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Cutting Edge: Critical Role of CXCL16/CXCR6 in NKT Cell Trafficking in Allograft Tolerance

Xiaofeng Jiang,*† Takeshi Shimaoka,‡ Satoshi Kojo,* Michishige Harada,* Hiroshi Watarai,* Hiroshi Wakao,* Nobuhiro Obokohchi,† Shin Yonehara,‡ Masaru Taniguchi,* and Ken-ichiro Seino2*

It is well-documented that certain chemokines or their receptors play important roles in the graft rejection. However, the roles of chemokines and their receptors in the maintenance of transplantation tolerance remain unclear. In this study, we demonstrate that blocking of the interaction between the chemokine receptor, CXCR6, highly expressed on Vα14+ NKT cells and its ligand, CXCL16, resulted in the failure to maintain graft tolerance and thus in the induction of acceleration of graft rejection. In a mouse transplant tolerance model, the expression of CXCL16 was up-regulated in the tolerated allografts, and anti-CXCL16 mAb inhibited intragraft accumulation of NKT cells. In vitro experiments further showed that blocking of CXCL16/CXCR6 interaction significantly affected not only chemotaxis but also cell adhesion of NKT cells. These results demonstrate the unique role of CXCL16 and CXCR6 molecules in the maintenance of cardiac allograft tolerance mediated by NKT cells. The Journal of Immunology, 2005, 175: 2051–2055.

The Vα14+ NKT cell is one of the immune regulatory cells expressing an invariant AgR α-chain encoded by a Vα14-ja281 gene segment (1). They recognize glycolipids such as α-galactosylsaccharide (α-GalCer)3 presented by CD1d (1). It has been reported that they play an important immune-regulatory role in the maintenance of transplant tolerance (2–4). In organ transplantation, when T cell costimulatory signals through CD40L or CD28 are blocked, peripheral transplant tolerance can be induced in wild-type (WT) recipients (5). However, the same treatment failed to maintain transplantation tolerance in NKT cell-deficient (NKT knockout; MNC, mononuclear cell).

regulation of autoimmune dis-

ease development (6, 7) and anterior chamber-associated immune deviation (ACAID) (8). However, the in vivo behavior of NKT cells in such immune-regulatory responses has not been fully understood.

It has been known that CXCR6, one of the chemokine receptors, is expressed at a high level on NKT cells even under physiological conditions as compared with other lymphocytes (9–11). Its ligand, CXCL16, is identical to the scavenger receptor that binds phosphatidylserine and oxidized lipoprotein (SR-PSOX), mainly expressed in macrophages and dendritic cells (9, 12–14). Interestingly, CXCL16 has unique structural properties, such as membrane-anchored protein with a mucin stalk, like fractalkine, and strict specificity to bind to CXCR6, although other cytokines are generally released as a soluble form and bind to more than one receptor (12). Similar to fractalkine, CXCL16 can also work as a soluble factor after cleaving by proteolytic enzymes (9).

Regarding their in vivo function, it has been reported that CXCL16-mediated CXCR6 activation is involved in pathogenic aortic smooth muscle cell proliferation or the development of experimental autoimmune encephalomyelitis (15, 16). However, the impact of CXCL16 and CXCR6 in vivo NKT cell function has not been elucidated. Thus, it is interesting to investigate the role of CXCL16 and CXCR6 molecules in the NKT cell-mediated transplantation tolerance, because various other chemokines and their receptors have been shown to play important roles in allogenic immune responses (17).

In this study, we examined the role of CXCL16/CXCR6 interaction on NKT cell function in a murine cardiac transplant model. The data indicate that, different from other chemokine receptors, CXCL16/CXCR6 is prerequisite for the maintenance of transplant tolerance mediated by NKT cells.

Materials and Methods

Mice

WT C57BL/6j (B6) (H-2b) and BALB/c (H-2d) mice were purchased from JapanCLEA. NKT cell-deficient (B6.129S-Jα18tm2umu, KO) mice were backcrossed to B6 mice by nine generations. All mice were bred and maintained.

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3 Abbreviations used in this paper: α-GalCer, α-galactosylsaccharide; WT, wild type; KO, knockout; MNC, mononuclear cell.

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in our animal facilities under specific pathogen-free conditions and used at 6–10 wk. The animals were used in accordance with the guidelines of the RIKEN Research Center for Allergy and Immunology and the animal committee of RIKEN approved the experiments.

