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Cutting Edge: JAM-C Controls Homeostatic Chemokine Secretion in Lymph Node Fibroblastic Reticular Cells Expressing Thrombomodulin

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The development and maintenance of secondary lymphoid organs, such as lymph nodes, occur in a highly coordinated manner involving lymphoid chemokine production by stromal cells. Although developmental pathways inducing lymphoid chemokine production during organogenesis are known, signals maintaining cytokine production in adults are still elusive. In this study, we show that thrombomodulin and platelet-derived growth factor receptor α identify a population of fibroblastic reticular cells in which chemokine secretion is controlled by JAM-C. We demonstrate that JAM-C–deficient mice and mice treated with Ab against JAM-C present significant decreases in stromal cell–derived factor 1α (CXCL12), CCL21, and CCL19 intranodal content. This effect is correlated with reduced naïve T cell egress from lymph nodes of anti–JAM-C–treated mice. The Journal of Immunology, 2011, 187: 000–000.

 naïve T lymphocyte recirculation is essential for immune function and is controlled by sequential interactions taking place between lymphocytes and endothelial cells, interstitial tissue, or lymphatic vessels. Although mechanisms regulating lymphocyte entry and exit from lymph nodes (LNs) were identified early on, the function of LN stromal cells such as fibroblastic reticular cells (FRCs) has been described only recently (1–3). FRCs provide adhesion and survival cues to naïve T cells and are localized around conduits in LNs (4, 5). They regulate the haptotactic intranodal lymphocyte migration through expression of cytokines and integrin ligands such as fibronectin, collagen, VCAM-1, or ICAM-1 (6, 7). Additionally, they control T cell access to the paracortex from the high endothelial venules (HEVs) forming preferential exit sites for T cells. Finally, they secrete chemokines that promote naïve T lymphocyte entry in LNs and counteract the egress-promoting signals delivered by sphingosine-1-phosphate secreted by lymphatic endothelial cells (8–10). As such, FRCs can be considered as immunoregulatory cells guiding the haptotactic migration of naïve T cells from the HEV entry sites to the efferent lymphatic exit sites (11). Despite such an essential function, their study has been hampered by the lack of surface markers allowing their isolation.

We have previously reported that JAM-C is highly expressed by LN endothelial and sinus lining cells, and that systemic treatment of mice with Abs against JAM-C affected adaptive immune response and leukocyte adhesion to the T cell area of LN sections (12–14). This suggested that JAM-C may deliver specific signals in these anatomical regions. In this study, we revisit the tissue distribution of JAM-C in LN stromal compartments and characterize a subset of FRCs expressing JAM-C. These stromal cells also express platelet-derived growth factor receptor α (PDGFRα) and thrombomodulin (Thbd), a new marker identified by means of mAb generation. Based on JAM-C, Thbd, and PDGFRα expression, we isolated and characterized this FRC subset, which differs from other subsets by its ability to secrete homeostatic chemokines. Using in vitro and in vivo approaches, we demonstrate that JAM-C controls homeostatic cytokine secretion in Thbd-expressing FRCs, providing to our knowledge, the first molecular basis for JAM-C immunoregulatory function.

Materials and Methods

Generation of mAbs against surface markers of LyEnd.5

The panel of LS Abs against LyEnd.5 was generated starting from membrane preparations of 180 × 10⁶ cells obtained using Polytron and differential centrifugation as previously described (15). Male Fisher rats were immunized by i.p. injection of 300 µl membrane preparation mixed with Ribi adjuvant.

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Abbreviations used in this article: DN, double-negative; DP, double-positive; FRC, fibroblastic reticular cell; HEV, high endothelial venule; LN, lymph node; PDGFRα, platelet-derived growth factor receptor α; SDF-1α, stromal cell-derived factor 1α; sIRNA, small interfering RNA; SLO, secondary lymphoid organ; Thbd, thrombomodulin.

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Two days after the final boost, splenocytes were fused to Sp2/0 cells, and hybridomas were selected in hypoxanthine/aminopterin/thymidine-containing medium. Resistant clones were screened by flow cytometry as described in Supplemental Fig. 1. The LS17-9 mAb described in this study is of IgG2a isotype and was purified using beads coupled to protein G (Thermo Scientific, Pierce) according to the manufacturer’s instructions.

