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Recruitment and Activation of Macrophages by Pathogenic CD4 T Cells in Type 1 Diabetes: Evidence for Involvement of CCR8 and CCL1

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Adoptive transfer of diabetogenic CD4 Th1 T cell clones into young NOD or NOD.scid recipients rapidly induces onset of diabetes and also provides a system for analysis of the pancreatic infiltrate. Although many reports have suggested a role for macrophages in the inflammatory response, there has been little direct characterization of macrophage activity in the pancreas. We showed previously that after migration to the pancreas, diabetogenic CD4 T cell clones produce a variety of inflammatory cytokines and chemokines, resulting in the recruitment of macrophages. In this study, we investigated mechanisms by which macrophages are recruited and activated by T cells. Analysis of infiltrating cells after adoptive transfer by the diabetogenic T cell clone BDC-2.5 indicates that large numbers of cells staining for both F4/80 and CD11b are recruited into the pancreas where they are activated to make IL-1β, TNF-α, and NO, and express the chemokine receptors CCR5, CXCγ3, and CCR8. Diabetogenic CD4 T cell clones produce several inflammatory chemokines in vitro, but after adoptive transfer we found that the only chemokine that could be detected ex vivo was CCL1. These results provide the first evidence that CCR8/CCL1 interaction may play a role in type 1 diabetes through macrophage recruitment and activation. The Journal of Immunology, 2007, 179: 5760–5767.

Materials and Methods

Mice

NOD and NOD.scid breeding mice were initially acquired from The Jackson Laboratory or the Barbara Davis Center for Childhood Diabetes and were housed in specific pathogen-free conditions at the University of Colorado Health Sciences Center for Laboratory Animal Care. NOD.scid mice were housed in sterile isolation cages. Mice in NOD.scid litters (6–10 days old) were used as recipients in adoptive transfer experiments. Breeding mice and experimental animals were monitored for development of disease by urine glucose. The 6.9 TCR transgenic (TCR-Tg) mouse was produced using TCR genes from a diabetogenic T cell clone, BDC-6.9 (8). All procedures used were in accordance with institutional IACUC guidelines and approved by the UCHSC Animal Care and Use Committee.
Culture and expansion of T cell clones

T cell clones were established from spleen and lymph nodes of diabetic NOD mice (9, 10) and were restimulated every 2 wk with a β cell granule membrane fraction obtained from β cell tumors as a source of Ag (11), irradiated NOD spleen cells as APCs, and EL-4 supernatant as a source of IL-2 in complete medium (CM). CM is DMEM supplemented with 44 mM sodium bicarbonate, 0.55 mM l-arginine, 0.27 mM l-asparagine, 1.5 mM l-glutamine, 1 mM sodium pyruvate, 50 μg/mL gentamicin sulfate, 50 μM 2-ME, 10 mM HEPES, and 10% FCS. Cell numbers were expanded for transfer experiments by subculturing 3–6 × 10^7 T cells 4 days after restimulation in a 5-fold volume of CM and additional IL-2. T cells were harvested, washed 3 times, and resuspended in HBSS for injection into young (≤10 days of age) NOD.scid recipients.

Assessment of cytokines and chemokines in vitro by intracellular staining

Production of cytokines and chemokines by T cells in vitro was analyzed by intracellular cytokine staining, as described previously (6). In brief, T cells were stimulated in plates coated with 1 μg/ml anti-CD3 Ab for 24 h before Ab staining. The cells were surface stained in 50–100 μl of staining buffer (PBS, 0.5% BSA) containing rat anti-CD4 or isotype control Ab for 30–45 min, and then washed and fixed in 2% formaldehyde. Cells were resuspended in permeabilization buffer (staining buffer plus 0.5% saponin), containing an insemination control or specific Ab mix for intracellular cytokines/chemokines. Polyclonal intracellular staining Abs used were obtained from R&D Systems and included polyclonal goat IgG anti-CCL1 (TCA-3), anti-CCL3 (MIP-1α), anti-CCL4 (MIP-1β), anti-CCL5 (RANTES), anti-CCL6 (C10), anti-CCL9/10 (MIP-1γ), and anti-CCL21 (SLC) as primary Abs, followed by FITC-rabbit anti-goat secondary Ab (Vector Laboratories). Monoclonal digoxigenin-RTAC-2, anti-lymphotactin, and Cy5-anti-digoxigenin secondary Abs were provided by B. Dorner (12).

