Chemokine Monokine Induced by IFN-γ/CXC Chemokine Ligand 9 Stimulates T Lymphocyte Proliferation and Effector Cytokine Production[1]

David Whiting,* George Hsieh,* James J. Yun,*† Anamika Banerji,* William Yao,* Michael C. Fishbein,† John Belperio,‡ Robert M. Strieter,‡ Benjamin Bonavida,§ and Abbas Ardehali[2]*

Monokine induced by IFN-γ (MIG; CXC chemokine ligand (CXCL)9) is important in T lymphocyte recruitment in organ transplantation. However, it is not known whether this chemokine, in addition to its chemotactic properties, exerts any effect on T lymphocyte effector functions. For in vivo studies, we used a previously characterized murine model of chronic rejection. The recipient mice were treated with anti-MIG/CXCL9 Ab; graft-infiltrating cells were analyzed for IFN-γ production. For in vitro studies, exogenous CXCR3 ligands were added to CD4 lymphocytes in MLRs, and the proliferative responses were measured. Separate experiments quantitated the number of IFN-γ-producing cells in MLRs by ELISPOT. Neutralization of MIG/CXCL9, in the in vivo model, resulted in significant reduction in the percentage of IFN-γ-producing graft-infiltrating T lymphocytes. In vitro experiments demonstrated that 1) exogenous MIG/CXCL9 stimulated CD4 lymphocyte proliferation in a MHC class II-mismatched MLR, 2) MIG/CXCL9 also increased the number of IFN-γ-producing CD4 lymphocytes in ELISPOT, 3) neutralization of MIG/CXCL9 in MLR reduced T lymphocyte proliferation, 4) IFN-γ-inducible protein 10/CXCL10 and IFN-inducible T cell α chemoattractant/CXCL11 had similar effects on T lymphocyte proliferation, 5) MIG/CXCL9 stimulated T lymphocyte proliferation in MHC class I- and total MHC-mismatched MLRs, 6) neutralization of CXCR3 reduced MIG/CXCL9-induced T lymphocyte proliferation and the number of IFN-γ-positive spots on ELISPOT, and 7) the proliferative effects of MIG/CXCL9 were mediated via an IL-2-independent pathway and were controlled by IFN-γ. This study demonstrates that MIG/CXCL9 stimulates T lymphocyte proliferation and effector cytokine production, in addition to its chemotactic effects. This novel observation expands our current understanding of MIG/CXCL9 biology beyond that of mediating T cell trafficking. ***The Journal of Immunology,*** **2004, 172: 7417–7424.**

Recruitment of alloantigen-primed T lymphocytes is an early and critical event in the process of allograft rejection. Several recent reports have demonstrated the importance of chemokines in trafficking T lymphocytes to the graft (1–6). Monokine induced by IFN-γ (MIG[3]; CXC chemokine ligand (CXCL)9), a member of the non-ELR (lacking a Glu-Leu-Arg motif in the N-terminal region) CXC chemokine family, specifically targets primed T lymphocytes (7). MIG/CXCL9 is produced by macrophages, APCs, and B cells (8). The chemoattractant effects of MIG/CXCL9 are mediated via a G protein-coupled receptor, CXCR3, which is expressed on memory and activated CD4 and CD8 lymphocytes, B cells, and NK cells (9).

The role of MIG/CXCL9 in alloimmune responses has been validated by several observations (10–12). MIG/CXCL9 is expressed in allogeneic skin grafts several days before completion of rejection; neutralization of MIG/CXCL9 inhibits T cell infiltration and delays skin allograft rejection (10). Chronic anti-MIG/CXCL9 therapy has also been shown to yield prolonged survival of MHC class II-disparate skin grafts (11). More recently, our group has shown that MIG/CXCL9 expression is up-regulated in the donor hearts of a murine model of cardiac allograft vasculopathy, correlates temporally with T lymphocyte recruitment, and precedes intimal thickening (12). Furthermore, neutralization of MIG/CXCL9 markedly reduces the severity of intimal lesions.

The current literature suggests that the predominant function of MIG/CXCL9 is the recruitment of primed T lymphocytes to the sites of inflammation. It is not known whether MIG/CXCL9 can potentiate the activation, proliferation, and differentiation of T lymphocytes in an alloimmune response.

The purpose of this study is to determine whether, in addition to its chemotactic effects, MIG/CXCL9 can enhance T lymphocyte alloimmune responses. The findings of this study demonstrate that the role of MIG/CXCL9 is not limited to recruitment of primed T lymphocytes to sites of inflammation; MIG/CXCL9 also potentiates T lymphocyte proliferation and cytokine production in allogeneic interactions.

*Department of Surgery, Division of Cardiothoracic Surgery, †Department of Pathology and Laboratory Medicine, ‡Department of Medicine, and §Department of Microbiology, Immunology, and Molecular Genetics, David Geffen School of Medicine, University of California, Los Angeles, CA 90095

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[2] Address correspondence and reprint requests to Dr. Abbas Ardehali, Division of Cardiothoracic Surgery, 62-246 Center for Health Sciences, University of California, Los Angeles, Medical Center, 10833 Le Conte Avenue, Los Angeles, CA 90095. E-mail address: aardehali@mednet.ucla.edu

[3] Abbreviations used in this paper: MIG, monokine induced by IFN-γ; CXCL, CXC chemokine ligand; CCL, CC chemokine ligand; IP-10, IFN-γ-inducible protein 10; ITAC, IFN-inducible T cell α chemoattractant.

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Materials and Methods

Animals

Female B6.C-H2^{bm12}, C57BL/6, B10.A-H2, B10.BR-H2k, and BALB/CJ mice, 6–12 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). The B6C.H-2^{bm12} and C57BL/6 strains differ at the I-A locus of MHC II but are identical at MHC I and minor MHC loci. The B10.A-H2 and B10.BR-H2k mice differ at the MHC I locus, whereas BALB/CJ and C57BL/6 strains are mismatched at all MHC loci. All animals received humane care in compliance with University of California, Los Angeles, guidelines and the Principles of Laboratory Animal Care published by the National Institute of Health.

