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CXCL12 Induction of iNOS in Human CD8 T Cells

Jonathan C. Choy¹, Tai Yi¹, Deepak A Rao¹, George Tellides⁴, Fox-Talbot K⁵, William M. Baldwin III⁵, and Jordan S. Pober^{1,2,3,*}

¹ Department of Immunobiology, Yale University School of Medicine, New Haven, CT, USA

² Department of Pathology, Yale University School of Medicine, New Haven, CT, USA

³ Department of Dermatology, Yale University School of Medicine, New Haven, CT, USA

⁴ Department of Surgery, Yale University School of Medicine, New Haven, CT, USA

⁵ Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Abstract

Background—We reported previously that inducible nitric oxide synthase (iNOS) expression in graft-infiltrating human T cells, which is confined to the bystander population, contributes to T cell-mediated rejection of allograft arteries in a humanized mouse model. Here, we examine if CXCL12, a chemokine thought to contribute to recruitment of bystander T cells, induces iNOS in human CD8 T cells.

Methods—Human CD8 T cells were treated with CXCL12 and iNOS expression examined. Also, human allograft arteries were immunohistochemically stained for iNOS and CD8, and adjacent sections stained for CXCL12 to determine their localization in human tissues.

Results—Resting human CD8 and CD4 T cells expressed the CXCR4, but not the CXCR7, receptor for CXCL12. Treatment with CXCL12 induced expression of both iNOS mRNA and protein in primary human CD8 T cells in a dose-dependent manner, but had no effect on CD4 T cells. Induction of iNOS expression in CD8 T cells was mediated by increased gene transcription. TCR-activated CD8 T cells rapidly down-regulated CXCR4 and this coincided with diminished ability of CXCL12 to induce iNOS in activated T cells. iNOS expression in infiltrating human CD8 T cells was spatially associated with CXCL12 expression both in the humanized mouse model of allograft artery rejection and in clinical specimens of coronary arteries displaying allograft vasculopathy.

Conclusions—CXCL12 induces iNOS expression in human CD8 T cells and this response may contribute to allograft rejection.

INTRODUCTION

Dysregulated production of nitric oxide (NO) is implicated in the pathogenesis of heart transplant failure, although the mechanisms by which this occurs in humans remains incompletely defined. NO has several biological properties that are concentration and cell source dependent. For instance, NO promotes T cell activation at low levels¹, but suppresses T cell responses at high concentrations². Within the vasculature, production of NO from

*Corresponding Author: Jordan S. Pober, MD, PhD, 10 Amistad St., Room 401D, New Haven, CT 06520-8089, Phone: (203)737-2292, Fax: (203)737-2293, E-mail: jordan.pober@yale.edu.

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endothelial cells is required for vascular homeostasis³, but production of NO by infiltrating immune cells leads to pathological changes in smooth muscle cell function⁴. Studies in mouse models have suggested that NO produced by inducible nitric oxide synthase (iNOS) in macrophages contributes to acute cardiac rejection by causing early graft damage and vascular permeability³, but can also inhibit the late development of allograft vasculopathy (AV) through inhibition of T cell responses⁵. The role of iNOS in allogeneic responses in human transplantation is less clear because iNOS expression is regulated very differently in human cells than in mouse/rat cells⁶, consistent with the fact that the human iNOS gene promoter possesses no homology to the mouse/rat iNOS promoter^{7, 8}.

In acutely rejecting human cardiac allografts, increased iNOS expression is associated with increased myocyte apoptosis⁹. Further, iNOS is expressed in infiltrating macrophages within clinical specimens of arteries affected by AV¹⁰. However, not all of the cells expressing iNOS in the affected vessels could be identified as macrophages (C. Lowenstein, personal communication). Using a humanized mouse model that lacks macrophages, we have shown that iNOS expression is confined to bystander human T cells (ie. graft-infiltrating T cells that are not activated through the T cell receptor [TCR]) and that iNOS in these cells contributes to vascular rejection¹¹. However, signals that induce iNOS in bystander human T cells and mechanisms by which iNOS expression is confined to bystander T cells remains unknown.

T cells may be recruited into tissues either by TCR signals or by chemokine signals delivered by vascular endothelium¹². In the case of an allograft, bystander T cells are presumed to have been recruited by chemokines. Therefore, chemokines are logical candidates to examine for actions on bystander T cells. CXCL12, also known as stromal cell derived factor-1 α , is a chemokine involved in B cell differentiation, hematopoiesis, mobilization and recruitment of hematopoietic stem cells to sites of tissue injury, and regulation of T cell responses^{13–16}. This chemokine may be involved in the pathogenesis of heart transplant rejection and associated AV through recruitment of leukocytes and progenitor cells into allografts^{17, 18, 19}.

