Monokine Induced by Interferon- γ (MIG/CXCL9) Is Derived from Both Donor and Recipient Sources during Rejection of Class II Major Histocompatibility Complex Disparate Skin Allografts

Michael B. Auerbach,* Naohiko Shimoda,*[†]
Hiroyuki Amano,*[†] Joshua M. Rosenblum,*[‡]
Danielle D. Kish,* Joshua M. Farber,[§] and
Robert L. Fairchild*^{†‡}

From the Department of Immunology,* and The Glickman Urological Institute,[†] Cleveland Clinic Foundation, Cleveland, Ohio; the Department of Pathology,[‡] Case Western Reserve University Medical School, Cleveland, Ohio; and the Inflammation Biology Section,[§] Laboratory of Molecular Immunology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

Chemokines, including monokine induced by interferon- γ (Mig/CXCL9), are produced both in allografts and during the direct T-cell infiltration that mediates graft rejection. Neither the specific production nor contribution of allograft donor versus recipient Mig in allograft rejection is currently known. C57BL/6 mice with a targeted deletion in the Mig gene were used as both skin allograft donors and recipients in a class II major histocompatibility complex-mismatched graft model to test the requirement for donor- versus recipient-derived Mig for acute rejection. B6.Mig^{-/-} allografts had a 10-day prolonged survival in B6.H-2^{bm12} recipients when compared with wild-type C57BL/6 allograft donors, and B6.H-2^{bm12} skin allografts had a 5-day prolonged survival in B6.Mig^{-/-} versus wild-type recipients. Transplantation of B6.Mig^{-/-} skin grafts onto B6.H-2^{bm12}.Mig^{-/-} recipients resulted in further prolonged allograft survival with more than 30% of the grafts surviving longer than 60 days. Prolonged allograft survival was also associated with delayed cellular infiltration into grafts but not with altered T-cell proliferative responses to donor stimulators. Immunohistochemical staining of allograft sections indicated that Mig is produced by both donor- and recipient-derived sources, but Mig from each of these sources appeared in different areas of the allograft tissue. These results therefore

demonstrate the synergy of donor- and recipient-derived Mig in promoting T-cell infiltration into allografts. (Am J Pathol 2009, 174:2172–2181; DOI: 10.2353/ajpath.2009.080516)

Acute allograft rejection is mediated by the coordinated infiltration of alloantigen-primed T cells into the graft and the expression of effector functions that destroy the vascular endothelium and the parenchymal tissue.^{1,2} Adhesion molecules and chemoattractant cytokines, chemokines, play major roles in directing primed T-cell recruitment and infiltration into allografts.^{3–5} The role of adhesion molecules in graft rejection is indicated by the ability of specific antibodies or the use of adhesion molecule-deficient graft recipients or donors to delay or inhibit acute allograft rejection in many animal models.^{6–8} Similarly, many studies have demonstrated the ability to delay or inhibit allograft rejection through administration of antibodies to specific chemokines or chemokine receptors.⁹ In addition, the use of graft recipients with targeted deletions in CXCR3 and CCR5 has supported a role for these receptors in promoting T-cell trafficking to mediate acute rejection.^{10,11} Although these studies indicate an important function of specific chemokines in directing T-cell infiltration into allografts, the induction and source of these chemokines during the rejection process remains poorly understood.

The CXCR3 ligands, Mig/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11, are potent chemoattractants for antigen-activated T cells.^{12,13} These chemokines are induced by interferon (IFN)- γ and are produced during many T-cell-mediated inflammatory responses including allograft rejection. Mig/CXCL9 is produced at low levels in skin and

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Address reprint requests to Robert L. Fairchild, NB3-59, Department of Immunology, Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195–0001. E-mail: fairchr@ccf.org.

heart allografts early after transplantation in mouse models but this production increases with alloantigen-primed T-cell infiltration and activity in the allograft.^{14,15} Consistent with animal models, the expression of Mig in biopsies from clinical renal and heart allografts is indicative of an ongoing acute rejection episode.^{16,17} In rodent models, treatment with Mig-specific antibodies delays T-cell infiltration and prolongs the survival of complete major histocompatibility complex (MHC)-mismatched skin allografts 3 to 5 days implicating a role for Mig in optimal T-cell recruitment into grafts.¹⁸ This is supported by the ability of chronic treatment of C57BL/6 recipients with Mig-specific antibodies to promote the survival of ~75% of single class II MHC-disparate B6.H-2^{bm12} full thickness trunk skin allografts until the treatment is stopped.¹⁹

Mig is produced by endothelial cells and macrophages during many inflammatory processes.²⁰ The production of Mig by donor- and recipient-derived sources during allograft rejection remains unclear and the relative contribution of each source in allograft rejection is untested. In the current study, we have used mice with a targeted deletion in the Mig gene as allograft donors and recipients to test these aspects of the skin allograft rejection process. The results indicate the production of Mig by both graft- and recipient-derived sources but the production of each source appears in different tissue locations and affects the time of T-cell graft infiltration during the acute rejection process.