Adoptive transfer of diabetes

For disease transfer experiments, expanded cell cultures were harvested and T cells (1 × 10^7) were injected i.p. into age-matched 6- to 10-day-old NOD.scid recipient mice. In some experiments, adoptive transfers were performed with spleen cells from diabetic NOD or TCR-Tg donors. Onset of diabetes was monitored by urine glucose screening and positive readings were confirmed by blood glucose measurement. Blood glucose concentration >15 mM for more than 1 day were considered diagnostic of overt diabetes.

Recovery of cells from pancreas

Approximately 1 week after adoptive transfer with T cell clones or at onset of diabetes (4–6 wk) following diabetic spleen cell transfers, recipient NOD.scid mice were sacrificed. As per methods described previously (2, 6), spleens and pancreata were removed and digested in PBS containing collagenase and GolgiPlug (BD Biosciences) in a 37°C water bath for 20–40 min. Single-cell suspensions of the digested pancreata and spleens were prepared, flash-spin to remove tissue debris, and then centrifuged at 300 × g for 10 min at 4°C to pellet. Cells were resuspended in CM/ GolgiPlug and incubated at 37°C for 3–5 h, and in the case of T cells, with or without PMA (100 ng/ml) and ionomycin (1 μg/ml).

Ex vivo analysis of cytokines, chemokines, and chemokine receptors

After culture ex vivo of cells isolated from the pancreas, cells were harvested and washed before resuscitation in staining buffer/GolgiPlug. Cells were washed in a 96-well round-bottom plate, 2 wells for each set of Ab combinations, one for specific Ab mix, and one for isotype control mix. Abs for surface staining included anti-CD4, anti-CD11b, and anti-CCR5 (BD Biosciences); anti-F4/80 (Caltag); and anti-CXCR3 and anti-CCR8 (R&D Systems). Surface and intracellular cytokine staining was performed as described for in vitro intracellular cytokine/chemokine staining, with the modification of adding GolgiPlug to all reagents until fixation and pre-culturing of staining buffer containing 2 μg/ml FcBlock (BD Biosciences) for 20 min, and then centrifuged at 300 × g for 10 min at 4°C to pellet. Cells were resuspended in CM/GolgiPlug and incubated at 37°C for 3–5 h, and in the case of T cells, with or without PMA (100 ng/ml) and ionomycin (1 μg/ml).

Analysis of NO from macrophages stimulated by T cell clones

Resting T cells (8 × 10^7) were cultured for 48 h in the presence of freshly isolated NOD thiglycolate-elicited peritoneal exudate cells (5 × 10^7) as APC and 10 μg of a membrane preparation from β tumor cells as Ag. As an indirect measurement of NO, nitrite ion concentration in the harvested supernatants was measured and averaged from triplicate wells using the one-step Griess reagent assay (13) and compared with nitrite standards. The same medium used in the cell culture (CM) was the zero standard to account for nitrite background.

