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Salmonella typhimurium Infection in Nonobese Diabetic Mice Generates Immunomodulatory Dendritic Cells Able to Prevent Type 1 Diabetes

Tim Raine,* Paola Zaccone,* Pietro Mastroeni, † and Anne Cooke2*

Infection, commencing across a wide age range, with a live, attenuated strain of Salmonella typhimurium, will halt the development of type 1 diabetes in the NOD mouse. The protective mechanism appears to involve the regulation of autoreactive T cells in a manner associated with long lasting changes in the innate immune compartment of these mice. We show in this study that autoreactive T cell priming and trafficking are altered in mice that have been infected previously by S. typhimurium. These changes are associated with sustained alterations in patterns of chemokine expression. We find that small numbers of dendritic cells from mice that have been previously infected with, but cleared all trace of a S. typhimurium infection are able to prevent the development of diabetes in the highly synchronized and aggressive cyclophosphamide-induced model. The effects we observe on autoreactive T cell trafficking are recapitulated by the immunomodulatory dendritic cell transfers in the cyclophosphamide model. The Journal of Immunology, 2006, 177: 2224–2233.

The effects of exposure to infectious agents on the subsequent development of autoimmunity represent a well-studied, but controversial field (1–3). Using a mouse model of type 1 diabetes (T1D), we recently demonstrated that infection with an attenuated strain of Salmonella typhimurium could halt the development of spontaneous T1D in the NOD mouse (4). Although the infection is cleared by the mice within 6 wk, the protection from T1D is long lasting. A number of different infectious agents have also been shown to inhibit development of T1D in these mice, including viral (5), bacterial (6), and helminthic (7) pathogens, but we believe that the effects of S. typhimurium infection are particularly interesting, as this infection shows strong protective effects even when exposure to the bacteria occurs well after the establishment of the initial leukocytic pancreatic infiltrates.

In our previous work, we demonstrated that autoreactive T cells remained present in the protected mice, because, even at late time points after infection, splenocytes from these mice could transfer T1D to immunodeficient recipient NOD.scid mice. When taken together with the observed patterns of Th1 cytokine secretion, these results indicated that neither deletion of autoreactive T cells, their deviation to a pattern of Th2 cytokine secretion, nor their regulation via the induction of IL-10-secreting regulatory T cells could explain the prevention of pancreatic islet destruction. Instead, we observed sustained alterations in the frequency of NKT cells and subset distribution of dendritic cells (DCs). DCs are known to play a role in a number of stages in a S. typhimurium infection, including the rapid phagocytosis of blood-borne Salmonellae (8) and contributing to the eradication of infection by the production of reactive nitrogen intermediates (9). Early in infection, their subset ratios are changed in the spleen, with an apparent expansion and redistribution of the CD8α+ population (10). DCs have also become well established as controllers of the T cell adaptive immune response, endowed with the capacity to prime naive T cells (11), but additionally to direct and coordinate the ongoing response through several means (12).

We investigated the role of Salmonella-modulated DCs in the protection from T1D. Using an in vivo approach, we report that DCs from previously infected NOD mice were protective against the development of diabetes in a model of cyclophosphamide (CY)-induced T1D, in which the disease process is accelerated and synchronized (13). DCs from noninfected littermate control mice did not afford any protection in this system. DCs generated in vitro from bone marrow or manipulated ex vivo have previously been used to protect in animal models from autoimmunity (14–18). However, we believe this to be the first study to show protection from an autoimmune disease using DCs that have been exposed in vivo to a physiological stimulus, but not using their unexposed counterparts. Additionally, in this study, we report evidence for changes in autoreactive T cell priming in the pancreatic lymph nodes (PLN) of previously infected mice and a block on their entry into the pancreas. This altered tissue distribution of autoreactive T cells is accompanied by changes in the expression patterns of certain chemokines, notably CCL21a. Finally, we link these differences in T cell trafficking to alterations in DCs after infection that we suggest might underlie the protection from T1D.