Materials and Methods

Animals

C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were purchased through Dr. Clarence Reeder, National Cancer Institute, Frederick, MD. B6.H-2^{bm12} mice and IP-10^{-/-} mice on the C57BL/6 (B6.IP-10^{-/-}) and on the BALB/c (BALB/c.IP-10^{-/-}) background were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice with a targeted deletion in the Mig gene on a C57BL/6 background (B6.Mig^{-/-}) were generated and characterized as previously reported.²¹ The targeted Mig gene deletion was bred onto the B6.C-H-2^{bm12} background to generate B6.H-2^{bm12}.Mig^{-/-} mice. To distinguish wild-type Mig from the gene with a neomycin gene insertion at the site of a deletion of sequences in exon 2 and intron 2, isolated tail DNA was amplified by polymerase chain reaction (PCR) using the Mig gene-specific oligonucleotide primers (5' to 3') 5'-CCCATTGCTAGAAACAACGG-3' and 5'-GTGGCAAACGCTCTCCTAAG-3' and the *neo* primer. The primers amplify a sequence of 532 bp from the wild-type Mig gene and a sequence of 630 bp from the genetically altered gene with the neomycin insertion. To distinguish wild-type C57BL/6 from B6.H-2^{bm12}, tail DNA was amplified by PCR using the I-A^b α chain primers: 5'-GGGACCCGCCGCGCTCACCAAGCC-3' and 5'-CTCGGCCCGCTTTTGCTCCAGGAA-3', followed by digestion of the resulting DNA with *Mbo*I that cleaves the B6.H-2^{bm12} but not the C57BL/6 product. B6.Mig^{-/-} and B6.H-2^{bm12}.Mig^{-/-} mice were bred at the Biological Re-

sources Unit of the Cleveland Clinic Foundation. Adult female mice of 7 to 12 weeks of age were used in these studies.

Transplantation

Skin grafting was performed using a modified version of the Billingham and Medawar protocol.²² Briefly, full-thickness trunk skin was prepared from donor ventral skin and cut into ~12-mm-diameter circles using a punch. Graft beds were prepared by carefully excising 14-mm-diameter circles of skin from the lateral dorsal thoracic wall of recipients. Recipients then received either allogeneic or syngeneic grafts that were covered with Vaseline gauze and an adhesive bandage. On day 7 after transplantation, the bandages and gauze were removed. Everyday thereafter, the grafts were visually monitored for rejection. Grafts were deemed rejected when >60% of the graft tissue was destroyed as assessed by visual examination.

Histology

Leukocyte infiltration into skin grafts was assessed by staining of prepared graft sections. Grafts were retrieved, fixed in 10% formalin, embedded in paraffin, and 8 μ m sections were stained with hematoxylin and eosin. Grafts were also embedded in OCT compound (Sakura Finetek, Torrance, CA), frozen at -80°C, and 8- μ m sections prepared for immunohistochemistry. The sections were placed on slides, fixed in acetone for 10 minutes, air-dried, immersed in phosphate-buffered saline (PBS) for 10 minutes, and incubated for 5 minutes at room temperature in 3% H₂O₂/PBS to inactivate internal peroxidase activity. After washing three times with PBS, the sections were covered for 1 hour at room temperature with the primary goat anti-Mig antibody (R&D Systems, Minneapolis, MN) or anti-CD4 mAb GK1.5 diluted to 10 μ g/ml in PBS/1% bovine serum albumin. Control slides were incubated with rat IgG. After washing, the slides were incubated for 20 minutes in biotinylated rabbit anti-goat IgG or biotinylated anti-rat IgG antibody diluted 1:300 in PBS/1% bovine serum albumin. The slides were rinsed three times with PBS, covered with streptavidin-horseradish peroxidase (DAKO, Carpinteria, CA) for 20 minutes, and washed three times with PBS. The substrate chromagen solution was prepared by dissolving a 10-mg tablet of 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) in 15 ml of PBS and 12 μ l of 30% H₂O₂ was added just before use. The substrate-chromagen solution was applied to the slides and incubated for 2 minutes and the slides were washed in dH₂O, counterstained with hematoxylin, rinsed with dH₂O, and immersed in 37 nmol/L NH₄OH for 10 seconds. Finally, the slides were dehydrated, viewed under light microscopy, and images were captured using ImagePro Plus (Media Cybernetics, Silver Spring, MD).

Mixed Lymphocyte Responses

Ten days after transplantation, cells in graft-draining lymph nodes from B6.H-2^{bm12} recipients of B6.Mig^{-/-} or

wild-type C57BL/6 skin allografts were tested for alloreactive T-cell priming by standard mixed lymphocyte responses. Recipient lymph node cell suspensions were washed three times and resuspended at 3×10^6 cells/ml in complete medium, RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Sigma), 2 mmol/L L-glutamine, 5×10^{-5} mol/L 2-mercaptoethanol, 10 mmol/L HEPES, and 20 μ g/ml gentamicin. Then 100- μ l aliquots were delivered to triplicate wells of a 96-well round-bottom tissue culture plate. Stimulator cells were prepared from wild-type C57BL/6, B6.Mig^{-/-}, B6.H-2^{bm12}, and BALB/c splenocytes. The stimulator cells were treated with 50 μ g/ml of mitomycin C for 30 minutes at 37°C. After washing three times, the stimulator cells were resuspended in culture media at 6×10^6 cells/ml and 100- μ l aliquots were delivered to the wells in the culture plate. After 48 hours, cultures were pulsed with 0.25 μ Ci of [³H]-thymidine, and 16 hours later the cultures were harvested onto fiber filter mats and the amount of ³H incorporation was determined by liquid scintillation counting.

Flow Cytometry

To detect the expression of CXCR3 on T cells and macrophages, cell suspensions were prepared from the spleens and graft-draining lymph nodes of graft recipient mice as well as control naïve mice. The cells were counted and 1×10^6 cell aliquots were stained with fluorescein isothiocyanate-conjugated anti-CD4, anti-CD8, or anti-macrophage F4/80 mAb and with phycoerythrin-conjugated anti-CXCR3 mAb (all antibodies from BD Biosciences, San Jose, CA) for 30 minutes on ice. The cells were washed and analyzed on a FACSCalibur (BD Biosciences) and data were analyzed using Flow Jo software (Tree Star, Ashland, OR).