Transplantation

Intra-abdominal heterotopic heart transplantation was performed using a modification of the method outlined by Corry et al. (13). Through a midline abdominal incision, the donor aorta was anastomosed to the recipient infrarenal abdominal aorta, and the donor pulmonary artery was anastomosed to the inferior vena cava. Function of the allografts was assessed by abdominal palpation and scored on a scale of 0–4 (4 = normal beating; 0 = absence of contractions).

Anti-MIG/CXCL9 and anti-CXCR3 serum preparation

Polyclonal goat anti-murine MIG/CXCL9 was produced by the immunization of goats with recombinant murine MIG/CXCL9 (R&D Systems, Minneapolis, MN). The goat was immunized in multiple intradermal sites with CFA followed by at least three boosts (14). Direct ELISA was used to evaluate titers. The specificity of the serum was analyzed when titers reached >1/1,000,000. The anti-MIG/CXCL9 serum was specific in sandwich ELISA without cross-reactivity to a panel of cytokines, including human and murine IL-Rα, IL-1, IL-2, IL-6, IL-4, TNF-α, IFN-γ, and members of the CXC and CC chemokine families (14). The polyclonal rabbit anti-murine CXCR3 serum was produced in similar fashion. The anti-CXCR3 serum demonstrated a specific neutralizing capacity in chemotaxis assays against murine MIG/CXCL9 and IFN-γ-inducible protein 10 (IP-10)/CXCL10 (14).

Experimental groups

The transplanted mice were divided into two groups. The first group heretofore designated as "anti-MIG/CXCL9 serum-treated mice" received 1.0-ml i.p. injections of anti-MIG/CXCL9 serum every other day starting on postoperative day 4 ($n = 5$). The second group, heretofore designated as "serum-treated mice" received 1.0-ml i.p. injections of goat serum (Invitrogen, Carlsbad, CA) every other day via a similar protocol ($n = 6$). All transplanted hearts were harvested on postoperative day 24. No immunosuppression was given.

Morphometric analyses

The explanted hearts underwent serial sectioning (5 μm thick) from the midventricular level to the base. Verhoff's staining was performed for morphometric analyses of arterial intimal lesions as previously reported (12). Three cross sections of the explanted hearts, spaced 200 μm apart, were examined. Vessel size was measured with the computer-based software, Optimas 6.0 (Media Cybernetics, Silver Spring, MD). Only vessels exceeding 80 μm in diameter were included. The intima was defined as the area bounded by the internal elastic lamina and the lumen. The lumen was defined as the clear region in the vessel. Lumenal (L) and intimal and lumenal areas (I + L) areas were traced, and the areas were quantitated with Optimas software. Intimal thickening was calculated according to the formula ((I + L) − L)/(I + L) and expressed as a percentage.

Immunohistochemistry

The basal segment of explanted hearts was embedded in OCT compound, frozen in 3-methyl-butane chilled with dry ice, and then stored at −70°C. The primary Abs used for immunohistochemistry were as follows: rat anti-mouse CD4 mAb (clone L3T4), rat anti-mouse CD8a (BD PharMingen, San Diego, CA), and rat anti-mouse MOMA-2 mAb for monocytes/macrophages (Serotec, Raleigh, NC). Immunohistochemistry was performed on 5-μm-thick cryostat sections. The sections were blocked with a 1/20 dilution of normal goat serum (Invitrogen), followed by a blocking step for endogenous avidin and biotin. Sections were then incubated with the primary Ab and the appropriate biotin-labeled secondary Ab. The avidin-biotin complex solution (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) followed by 3,3′diaminobenzidine was then applied. Counterstaining was performed with Harris hematoxylin (Fisher Chemicals, Fairlawn, NJ). The perivascular and intimal regions were graded by two blinded observers on a scale of 0–4 (0 = no staining; 1 = scattered individual cells; 2 = focal cluster of cells; 3 = multifocal cluster of cells; 4 = diffuse cluster of cells).

Intracellular cytokine analyses

Hearts were minced and digested for 2 h in collagenase D (2 mg/ml; Worthington Biochemicals, Lakewood, NJ) in RPMI 1640 medium with 10% FBS. Isolated cells were counted after lysis of erythrocytes. The leukocytes were then resuspended in growth medium RPMI 1640 including 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM HEPES, 5×10^{-5} M 2-ME, 100 mg/ml streptomycin, 100 U/ml penicillin, and 10% heat-inactivated FBS at a concentration of 1.5×10^6 cells/ml. For intracellular cytokine evaluation, graft-infiltrating cells were stimulated with BD PharMingen activation mixture with Golgi Plug (containing PMA, ionomycin, and brefeldin A) by adding 2 μl of mixture to each milliliter of growth medium. The cells were incubated for 4 h at 37°C. Samples were washed, fixed, and permeabilized with Cytofix/Cytoperm solution (BD Biosciences, San Diego, CA) for 30 min at 4°C. The cells were then washed in permeabilizing buffer, and incubated with PE-labeled IFN-γ mAb (BD PharMingen) for 15 min at 4°C. After two washes with permeabilizing buffer, the cells were analyzed via FACS. The intracellular cytokine assay on graft-infiltrating cells was performed once for each donor heart ($n = 5$ for anti-MIG/CXCL9 serum-treated group, and $n = 6$ for the serum-treated mice).

Mixed leukocyte reaction

Spleens were harvested and minced. The cell suspension was then passed through a nylon mesh filter and treated with a hypotonic red cell lysis buffer (Sigma-Aldrich, St. Louis, MO). The cells were resuspended in growth medium. CD4 lymphocyte enrichment was performed by negative isolation (StemCell Technologies, Vancouver, British Columbia, Canada). The enriched CD4 lymphocytes were analyzed by FACS and found to be >94% pure, with <0.5% T lymphocyte cross-contamination. A total of 8×10^5 CD4 lymphocyte or splenocyte responder cells was incubated with irradiated stimulator cells (2000 rad) in 96-well U-bottom plates. Control MLRs were set up using syngeneic stimulator cells. The MLRs were incubated for 72 h and then pulsed with 0.5 μCi of [^{3}H]thymidine (Amersham, Cleveland, OH) for 14 h. The cells were harvested with a semiautomated cell harvester and counted on a beta scintillation counter. All MLRs were performed in triplicate, and repeated three to six times, as noted.