In this report, we examined the possibility that an additional mechanism by which CXCL12 may be immune-stimulatory is through iNOS induction in human T cells. We find that CXCL12 induces iNOS expression in primary human CD8 T cells, but not in CD4 T cells, and that down-regulation of its receptor after TCR activation coincides with the inability of activated CD8 T cells to express iNOS in response to CXCL12. Further, we show that iNOS is expressed in CD8 T cells in human arteries with AV, and that CXCL12 is also present in the same regions as iNOS-expressing CD8 T cells.

METHODS

Human Cell Isolation

Human cells were obtained with approval of appropriate Institutional Review Boards. Endothelial cells, PBMC, and T cells were isolated as described²⁰. T cell isolation involved positive isolation with Dynabeads (Invitrogen, Carlsbad, CA).

Cell Culture

Experiments were performed in RPMI 1640 + 0.3% BSA to avoid serum-induced increases in iNOS expression¹¹. T cells were treated with recombinant CXCL12/SDF-1 α (R&D Systems, Minneapolis, MN). CD8 T cells were activated either with endothelial cells and phytohemagglutinin (PHA; Sigma) or plate bound anti-CD3 and soluble anti-CD28 (eBioscience, San Diego, CA) for 24h.

Quantitative RT-PCR

RNA was isolated using a RNeasy Minikit (Qiagen, Valencia, CA). Taqman real time RT-PCR was performed as described⁴ using validated primer/probe sets (Applied Biosystems, Foster City, CA). Transcript data were normalized to either CD3ε or GAPDH.

Flow Cytometry

Intracellular flow cytometry for iNOS was performed as described¹¹ using a FITC-conjugated mouse anti-iNOS (1:1000; BD Biosciences, San Diego, CA) and cell surface staining was performed with a PE-conjugated mouse anti-CXCR4 (1:50; eBiosciences) or rabbit anti-CXCR7 (10 µg/mL; Abcam, Cambridge, MA).

Reporter Gene Assay

Plasmid containing the wild-type -7.2 kb region of the human iNOS promoter attached to a luciferase reporter gene (hiNOS-luc.) was provided by Dr. David Geller (University of Pittsburgh, Pittsburgh, PA), transfected into T cells and luciferase assays performed¹¹.

Clinical Specimens of AV

Coronary arteries were dissected from four cardiac transplants with advanced vasculopathy that were explanted to be replaced with a second transplant. The hearts were explanted 12.3 to 18.3 years after transplantation. Non-diseased coronary arteries were obtained from age-matched cadaver organ donors. Protocols were approved by the institutional review boards of Johns Hopkins University, Yale University and the New England Organ Bank.

Humanized SCID/beige Mouse Model of Coronary Artery Rejection

All animal procedures were approved by the Yale Animal Care and Use Committee. Human coronary artery segments were transplanted into SCID/beige mice, mice adoptively transferred with human PBMC, and arteries harvested after four weeks as described²¹.

Immunohistochemistry

Immunohistochemistry was performed on frozen sections (6 µm) as described¹¹ with rabbit anti-iNOS (1:1000; Chemicon, Temecula, CA) and monoclonal anti-CD8 (1:50; Dako, Carpinteria, CA), or monoclonal anti-CXCL12 (10 µg/mL, R&D Systems). Human skin was used as a positive control (data not shown)²².

Statistical Analysis

In experiments examining the induction of iNOS expression by CXCL12, a one-way ANOVA was performed followed by a Bonferroni post-hoc analysis. Data are expressed as the average ± SD and an alpha value of less than 0.05 was considered significant.

RESULTS

CXCL12 induces iNOS expression in human CD8 T cells

CXCL12 signals through either of two receptors, CXCR4^{23, 24} or CXCR7²⁵. Flow cytometry on freshly isolated human CD8 and CD4 T cells confirmed homogeneous expression of CXCR4 on both human T cell subsets (Fig. 1A). CXCR7 expression was not detected on CD8 or CD4 T cells (Fig. 1B), although its expression could be detected on HeLa cells, as described previously²⁵ (data not shown).