ELISPOT Assay

Priming of alloantigen-specific T cells from skin allograft recipients was also tested by enumerating IFN- γ -producing T cells in graft-draining lymph nodes using ELISPOT assays as previously described.⁸ Briefly, ELISA spot plates (Unifilter350; Polyfiltronics, Rockland, MA) were coated with 2 μ g/ml of anti-IFN- γ mAb and blocked with 1% bovine serum albumin/PBS. Lymph node cell suspensions from recipients of skin allografts were prepared on days 12 or 14 after transplant and used as responder cells. Spleen cells from wild-type C57BL/6 or B6.C-H-2^{bm12} mice were prepared and treated with mitomycin C for use as stimulator cells in the assay. Responder and stimulator cells (1:2) were cultured in serum-free HL-1 medium (BioWhittaker, Walkersville, MD) supplemented with 1 mmol/L L-glutamine. After 24 hours of cell culture the cells were removed by extensive washing with PBS and PBS/0.025% Tween 20. Biotinylated anti-IFN- γ mAb (2 μ g/ml) was added for 6 hours at room temperature and streptavidin-conjugated alkaline phosphatase was added to each well. After 2 hours, the plates were washed with PBS, and nitro blue tetrazolium-5-bromo-4-

chloro-3-indolyl substrate (Kirkegaard & Perry, Gaithersburg, MD) was added for the detection of IFN- γ -producing cells. The resulting spots were counted with an ImmunoSpot Series I analyzer (Cellular Technology Ltd., Cleveland, OH) that was designed to detect ELISA spots with predetermined criteria for spot size, shape, and colorimetric density.

Results

Extended Survival of Mig-Deficient Allografts

The role of allograft-derived Mig in acute skin allograft rejection was first tested by transplanting full thickness trunk skin grafts from wild-type C57BL/6 or B6.Mig^{-/-} donors to B6.H-2^{bm12} recipients. Whereas all wild-type allografts were rejected by day 14 after transplant, survival of B6.Mig^{-/-} allografts was extended to day 24 after transplant (Figure 1 and Table 1, group 1 versus group 2). When B6.Mig^{-/-} mice were used as recipients of B6.H-2^{bm12} skin allografts, allograft survival was again significantly extended from day 14 to day 19 after transplant when compared with rejection by wild-type recipients (Table 1, group 1 versus group 3). These results suggested that both donor- and recipient-derived sources of Mig were important during rejection of class II MHC disparate skin allografts.

B6.Mig^{-/-} skin allograft rejection was also tested in other MHC disparate donor $\rightarrow$ recipient combinations (Table 1). Survival of the B6.Mig-deficient allografts was modestly extended when transplanted to a single class I MHC-disparate B6.H-2^{bm1} recipient (group 4 versus group 5). When transplanted to complete MHC-mismatched BALB/c recipients, survival of B6.Mig-deficient and wild-type allografts was not significantly different (group 6 versus group 7).

Figure 1. Acute rejection of single class II MHC-mismatched skin allografts from Mig wild-type and Mig^{-/-} donors. Groups of five B6.H-2^{bm12} mice received full thickness skin allografts from wild-type C57BL/6 (open squares) and B6.Mig^{-/-} (filled circles) donors. Mig^{-/-} grafts survived significantly longer than wild-type grafts (23.5 ± 2 days versus 14 ± 0.5 days; $P < 0.025$).

Table 1. Rejection of Skin Allografts in the Absence of Donor- or Recipient-Derived Mig/CXCL9

Group*	Graft recipient	Graft donor	MHC disparity	Time of rejection
1	B6.H-2 ^{bm12}	C57BL/6	Class II	14.0 ± 0.5
2	B6.H-2 ^{bm12}	B6.Mig ^{-/-}	Class II	23.5 ± 2.0
3	B6.Mig ^{-/-}	B6.H-2 ^{bm12}	Class II	19.3 ± 2.0
4	B6.H-2 ^{bm1}	C57BL/6	Class I	14.5 ± 1.0
5	B6.H-2 ^{bm1}	B6.Mig ^{-/-}	Class I	18.8 ± 2.0
6	BALB/c	C57BL/6	Class I and II	13.0 ± 1.0
7	BALB/c	B6.Mig ^{-/-}	Class I and II	14.1 ± 1.5

*Groups of five recipients were transplanted with full thickness skin grafts from the indicated donors and time of rejection in days was determined.

Delayed Cellular Infiltration into Mig-Deficient Skin Allografts

A potential mechanism that could account for the extended survival of Mig^{-/-} skin allografts on B6.H-2^{bm12} recipients was the impaired infiltration of alloantigen-primed effector T cells into the Mig-deficient graft. To begin to address this possibility tissue sections of wild-type C57BL/6 and B6.Mig^{-/-} skin grafts were retrieved from B6.H-2^{bm12} recipients at day 12 after transplantation and sections were stained with hematoxylin and eosin (H&E). Examination of sections from wild-type allografts indicated intense mononuclear cell infiltration consistent with the rejection of the allografts (Figure 2a). In contrast, the B6.Mig^{-/-} skin allografts harvested at day 12 indicated little to no cellular infiltration (Figure 2b) and the dermis looked similar to an isograft retrieved at day 12 after transplant (Figure 2d). When B6.Mig^{-/-} skin allografts were retrieved from B6.H-2^{bm12} recipients at day 20 after transplant, intense mononuclear cell infiltration into the allografts was evident, again consistent with the impending rejection of the allografts (Figure 2c). Immunohistochemical staining performed on day 20 tissue sections revealed that the B6.Mig^{-/-} graft-infiltrating

cells were primarily CD4⁺ T cells and macrophages with no infiltrating CD8⁺ T cells evident (data not shown), which are characteristics of the graft infiltrate in this allo-immune response.^{18,19}