Results

Macrophages are recruited to the pancreas by pathogenic CD4 T cells

To investigate the activity of CD4 T cell clones in vivo following adoptive transfer, pancreatic tissue was removed at various time points after transfer (but before onset of diabetes) and single cell suspensions of the infiltrating immune cells were analyzed by flow cytometry. We found that in addition to producing inflammatory cytokines in the pancreas, the pathogenic T cell clones recruited substantial numbers of macrophages, detectable at 2, 4, and 6 days after transfer (6). Similar data are illustrated in Fig. 1 in a experiment in which the diabetogenic T cell clone BDC-2.5, or a nonpathogenic T cell clone BDC-2.4, were transferred to young NOD.scid recipients and a week later, cells were isolated from the pancreas and analyzed ex vivo for the presence of macrophages (Fig. 1A). Comparison of the percentages of CD11b^+ cells in pancreas after transfer of the two clones indicates that whereas substantial numbers of macrophages were observed in pancreas of mice receiving the BDC-2.5 T cell clone, the numbers of CD11b^+ macrophages were significantly lower in animals receiving the BDC-2.4 T cell clone. This result was confirmed by quantification of NO production by macrophages at 6 days after transfer, in which the diabetogenic T cell clone BDC-2.5, or a nonpathogenic control, the Th1 T cell clone, BDC-2.4. Macrophage infiltration could be detected as early as 2 days after T cell clone transfer, but was considered optimal at 6 days. Because recipient mice start becoming diabetic at about 1 week after transfer, we did not go out to further time points. After removal and digestion of pancreata, cells were stained for the CD11b macrophage marker and analyzed by flow cytometry. Cumulative data from two separate experiments was used for the bar graph, and error bars represent SEM from 4 to 5 mice for each clone. *p = 0.00015 × 2-tailed t test. B, F4/80 vs CD11b surface staining on pancreatic single-cell suspensions after transfer of BDC-2.5.
Macrophages in pancreas of BDC-2.4 recipients were quite low, similar to those found in uninjected controls (data not shown). These results suggest that macrophage recruitment is linked with the pathogenic T cell response in autoimmune diabetes. Because CD11b is a marker for both macrophages and dendritic cells, we performed two-color staining on cells recruited to the pancreas for CD11b and for F4/80. Although expressed at lower levels on activated macrophages, the F4/80 Ag is commonly considered to be the most macrophage-specific marker (14). We found that almost all of the CD11b+ cells recruited to the pancreas also stained for the F4/80 Ag (Fig. 1B), and therefore to identify macrophages in subsequent experiments, we used the brighter staining anti-CD11b Ab, which is a particularly valuable reagent in four-color staining from the pancreas ex vivo. It is possible, however, that the CD11b+ F4/80+ cells may include some dendritic cells.

Macrophages recruited to the pancreas are activated to produce inflammatory cytokines

To test the hypothesis that macrophages are activated by diabetogenic T cells upon recruitment to the pancreas, we analyzed the CD11b-staining population recovered from the pancreas ex vivo. It is possible, however, that the CD11b+ F4/80+ cells may include some dendritic cells.

Activated macrophages in the pancreas also produce chemokines

We also wanted to know whether macrophages recruited to the pancreas after adoptive transfer of T cells were a significant source of inflammatory chemokines. We found that whereas CD11b+ cells isolated from the spleen produced very low levels of chemokines, there were substantial amounts of three chemokines found in macrophages recruited to the pancreas. Fig. 3 shows intracellular staining in CD11b+ cells for CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL6 (C10). 1 wk after transfer of the diabetogenic CD4 T cell clone BDC-2.5. As also illustrated in this figure, no staining was detected in macrophages for CCL1, CCL9/10, or CCL5. This result demonstrates that the macrophages recruited by pathogenic T cells are an important source of at least three inducible chemokines in the pancreas during disease.

**NO is another product of macrophages activated by diabetogenic CD4 T cells**

NO can function either as an important mediator of positive cellular outcomes such as proliferation and activation, or as a signaling mediator leading to cell death (17). The deleterious effects of NO include its functioning as an intermediate in the production of reactive oxygen and reactive nitrogen species (ROS and RNS). The exact role of NO in T1D appears to be complicated (17), but its presence in combination with inflammatory cytokines, particularly IL-1β, is a strong predictor of islet-cell cytotoxicity (18, 19). Because macrophages are an important source of NO, we measured the ability of a panel of pathogenic CD4 T cell clones to stimulate NO production by macrophages, using the concentration of nitrite ions as an indirect measure of NO. By stimulating T cell clones with Ag, we are able to observe the effects on macrophages cocultured with the T cells and thus exposed to T cell-derived inflammatory cytokines. The data shown in Fig. 4 show that the ability to stimulate macrophage production of this important inflammatory mediator is a property of diabetogenic CD4 Th1 T cells. In contrast, a Th2 T cell clone, 2.5Tg/T2-X, used as a control, was unable to stimulate NO production by macrophages, correlating with its inability to transfer disease to the NOD.scid mouse.