Since human T cells express CXCR4 and respond to CXCL12, we examined whether CXCL12 could induce iNOS expression in primary human T cells. CXCL12 dose dependently induced

a low level of iNOS mRNA expression in human CD8 T cells (Fig. 2A). Over a series of seven experiments with seven different donors, there was a significant induction of iNOS mRNA at 1, 10, and 100 ng/mL CXCL12 (1.52 ± 0.21 , 1.81 ± 0.43 , and 1.58 ± 0.37 fold induction, respectively; $p < 0.01$). At 100 ng/mL CXCL12, there was donor-related variability in the induction of iNOS expression. In two donors, there was an increase in the induction of iNOS as compared to lower concentrations of CXCL12, but in the other five donors iNOS induction at 100 ng/mL CXCL12 was less than that observed at 10 ng/mL of this cytokine. The dose response curve of iNOS induction in response to CXCL12 was biphasic, consistent with the concentration-dependence of T cells to CXCL12 in chemotactic assays²³. Interestingly, iNOS expression was not induced in CD4 T cells in response to CXCL12 (data not shown).

iNOS protein expression was then examined by intracellular flow cytometry. CXCL12 increased iNOS protein expression in primary human CD8 T cells (Fig. 2B). Similar to iNOS mRNA expression, the induction of iNOS protein by CXCL12 was biphasic in most cases, with maximal iNOS protein expression observed at 10 ng/mL CXCL12 and a relative reduction observed in response to 100 ng/mL CXCL12. Over a series of six experiments with different donors, CXCL12 induced a significant increase in the number of iNOS-expressing CD8 T cells as compared to untreated controls ($28 \pm 16\%$ of iNOS-expressing T cells in untreated controls as compared to $42 \pm 19\%$ and $44 \pm 22\%$ in response to 1 and 10 ng/mL CXCL12, respectively; $p < 0.03$).

iNOS expression is regulated largely at the level of gene transcription⁷. To determine whether CXCL12 induces iNOS expression in human CD8 T cells through increased gene transcription, iNOS promoter activity was examined in CD8 T cells in response to CXCL12. CD8 T cells were transfected with an iNOS promoter-luciferase reporter construct, treated with CXCL12, and luciferase activity measured after 16h. CXCL12 significantly induced the activity of the iNOS promoter in transfected human CD8 T cells (Fig. 2C; $p < 0.02$).

CXCR4 expression is down-regulated in activated T cells

We showed previously that iNOS is only expressed by bystander T cells in part because TCR signals actually suppress expression¹¹. To determine whether TCR-activated CD8 T cells become refractory to induction of iNOS by CXCL12 through CXCR4 down-regulation, we examined the expression of CXCR4 in activated CD8 T cells. CD8 T cells were activated with endothelial cells and PHA for 24h, and CXCR4 protein expression examined by flow cytometry. In this system PHA activates the TCR on all human T cells and endothelial cells provide co-stimulation, thereby leading to activation of the majority of T cells in the culture. Cell surface and mRNA expression of CXCR4 was decreased in CD8 T cells activated with endothelial cells and PHA (Fig. 3A and B). Similar to activation with endothelial cells and PHA, CD8 T cells activated with anti-CD3/CD28 also down-regulated expression of CXCR4 (Fig. 3B). Finally, to determine whether activated human T cells were able to express iNOS in response to CXCL12 at a time point when CXCR4 is down-regulated, CD8 T cells that were resting or had been activated with anti-CD3/CD28 for 24h were treated with CXCL12 and iNOS mRNA expression determined 16h later. CXCL12 induced iNOS expression in resting CD8 T cells but was not able to induce iNOS expression in T cells that had been activated by TCR stimulation (Fig. 3C).

iNOS expression in CD8 T cells is spatially associated with CXCL12 expression in human allograft arteries

We used immunohistochemical staining of CD8, iNOS and CXCL12 to assess their association in human artery specimens generated in our humanized mouse model of T cell-mediated allograft artery rejection. In this model, a few iNOS-expressing T cells are detectable at 2 weeks and are pronounced at 4 weeks after adoptive transfer with PBMC. In four week specimens,

iNOS expression was observed in a subset of CD8 T cells within the intima of three of three arteries examined and CXCL12 expression was observed in nearby endothelium, medial smooth muscle cells, and in infiltrating intimal cells that likely represent T cells (Fig. 4A). Some CXCL12 was also expressed in medial smooth muscle cells and luminal endothelium of control arteries harvested from mice that did not receive human T cells (Fig. 4A).