**Flow cytometry and histology**

Abs are listed in Supplemental Table I. Data were acquired on a BD LSRII SORP using BD FACSDiva software (BD Biosciences) and analyzed with FlowJo 7.2.2 software (Tree Star). For FRC population sorting, cells were suspended in PBS containing EDTA (5 mM), filtered through a 70-μm cell strainer (BD Biosciences), washed twice, and plated in DMEM medium containing 10% FCS. After 24 h, nonadherent cells were removed. When cells reached 80% confluency, cells were split 1:2 and used within 15 passages. For experiments using small interfering RNA (siRNA), cells were transfected using Lipofectamine RNAiMax (Invitrogen) and siRNA against JAM-C (J-044688-09 and J-044688-10; Dharmacon) or control nontargeting siRNA pool (D-0011810-10-05; Dharmacon). Medium was changed after 24 h and cells were counted at the time of supernatant harvesting to control growth.

**RT-PCR analysis**

Total RNAs were isolated using an RNeasy Micro kit (Qiagen) and reverse transcribed using SuperScript II kit (Invitrogen). Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems), and primer pairs are listed in Supplemental Table II. Quantification was performed using GAPDH as the housekeeping gene (see Fig. 2) or using several internal controls as described in Vandesompele et al. (17) (Supplemental Fig. 5E–5P).

**Homing/egress assays**

Mice were injected with 13H33 Ab (100 μg) or isotype control 9B5 (100 μg) during 3 d before processing with homing and egress assays. For homing assays, 1 × 10^6 Ly5.1 naive lymphocytes were i.v. injected into Ly5.2 mice and the percentage of recovered T lymphocytes from secondary lymphoid organs (SLOs) after 3 h was quantified by flow cytometry. Egress assays were performed essentially as previously described (18). Briefly, 10 × 10^5 calcine-labeled naive lymphocytes were i.v. injected into 13H33-pretreated mice. After 24 h, half of the mice were injected with anti-CD62L (MEL-14) mAb to block de novo lymphocyte entry in LNs. Eighteen hours later, the percentage of calcine-labeled lymphocytes in SLOs was determined. Results are expressed as a retention index, calculated as the ratio of calcine-labeled cells found in SLOs of MEL-14–treated mice divided by the migrated cells found in SLOs of control mice.

**Statistical analysis**

Statistical significance was determined with a nonparametric Mann-Whitney U test or one-way ANOVA with Bonferroni posttest using Prism software.
Results and Discussion

Thbd is a new marker of FRCs expressing JAM-C

To reveal heterogeneity between JAM-C–expressing cells of LNs, we generated a panel of mAbs directed against surface markers of the sinus-derived JAM-C–expressing cell line LyEnd-5. The mAbs were tested by flow cytometry for their ability to recognize the LyEnd-5 cells but not the JAM-C–expressing vascular cell line bEnd-2. Among 408 hybridomas, only 11% of the reactivities were specifically directed against the LyEnd-5 cell line (Supplemental Fig. 1A). Among these, the mAb LS17-9 was selected for further studies and found to be specifically directed against mouse Thbd (Supplemental Fig. 1B–E).

The Thbd expression profile on LN sections was then studied by immunohistochemistry and compared with JAM-C. We found that Thbd was not expressed by HEVs expressing JAM-C and partially colocalized with JAM-C on fibroblastic stromal cells of LNs (Fig. 1A). Similarly, a partial colocalization with PECAM-1 and Lyve-1 was observed, indicating that Thbd expression on endothelial and lymphatic cells is heterogeneous (Supplemental Fig. 2A). To determine whether fibroblastic expression of Thbd was associated to chemokine-secreting cells in the T cell area, LS17-9 staining was combined with CCL21. As shown in Fig. 1B, we found a close association between CCL21 and Thbd expression, indicating that Thbd is most likely expressed by FRCs. To address this issue, LN sections of chimeric irradiated ubiquitin promoter-GFP transgenic animals reconstituted with wild-type bone marrow cells were stained with the LS17.9 mAb. In these mice, the GFP-expressing cells in the T cell paracortex were stained with LS17.9 mAb, demonstrating that Thbd is a new marker of FRC heterogeneity (Supplemental Fig. 2B).