**Recruitment and activation of macrophages is a manifestation of T cell effector function in other diabetes induction and spontaneous disease models**

To determine whether macrophage recruitment and activation was a common feature of T1D pathogenesis, we analyzed macrophages isolated from the pancreas in several adoptive transfer and two

![FIGURE 2.](#) Inflammatory cytokine production in macrophages recruited to the pancreas by a pathogenic T cell clone. Expanded cultures of the BDC-2.5 T cell clone or the nonpathogenic BDC-2.4 Th1 T cell clone were injected into young NOD.scid recipients. At 4–6 days after transfer, pancreatic single-cell suspensions were incubated in the presence of Brefeldin A and were stained for surface expression of CD11b and for intracellular cytokine protein. TNF-α (A) or IL-1β (B) production by these macrophages was analyzed by flow cytometry. Histograms gated on CD11b+ cells are used to indicate the proportion of macrophages staining positive for the indicated cytokine protein. The data shown are representative of two independent experiments with similar results.
spontaneous models of T cell-mediated autoimmune diabetes (Fig. 5).  

Fig. 5A shows inflammatory cytokine production by macrophages in two commonly used adoptive transfer systems involving spleen cells or in transfers with defined T cell clones. Spleen cell transfers included either heterogeneous populations of spleen cells from diabetic NOD mice or a quasi-clonal population of spleen cells obtained from the widely used BDC-2.5 TCR-Tg mouse. These systems were compared with transfers with two T cell clones, either a clone from our panel of diabetogenic CD4 T cell clones, or the pathogenic insulin-reactive CD8 T cell clone G9 described by Wong et al. (20). These different transfer models exhibit varying kinetics of disease induction in young NOD.scid recipients, but all depend on a population of pathogenic T cells to transfer disease. As a control, a CD4 Th2 T cell clone, 2.5Tg/T2-X, was used. The 2.5Tg/T2-X clone does not induce diabetes in NOD.scid recipients (21), nor does it lead to infiltration and cytokine production by macrophages (Fig. 5B). In Fig. 5C are results from analysis of macrophage inflammatory cytokine production in two spontaneous models of disease, either a spontaneously diabetic wild-type NOD mouse or a prediabetic 6.9 TCR-Tg/NOD.scid mouse (a TCR-Tg produced from a second diabetogenic CD4 T cell clone in our panel, BDC-6.9; see Ref. 8). Although mouse models for T1D differ in some respects and mechanisms of pathogenesis vary with the induction method, these results suggest that a common feature of disease mediated by pathogenic T cells, in both spontaneous and transfer models of diabetes, is a large number of infiltrating macrophages, many of which are producing inflammatory cytokines. The NOD spontaneous mouse model exhibits a longer time frame for disease onset, which likely explains the smaller numbers of macrophages in the pancreas at any given time point, even though a large percentage of them are producing inflammatory cytokine. Our findings lend support to the theory that the macrophage is a major effector cell for β cell destruction through its cytotoxic activity (22). The fact that this macrophage activity is dependent on pathogenic T cells is suggested by the lack of activated macrophages recruited to the pancreas by either a Th2 control clone (Fig. 5B) or a nonpathogenic CD4 Th1 clone (Fig. 2).
Macrophages recruited to the pancreas express chemokine receptors

As illustrated in Fig. 1, our results have demonstrated that adoptive transfer of diabetogenic CD4 T cell clones leads to the accumulation of large numbers of macrophages in the pancreas. For macrophages to be recruited to an inflammatory site in which T cells are producing cytokines and chemokines, they must express chemokine receptors. To investigate further the mechanisms by which macrophages are recruited to the pancreas during T1D, we examined the expression of three chemokine receptors that have been implicated in autoimmune diseases. CCR5 has been shown previously to be necessary for development of T1D (23) as a receptor for MIP-1α, MIP-1β, MIP-1γ, and RANTES, all of which are chemokines produced at the protein level by the pathogenic CD4 T cell clones in response to TCR stimulation in vitro (Table I).