Materials and Methods

Mice, drugs, and bacteria

NOD, BDC2.5NOD, and GFP.BDC2.5NOD mice were bred and maintained under barrier conditions in the Pathology Department, University of Cambridge animal facilities. GFP.NOD mice were used to bre...
GFP.BDC2.5NOD mice were a gift from D. Kioussis (National Institute of Medical Research, London). Briefly, GFP-S65T plasmid DNA was obtained from Quantum. The GFP cDNA was isolated by digestion with Sau3A1-EcoRV before treatment with Klenow polymerase. The blunt GFP cDNA was then cloned into the Smal site of the human CD2 VA vector. For the generation of transgenic mice, the injection fragment was digested away from the vector sequences (KpnI-Xbal for human CD2-GFP) and gel purified. The isolated fragment was further purified using Elutip columns.

Protocols of fertilized oocytes from NOD mice were injected with the purified DNA at a concentration of 1–2 μg/ml TE buffer. Transgenic founders were detected by Southern blot analysis of genomic tail DNA. All animal work was conducted after approval by the Ethical Review Committee of the University of Cambridge. CY (Baxter) was given at 200 mg/kg i.p. Mice given CY were monitored daily for glycosuria and sacrificed after positive readings. For some experiments, groups of NOD mice were given CY and sacrificed at various time points after dosing, for analysis of splenocyte populations by flow cytometry. S. typhimurium SL3261 were grown and prepared for i.v. injection at 5 × 10⁶ CFU, as described previously (4). Salmonella Ags were prepared, as described previously (4).

Cellular purification

Splenetic DCs were prepared using CD11c microbeads (Miltenyi Biotec), according to the manufacturer’s instructions. Briefly, spleens were cut into fragments and digested in medium containing Liberase CI (Boehringer Mannheim). DC-T cell complexes were then disrupted by the addition of EDTA (0.1 M (pH 7.3)). Splenic fragments were forced through a nylon cell strainer (BD Biosciences), and the resulting suspension was washed. Cell pellets were resuspended in cold buffer (PBS, 0.5% BSA, 2 mM EDTA, 0.5% FCS, and 10% CD11c microbeads/cell). All incubations were performed for 20 min at 4°C with constant agitation using a rotator. Labeled cells and surplus beads were then removed from cell suspensions using a magnet supplied by the manufacturer in a series of repeat depletion steps. Unlabeled cells were left free and used for subsequent cell transfers.

In all experiments involving DC transfers, 5 × 10⁵ purified DCs were injected i.v. into a lateral tail vein 48 h after the administration of CY. CD4⁺ T cells were prepared from single-cell suspensions of splenocytes incubated with a biotin-conjugated Ab depletion mixture for non-CD4 lymphocytes. For some experiments, CD4⁻ T cell complexes were disrupted by the addition of 0.1 M EDTA (pH 7.3). Cells were stained in buffer (PBS containing 2% BSA and 0.05% NaN₃) with combinations of PE, PerCP, allophycocyanin, or biotinylated conjugates of: anti-CD3 (2C11), anti-CD4 (RM4-5), anti-CD25 (PC61), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-CCR5 (C34-3448) (all from BD Pharmingen), CD279 (4B12; AbCam), CXCRI (220803; R&D Systems), pan MHC-II (OX6; Serotec), and appropriate isotype control Abs. Biotinylated Abs were detected with a streptavidin-allophycocyanin-Cy7 conjugate. Cells were then washed and analyzed using a BD LSR I flow cytometer (BD Biosciences).

Analysis of chemokine expression

Spleens, PLN, and pancreata were harvested into RNAlater (Qiagen). mRNA extraction was performed using the RNaseasy Kit (Qiagen), according to the manufacturer’s protocol. Oligo-dT N20 digests were performed (Qiagen) to remove any residual contaminating genomic DNA. All final RNA yields and purities were checked by measuring A₂₆₀/A₂₈₀ values using a quartz cuvette and spectrophotometer (model DU-62; BD Biosciences).

Quantitative RT-PCR was performed as a one-step process using preprepared RNA and SYBR-green-based detection of PCR products after the reverse-transcriptase step, according to the manufacturer’s protocols (Qiagen). Amplification and detection were performed using an ABI 7500 Real-Time PCR System (Applied Biosystems). Reactions were run using QuantiTect reverse transcriptase primers (Qiagen) for genes of interest. These were CCL2, CCL3, CCL4, CCL5, CCL17, CCL19, CCL20, CCL21a, CCL22, CXCL10, and CXCL16. Reactions were set up in triplicate on 96-well plates and SYBR-green levels in each well normalized to levels of the passive reference dye, ROX, to correct for pipetting errors. After amplification, melting curve analysis was used to check for contamination and primer dimerization. Average cycle threshold (Ct) values were determined for each reaction by determining the cycle number at which SYBR-green fluorescence crossed a threshold level set during the log-linear amplification phase, and ΔCt values determined by comparison with the reference gene hypoxanthine guanine phosphoribosyltransferase (HPRT) as:

\[
\Delta C_t = C_t \text{gene of interest} - C_t \text{HPRT}
\]

with:

\[
SD_{\Delta C_t} = \sqrt{SD_{C_t \text{gene of interest}} + SD_{C_t \text{HPRT}}}
\]