The expression of the chemokine receptor binding Mig as well as IP-10 on allograft recipient T cells and macrophages was then investigated. Groups of wild-type C57BL/6 mice received skin grafts from B6.H-2^{bm12} and on days 7 and 14 after transplant splenic and graft-draining lymph node cell suspensions were prepared and stained with antibodies to detect CXCR3 expression on CD4 and CD8 T cells and macrophages. An increased frequency of CD4 T cells expressing CXCR3 was observed on day 7 after transplant in the graft-draining lymph nodes of the allograft recipients versus ungrafted mice (19.5% versus 6.5% of total CD4 T cells, respectively) and these frequencies returned to naïve levels by day 14 after transplant (Figure 3a). In contrast, the frequency of CXCR3⁺ CD4⁺ T cells in the spleen of allograft recipients decreased from 22.7% of the total CD4 T-cell population observed in naïve mice to 6.5% on day 7 after transplant but returned to naïve levels by day 14 after transplant. Although few CD8 T cells were observed infiltrating B6.H-2^{bm12} skin allografts, there was also an in-

Figure 2. Cellular infiltration into single class II MHC-mismatched skin allografts from Mig wild-type and Mig^{-/-} donors. B6.H-2^{bm12} mice received full thickness skin allografts from wild-type C57BL/6 and B6.Mig^{-/-} donors. Wild-type allografts (A) and B6.H-2^{bm12} isografts (D) were retrieved at day 12 after transplant and Mig^{-/-} allografts were retrieved at days 12 (B) and 20 (C) after transplant. Sections were prepared and stained with H&E. Original magnifications, ×100.

Figure 3. Increase in CD4 and CD8 T cells expressing CXCR3 in the graft-draining lymph nodes of wild-type C57BL/6 recipients of B6.H-2^{bm12} skin allografts. Spleen and pooled lymph node (bilateral, axillary, and inguinal) cell suspensions were prepared, stained with fluorescein isothiocyanate-conjugated anti-CD4 (A), anti-CD8 (B), or anti-macrophage F4/80 (C) mAb and with phycoerythrin-conjugated anti-CXCR3 mAb and analyzed by two-color flow cytometry. Numbers in each quadrant indicate percentages of unstained and stained cells for each of the indicated antibodies.

crease in the frequency of CD8 T cells expressing CXCR3 in the graft-draining lymph nodes as well as in the spleen on day 7 after transplant (Figure 3b). In addition, a small population of F4/80⁺ cells expressing CXCR3 was observed in the lymph nodes of allograft recipients and in the spleen of both naïve mice and allograft recipients (Figure 3c).

T-Cell Proliferative Responses in B6.H-2^{bm12} Recipients of Wild-Type and B6.Mig^{-/-} Skin Allografts

The priming of alloantigen-reactive T cells in B6.H-2^{bm12} recipients of class II MHC disparate wild-type and Mig-deficient allografts was tested by mixed lymphocyte reactions. At day 10 after transplant skin allograft draining lymph node cell (LNC) suspensions were prepared and cultured with mitomycin C-treated spleen cells from allograft donor C57BL/6, third-party allogeneic BALB/c, and recipient B6.H-2^{bm12} mice. Equivalent proliferative reactivity was observed when LNCs from recipients of wild-type C57BL/6 and B6.Mig^{-/-} skin allografts were cultured with donor alloantigen-presenting cells (Figure 4). Proliferative reactivity to autologous and to third-party allogeneic stimulator cells was low in both of the groups of test responder LNCs. These results indicated equivalent levels of class II alloantigen reactive T-cell priming in response to wild-type and Mig-deficient skin allografts.

Mig Is Derived from Both Donor and Recipient Sources during Rejection of Mig-Deficient Skin Allografts

Previous studies have indicated that Mig production in the graft promotes recipient C57BL/6 T-cell infiltration into B6.H-2^{bm12} skin allografts.^{18,19} Initial experiments using Northern blot hybridization of RNA isolated from rejecting

wild-type C57BL/6 and B6.Mig^{-/-} allografts on B6.H-2^{bm12} recipients indicated strong expression of Mig mRNA in both grafts at the time of rejection (data not shown). Therefore, the presence of Mig protein in skin allografts from wild-type C57BL/6 and B6.Mig^{-/-} donors was directly tested by immunohistochemical staining for Mig. In wild-type allografts retrieved from B6.H-2^{bm12} recipients at day 12 after transplant, Mig protein was clearly evident in the lower areas of the epidermis, in vessels in the upper and lower dermis and in cells scattered throughout the dermis (Figure 5, a and d). In contrast, Mig^{-/-} allografts retrieved at day 12 after transplant had no Mig protein evident in the epidermis or upper dermis (Figure 5b). However, there was a small number of vessels in the extreme lower dermis with

Figure 4. T cells from B6.H-2^{bm12} recipients of wild-type and Mig^{-/-} skin allografts have similar proliferative responses *in vitro*. B6.H-2^{bm12} mice received full thickness skin allografts from wild-type C57BL/6 and B6.Mig^{-/-} donors. On day 10 after transplant, lymph node cells were prepared and were cultured with mitomycin C-treated splenocytes from syngeneic (black bars), wild-type C57BL/6 (white bars), and third-party allogeneic BALB/c (stippled bars) mice. After 48 hours, cultures were pulsed with 1 μ Ci of ³H-thymidine and 20 later cultures were harvested onto fiber filter mats and ³H incorporation was determined by liquid scintillation counting. The data are presented as the mean incorporation $\pm$ SD and are representative of three individual experiments.