To refine the cellular distribution of Thbd and JAM-C on LN stromal cells, we used a collagenase-based protocol and flow cytometry. As expected from immunohistochemistry results, Thbd expression was restricted to the non-hematopoietic CD45⁻ compartment and was heterogeneous by endothelial and lymphatic cells expressing PECAM-1 and Lyve-1, respectively (Supplemental Fig. 2C). When gated on the fibroblastic compartment CD45⁻ PECAM-1⁻ Lyve-1⁻ and combined with the FRC marker PDGFRα (19), we found that Thbd divided FRCs into three subsets: one minor population expressing intermediate levels of PDGFRα and Thbd, and two major subsets: double-negative (DN) (PDGFRα⁻ Thbd⁻) and double-positive (DP) (PDGFRα⁺ Thbd⁺) subsets, referred to as FRCsDN and FRCsDP, respectively (Fig. 1C, top right panel).

![FIGURE 2. Functional features of FRC subsets. A. Graph representing adhesion of lymphocytes to primary stromal cells under the indicated conditions. B. Transmigration of naïve T cells toward medium containing FCS (gray bar) or medium containing stromal cells (white bar). The negative control consists of medium without FCS added in the lower chamber (black bar). A and B. Results are expressed as percentage of input ± SEM. Representative data from three independent experiments are shown. C. Quantitative PCR analysis of the homeostatic chemokines SDF-1α, CCL19, and CCL21 mRNA expression in sorted FRC subsets. Results are normalized to GAPDH. Data represent means ± SEM. Representative data from three independent experiments are shown. **p < 0.01, ***p < 0.001.](http://www.jimmunol.org/)

![FIGURE 3. Anti–JAM-C treatment affects homeostatic chemokine secretion by FRCs in lymph nodes. A. Intranasal SDF-1α and CCL21 contents were quantified by ELISA in LNs from mice treated during 3 d with isotype-matched control mAb (filled bars) or anti–JAM-C mAb (open bars) (n = 6 mice/group). Results are expressed as mean values ± SEM. One representative experiment is shown. **p < 0.01, ***p < 0.001. B. Confocal images of frozen LN tissue sections obtained from isotype control mice (left panel) or anti–JAM-C–treated mice (right panel). Sections were stained with the indicated markers. Original magnification ×25. C. Mean fluorescence intensities (MFI) for CCL21, ERTR-7, and Lyve-1 stainings obtained on 10–20 LN sections from isotype control mice (filled bars) or anti–JAM-C–treated mice (open bars). ***p > 0.001. D. Left panel, Homing of naïve T lymphocytes to LNs of treated mice is not affected. Right panel, Retention index of T lymphocyte in LNs of anti–JAM-C–treated mice is increased as compared with control (n = 6 mice/group). **p < 0.01. Results are expressed as mean values ± SEM. Representative data from three independent experiments are shown.](http://www.jimmunol.org/)
Expression of JAM-C was restricted to FRCs\textsuperscript{DP} (Fig. 1C, lower panels).

FRC\textsuperscript{DP} support naive T cell adhesion and secrete homeostatic chemokines

To investigate the function of FRCs, primary stromal cells from LNs were isolated by long-term culturing of primary adherent cells. All isolated cell lines had a typical fibroblastic morphology and four of five cell lines expressed PDGFR\(\alpha\), Thbd, and JAM-C, which was the expected phenotype for FRCs\textsuperscript{DP} (Supplemental Fig. 3). Consistent with previous reports, they all expressed high levels of VCAM-1 and gp38 and variable levels of ICAM-1 (4, 20). We thus tested FRCs\textsuperscript{DP} for their ability to support naive T cell adhesion and found that 22% of naive T lymphocytes adhered to the stromal cells whereas the adhesion level of activated T cells was only 4% (Fig. 2A). Adhesion was reduced by 30% when naive T cells were pretreated with pertussis toxin, suggesting the involvement of cytokine secretion in the preferential adhesion of naive T cells to FRCs\textsuperscript{DP}. This was consistent with the 2-fold increase in naive T cell transmigration toward stromal cell-conditioned medium as compared with control medium (Fig. 2B), suggesting that FRCs\textsuperscript{DP} secrete homeostatic chemokines acting on naive T cells. FRCs\textsuperscript{DP} and FRCs\textsuperscript{DN} were then freshly isolated by cell sorting and tested by quantitative reverse PCR for SDF-1\(\alpha\) (data not shown). This shows that Thbd and PDGFR\(\alpha\) are two valuable markers to isolate FRC\textsuperscript{DP}, secreting homeostatic chemokines from LNs.