Chemokine stimulation/neutralization

Recombinant MIG/CXCL9, IP-10/CXCL10, IFN-inducible T cell α chemoattractant (ITAC)/CXCL11, or eotaxin/CC chemokine ligand (CCL)11 (R&D Systems) was added to each well at varying concentrations at the start of MLR. In separate experiments, neutralization experiments were performed by adding 8 μg/ml anti-MIG/CXCL9 (15) or anti-CXCR3 neutralizing Ab or control Ab (R&D Systems) at the start of the MLR.

ELISPOT

ELISPOT assays for murine IFN-γ were performed according to the manufacturer's guidelines (BD Biosciences). Briefly, 200,000 cells from a 48-h MLR were plated on 96-well plates that had been previously coated with a goat anti-murine IFN-γ Ab overnight. The cells were incubated for 24 h. The wells were then washed and reacted with a biotinylated goat anti-murine IFN-γ Ab. The spots were visualized with 3-amino-9-ethylcarbazole chromogen (Sigma-Aldrich). Visualization and analysis was performed using Immunospot Series 1 Analyzer (Cellular Technology, Cleveland, OH). All assays were performed in triplicate and were repeated three times.

IFN-γ neutralization

IFN-γ-neutralizing experiments were performed by adding 2 μg/ml goat anti-mouse IFN-γ-neutralizing Ab (R&D Systems) to allogeneic MLR. Control experiments were performed with goat IgG using a similar protocol. Our preliminary experiments have shown that addition of this concentration of Ab will adequately neutralize the endogenous IFN-γ produced in this MLR.

ELISA

IFN-γ, IL-2, and IP-10/CXCL10 protein levels were measured in the cell culture supernatant using the Quantikine ELISA kit according to the manufacturer's instructions (R&D Systems). MIG/CXCL9 protein levels were measured with ELISA using a biotinylated anti-mouse MIG/CXCL9 detection Ab and anti-mouse MIG/CXCL9 capture Ab (R&D Systems).

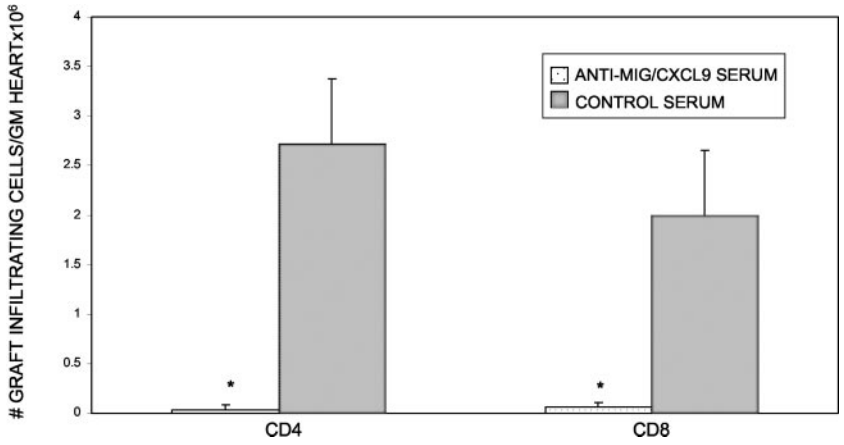

FIGURE 1. FACS analysis of graft-infiltrating cells of donor hearts from anti-MIG/CXCL9 serum and control serum-treated mice. Graft-infiltrating cells were isolated from donor hearts and stained for surface markers. There was a significant reduction in CD4 and CD8 lymphocytes per gram of heart. Each mouse represents one experiment; anti-MIG/CXCL9 serum-treated group, $n = 5$; control serum-treated group, $n = 6$; $*$, $p < 0.05$.

Briefly, wells were coated with 100 μl/well anti-mouse MIG/CXCL9 capture Ab at concentration of 0.8 μg/ml overnight. The plates were washed, and MLR supernatants were added to the wells and placed on an orbital shaker at 500 rpm for 2 h. The biotinylated anti-mouse MIG/CXCL9 detection Ab was then added, followed by peroxide-tetramethylbenzidine substrate (R&D Systems). OD was determined using an Emax microplate reader (Molecular Devices, Sunnyvale, CA) set to 450 nm with wavelength correction at 550 nm.

Flow cytometry

Surface labeling of cells was performed using primarily labeled Abs at concentrations of 0.12 μg per million cells. FITC- and PE-labeled CD4 and CD8 Abs were purchased from BD PharMingen. Rabbit anti-mouse CXCR3 was followed with FITC-labeled goat anti-rabbit secondary Ab (Zymed, San Francisco, CA). FACS analysis of labeled cells was conducted on an EPICS XL-MCL flow cytometer (Coulter, Miami, FL).

Statistics

Data are presented as means $\pm$ SEM. Student's *t* test was used to compare the mean values of cell number, protein level, and intimal thickening between different groups. For other in vitro studies, one-way ANOVA was performed and a two-tailed paired Student's *t* test was used to compare control and experimental values. To avoid spurious significance due to multiple statistical comparisons, Bonferroni correction was used. A value of $p < 0.05$ was considered significant.