Figure 5. Intragraft production of Mig in wild-type and Mig^{-/-} skin allografts. B6.H-2^{bm12} mice received full thickness skin allografts from wild-type C57BL/6 and B6.Mig^{-/-} donors. Wild-type allografts (**A**) were retrieved at day 12 after transplant and Mig^{-/-} allografts were retrieved at days 12 (**B** and **E**) and 20 (**C** and **F**) after transplant. Frozen sections were prepared and stained with Mig-specific antibodies (**A–C**, **E**, and **F**) for immunochemistry. **D**: Wild-type allograft sections were also stained with biotinylated rabbit anti-goat IgG alone. Original magnifications: $\times 100$: (**A–C**, **E**); $\times 200$ (**D**, **F**).

associated cells that stained positively for Mig in these allografts at this time point (Figure 5e). At day 20 after transplant, the number of vessels as well as the intensity of staining for Mig was increased in the vessels in the lower dermis of the Mig^{-/-} allografts but there remained little to no detectable Mig staining at other locations in the allograft tissue (Figure 5, c and f).

To further investigate the localization of donor- versus recipient-derived Mig during skin allograft rejection, the presence of Mig protein in rejecting B6.H-2^{bm12} skin allografts on B6.Mig^{-/-} recipients was examined. At day 12 after transplant several days before rejection, B6.H-2^{bm12} skin allografts had intense staining of Mig protein in the lower epidermis as well as several places with light Mig staining in the dermis including some vessels (Figure 6, a and c). At day 18 (eg, the time of rejection), the intensity of the Mig staining was increased in the dermis of these allografts (Figure 6, b and d).

Skin Allograft Rejection in the Absence of Mig

Because Mig was derived from both donor and recipient sources, the rejection of C57BL/6 skin allografts by B6.H-2^{bm12} recipients in the complete absence of Mig was tested. B6.H-2^{bm12} mice were crossed with the B6.Mig^{-/-} mice to generate B6.H-2^{bm12}.Mig^{-/-} mice. When B6.Mig^{-/-} skin was transplanted to B6.H-2^{bm12}.Mig^{-/-} recipients, allograft rejection was not observed until after day 20 after transplant and one-third of the allografts survived longer than 60 days after transplantation (Figure 7). At day 14 after transplant, the time of wild-type allograft rejection, B6.Mig^{-/-} allografts retrieved from B6.C-H-2^{bm12}.Mig^{-/-} recipients had marked decreases in CD4 T cell and other mononuclear cells infiltrating into the allograft (Figure 8, b and d) when compared with the rejecting wild-type allografts (Figure 8, a and c).

To compare the levels of alloreactive T-cell priming in the graft-draining lymph nodes of each set of recipients, ELISPOT assays to enumerate donor-specific T cells producing IFN- γ were performed. B6.C-H-2^{bm12}.Mig^{-/-} recipients of B6.Mig^{-/-} skin allografts had slight but not significantly different decreases in donor-reactive T cells producing IFN- γ when compared wild-type B6.H-2^{bm12} recipients of wild-type C57BL/6 skin allografts (Figure 9).

Figure 6. Intragraft production of Mig in wild-type skin allografts from Mig-deficient recipients. B6.Mig^{-/-} mice received full thickness skin allografts from B6.H-2^{bm12} donors and grafts were retrieved at days 12 (**A** and **C**) and 18 (**B** and **D**) after transplant. Frozen sections were prepared and stained for the presence of Mig protein by immunohistochemistry. Original magnifications: $\times 100$ (**A**, **B**); $\times 200$ (**C**, **D**).

Figure 7. Rejection of single class II MHC-mismatched skin allografts in the absence of donor- or recipient-derived Mig. Groups of six B6.H-2^{bm12} mice received full thickness skin allografts from wild-type C57BL/6 (open squares) and B6.H-2^{bm12}.Mig^{-/-} mice received full thickness skin allografts from B6.Mig^{-/-} (filled circles) donors. The allografts survived significantly longer in the absence of Mig ($P < 0.001$).

Alloimmune Responses to MHC-Mismatched Skin Allografts in the Absence of Recipient IP-10

Because the survival of B6.C-H-2^{bm12} skin allografts was prolonged when C57BL/6 recipients were deficient in the production of Mig, the rejection of skin allografts in recipients deficient in the CXCR3 ligand IP-10 was also investigated. Single class II MHC mismatched B6.C-H-2^{bm12} skin allografts were rejected by wild-type C57BL/6 recipients by day 16 after transplant (Figure 10). In contrast, B6.IP-10^{-/-} recipients rejected the B6.C-H-2^{bm12} allografts by day 10 after transplant. B6.Mig^{-/-} recipients rejected complete MHC-mismatched BALB/c and BALB/c.IP-10^{-/-} skin allografts at equivalent times, on day 13 to 14 after transplant (Figure 10 and data not shown).

A potential mechanism for the accelerated rejection of the single class II MHC mismatched skin allografts in IP-10-

Figure 9. Frequency of donor-specific IFN-γ-producing cells in recipients during rejection of skin allografts in the absence of Mig. Wild-type B6.H-2^{bm12} mice received full thickness skin allografts from wild-type C57BL/6 and B6.H-2^{bm12}.Mig^{-/-} mice received full thickness skin allografts from B6.Mig^{-/-} donors. On day 12 after transplant lymph node cells from the graft recipients and from naive wild-type B6.H-2^{bm12} mice were analyzed by ELISPOT to enumerate donor-specific T cells producing IFN-γ. The results are representative of two individual experiments.

deficient recipients was an increased alloreactive T-cell response induced in the absence of IP-10. This was investigated by performing ELISPOT assays to enumerate donor-specific T cells producing IFN-γ in wild-type C57BL/6 versus B6.IP-10^{-/-} recipients of the B6.C-H-2^{bm12} allografts. Consistent with the accelerated rejection of the skin allografts, B6.IP-10^{-/-} recipients had a 2.5-fold increase in the number of donor-reactive CD4 T cells producing IFN-γ (Figure 11).