JAM-C controls FRC\textsuperscript{DP} chemokine secretion in vitro and in vivo

To explore the mechanisms controlling chemokine secretion in FRCs\textsuperscript{DP}, homeostatic chemokine secretion was assessed in primary stromal cells. Constitutive secretion of SDF-1\(\alpha\) was detected and significantly reduced when expression of JAM-C was silenced by means of two different siRNAS, which did not affect stromal cell growth or survival (Supplemental Fig. 5A–C). To extend our findings to the physiological function of JAM-C expression in LNs, intranodal homeostatic chemokine content was determined in mice treated with mAbs directed against JAM-C. The intranodal chemokine content of treated mice was significantly reduced, ranging from 25 to 30% reduction for SDF-1\(\alpha\) to 50% reduction for CCL21 (Fig. 3A). This was further confirmed by in situ staining for CCL21 on LN sections obtained from control and anti-JAM-C–treated mice showing a loss in CCL21 staining in the T cell area (Fig. 3B). This effect did not result in overall changes of fibroblastic and lymphatic network organization as demonstrated by quantification of ERTR-7 and Lyve-1 stainings (Fig. 3C, Supplemental Fig. 6). Such a result was in agreement with the decreased intranodal chemokine content observed in Jam-C\textsuperscript{−/−} mice (Supplemental Fig. 5D). To determine whether the regulation of chemokine secretion by JAM-C occurred at the translational level, transcripts encoding homeostatic chemokines were quantified in total LNs and in FRCs\textsuperscript{DP} isolated from control and anti-JAM-C–treated mice. No change in relative quantities of mRNA encoding chemokines present in total LNs or sorted FRCs from anti-JAM-C–treated mice as compared with controls was observed (Supplemental Fig. 5E, 5F). This suggested that anti-JAM-C treatment affected the posttranslational regulation of chemokine secretion in agreement with changes in SDF-1\(\alpha\) intracellular distribution observed in FRCs silenced for JAM-C expression (data not shown). We therefore tested whether naive T lymphocyte homeostasis in LNs was altered by anti-JAM-C treatment. To this end, homing and egress assays were performed in mice pretreated during 3 d with control Ab or Abs directed against JAM-C. Although no difference in T lymphocyte homing into peripheral LNs was observed, there was a 2-fold increase in T lymphocyte retention in LNs without changes in the overall distribution of T and B cells (Fig. 3D, Supplemental Fig. 7). This suggested that JAM-C indirectly affected lymphocyte trafficking by controlling the intranodal homeostatic chemokine content, which in turn regulated intranodal lymphocyte velocity and delayed naive T lymphocyte exit (21).

In summary, our data support a model in which adhesion molecules involved in intercellular contacts contribute to chemokine secretion in FRCs. Using a new mAb directed against mouse Thbd, we provide a simple method to freshly isolate FRCs from LNs. This allows studying cell-autonomous signals controlling lymphoid chemokine secretion in FRCs. Such signals must differ from the reported bidirectional interaction occurring between hematopoietic lymphoid tissue inducer cells and non-hematopoietic lymphoid tissue organizer cells that are needed to induce chemokine secretion during LN organogenesis (22). Indeed, in our experimental setup in vitro, JAM-C signaling occurs between neighboring cells without the need of counterinteraction between stromal and hematopoietic cells. Additionally, LN development occurs normally in Jam-C\textsuperscript{−/−} mice, suggesting that JAM-C is regulating rather than inducing homeostatic chemokine expression in stromal cells. We thus propose that JAM-C, which is not expressed on mature hematopoietic cells in mice, provides a cell-autonomous signal to FRCs that contributes to homeostatic chemokine secretion.

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Disclosures

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