Results

MIG/CXCL9 neutralization decreased the severity of intimal thickening, attenuated T lymphocyte recruitment, and altered the cytokine profile of graft-infiltrating T lymphocytes

Intimal thickening is the principal manifestation of the alloimmune response in this murine model of chronic rejection. As previously reported, the donor hearts from the anti-MIG/CXCL9 serum-treated mice had significantly decreased intimal thickening (6.8 $\pm$ 2.0%) when compared with the donor hearts from control serum-treated mice (36.5 $\pm$ 5.9%; $p < 0.05$) (12). The mean vessel counts in anti-MIG/CXCL9 serum-treated mice and control serum-treated mice were equivalent (9.4 $\pm$ 0.75 vs 10 $\pm$ 0.93, respectively; $p > 0.05$). MIG/CXCL9 neutralization also significantly reduced the number of graft-infiltrating CD4 and CD8 lymphocytes (anti-MIG/CXCL9 serum-treated group: CD4 lymphocytes, $0.03 \pm 0.01 \times 10^6$, and CD8 lymphocytes, $0.06 \pm 0.01 \times 10^6$; control serum-treated group: CD4 lymphocytes, $2.72 \pm 0.6 \times 10^6$, and CD8 lymphocytes, $2.00 \pm 0.7 \times 10^6$) (Fig. 1). The reduction in the number of graft-infiltrating CD8 lymphocytes with MIG/CXCL9 neutralization was in contrast to our prior report (12). This observation may be related to the doubling of the dose of anti-MIG/CXCL9 serum that was used in the current study. Immunohistochemical stains also confirmed that there was a marked reduction in the number of graft-infiltrating CD4, CD8, and MOMA-2^+ cells in the anti-MIG/CXCL9 serum-treated group (data not shown).

We next sought to determine whether the function of the recruited lymphocytes is altered in the anti-MIG/CXCL9 serum-treated group. The graft-infiltrating T lymphocytes from donor hearts recovered from anti-MIG/CXCL9 serum-treated and control serum-treated mice were analyzed for the presence of intracellular cytokines. The number of IFN-γ-expressing, graft-infiltrating CD4 lymphocytes was markedly lower in the donor hearts of mice

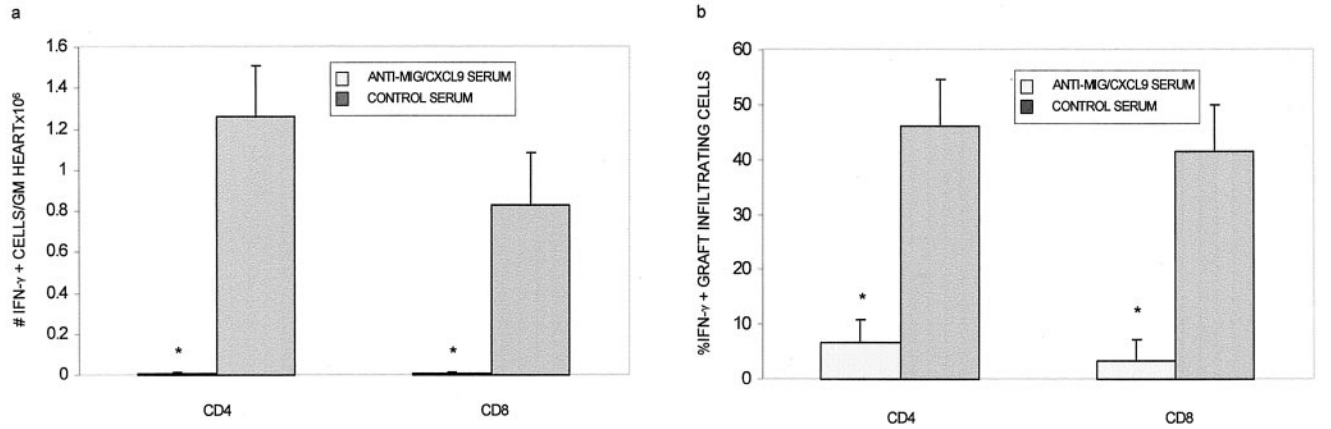

FIGURE 2. Multicolor FACS analysis of graft-infiltrating cells expressing intracellular IFN-γ. Graft-infiltrating cells were isolated from donor hearts, fixed, and permeabilized. The cells were then stained for cell surface markers and intracellular IFN-γ. *a*, The number of IFN-γ-producing CD4 and CD8 lymphocytes was markedly lower (>100-fold) in donor hearts of anti-MIG/CXCL9-treated mice when compared with control serum-treated mice. *b*, Treatment of recipients with anti-MIG/CXCL9 serum resulted in a significant reduction in the percentage of graft-infiltrating, IFN-γ-producing CD4 and CD8 lymphocytes. Anti-MIG/CXCL9 serum-treated group, $n = 5$; control serum-treated group, $n = 6$; $*$, $p < 0.05$.

Table I. *Addition of exogenous MIG/CXCL9 to allogeneic MLRs stimulates T lymphocyte proliferation*[a]

		MIG/CXCL9 Concentration (μg/ml)			
cpm	Syngeneic	0	0.04	0.2	0.4
Splenocytes	7,085 ± 633	27,878 ± 1381	57,949 ± 8,008[b]	70,956 ± 9,612[b]	74,504 ± 8,908[b]
CD4 T lymphocytes	6,063 ± 2755	32,922 ± 12,907	36,517 ± 599	69,994 ± 25,864[b]	72,694 ± 24,117[b]

[a] Addition of increasing concentrations of exogenous MIG/CXCL9 to allogeneic MLR resulted in a concentration-dependent increase in cpm ($n = 6$ experiments, each with triplicate wells). Results expressed as mean cpm ± SEM.
[b] $p < 0.05$ vs 0 concentration of MIG/CXCL9.

treated with anti-MIG/CXCL9 serum compared with those treated with control serum (0.002 ± 0.002 vs $1.26 \pm 0.58 \times 10^6$ cells/g of heart, respectively; $p < 0.05$) (Fig. 2*a*). The number of IFN-γ-expressing, graft-infiltrating CD8 lymphocytes was also lower in donor hearts of anti-MIG/CXCL9 serum-treated recipients (0.002 ± 0.002 vs $0.83 \pm 0.40 \times 10^6$ cells/g of heart; $p < 0.05$) (Fig. 2*a*). More importantly, the percentage of CD4 and CD8 lymphocytes capable of producing IFN-γ (number of IFN-γ-positive CD4 or CD8 lymphocytes/number of infiltrated CD4 or CD8 lymphocytes, corrected per gram of heart tissue) was also markedly reduced with MIG/CXCL9 neutralization (CD4 lymphocytes: 6.7 ± 6.1 vs $46.2 \pm 9.7\%$; CD8 lymphocytes: 3.3 ± 3.0 vs $41.4 \pm 7.9\%$; $p < 0.05$) (Fig. 2*b*). These findings suggest that MIG/CXCL9 1) participates in the recruitment of T cells capable of producing IFN-γ, and/or 2) activates T cells to become capable of producing IFN-γ. Despite lack of information on the allospecificity of these infiltrating cells, a reduction in the number of IFN-γ-producing cells (and the total intragraft IFN-γ level) has important implications in this model (12).