Reagents
Fluorescence-conjugated or unconjugated mAbs were purchased from BD Biosciences. CXCL16–Fc fusion protein and control Fc were generated as described previously (9). Rat IgG was purchased from Serotec. Anti-mouse CXCL16 mAb (rat IgG1) was generated as described previously (18). Mouse CXCL16-specific polyclonal goat IgG and goat control IgG used for immunohistochemical staining were purchased from R&D Systems. α-GaLCer was kindly provided by Kimi Brewery.

Heart transplantation
WT BALB/c or B6 mice were used as donors and WT or NKT KO B6 mice were used as recipients. Intra-abdominal heterotopic heart transplantation was performed according to the technique previously described (19). Rejection was defined as a complete cessation of palpable beat and was confirmed by direct visualization after laparotomy. For tolerance induction, the recipients were i.v. injected 0.25 mg/mouse anti-CD40L mAb on day 0 and i.p. injected on days 2 and 4 posttransplant. Anti-CXCL16 mAb or control Ab (0.2 mg/mouse) was i.p. injected daily for 14 days posttransplant and every 5 days until 40 days thereafter or until rejection was complete. For analyses of real-time PCR, immunohistochemistry, and in situ hybridization, the grafts or spleens were harvested as follows: in the syngenic group and the group with treatment of anti-CD40L mAb and control Ab (rat IgG), they were harvested 60 days after transplant; and in the group with treatment of anti-CD40L plus anti-CXCL16 mAbs, the grafts and spleens were obtained at the time point of rejection or 60 days after transplant.

Real-time RT-PCR analysis
At the indicated time points, cardiac allografts were harvested and total RNA was extracted using Isogen (Nippon Gene). After cDNA synthesis, the quantity of mRNA for rearranged Va14-Jα281 and CXCL16 was examined with the ABI PRISM 7000 Sequence Detector System (Applied Biosytems), which was normalized using 18S expressions. Real-time PCR primers and probes for Va14-Jα281 were prepared as described previously (20) and those for CXCL16 were purchased from Applied Biosytems. The 18S-normalized value of syngenic transplant group was designated as the calibrator and expressed as 1. Final relative quantity of mRNA was expressed relative to the calibrator.

Immunohistochemical analysis and in situ hybridization
Anesthetized mice were perfusion-fixed with a tissue fixative (GenoStaff) and 6 μm of the neighboring frozen sections were prepared after being embedded in paraffin. For immunohistochemical analysis, the slides were stained overnight at 4°C with 1 μg/ml goat anti-mouse CXCL16 or control Ab in blocking reagent. The slides were then incubated for 2 h with HRP-labeled anti-goat IgG at room temperature and incubated with Fluorophore Tyramide (PerkinElmer Life Sciences). Subsequently, the slides were incubated with anti-fluorescein-HRP (PerkinElmer) for 30 min at room temperature and immersed in diamobenzidine-H2O2. For in situ hybridization, a 0.35-kb fragment of mouse rearranged Va14-Jα281 gene, amplified with the above-mentioned PCR primers, was subcloned into the pBluescript2 KS vector (Stratagene) and was used for the generation of RNA probes. Digoxigenin-labeled RNA probes were prepared with DIG RNA Labeling Mix (Roche). Hybridization was performed as described (21) with some modifications. Coloring reactions were performed with NBT/5-bromo-4-chloro-3-indolyl phosphate, an alkaline phosphatase color substrate, and tissue sections were counterstained with Kernechtrot stain solution (Muto Pure Chemicals).

Cell adhesion assay
A total of 1 × 10⁶ liver mononuclear cells (MNCs; the percentage of CD1d-tetramer⁺ NKT cells was 15.5–21.6%) were transferred to 6-well plates where 1 × 10⁶ CHO-CXCL16 cells or control CHO cells were preseeded. After incubation at 37°C in 5% CO₂ for the indicated times, nonadherent cells were removed by washing, and adherent cells were collected and stained with anti-TCRβ mAb and CD1d tetramer. The number of NKT cells attached to the preseeded cells was estimated using FACSCalibur. In the blocking experiment, anti-CXCL16 mAb was added (20 μg/ml).