For comparison of expression levels between tissues of individual mice, ΔCt values were compared to generate ΔΔCt values as follows:

\[
\Delta \Delta C_t = \Delta C_t \text{tissue of interest} - \Delta C_t \text{tissue of reference}
\]

and fold change in expression expressed as:

\[
\text{fold change}_{\text{tissue of interest}} = 2^{-\Delta \Delta C_t}
\]

Values are reported as 2⁻ΔΔCt ± SD, according to the methods described by Livak and Schmittgen (19).

Statistics

Appropriate statistical tests were performed on all data, as described in the figure legends.

Results

DCs from mice previously infected with S. typhimurium can prevent diabetes in an accelerated, synchronized model

Our previous observations suggested that in NOD mice protected from T1D after a S. typhimurium infection, alterations within the innate immune compartment might be responsible for exerting...
To understand better the phenotype of the DCs used for these transfers, we used flow cytometry and cell culture. No differences were seen in the parameters studied between the DCs of NOD mice exposed to *S. typhimurium* infection 8 wk earlier and their noninfected littermate controls, both in terms of activation marker expression and cytokine levels in culture supernatants after 24 h of exposure to an inflammatory stimulus (Fig. 2).

**DCs from S. typhimurium-exposed mice do not bear detectable S. typhimurium Ags**

Although we find that NOD mice clear the *S. typhimurium* infection within 6 wk, we determined whether the transferred DCs harbored surviving *Salmonellae*. There were, as expected, no surviving bacteria detectable by bacterial growth on agar plates in any of the DC preparations taken at the time of our transfer studies. To further exclude carryover of Ag, we used a reporter population of presensitized T cells from NOD mice infected with *S. typhimurium* 8 wk earlier, which will mount a strong anti-*Salmonella* memory response to an antigenic extract from *S. typhimurium* (4). As shown in Fig. 3, when these *Salmonella*-immune T cells were mixed with purified populations of splenic DCs from previously infected and control NOD mice, no T cell response was observed in the absence of exogenously added *Salmonella* Ags. Both populations of DCs could present exogenously added *Salmonella* Ags with equal efficiency, and the response was *Salmonella* specific, because T cells from the noninfected mice did not respond. This indicates that DCs from previously infected mice do not present any *Salmonella* Ags that might have remained after the clearance of the live infection.

**Impact of CY on DC populations in NOD mice**

To examine the effects that CY might be having on DCs in the recipient mice, we performed a series of serial time course analyses in cohorts of NOD mice given a single dose of CY. As expected, we found a decrease in splenic cellularity, recovering to normal levels by day 9 after dosing (Fig. 4). However, the relative decrease in cell number was not more significant for DCs

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**FIGURE 1.** DCs from mice exposed to a prior *S. typhimurium* infection reduce the incidence of T1D in CY-treated mice. Ten- to 12-wk-old NOD mice were given CY, and 48 h later, 5×10⁵ splenic DCs from 16-wk-old female NOD mice that had been exposed to *S. typhimurium* infection 8 wk previously (○), or their nonidiabetic littermate controls (▲). A third group received only CY, but no cells (■). The reduction in incidence in the recipients of *Salmonella* DCs was significant when compared with the recipients of control DCs (p = 0.002) and mice receiving no cells (p = 0.014). Results are from one experiment, n = 7 mice per group, representative of four repeat experiments. Two-sided p values are by a Mantel-Haenszel test of Kaplan Meier survival curves.

