplanted animals showed sustained or even increased expression of these molecules during further follow-up. From day 9 on, VCAM-1 was upregulated in skin and liver, and ICAM-1 was upregulated in skin, liver, and colon of allogeneic transplanted mice. These 2 CAMs were shown to be the major players in T-cell guidance, because they were expressed in virtually all GVHD organs of allogeneic recipients throughout the entire observation period. Other CAMs, unexpectedly, seemed less important. ICAM-2 was only mildly upregulated in skin, and PECAM-1 was upregulated only at later time points in colon. It is interesting to note that the expression of MadCAM-1 (which serves as a ligand for α4β7+ T cells, which are essential for the induction of intestinal GVHD [19]) on intestinal high endothelial venules was only temporarily increased in the colon around day 9. This suggests that other ligands for α4β7 integrin like VCAM-1 [36] or the chemokine receptor CCR5 [7] must be equally important or even more important for the homing of α4β7+ T cells to intestinal organs than MadCAM-1 in our model. However, we cannot exclude that MadCAM might be more important for GVHD induction in other strain combinations (eg, B10.BR → CBA or B10.BR → C57BL/6 [19]). E-selectin was unspecifically upregulated in allogeneic and syngeneic transplanted mice at later time points in all organs. This, together with the finding that E-selectin+/- knockout mice show only a mild impairment in recruitment of leukocytes to sites of inflammation [37], suggests that E-selectin plays no important role in the development and maintenance of GVHD.

Similar to the expression of CAMs, increased expression of the costimulatory molecule B7-2 after irradiation in the intestinal organs disappeared in syngeneic transplanted animals on day 9. However, in the allogeneic group, B7-2 remained increased in the il-
eum and colon and became upregulated in the skin on day 22. B7-2 has been reported to be preferentially expressed over B7-1 on dermal dendritic cells, at least in T cell–mediated skin disorders such as psoriasis [35]. Indeed, costimulation via B7-1 seemed to be less important in our model, because B7-1 was virtually unaffected by irradiation and increased only in the skin and ileum of allogeneic transplanted mice on day 22. The biologic significance of these differences in B7-1 and B7-2 expression for GVHD remains to be determined. Blocking studies with monoclonal antibodies against B7-1 and B7-2 have shown that both molecules are important for optimal CD4+ and CD8+ T-cell expansion in murine GVHD. [12] Blockade of the B7 family could significantly reduce GVHD-associated lethality, but it did not result in complete abrogation of the disorder [13].

Our results of an increased expression of apoptotic markers in GVHD target organs indicate that the Fas/Fas-L pathway, as well as molecules such as Bax and Bel-2, seem to be involved in the pathogenesis of GVHD [38]. This was recently confirmed by gene expression profiling of skin affected by GVHD [39]. It is interesting to note that we could not observe a gross dysbalance in expression of proapoptotic and antiapoptotic proteins in GVHD target organs, whereas in spleen the pattern was skewed toward a preferential expression of Bax. This most likely reflects a dysregulated Bax/Bel-2 ratio in peripheral T cells; this has been reported to occur in T cells early after transplantation and results in an increased sensitivity of these cells to apoptotic cell death [40]. However, because our RT-PCR data provide no information about which types of cells express these markers and whether their expression finally results in an increased rate of apoptotic tissue cells, further studies are needed to refine the role of apoptosis in GVHD.

An increase in α4 integrin–positive and LFA-1+ cells was observed in the skin and liver of the allogeneic group on day 9 and in the ileum and colon on day 22. In ileum, colon, and liver, these 2 molecules were almost exclusively expressed on activated lymphocytes and on only a few macrophages in the lamina propria of the ileum and colon. In contrast, in skin, a substantial proportion of α4 integrin–positive and LFA-1+ cells exhibited a dendritic morphology. Thus, at least in skin, α4 integrin and LFA-1 seem to be expressed not only on activated T cells, but also on a number of non–T cells during GVHD. Although the increase in α4 integrin and LFA-1 expression was paralleled by an increased expression of CD4 and CD8 in GVHD organs and although the clinical onset of GVHD symptoms in allogeneic transplanted mice coincided with infiltration of α4 integrin–positive and LFA-1+ cells, it is impossible to say whether this increased expression is the cause or the effect of GVHD induction. Several studies in mice and humans have shown that by selective blockade of α4 integrin or LFA-1, the onset or lethality of GVHD can be delayed [10] or reduced [11]. However, in all these trials, GVHD could not be completely abrogated, and in patients after BMT, the beneficial effect of LFA-1 blockade on GVHD was thwarted by an increased rate of infectious complications [41]. Therefore, increased expression of α4 integrin and LFA-1 on different cell types rather seems to be secondary in GVHD, and intervention at this stage will not result in prevention of the disease.

To summarize our data in a downstream model of events, we found that after transplantation across miHAg barriers, CAMs and adhesion molecules are expressed in a tissue-specific manner and exert different functions at different time points after transplantation. We were able to distinguish between irradiation-induced and alloreactivity-related effects. In the intestine, irradiation induces upregulation of VCAM-1, ICAM-1, and B7-2. Because this early upregulation coincides with the time slot of irradiation-induced aplasia, it does not result in significant tissue T-cell invasion. However, expression of these molecules will provide an important costimulatory signal to either naive (VCAM-1 and ICAM-1) or antigen-experienced (B7 family) donor T cells [42]. Interaction of VCAM-1 and ICAM-1 in intestinal organs with their respective ligands on naive donor T cells, which have been reported to be the predominant GVHD-inducing population [43], could be one of the earliest events in GVHD development. Therefore, our findings underline the importance of gut mucosal damage caused by the conditioning regimen in GVHD pathophysiology [44], whereas in skin and liver, no such irradiation-related effects could be observed.

Whereas in syngeneic transplanted mice, these irradiation-induced alterations disappear between day 9 and 22, the presence of an alloreponse, indicated by the systemic expansion of Vβ3+ T cells in the allogeneic group, results not only in the maintenance of these changes, but additionally in the initiation of a whole cascade of proinflammatory signaling events. Upregulation of VCAM-1 and ICAM-1 occurs at these stages in organs in which they have not been previously expressed, and, to a lesser extent, expression of ICAM-2 is increased in skin, as is that of PECAM-1 and MadCAM-1 in colon. This process also includes increased expression of costimulatory molecules such as B7-1 and B7-2, as well as proapoptotic and antiapoptotic molecules, and results in the recruitment of activated donor T cells into GVHD target organs. However, because many of these events seem to be redundant or easily bypassed in case of blockade, it is unlikely that interference with a particular pathway will result in significant amelioration of the disease at this stage of GVHD.

In conclusion, early coblockade of VCAM-1 and
ICAM-1 is an interesting strategy that has been shown to result in a 70% reduction of active inflammation in Crohn disease [45]. To our knowledge, this approach has not been tested in GVHD models. Alternatively, the use of radioprotective substances such as keratinocyte growth factor [46] may help to diminish the GVHD-facilitating capacity of the conditioning regimen.

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