Statistical analysis
Comparisons were performed using Mann-Whitney U test. A value of p < 0.05 was considered significant.

Results and Discussion

Effects of CXCL16 blockade on allograft survival
Treatment of B6 WT recipients with anti-CD40L mAb induced long-term BALB/c cardiac allograft survival, since 10 of 11 animals survived over 100 days (Fig. 1). In contrast, in B6 NKT KO recipients, only 3 of 11 allografts survived over 100 days (n = 11, p < 0.01 vs anti-CD40L treated WT recipients, Fig. 1). Interestingly, injection of anti-CXCL16 mAb to the WT recipients significantly shortened the allograft survival time, similar levels to that observed in NKT KO recipients (n = 5, p < 0.01 vs control Ab-treated recipients, Fig. 1). These results suggest that anti-CXCL16 mAb affected NKT cell trafficking in the recipients and impaired the graft acceptance.

Intragraft accumulation of NKT cells mediated by CXCL16/CXCR6
To understand the cellular events in the graft rejection induced by anti-CXCL16 mAb, we attempted to quantify the accumulation of NKT cells and the expression of CXCL16 in the transplanted cardiac allografts. The grafts were harvested from groups treated with or without anti-CXCL16 mAb and were estimated for amounts of the invariant Vα14-Jα281 and CXCL16 mRNAs by a quantitative real-time RT-PCR. The expression of CXCL16 in the cardiac allografts was higher both in the groups treated with and without anti-CXCL16 mAb than in the syngenic grafts (p < 0.05, Fig. 2). In contrast, the expression of Vα14-Jα281 mRNA was significantly higher in the accepted cardiac allografts than that in syngenic grafts (p < 0.05, Fig. 2). These results indicate that allogeneic heart transplantation plus tolerance induction with CD40L-blockade induces an expression of CXCL16 in the allogeneic grafts, and subsequently recruits NKT cells in the tolerated grafts. Consistently, the administration of anti-CXCL16 mAb led to an 8-fold decrease in Vα14-Jα281 gene expression in the grafts (Fig. 2). Thus, the blocking with anti-CXCL16 mAb impeded the accumulation of NKT cells, resulting in the failure of maintenance of tolerance. This up-regulation of CXCL16 and accumulation of NKT cells observed in the accepted grafts was not seen in the spleens (p > 0.05 vs syngenic, Fig. 2), indicating that the recruitment of NKT cells in the grafted tissue is important.

FIGURE 1. Administration of anti-CXCL16 mAb impaired the prolonged cardiac allograft survival induced by anti-CD40L mAb. Anti-CD40L mAb measuring 0.25 mg/mouse was i.v. injected on day 0 and i.p. injected on days 2 and 4 posttransplant. Anti-CXCL16 mAb or control Ab (rat IgG) measuring 0.2 mg/mouse was i.p. injected daily for 14 days posttransplant, and every 5 days until 40 days thereafter or until rejection was completed.
To confirm the results of real-time RT-PCR, we further performed immunohistological and in situ hybridization analyses. In keeping with the results of real-time RT-PCR analysis, graft-infiltrating cells in the cardiac allografts, regardless of the graft acceptance or rejection, showed a significant staining of CXCL16, while those from syngenic grafts showed no staining (Fig. 3). In situ hybridization of the grafts with a specific probe for the Vα14-Jα281/H925114-J/H925281 gene detected significant signals in the accepted grafts (Fig. 3). However, no Vα14-Jα281 signals were detected in grafts from recipients treated with anti-CXCL16 mAb, confirming that CXCL16 blockade inhibited NKT cell migration in the grafts and resulted in the failure of tolerance induction.