Clinical specimens of human coronary arteries with AV (six arteries from four individuals) were similarly analyzed. iNOS has been shown previously to be expressed by macrophages in AV¹⁰, but its expression in T cells was not reported. Consistent with previous reports, we observed iNOS expression in some cells that did not express CD8, which are most likely to be macrophages (not shown). Significantly, iNOS expression was observed in a subset of infiltrating CD8 T cells within the intima and media of human arteries with AV (Fig. 4B). In these arteries, CXCL12 was also expressed in regions that coincided with iNOS expression in CD8 T cells (Fig. 4B). Chemokine staining was observed in luminal and microvascular endothelium, as well as in infiltrating cells that morphologically resembled leukocytes. However, unlike findings from our humanized mouse model, CXCL12 was not prominently expressed in medial smooth muscle cells.

DISCUSSION

In the current report, we show that CXCL12 induces iNOS expression in human CD8 T cells, and that CXCL12 induction of iNOS can be prevented in activated T cells most likely through down-regulation of CXCR4. We also demonstrate the expression of iNOS in infiltrating CD8 T cells is spatially associated with CXCL12 expression in human allograft arteries. Taken together with our previous data showing that iNOS expression in bystander human T cells plays an immune-stimulatory role in allograft arteries^{4, 11}, these results suggest that CXCL12 may contribute to T cell activation in human allografts through induction of iNOS in CD8 T cells.

iNOS expression has been observed in human macrophages during various pathologies²⁶. However, the signals that induce iNOS in these leukocytes are unknown since many studies have been unable to induce the expression of this enzyme in human macrophages in vitro, even in response to signals that robustly induce expression of this enzyme in mouse/rat macrophages^{27, 28}. iNOS expression is induced in T cell lines in response to hypoxia²⁹, but protein inducers of iNOS expression in primary human T cells have not been identified to this point. Our findings show that CXCL12 is an inducer of iNOS expression in human CD8 T cells. Although the average fold induction of iNOS mRNA was relatively small, this was consistent with the significant increase in iNOS protein expression in response to CXCL12 that resulted in a large proportion of the T cell population expressing iNOS protein. Also, this low level of expression is consistent with the immune stimulatory effects of iNOS in bystander T cells that are mediated by the production of a low level of NO¹¹. Additional protein inducers of iNOS in T cells remain to be defined. We have examined the ability of a number of cytokines and growth factors to induce iNOS, including IFN γ , TNF, CD40L, and IL-6 (data not shown), and none of these factors has induced iNOS expression in human T cells. Determination of the effects of other chemokines on iNOS expression in T cells will require subset purification because many chemokine receptors are restricted in expression and are found only on specific T cell subsets.

At present, it is unclear what intracellular signaling pathways are involved in CXCL12 induction of iNOS expression. We have been unable to demonstrate a role for NF- κ B in the response to CXCL12 through promoter analysis experiments (data not shown), a difference from our previous description of an endothelial cell-derived factor that is also able to induce

iNOS in T cells¹¹. This difference suggests that there is likely more than one factor that induces iNOS in T cells within human blood vessels.

Our data indicate that down-regulation of CXCR4 may leave activated T cells refractory to the induction of iNOS by CXCL12. CXCR4 surface expression is known to be down-regulated early after TCR activation³⁰, and these findings are consistent with a recent report from our laboratory that TCR signals in CD4 effector memory T cells blocked CXCL12-induced transmigration³¹. Moriuchi et al.³² have reported that TCR activation can induce CXCR4 promoter activation, but the mRNA or protein expression was not determined in this study. Also, CXCR4 expression is rapidly decreased immediately after TCR stimulation and recovers after 3 days. Therefore, differences in the reported CXCR4 expression in T cells may be a result of the time-point studied. Interestingly, CXCR4 has been shown to potentiate TCR-mediated signaling in T cells³³. This report focused on early signaling events, indicating that CXCR4 may potentiate TCR signals immediately after stimulation before it is down-regulated. However, the effects of CXCL12 signaling through CXCR4 may also potentiate immune responses through actions on bystander CD8 T cells as we have determined that iNOS expression is confined to this T cell subset. Activation of bystander T cells has been described during viral infections^{34, 35} and posited to augment antigen-specific responses through the provision of soluble factors^{36, 37}. Bystander T cell production of NO may act in a similar manner to potentiate allogeneic responses. This response may be self-limiting as NO has been reported to inhibit CXCL12 production in a mouse model of *Plasmodium* infection³⁸.