To determine the effect of MIG/CXCL9 neutralization on T lymphocyte stimulation in vivo, we harvested the spleens from both experimental groups at the time of allograft harvest and performed allogeneic MLRs. Because we noted variations in these MLRs, we normalized the results to control MLRs using splenocytes from nontransplanted recipient mice. We did not detect any difference in the proliferative responses of splenocytes derived from anti-MIG/CXCL9 serum vs control serum-treated mice (0.35 ± 0.065 vs 0.43 ± 0.097; $p =$ NS). The absolute value of the proliferative response of splenocytes from anti-MIG/CXCL9 serum vs control serum-treated mice were $9,754 \pm 11,038$ and $11,983 \pm 3,344$ cpm, respectively ($p =$ NS).

Exogenous MIG/CXCL9 stimulated CD4 lymphocyte proliferative responses in a MHC class II-mismatched allogeneic MLR

To investigate the effect of MIG/CXCL9 on T lymphocyte proliferation in vitro, we first measured the amount of endogenous MIG/CXCL9 in MLRs. MIG/CXCL9 concentration in allogeneic and syngeneic MLRs were 164 ± 20 and 29 ± 5 pg/ml, respectively. It is also known that 0.1–0.3 μg/ml MIG/CXCL9 is needed to elicit chemotaxis of activated T lymphocytes in vitro (R&D Systems). Because secreted chemokines can be bound to extracellular proteoglycans (16) and may therefore be underestimated by ELISA, we decided to use the concentration range that is known to induce chemotaxis in vitro (0.04–0.4 μg/ml) for the in vitro studies.

To study the effect of MIG/CXCL9 on T lymphocyte proliferation in vitro, we added exogenous recombinant MIG/CXCL9 to allogeneic and syngeneic MLRs, as well as isolated CD4 lymphocyte MLRs. Addition of MIG/CXCL9 resulted in a significant rise in the proliferative response of splenocytes and CD4 lymphocytes in allogeneic MLR, in a concentration-dependent fashion (triplicate wells, $n = 6$) (Table I). Addition of MIG/CXCL9 to syngeneic MLRs did not stimulate a proliferative response, thereby excluding a nonspecific proliferative effect of the exogenous MIG/CXCL9 (Table I). Moreover, addition of a control chemokine, eotaxin/CCL11 (23 ng/ml) did not affect allogeneic T lymphocyte proliferation (eotaxin/CCL11-treated wells, $33,115 \pm 2405$, vs control wells, $30,709 \pm 1555$ cpm; $p =$ NS).

Exogenous MIG/CXCL9 also increased the number of IFN-γ-producing cells in ELISPOT

To determine whether MIG/CXCL9 also affects T lymphocyte effector functions, we studied the production of IFN-γ in an allogeneic MLR in the presence of increasing concentrations of exogenous MIG/CXCL9. Addition of exogenous MIG/CXCL9 resulted

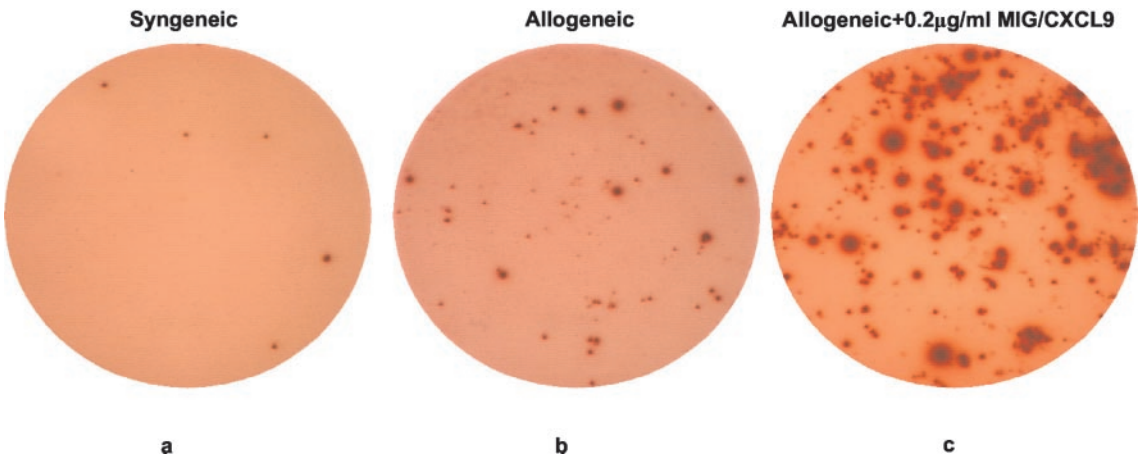

FIGURE 3. Representative ELISPOT wells from 72-h syngeneic (*a*), allogeneic (*b*), and allogeneic plus 0.2 μg/ml MIG/CXCL9 (*c*) MLRs. The number of cells expressing IFN-γ was markedly increased with addition of exogenous MIG/CXCL9 to the allogeneic MLR (see Table II) ($n = 6$ with triplicate wells).