Adhesion of NKT cells to membrane-bound CXCL16
Unlike other chemokines, CXCL16 is expressed on the cell membrane and works as an adhesion molecule to CXCR6-expressing cells (18). Thus, we examined adhesion of NKT cells to membrane-bound CXCL16 in vitro. CHO cells transfected with CXCL16 cDNA (CHO-CXCL16) were prepared as described previously (13) and the cell surface expression was confirmed by FACS (data not shown). Liver MNCs were cocultured with control CHO or CHO-CXCL16 cells, and were examined for their attachment by counting cell numbers of CD1d-tetramer+ NKT cells in the adherent cells. The results indicated that NKT cells selectively attached to the CHO-CXCL16 cells, which was inhibited to the control level by the addition of anti-CXCL16 mAb (Fig. 4). Because it has been reported that NKT cells can migrate to soluble CXCL16 (10), the results indicate that the CXCL16/CXCR6 can induce not only migration but also adhesion of NKT cells. However, several chemokines other than CXCL16, such as SDF-1 or CXCL9, can also induce chemotaxis in NKT cells (10). Thus, those non-CXCL16 chemokines and their receptor pairs might also be involved in the accumulation of NKT cells in vivo: CXCR6-deficient (cxcr6gfp/gfp) mice indicated that CXCR6 controls an accumulation of NKT cells in vivo: CXCR6-deficient mice showed a selective and severe reduction of hepatic NKT cells (22). Re-evaluation of the principal experiments in this study using such gene-manipulated mice that lack CXCL16/CXCR6 interaction can verify these results. Such a study is now under way at our laboratory.

To investigate whether blocking of the CXCL16 and CXCR6 interaction would also affect the cytokine production...
by NKT cells, we injected 200–1000 μg of anti-CXCL16 mAb or control Ab into mice receiving 2 μg of α-GalCer and subsequently measured IFN-γ and IL-4 levels in the serum. However, no significant changes in the level and time course of cytokine production with the CXCL16 blockade were observed (our unpublished data). Therefore, it appears that interaction of CXCL16 and CXCR6 molecules mainly influence the trafficking of NKT cells but not their cytokine production.

Regarding the role of accumulated NKT cells in the tolerated allografts, their precise mechanisms on regulatory function have not been clarified so far. However, it is noted that there exist CXCL16-expressing infiltrating cells in the tolerated grafts (Fig. 3) as previously reported by several investigators that APCs, including dendritic cells and macrophages which can express CXCL16 (9, 12–14), are migrated into long-surviving allografts (24). The existence of CXCL16-expressing cells in the tolerated grafts (23). The existence of CXCL16-expressing infiltrating cells in the tolerated allografts indicates that there is an inflammation to some extent, which is in line with the observation that sole CD40L blockade therapy cannot prevent chronic rejection of the allografts (24). Therefore, our present findings may not be relevant to explain the mechanism of tolerance induced by relatively stronger immune suppression with no cellular infiltrates. However, we believe that the present findings contribute to clarifying the mechanisms underlying NKT cell-mediated immune suppression shown in various experimental settings such as autoimmune disease models or ACAID.

In fact, some reports have shown that NKT cells accumulated at the site of immune regulation. For example, it has been demonstrated that, in female NOD mice, therapy with α-GalCer specifically recruited NKT cells to the islets and the draining pancreatic lymph nodes, and ameliorated the development of diabetes (7). Furthermore, Oh et al. (25) have recently reported that NKT cells migrated into H-Y-mismatched skin grafts whose acceptance was shown to require CD1d-NKT cell interaction. However, it has not been clarified which chemokine/receptor interaction is essential for the trafficking of NKT cells to the site of immune regulation. Therefore, it would be important to investigate the contribution of CXCL16/CXCR6 to the accumulation of NKT cells in the immune-regulatory responses. In this regard, it is important to note that, in the ACAID system, blocking CXCL16/CXCR6 with anti-CXCL16 mAb completely impeded the suppression of subsequent delayed-type hypersensitivity induction (K. Sonoda, Kyushu University, personal communication). Although, it is not clear where NKT cells accumulate in ACAID, this information combined with the present data strongly suggest that CXCL16/CXCR6 is generally involved in the immune-regulatory responses controlled by NKT cells.

Given the fact that there seems to be an inflammation to some extent in the tolerated grafts, it is highly conceivable that the accumulated NKT cells are chronically stimulated. Because recent findings have indicated that chronic or repeated stimulation of NKT cells renders them immune regulatory (Ref. 26 and our unpublished data), NKT cells in the tolerated allografts may regulate the transplant tolerance in this manner. Consistently, we have recently found that NKT cells in the tolerated recipients changed their cytokine profile to produce higher IL-10, one of immune-regulatory cytokines, than those from recipients undergoing rejection (K. Seino et al., manuscript in preparation). Further studies elucidating more detailed cytokine profile of NKT cells and their interaction with other immune cells are now under way to clarify these issues.

Disclosures

The authors have no financial conflict of interest.

References