Table I. Chemokines produced by pathogenic CD4 Th1 T cell clones

<table>
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Diabetogenic T cells secrete numerous macrophage chemotactants in vitro but only CCL1 in the pancreas

Chemokines are potent chemoattractant and possible activators of macrophages in inflammatory sites, and it has been reported that mRNA for several inflammatory chemokines can be detected in whole pancreas after disease transfer (26). We reported previously that a variety of chemokines can be detected both at the mRNA and at the protein level in a panel of seven CD4 diabetogenic T cell clones (6). Table I is a list of all the chemokines tested in three of...
the CD4 Th1 T cell clones; those that tested positive at the mRNA level were confirmed by intracellular staining. These results indicate that at least seven chemokines are made by diabetogenic T cells, all of which are documented chemoattractants for macrophages (27–31). In vitro, these chemokines are produced only upon TCR-mediated activation, with the exception of RANTES, a potent T cell chemokine that is present constitutively in unstimulated T cell clones.

Because macrophages and T cells migrating to the pancreas produce multiple inflammatory mediators, and as several chemoattractant receptors capable of binding multiple ligands are found on macrophages, it is not immediately apparent whether there is any one receptor-ligand pairing that is of particular significance in the developing disease process. Our results indicate that macrophages recruited to the pancreas express at least three chemokine receptors thought to be relevant to disease (Fig. 6), the most interesting of which may be CCR8 because this chemokine receptor, unlike others, is known to bind only one ligand, the chemokine CCL1 (or TCA-3) (25). To further investigate the possible role of CCR8 on macrophages in T1D, we investigated whether diabetogenic CD4 T cell clones produce the ligand for this chemokine receptor in vivo. We adoptively transferred the T cell clone BDC-2.5 to NOD.scid mice and after removal spleen and pancreas 6 days later, we analyzed the T cells by intracellular staining for production of chemokines. Although in vitro the T cells can be induced to make several inflammatory chemokines, including CCL3 (MIP-1α), CCL4 (MIP-1β), CCL9/10 (MIP-1γ), CCL6 (C10), CCL5 (RANTES), and CCL1 (TCA-3) (6), the only chemokine that could be detected in CD4 T cells in the pancreas ex vivo was CCL1 (Fig. 7). The few T cells that were found in the spleen were negative for all chemokines tested (data not shown). The production of CCL1 by pathogenic T cells in the pancreas, together with the expression of CCR8 on macrophages recruited to the pancreas, provide the first evidence that CCR8/CCL1 interaction may play a role in T1D.