Discussion

The rejection of single class II MHC disparate skin allografts in the C57BL/6 and B6.H-2^{bm12} combination is strongly promoted by the production of Mig. Previous studies from this laboratory demonstrated the survival of

Figure 8. Cellular infiltration into single class II MHC-mismatched skin allografts in the absence of Mig. B6.H-2^{bm12} mice received full thickness skin allografts from wild-type C57BL/6 (A and C) and B6.H-2^{bm12}.Mig^{-/-} mice received full thickness skin allografts from B6.Mig^{-/-} (B and D) donors. The allografts were retrieved at day 12 after transplant. Formalin-fixed sections were stained with H&E (A and B) and frozen sections were prepared and stained with anti-CD4 mAb for immunohistochemical detection of infiltrating CD4⁺ T cells (C and D). Original magnifications, ×100.

Figure 10. Rejection of single class II MHC-mismatched skin allografts in the absence of recipient-derived IP-10. Groups of five wild-type C57BL/6 and B6.IP-10^{-/-} mice received full thickness skin allografts from B6.H-2^{bm12} donors. In addition a group of B6.Mig^{-/-} mice received full thickness skin allografts from BALB/c.IP-10^{-/-} donors.

B6.H-2^{bm12} skin grafts on C57BL/6 recipients for as long as the recipients were treated with Mig-specific antibodies.¹⁹ Furthermore, T cells from B6.IFN- γ ^{-/-} recipients do not infiltrate B6.H-2^{bm12} allografts despite detectable priming of graft reactive T cells consistent with the requirement for IFN- γ production to induce Mig production in the allograft site and direct T-cell graft infiltration. Mig is produced by endothelial cells, macrophages, and dendritic cells and each is a potential source of the T-cell chemoattractant during rejection. *In situ* hybridization studies indicated cardiac graft endothelial cells and graft-infiltrating neutrophils and macrophages as primary sources of Mig in vascularized cardiac allografts.¹⁵ However, the importance of donor versus recipient cells in providing Mig to direct T-cell graft infiltration during rejection has not been tested.

The current results demonstrate the production of Mig by both donor- and recipient-derived sources. Delayed rejection of Mig-deficient skin allografts by wild-type B6.H-2^{bm12} recipients and rejection of B6.H-2^{bm12} skin allografts by Mig-deficient recipients suggests an important role for each source during rejection. During rejection of Mig-deficient skin allografts, the chemokine protein was clearly present in the vascular endothelium of the lower dermis and was associated with cells around the vessels. These results are consistent with the neovascularization of the skin allograft by recipient endotheli-

um²³⁻²⁵ and the importance of this source of Mig in directing T-cell infiltration into the allograft. In contrast, Mig protein was not detected in vascular structures in the upper dermal vascular plexus of Mig^{-/-} allografts and there was little to no detectable T-cell infiltration into this area of the allograft until late in the delayed rejection of the grafts. These results suggest that, at least in this strain combination, Mig production by graft endothelium may be required for optimal penetration of the alloantigen-primed CD4⁺ T cells and macrophages through the vasculature. At the time Mig^{-/-} allografts were rejected, however, there were many T cells and macrophages in the upper dermal plexus and the epidermis suggesting that Mig does not mediate T-cell infiltration through the graft parenchyma to the epidermis once the T cells clear the vascular barrier.

Several studies have indicated that the dermal microvasculature is the critical target of alloantigen-primed T cells during rejection of skin allografts.^{23,26,27} Once these T cells interact with and destroy the endothelium, penetration of the parenchyma is proposed to be a secondary event in the rejection process. The factors directing the migration of the endothelium penetrating T cells and macrophages from the lower dermis to the epidermis remain unclear at this time. Previous studies from this laboratory have indicated that basal keratinocytes are a primary source of IP-10 during skin allograft rejection.²⁸ However, IP-10 antibodies have little effect on skin allograft survival in any donor recipient strain combination tested suggesting that IP-10 does not direct this infiltration.

The results raise questions concerning the source of the allogeneic stimulus (ie, class II MHC) that activates primed T cells to produce IFN- γ and induce Mig production in the Mig^{-/-} graft vessels. Recipient endothelial cells in allografts present allopeptides via the indirect presentation pathway that promote rejection of the graft.²⁹ However, recent studies in this laboratory have failed to support a role for activation of T cells through the indirect allorecognition pathway in the B6.H-2^{bm12} → C57BL/6 combination. Recipient priming with an 18-mer peptide of the B6.H-2^{bm12} I-A α chain that includes the three amino acid differences between the two strains does not activate C57BL/6 T cells or accelerate graft rejection (R. Fairchild, unpublished results). Furthermore, peptide reactive T cells are not detectable in the lymph nodes or spleens of C57BL/6 recipients of B6.H-2^{bm12} skin or heart allografts. Because both donor and recipient endothelial cells constitute the vessels penetrating the skin and the delay in rejection of the Mig^{-/-} allografts is concurrent with the growth of new vessels into the allograft, an alternate and more likely possibility is that IFN- γ induced by activation of alloantigen-primed T cells interacting with donor endothelial cells stimulates the recipient-derived Mig^{+/+} endothelial cells and macrophages to produce Mig. It remains unclear, however, why alloantigen-primed T-cell recognition of allogeneic class II MHC on vessels toward the upper regions of the dermis does not induce IFN- γ production that in turn stimulates recipient-derived cells (ie, endothelial cells and/or macrophages) to produce Mig. Recipient-derived endothelial