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**FIGURE 2.** A–F. Phenotypic analysis of DCs as used in transfer experiment. Single-cell suspensions were prepared from 16-wk-old female NOD mice infected with *S. typhimurium* at 8 wk of age (n = 5) and their noninfected, nondiabetic littermate controls (n = 5). A–C. Flow cytometric analysis of surface expression levels of A, MHC class II; B, CD80; C, CD86 expressed as geometric mean fluorescence intensity on CD11c+ cells. Values are for individual mice; bars represent arithmetic means. p values by Mann-Whitney U testing were nonsignificant in all instances. D–F. Purified DCs from individual mice previously exposed to *S. typhimurium* (■) and littermate controls (▲) were cultured in triplicate wells at 5×10⁴ cells/well in medium alone or in the presence of 200 ng ml⁻¹ LPS. Supernatants were harvested at 24 h and analyzed for levels of TNF-α (D), IL-6 (E), and IL-10 (F) by cytometric bead array. All results shown are means ± SEM for mean values from triplicate wells (i.e., means of means). Statistical testing by two-sided Student’s t test shows LPS up-regulated expression of all cytokines (p < 0.0001), but does not show any significant differences between DCs taken from the two different groups of mice.
Alterations in immune function of cells of the innate immune system might be expected to lead to differences in Ag presentation and might therefore influence autoreactive T cells. To examine the potential influence of DC alterations on such T cells, we transferred CFSE-labeled splenocytes from the BDC2.5.NOD transgenic mouse (20) into previously infected and noninfected littermate control groups of NOD mice and then studied expansion, expression of T cell activation markers, and cytokines after 72 h. Before transfer, the CFSE-labeled CD4+ T cell population consisted mostly of cells with a naive phenotype, expressing CD62L and intermediate levels of CD44 (Fig. 5). In a typical transfer experiment, <10% of transferred CD4+ T cells expressed surface CD25 (data not shown). Seventy-two hours after cell transfer, recipient mice were sacrificed, and donor CD4+ T cells were identified in the harvested organs based upon CFSE labeling. Dilution of the CFSE label, corresponding to cell proliferation, was restricted to CD4+ T cells in the PLN of recipient mice. In the PLN, undivided CD4+ cells include a population of Ag-inexperienced cells that retain expression of the surface marker CD62L (Fig. 5, A and B). These cells are CD25+ and CD44+, but upon initial activation, will cleave surface CD62L and up-regulate CD44 (Fig. 5C). Dividing cells are almost entirely CD62LlowCD44high and include both CD25+ and CD25− populations (Fig. 5C). Overall, the proliferation kinetics were apparently similar in both groups of recipient mice, with similar percentages of cells showing dilution of the CFSE label (Fig. 5, A and B). Interestingly, we found that the nonproliferating (CFSEhigh) population of CD4+ BDC2.5.NOD T cells in the PLN of mice that had been infected with S. typhimurium 8 wk previously contained a significantly larger population of naive CD62LhighCD25+CD44int cells (Fig. 5E) than the equivalent population in the noninfected control littermates. This suggests that less effective activation was occurring in these mice, despite the apparently similar proliferation kinetics. However, we also found that in the PLN of the S. typhimurium-infected mice, a larger fraction of the proliferating CD4+ BDC2.5.NOD cells stained for intracellular IFN-γ after nonspecific restimulation in vitro (data not shown). This may reflect bystander activation in a strongly Th1-polarized environment.