Discussion
Our studies on the activity of macrophages in the inflammatory site have indicated that recruitment and activation of macrophages is a key component of pathogenic CD4 T cell effector function. First, comparison of the numbers of infiltrating macrophages after adoptive transfer of a nonpathogenic vs a pathogenic CD4 T cell clone indicates that macrophage recruitment is linked to pathogenicity. Second, inflammatory cytokine production by macrophages, which is stimulated by diabetogenic CD4 T cells, was investigated ex vivo from the pancreas. Our data demonstrate that TNF-α and IL-1β are made by macrophages in the pancreas after transfer of the T cell clone, BDC-2.5, but not with a nonpathogenic T cell clone. These findings were extended and confirmed by our data on recruitment and activation of macrophages recruited to the pancreas in other T cell-mediated models of T1D (Fig. 5). A third important finding was that macrophages recruited and activated by
diabetogenic CD4 T cells in the pancreas are a key source of inflammatory chemokines. The chemokines produced in the pancreas during disease progression are of the inducible category of chemoattractants, present in peripheral tissues during inflammation. As there has been little investigation of macrophage chemokines in autoimmunity, this observation now serves as a basis for future study into the role of macrophage-derived chemoattractants. For example, it may be that production of chemokines such as CCL3, CCL4, and CCL6 by macrophages in the disease site provides another mechanism by which inflammation is augmented, serving to further recruit and/or boost the activation of other inflammatory cells. Another intriguing question with regard to chemokines made by macrophages is whether different chemokines, or levels of cytokines/chemokines, represent heterogeneity among macrophages recruited to the pancreas, such as is suggested by the dot plots in Fig. 3 indicating that CD11b<sup>high</sup> vs CD11b<sup>dim</sup> cells may be secreting different chemokines. A fourth aspect of macrophage function in the pancreas is the NO production that is stimulated by pathogenic CD4<sup>+</sup> T cell clones. NO is produced by islet β cells and is a mediator that combines with IL-1β and TNF-α to cause β cell death, at least in vitro (15, 19). Our work points to macrophages as another important source of NO in the pancreas during progression to disease.

The fact that the infiltrating macrophages express CCR5, CXCR3, and CCR8, three chemokine receptors implicated in inflammation, suggests that chemokines secreted by the CD4 T cells could be one mechanism whereby pathogenic CD4 T cells recruit, and perhaps help to activate, macrophages. Our data regarding CCL1 (TCA-3) production by CD4 T cells and expression of CCR8 on macrophages in the pancreas may be especially significant in this regard. The role of this ligand/receptor pair in the chemoattractant system is still being delineated and CCR8 has been studied more commonly in its role as a chemoattractant receptor on neutrophils, eosinophils, and Th2 T cells (27, 32, 33). More recently, however, it has been reported that phagocytic macrophages and activated microglial cells express CCR8 in CNS infiltrates in experimental allergic encephalitis, and that these CCR8<sup>+</sup> cells respond to CCL1 (7, 34). Our results raise the possibility that production of CCL1 by pathogenic Th1 T cells in the pancreas is a means of recruiting CCR8-positive cytotoxic macrophages. Although substantially higher on macrophages in the pancreas after adoptive transfer of pathogenic T cells, CCR8 is also expressed on macrophages in the spleen and it may be that chemotraction of these cells (or dendritic cells) to the pancreas depends initially on CCR8. The levels of CCR5, and possibly CXCR3, are increased on macrophages after traffic to the target organ (35, 36) and may serve to “anchor” macrophages and other responding cell types in the pancreas. CCR5 and CXCR3 are both up-regulated by IFN-γ and TNF-α in tissue-specific inflammation (35). The ligands for CCR5 (CCL3, CCL4, CCL5) are very common in inflammatory sites and although ligands for CXCR3 have been shown by others to be expressed by inflamed islets (24), they were not produced by the diabetogenic T cell clones. The non-specificity of CCR5 interactions, as well as the apparent lack of CD4 T cell chemokines for CXCR3, underscore the potential importance of our data implicating the highly specific interaction that occurs between the T cell chemokine CCL1 and the macrophage receptor CCR8.

In summary, these various findings would suggest that the recruitment of macrophages is directed first through the action of cytokines and chemokines produced by pathogenic T cells, as depicted in Fig. 8. In particular, the inflammatory cytokine TNF-α is a central player in the activity of diabetogenic CD4<sup>+</sup> T cell clones (6), and was found to be responsible for up-regulation of CCL1 and CCR8 in inflamed CNS tissue (7, 34). T cell chemokines such as CCL1 attract macrophages to the pancreas through CCR8 and lead to up-regulation of additional chemokine receptors (CCR5, CXCR3) on macrophages. Macrophages are in turn activated by T cell-derived cytokines such as TNF-α and IFN-γ to become cytotoxic effector cells that produce NO as well as inflammatory cytokines and chemokines, thereby contributing to the overall inflammatory milieu and recruitment of other immune cells.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