Table II. *Addition of exogenous MIG/CXCL9 to allogeneic MLRs increases IFN-γ-positive spots in ELISPOT*[a]

IFN-γ^+ Spots/200,000 Splenocytes	Syngeneic	MIG/CXCL9 Concentration (μg/ml)			
		0	0.04	0.2	0.4
Splenocytes	2.1 ± 0.37	98.5 ± 9.7	79.9 ± 11.9	283.8 ± 35.1[b]	209.5 ± 35.7[b]
CD4 T lymphocytes	2.8 ± 0.92	17.6 ± 3.5	22.5 ± 6.2	70.3 ± 8.8[b]	59.1 ± 12.8[b]

[a] Increasing concentrations of exogenous MIG/CXCL9 in allogeneic MLR resulted in an increase in IFN-γ-positive spots ($n = 4$ experiments, each with triplicate wells). Results expressed as mean IFN-γ-positive spots ± SEM.
[b] $p < 0.05$ vs 0 concentration of MIG/CXCL9.

in a significant increase in the number of IFN-γ-positive spots by ELISPOT assay (Fig. 3, Table II). The stimulatory effect of MIG/CXCL9 on the number of IFN-γ-producing cells was evident in splenocyte MLRs, as well as isolated CD4 lymphocyte MLRs (Table II). However, addition of exogenous MIG/CXCL9 was associated with a trend in increased IFN-γ level in MLR supernatants by ELISA, but did not reach statistical significance (MIG/CXCL9 addition, 2440 ± 124 μg/ml; control, 1846 ± 421 μg/ml; $p >$ 0.05; $n = 4$ in triplicate).

Neutralization of MIG/CXCL9 in MLR reduced T lymphocyte-proliferative responses and the number of IFN-γ spots in ELISPOT

We next sought to determine whether endogenous MIG/CXCL9 production participates in the stimulation of splenocytes in allogeneic MLR. The addition of 8 μg/ml anti-MIG/CXCL9 neutralizing Ab to allogeneic MLRs resulted in a significant reduction in mean cpm vs the addition of 8 μg/ml control IgG (17,107 ± 1,031 vs 27,869 ± 5,384; $p < 0.05$). Addition of anti-MIG/CXCL9 neutralizing Ab also decreased the number of IFN-γ spots in ELISPOT (anti-MIG/CXCL9 Ab-treated wells, 10.3 ± 11.7, vs control serum-treated wells, 132.3 ± 86.3; $p < 0.05$), and total IFN-γ level in MLR supernatant by ELISA (anti-MIG/CXCL9 Ab-treated wells, 913 ± 31, vs control serum-treated wells, 2360 ± 313 pg/ml; $p < 0.05$). These findings suggest that endogenous MIG/CXCL9 stimulates the proliferation of splenocytes and enhances cytokine production, in an allogeneic MLR.

IP-10/CXCL10 and ITAC/CXCL11 also enhanced T lymphocyte proliferation in vitro

To determine whether other CXCR3 ligands also stimulate allogeneic T lymphocyte proliferation, we added varying concentrations of IP-10/CXCL10 and ITAC/CXCL11 to the MHC II-mismatched MLR. The concentration of endogenous IP-10/CXCL10 in allogeneic and syngeneic MLRs were 162.9 ± 9.1 and 24.7 ± 5.4 pg/ml, respectively. For similar reasons as the MIG/CXCL9 experiments, we used higher concentrations of exogenous IP-10/CXCL10 for in vitro experiments, a concentration range that is known to induce chemotaxis in vitro (17). The ITAC/CXCL11 concentration range was based on in vitro chemotaxis assays (18).

Addition of both IP-10/CXCL10 and ITAC/CXCL11 stimulated T lymphocyte proliferation in vitro. Maximal proliferation occurred with the addition of 0.25 μg/ml IP-10/CXCL10, and 0.08 μg/ml ITAC/CXCL11 (Tables III and IV). This observation suggests that all CXCR3 ligands can stimulate allogeneic T lymphocyte proliferation in MLRs. Neutralization of IP-10/CXCL10 at a concentration known to block the activity of this chemokine was not associated with a significant decline in T lymphocyte proliferation, but a trend was noted (anti-IP-10/CXCL10-treated wells, 25,639 ± 3,024 cpm; control wells, 29,376 ± 2,629 cpm; $p =$ NS). The discrepancy in the effect of MIG/CXCL9 vs IP-10/CXCL10 neutralization may be related to this specific model. MIG/CXCL9 is known to be critical in the allogeneic responses of this MHC class II-mismatched model (10).

MIG/CXCL9 stimulated allogeneic T lymphocyte proliferation in MHC class I- and total MHC-mismatched allogeneic MLRs

To determine whether the proliferative effects of MIG/CXCL9 on T lymphocytes can be extended to other models, we added this chemokine to MHC class I- and total MHC-mismatched allogeneic MLRs. MIG/CXCL9 stimulated allogeneic T lymphocyte proliferation in both models, suggesting that the proliferative effects of MIG/CXCL9 on T lymphocytes can occur in MHC class I-, II-, or totally mismatched combinations (Table V).

CXCR3$^+$ cells were preferentially expanded by the addition of MIG/CXCL-9

Some splenic CD4 and CD8 lymphocytes from mice that have not been transplanted express CXCR3: 8.0 ± 0.8% of CD4 lymphocytes and 27.1 ± 1.2% of CD8 lymphocytes were positive for CXCR3 expression. Following allogeneic stimulation in MLR, there was significant up-regulation of CXCR3 on both CD4 and CD8 lymphocytes (percentage of CD4 lymphocytes expressing CXCR3, 30.2 ± 7.7; percentage of CD8 lymphocytes expressing CXCR3, 39.4 ± 3.5), consistent with a prior report (19). We next investigated the effect of a neutralizing CXCR3 Ab on MIG/CXCL9-induced proliferation of T lymphocytes in MLRs. Neutralization of CXCR3 in MLR reduced T lymphocyte proliferation, both in the presence and absence of MIG/CXCL9 (Table VI). Moreover, addition of anti-CXCR3 to MLRs (in the presence or absence of MIG/CXCL9) also markedly decreased the number of IFN-γ-positive spots on ELISPOT (Table VI). These observations

Table III. *Addition of exogenous IP-10/CXCL10 to allogeneic MLRs stimulates splenocyte proliferation*[a]

cpm	Syngeneic	IP-10/CXCL10 Concentration (μg/ml)				
		0	0.01	0.05	0.1	0.25
Splenocytes	9,635 ± 633	29,376 ± 2629	31,800 ± 3,555	62,609 ± 5,653[b]	99,270 ± 10293[b]	123,351 ± 14,132[b]

[a] Addition of increasing concentrations of exogenous IP-10/CXCL10 to allogeneic MLR resulted in a concentration-dependent increase in cpm; $n = 3$ experiments, each with triplicate wells. Results are expressed as mean cpm ± SEM.
[b] $p < 0.05$ vs 0 concentration MIG/CXCL9.