Figure 11. Frequency of donor-specific IFN- γ -producing cells in recipients during rejection of B6.H-2^{bm12} skin allografts in the absence of recipient IP-10. Wild-type C57BL/6 and B6.IP-10^{-/-} mice received full thickness skin allografts from B6.H-2^{bm12} donors. On day 14 after transplant lymph node cells from the graft recipients were analyzed by ELISPOT to enumerate donor-specific T cells producing IFN- γ . The results are representative of two individual experiments.

cells may not traffic far enough to become a component of the vessels in the upper dermis. The absence of Mig in this area of the allograft also suggests that T-cell penetration of the allograft vasculature must precede recipient-derived macrophage infiltration and is supported by the low or absent macrophage infiltration observed until T cells infiltrate the allograft.

One surprising finding from these studies was the rejection of the class II MHC disparate allografts in the absence of Mig. Our previous studies had indicated that ~75% of B6.H-2^{bm12} skin allografts survived for as long as Mig-specific antibodies were administered to C57BL/6 recipients.¹⁹ In contrast, only 33% of B6.Mig^{-/-} skin allografts survived long term on B6, H-2^{bm12}.Mig^{-/-} recipients. However, the B6.Mig^{-/-} allografts that were rejected by these recipients did not begin to do so until after day 20 after transplant whereas the B6.Mig^{-/-} allografts began rejecting on day 16 by wild-type B6.H-2^{bm12} recipients. These results indicate the importance of Mig in promoting T-cell graft infiltration but also suggest other mechanisms that can compensate to direct this infiltration in the absence of donor or recipient Mig.

It is becoming clear that chemokines may influence the development of T cells during antigen priming with respect to the magnitude and phenotype of the responses generated.^{26,30} The absence of Mig production by graft-derived antigen-presenting cells had no apparent effect on alloreactive T-cell priming because equivalent proliferative responses and eventual infiltration into the allograft were observed in recipients of Mig^{+/+} and Mig^{-/-} skin allografts. Previous studies from this laboratory had also indicated that treatment with Mig-specific antibodies had no detectable effect on alloreactive T-cell responses in C57BL/6 recipients of B6.H-2^{bm12} skin allografts.¹⁸ In contrast, Whiting and co-workers³¹ have reported that treatment with Mig-specific antiserum decreases the priming of alloreactive T cells in heart allograft recipients. It is possible that the stronger allogeneic stimulus provided by skin allografts that includes increased ischemic time and increased number of graft dendritic cells circumvents a requirement for Mig during T-cell priming.

Similar to results observed with anti-Mig antibody treatment, the absence of donor or recipient Mig had little effect on the rejection of complete MHC-mismatched skin allografts. Furthermore, rejection of complete MHC-mismatched skin allografts when both donor and recipient are unable to produce Mig is also not altered (H. Amano, data not shown). These results indicate that a stronger alloreactive immune response than that induced to a single class II MHC antigen negates the need for Mig-directed T-cell penetration through the allograft vascular endothelium and this recruitment is compensated by other mechanisms. These mechanisms may include recruitment directed by other chemokines or complement cleavage components that are chemotactic for T cells and other leukocytes. As discussed above, IP-10 is an unlikely candidate but the induction and role of I-TAC in skin allograft rejection remains untested. In addition, the expression of CCR5 and its ligands may play more of a role in skin allograft rejection in the absence of Mig.

Collectively, the results of this study have used a model that is strictly dependent on the production of Mig to show that the chemokine is produced by both donor- and recipient-derived sources and that production by one source is sufficient to promote T-cell infiltration and rejection of the allograft. The sites of Mig production by donor- and recipient-derived sources are considerably different. In Mig-deficient allografts, Mig is provided by recipient-derived endothelial cells in vessels revascularizing the skin allograft and by graft-infiltrating macrophages. Graft donor-derived Mig is evident in the upper dermal vascular plexus when wild-type allografts are transplanted and mediates more rapid infiltration of primed T cells into the graft and rejection.