We have also observed the spatial association of iNOS-positive CD8 T cells with CXCL12 expression in human allograft arteries. To our knowledge, this is the first description of iNOS expression within T cells in allografts. Previous studies have reported the expression of CXCL12 mRNA in biopsies from cardiac transplants¹⁷, as well as in biopsies from human renal transplants undergoing chronic rejection³⁹. Further, increased CXCL12 mRNA and protein expression is associated with ischemic injury of cardiac transplants⁴⁰. This suggests that ischemia may be a biologically relevant initiator of CXCL12 expression in transplanted organs. Alternatively, additional mechanisms, such as cytokines, regulate CXCL12 and this may be pertinent to allograft rejection⁴¹.

The role of CXCL12 in cardiac allograft rejection and failure is not well understood, and is likely to be complex. In a mouse aortic allograft model, antibody neutralization of CXCL12 reduces the development of intimal hyperplasia¹⁸. One mechanism by which CXCL12 contributes to AV in this model is posited to involve recruitment of host stem cells that contribute to intimal hyperplasia. Other mechanisms are also likely to contribute due to the pleiotropic nature of this chemokine. Our observed induction of iNOS in human CD8 T cells by CXCL12 in vitro, along with the spatial association of iNOS-positive CD8 T cells with CXCL12 production in human allograft arteries, suggests that CXCL12 is a physiologically relevant inducer of iNOS in human CD8 T cells that may contribute to the activation of T cells within heart transplants.

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Figure 1. CXCL12 receptor expression on human CD8 T cells

CXCR4 and CXCR7 expression on freshly isolated human CD8 and CD4 T cells was examined by flow cytometry. Black line = IgG, Shaded area = CXCR4 or CXCR7.

Figure 2. CXCL12 induces iNOS expression in human CD8 T cells

A Human CD8 T cells were treated with varying concentrations of CXCL12. After 16h, RNA was isolated and iNOS mRNA expression quantified by qRT-PCR. Data are the average $\pm$ SD of seven independent experiments with different donors (* $p < 0.01$). **B.** Human CD8 T cells were treated with varying concentrations of CXCL12 for 24h, and iNOS protein expression examined by intracellular flow cytometry. Black line = IgG, Shaded area = iNOS. **C.** Human CD8 T cells were transfected with an iNOS-luciferase reporter construct and treated with CXCL12 (10 ng/mL) for 16h. Cells were lysed and iNOS promoter activity quantified with a luciferase assay (* $p < 0.02$). Data are the average $\pm$ SD of four independent experiments.

Figure 3. Down-regulation of CXCR4 in activated CD8 T cells coincides with insensitivity to CXCL12-induced iNOS expression

A Human CD8 T cells were cultured alone or with endothelial cells + varying concentrations of PHA for 24h. Surface expression of CXCR4 was examined by flow cytometry. Black line = IgG, Shaded area = CXCR4. **B.** CD8 T cells were activated with either endothelial cells and varying concentrations of PHA, or with varying concentrations of plate bound anti-CD3 and CD28 (1 $\mu\text{g/mL}$). After 24h, RNA was isolated and CXCR4 mRNA expression quantified by qRT-PCR. **C.** Human CD8 T cells were activated with anti-CD3/28 for 24h, and then subsequently left untreated or treated with CXCL12 (10 ng/mL) for 16h. RNA was isolated

and iNOS mRNA expression quantified by qRT-PCR. Presented data is representative of two independent experiments.

Figure 4. iNOS is expressed by CD8 T cells in human allograft arteries and is observed in areas containing CXCL12

A. Arteries from a humanized mouse model of AV (Experimental AV; n=3) in which human arteries are interposed into the infrarenal aorta of a SCID/beige mouse followed by adoptive transfer of allogeneic T cells were immunohistochemically stained for iNOS (Red) and CD8 (Blue). Light, diffuse staining was interpreted as background and strong, focal staining as iNOS-positivity. Adjacent sections were stained for CXCL12 (Red) and nuclei counterstained with hematoxylin. Arrows depict iNOS-expressing CD8 T cells. Control arteries from SCID/beige mice that were not adoptively transferred with allogeneic PBMC were also stained for CXCL12. Magnification = 200X. **B.** Clinical samples of AV (n=6 arteries from 4 individuals)

were also stained as described above. The top panels correspond to a magnification of 200X. The bottom panels are high power (400X) magnifications depicting areas outlined in the top panels. Arrows depict iNOS-expressing CD8 T cells, solid arrowheads depict CD8 T cells that do not express iNOS, and open arrowheads depict CXCL12 expressing cells that morphologically resemble leukocytes. Clinical samples of arteries without AV (n=3) were stained for CXCL12 (Red) and the nuclei counterstained with hematoxylin.