Table IV. *Addition of exogenous ITAC/CXCL11 to allogeneic MLRs stimulates splenocyte proliferation*[a]

		ITAC/CXCL11 Concentration (μg/ml)				
cpm	Syngeneic	0	0.008	0.04	0.08	0.2
Splenocytes	9,635 ± 633	29,376 ± 2629	36,096 ± 4296	47,421 ± 4475[b]	57,032 ± 7580[b]	31,800 ± 3,555[b]

[a] Addition of increasing concentrations of exogenous ITAC/CXCL11 to allogeneic MLR resulted in a concentration-dependent dependent increase in cpm ($n = 3$ experiments, each with triplicate wells). Results are expressed as mean cpm ± SEM.
[b] $p < 0.05$ vs 0 concentration MIG/CXCL9.

suggest that 1) endogenous MIG/CXCL9-CXCR3 interaction participates in T lymphocyte proliferation/effector cytokine production, and 2) the stimulatory effects of exogenous MIG/CXCL9 are mediated via CXCR3.

The proliferative effects of MIG/CXCL9 were mediated via an IL-2-independent pathway and are controlled by IFN-γ

To define the mechanism of MIG/CXCL9-induced T cell proliferation, we first analyzed the IL-2-IL-2R pathways. Addition of MIG/CXCL9 to the MLR did not result in increased percentage of CD4 lymphocytes expressing CD25. We could not detect a significant change in mean fluorescent intensity of CD25 on CD4 lymphocytes either. Moreover, addition of MIG/CXCL9 did not affect the IL-2 level in MLR supernatant (data not shown). Collectively, these observations suggest that the stimulatory effect of MIG/CXCL9 on T lymphocyte proliferation is independent of IL-2.

Because IFN-γ is known to down-regulate T lymphocyte proliferation, we next asked the question whether neutralization of IFN-γ in MLR would further enhance the effects of MIG/CXCL9 on T lymphocyte proliferation (20). Addition of IFN-γ-neutralizing Ab to the MLRs resulted in further increase in MIG/CXCL9-induced T lymphocyte proliferation (34,456 ± 5,276 vs 49,864 ± 3,924 cpm; $p < 0.05$). This finding is of notable interest in that the observed stimulatory effect of MIG/CXCL9 on T lymphocyte proliferation occurs despite increasing IFN-γ production and its down-regulatory effects on T lymphocyte proliferation.

Discussion

The findings of this study demonstrate that 1) MIG/CXCL9 stimulates allogeneic T lymphocyte proliferation, 2) MIG/CXCL9 increases the number of IFN-γ-producing T lymphocytes both in vivo and in vitro, 3) the effects of MIG/CXCL9 are mediated via $CXCR3^+$ cells, are independent of IL-2, and are controlled by IFN-γ, and 4) the effects of MIG/CXCL9 on T lymphocyte proliferation occur in MHC class I-, II-, and totally mismatched models. Taken together, these observations suggest that MIG/CXCL9, in addition to its chemotactic properties, is also involved in T lymphocyte proliferative responses and generation of effector functions.

The common functional feature of the ever-expanding family of chemokines is their ability to navigate cells. Cell migration involves recognition of chemokine concentration gradients and development of cell polarity (21). Directional cell migration by cytoskeletal rearrangement homes the cell to its intended location, be it either inflamed tissue or secondary lymphoid organs. The spatial and temporal expression of inflammatory and homeostatic chemokines provides a highly organized framework for trafficking of leukocyte subsets. Although cell traffic control remains the central task of chemokines, there is increasing evidence that a variety of other functions such as lymphocyte differentiation and effector functions may also be modulated by chemokines. It has been shown that macrophage-inflammatory protein-1α/CCL3, macrophage-inflammatory protein-1β/CCL4, and RANTES/CCL5 exert positive regulatory effects on Th1 lymphocyte differentiation (22, 23). This effect is mediated both directly and indirectly via induction of IL-12 expression. In contrast, monocyte chemoattractant protein-1/CCL2 polarizes T lymphocyte differentiation to Th2 cells by suppressing IL-12 expression and stimulating IL-4 expression (24, 25). Moreover, monocyte chemoattractant protein-1/CCL2 appears to have a direct effect on T lymphocyte differentiation toward Th2 responses. Among CXCR3 ligands, the extrachemotactic properties of IP-10/CXCL10 have been the subject of several studies. IP-10/CXCL10 has been shown to be important in generation of tumor-specific T cells and protective immunity in an IL-12 gene therapy model (26). Neutralization of IP-10/CXCL10 following acute *Toxoplasma gondii* infection resulted in impairment of Ag-specific T cell generation (27). More recently, it has been shown that IP-10/CXCL10 is important in generation of T lymphocyte effector functions, using IP-10/CXCL10 knockout mice (28). These studies highlight the important role of some chemokines in T lymphocyte differentiation that is similar to the role of several classical cytokines.

MIG/CXCL9 is an inflammatory chemokine that is induced by IFN-γ. The role of MIG/CXCL9 in the recruitment and retention of activated T lymphocytes has been demonstrated in several inflammatory conditions (29, 30). Furthermore, several elegant studies have implicated MIG/CXCL9 as a critical mediator of primed T lymphocyte trafficking in skin and cardiac transplant models (6, 10, 11). These reports have focused on and proven that MIG/CXCL9 is needed for T lymphocyte recruitment to the graft. The findings of the current study confirm these observations in a model of cardiac allograft vasculopathy and expand our understanding of the functional role of MIG/CXCL9 in T lymphocyte regulation.