References

- Hall B: Cells mediating allograft rejection. *Transplantation* 1991, 51:1141–1151
- Rosenberg AS, Singer A: Cellular basis of skin allograft rejection: an in vivo model of immune-mediated tissue destruction. *Annu Rev Immunol* 1992, 10:333–358
- Fuggle SV, Sanderson JB, Gray DWR, Richardson A, Morris PJ: Variation in expression of endothelial adhesion molecules in pretransplant and transplanted kidneys: correlation with intragraft events. *Transplantation* 1993, 55:117–123
- Pelletier RP, Ohye RG, VanBuskirk AM, Sedmak DD, Kincade P, Ferguson RM, Orosz CG: Importance of endothelial VCAM-1 for inflammatory leukocytic infiltration in vivo. *J Immunol* 1992, 149:2473–2481
- von Andrian U, Mackay CR: T-cell function and migration. *N Engl J Med* 2000, 343:1020–1034
- Nicolls MR, Gill RG: LFA-1 (CD11a) as a therapeutic target. *Am J Transplant* 2006, 6:27–36
- Isobe M, Yagita H, Okumura K, Ihara A: Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. *Science* 1992, 255:1125–1129
- Zhang Q-W, Kish DD, Fairchild RL: Absence of allograft ICAM-1 attenuates alloantigen-specific T cell priming, but not primed T cell trafficking into the graft, to mediate acute rejection. *J Immunol* 2003, 170:5530–5537
- Hancock WW, Wang I, Ye O, Han R, Lee I: Chemokines and their receptors as markers of allograft rejection and targets for immunosuppression. *Curr Opin Immunol* 2003, 15:479–486
- Gao W, Faia KL, Csizmadia V, Smiley ST, Soler D, King JA, Danoff TM, Hancock WW: Beneficial effects of targeting CCR5 in allograft recipients. *Transplantation* 2001, 72:1199–1205
- Hancock WW, Lu B, Gao W, Csizmadia V, Faia K, King JA, Smiley ST, Ling M, Gerard NP, Gerard C: Requirement of the chemokine receptor CXCR3 for acute allograft rejection. *J Exp Med* 2000, 192:1515–1520
- Liao F, Rabin RL, Yannelli JR, Koniaris LG, Vanguir P, Farber JM: Human Mig chemokine: biochemical and functional characterization. *J Exp Med* 1995, 182:1301–1314
- Loetscher M, Gerber B, Loetscher P, Jones SA, Piali L, Clark-Lewis I, Baggiolini M, Moser B: Chemokine receptor specific for IP-10 and Mig: structure function and expression in activated T-lymphocytes. *J Exp Med* 1996, 184:963–969
- Kondo T, Novick AC, Toma H, Fairchild RL: Induction of chemokine gene expression during allogeneic skin graft rejection. *Transplantation* 1996, 61:1750–1757
- Miura M, Morita K, Kobayashi H, Hamilton TA, Burdick MA, Strieter RM, Fairchild RL: Monokine induced by IFN- γ is a dominant factor directing T cells into murine cardiac allografts during acute rejection. *J Immunol* 2001, 167:3494–3504
- Fahmy ND, Yamani MH, Starling RX, Ratliff NB, Young JB, McCarthy PM, Feng J, Novick AC, Fairchild RL: Chemokine and chemokine receptor gene expression indicates acute rejection of human cardiac transplants. *Transplantation* 2003, 75:72–78
- Melter M, Exeni A, Reinders MEJ, Fang JC, McMahon G, Ganz P,

- Hancock WW, Briscoe DM: Expression of the chemokine receptor CXCR3 and its ligand IP-10 during human cardiac allograft rejection. *Circulation* 2001, 104:2558–2564
18. Koga S, Auerbach MD, Engeman TM, Novick AC, Toma H, Fairchild RL: T cell infiltration into class II MHC-disparate allografts and acute rejection is dependent on the IFN- γ -induced chemokine Mig. *J Immunol* 1999, 163:4878–4885
19. Kobayashi H, Novick AC, Toma H, Fairchild RL: Chronic antagonism of Mig inhibits cellular infiltration and promotes survival of class II MHC disparate skin allografts. *Transplantation* 2002, 74:387–395
20. Farber JM: Mig and IP-10: CXC chemokines that target lymphocytes. *J Leukoc Biol* 1997, 61:246–257
21. Park MK, Amichay D, Love P, Wick E, Liao F, Grinberg A, Rabin RL, Zhang HH, Gebeyehu S, Wright TM, Iwasaki A, Weng Y, DeMartino JA, Elkins KL, Farber JM: The CXC chemokine murine monokine induced by IFN- γ (CXC chemokine ligand 9) is made by APCs, targets lymphocytes including activated B cells, and supports antibody responses to a bacterial pathogen in vivo. *J Immunol* 2002, 169:1433–1443
22. Billingham RE, Medawar PB: The technique of free skin grafting in mammals. *J Exp Biol* 1951, 28:385–402
23. Dvorak HF, Mihm Jr MC, Dvorak AM, Barnes BA, Manseau EJ, Galli SJ: Rejection of first-set skin allografts in man, the microvasculature is the critical target of the immune response. *J Exp Med* 1979, 150:322–337
24. Jooste SV, Colvin RB, Soper WD, Winn HJ: The vascular bed as the primary target in the destruction of skin grafts by antiserum. I. Resistance of freshly placed xenografts of skin to antiserum. *J Exp Med* 1981, 154:1319–1331
25. Jooste SV, Colvin RB, Winn HJ: The vascular bed as the primary target in the destruction of skin grafts by antiserum. II. Loss of sensitivity to antiserum in long-term xenografts of skin. *J Exp Med* 1981, 154:1332–1341
26. Abdi R, Smith RN, Makhoul L, Najafian N, Luster AD, Auchincloss Jr H, Sayegh MH: The role of CC chemokine receptor 5 (CCR5) in islet allograft rejection. *Diabetes* 2002, 51:2489–2495
27. Dvorak HF, Mihm Jr MC, Dvorak AM, Barnes BA, Galli SJ: The microvasculature is the critical target of the immune response in vascularized skin allograft rejection. *Transplantation* 1980, 74:280–284
28. Watarai Y, Paolone D, Koga S, Tannenbaum C, Hamilton TA, Fairchild RL: Intra-graft chemokine expression during rejection of skin grafts with multiple minor histocompatibility differences. *J Immunol* 2000, 164:6027–2033
29. Valujskikh A, Lantz O, Celli S, Matzinger P, Heeger PS: Cross-primed CD8 T cells mediate graft rejection via a distinct effector pathway. *Nat Immunol* 2002, 3:844–851
30. Nozaki T, Amano H, Bickerstaff A, Orosz CG, Novick AC, Tanabe K, Fairchild RL: Antibody-mediated rejection of cardiac allografts in CCR5-deficient recipients. *J Immunol* 2007, 179:5238–5245
31. Whiting D, Hsieh G, Yun JJ, Banerji A, Yao W, Fishbein M, Belperio J, Strieter RM, Bonavida B, Ardehali A: Chemokine monokine induced by IFN- γ /CXC chemokine ligand 9 stimulates T lymphocyte proliferation and effector cytokine production. *J Immunol* 2004, 172:7417–7424