Table V. *Addition of exogenous MIG/CXCL9 to MHC I-mismatched or total MHC-mismatched MLRs stimulates T lymphocyte proliferation*[a]

		MIG/CXCL9 Concentration (μg/ml)				
cpm	Syngeneic	0	0.04	0.2	0.4	0.5
MHC I mismatch	6,637 ± 443	11,386 ± 962	12,969 ± 1303	20,973 ± 3246[b]	21,014 ± 2611.5[b]	23,267 ± 2826[b]
MHC I & II mismatch	11,704 ± 2,367	56,363 ± 6500	58,536 ± 18,043	96,766 ± 13,092[b]	91,561 ± 11,572[b]	87,529 ± 11,286[b]

[a] Addition of increasing concentrations of exogenous MIG/CXCL9 to allogeneic MLR resulted in a concentration-dependent increase in cpm ($n = 3$ experiments, each with triplicate wells). Results are expressed as mean cpm ± SEM.
[b] $p < 0.05$ vs 0 concentration MIG/CXCL9.

Table VI. *Addition of anti-CXCR3 neutralizing Ab attenuates MIG/CXCL9-induced proliferation and the number of IFN-γ spots*

	Proliferative Response (cpm)	IFN-γ^+ Spots in ELISPOT
Allogeneic MLR + 0.4 μg/ml MIG/CXCL9 + anti-CXCR3 Ab	21,936 ± 1163[a]	46.2 ± 3.8[a]
Allogeneic MLR + 0.4 μg/ml MIG/CXCL9 + IgG	74,504 ± 8,908	259.8 ± 20.2
Allogeneic MLR + anti-CXCR3 Ab	21,231 ± 2230[a]	41.8 ± 9.2[a]
Allogeneic MLR + IgG	27,878 ± 1381	98.5 ± 9.7

[a] Addition of anti-CXCR3 neutralizing Ab to allogeneic MLR (with or without MIG/CXCL9 addition) reduced splenocyte proliferation and IFN-γ spots in ELISPOT; $n = 3$ experiments, each with triplicate wells; $p < 0.05$ vs IgG.

This study suggests that MIG/CXCL9 stimulates T lymphocyte proliferation and enhances T lymphocyte effector cytokine production. The mechanism by which MIG/CXCL9 affects T lymphocyte proliferation and effector functions is similar to T lymphocyte costimulation. Chemokines are known to function as T lymphocyte costimulatory molecules. For example, stromal cell-derived factor-1/CXCL12 acts as a costimulator for T cell activation (31). IP-10/CXCL10 may also enhance effector T lymphocyte generation via costimulation (28).

CXCR3 is the common receptor for IFN-γ-inducible CXC chemokines and mediates chemotaxis. The findings of this study suggest that MIG/CXCL9-induced stimulatory effects on T lymphocyte proliferation and effector cytokine production is also mediated via CXCR3. Recent reports have elucidated the intracellular signaling pathways following chemokine-chemokine receptor interaction that lead to cell migration (21, 32). Chemokine receptor dimerization followed by activation of the Janus kinase/STAT pathway mediate changes in cellular cytoskeleton and promotes directional migration (32). In contrast, there is limited information on the signaling cascade responsible for the cellular responses other than chemotaxis. The positive regulatory effects of RANTES/CCL5-CCR5 interaction on T cell stimulation has been shown to be mediated via increases in phosphotyrosine levels and induction of intracellular calcium flux (33). The intracellular signals linking the MIG/CXCL9-CXCR3 interaction to T cell proliferation and effector cytokine production are currently unknown.

IP-10/CXCL10 has been shown to have opposing effects on T lymphocyte IFN-γ production and proliferative response (34). Gangur et al. (34) have shown that IP-10/CXCL10 augments mononuclear cell IFN-γ production, but inhibits cell proliferation, via a reduction in IL-2 synthesis. In contrast, the current study suggests that MIG/CXCL9 promotes allogeneic T lymphocyte proliferation and IFN-γ production. These processes are independent of IL-2, but are regulated by IFN-γ. The variable effects of chemokines of the same family on T cell proliferation have been reported (35, 36). The findings of this study further underscore the variable effects of chemokines on effector functions depending on the studied function and the context of the immune response.

It is notable that we could not detect a decline in the proliferative responses of splenocytes derived from anti-MIG/CXCL9 serum-treated mice when compared with control serum-treated mice. Similar observations were also noted by Miura et al. (6). One possible explanation is that the majority of primed CXCR3-expressing T lymphocytes in control serum-treated mice have homed to the allograft. As a result, the number of alloantigen-specific T lymphocytes in the spleen of control serum-treated mice is fewer than that in anti-MIG/CXCL9 serum-treated mice. In a MLR, the proliferative response is determined by the number of alloantigen-specific T lymphocytes. Hence, the reduction in the proliferative response by MIG/CXCL9 neutralization may not be detectable. Isolation of alloantigen-specific T lymphocytes from the spleen of both groups of mice and assaying their proliferative responses in a MLR may be a strategy to address this tissue.

The link between the chemotactic properties of MIG/CXCL9 and its effect on T lymphocyte stimulation underscores the efficiency of the immune system to localize and maximize the inflammatory responses. Chemokine/chemokine receptor-directed strategies have proven practical and effective in several experimental models of transplantation (5, 10, 12). Understanding the extrachemotactic properties of chemokines highlights the potential of such strategies in a variety of inflammatory and immune-mediated disease processes. Interventions directed at the chemokine-chemokine receptor interaction may not only prevent trafficking of the primed leukocytes to their target areas, but may also alter the leukocytes' functional efficacy.

In conclusion, this study expands our current understanding of MIG/CXCL9 biology in inflammation. These findings suggest that, in addition to recruitment of CXCR3-expressing cells, MIG/CXCL9 also stimulates the effector function of recruited leukocytes and promotes T cell proliferation. These observations render further support to chemokine/chemokine receptor-directed strategies to control the inflammatory processes.

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