Previously, we have shown that despite the block on the development of T1D in mice infected with S. typhimurium, pancreatic infiltrates still develop in these mice (4). Therefore, we used flow cytometry to determine whether any of the transferred CFSE-labeled BDC2.5.NOD splenocytes were among the pancreatic infiltrating lymphocytes. Although we could observe these cells in the pancreas of the noninfected control mice 72 h after transfer, we were consistently unable to detect any CFSE+ T cells in the pancreas of mice that had been infected with S. typhimurium 8 wk previously (Fig. 6). This was despite the presence of similar relative levels of transferred cells within the PLN of both groups of recipient mice (Fig. 6D). Taken together, we regard these changes to indicate that in mice previously infected with S. typhimurium, autoantigen presentation is somehow altered within those lymph nodes known to be important for T cell priming in T1D (21). Consequently, autoreactive T cells receive subtly different signals.
A total of $1 \times 10^7$ CFSE-labeled BDC2.5.NOD splenocytes was transferred into 16-wk-old female NOD mice that had been exposed to *S. typhimurium* infection 8 wk previously or their nondiabetic littermate controls. The mice were sacrificed 72 h later, and proliferation restricted to the PLN was confirmed during phenotypic analysis by FACS. A and B, Representative FACS data showing levels of CD62L expression on transferred CD4$^+$ cells in PLN of control (A) and previously infected (B) mice. Plots are gated on CD4$^+$ CFSE$^{int}$-high cells; percentages shown are for events falling within indicated quadrants. C, Representative data for CD25/CD44 expression on PLN CFSE$^{int}$-high T cell populations in B. CD4$^+$ cells falling within the upper right, lower right, and lower left quadrants of B (respectively representing undivided CD62L$^{high}$ cells, undivided CD62L$^{low}$ cells, and CD62L$^{low}$ cells that have divided) are shown in the correspondingly situated panel. Note that a similar distribution pattern is seen for the same populations in A. D, Representative data for CD44/CD62L expression in the CD4$^+$ population of BDC2.5.NOD splenocytes after CFSE labeling, immediately before transfer. E, Comparison of activation status of T cells 72 h after transfer in the two groups of mice. Transferred T cells were identified as CD4$^+$ CFSE$^{int}$-high, and the percentage of them showing a naive phenotype (defined as CD62L$^{high}$CD44$^{int}$CD25$^-$) expressed as a percentage of the whole population. The percentage of CD4$^+$ T cells with a similar phenotype in the cell population before transfer is shown for comparison (pre-transfer). Results plotted are for individual mice from one experiment, representative of two separate studies. Lines indicate arithmetic means, and $p$ values are by two-sided Mann-Whitney $U$ test.
that result in a diminished ability of these cells to access the tissues of the pancreas.

Changes in chemokine expression patterns in S. typhimurium-exposed NOD mice

CFSE-labeled BDC2.5.NOD splenocytes were examined by flow cytometry after transfer into groups of nondiabetic control and previously infected NOD mice for expression of chemokine receptors known to be important for pancreatic infiltration. Within the PLN, there were no significant differences between the two groups in levels of expression of CCR5, CCR7, or CXCR3 on either CFSE$^+$CD4$^+$ or endogenous (CFSE$^-$) CD4$^+$ T cells (Fig. 7, A–C, and data not shown). CCR5 and CCR7 were found to be expressed at much higher levels on T cells within the pancreatic infiltrates than on T cells resident in the PLN, underlining the importance of these chemokine receptors for homing to the pancreas and for entry into the infiltrates (data not shown).

To examine potential changes in the pattern of chemokine expression within the tissues of NOD mice after S. typhimurium infection, mRNA was extracted from samples of spleen, PLN, and pancreas harvested from female mice 8 wk after infection and their nondiabetic littermate controls. This was used as the template in a series of real-time RT-PCR studies, using primers designed to amplify chemokine gene sequences for a panel of chemokines, and expression of each gene in each tissue normalized to that of the constitutive expressed enzyme HPRT. Using this approach, comparison was made in each mouse studied between the normalized expression level of each gene between the different tissues. For most chemokines, no consistent differences in expression patterns were found when comparing mice in the noninfected control group with those previously infected with S. typhimurium.
typhimurium. However, in the case of CXCL10 and CCL21a, comparison of pancreatic to PLN expression showed considerably higher relative pancreatic expression in the case of noninfected mice than in mice previously exposed to infection (Fig. 7, D and E). For CCL21a, this amounted to a complete switch in the direction of the CCL21a gradient, with higher pancreatic than PLN expression in all control mice examined, and higher PLN than pancreatic expression in all previously infected mice studied.

Autoreactive T cell trafficking in CY-treated mice can be modulated by DCs from mice previously infected with S. typhimurium

To determine whether the altered trafficking of transferred autoreactive T cells in Salmonella-exposed mice was related to the observed immunomodulatory effects of DCs on T1D, we used a transfer system. Forty-eight hours after being given a dose of CY, NOD mice were injected with DCs from mice that had been infected with S. typhimurium 8 wks previously, at a dose established above to protect from diabetes, or with DCs from noninfected littermate control mice. Two hours later, splenocytes from a GFP,BDC2.5NOD mouse that expresses GFP on all T cells were injected i.v. into the CY/DC-treated mice. After a further 72 h, the mice were sacrificed, and their PLN and pancreata were examined by flow cytometry for the presence of autoreactive T cells. Mice that had received DCs from a previously infected donor consistently showed a significant relative excess of GFP<sup>+</sup>CD4<sup>+</sup> T cells within their PLN and a relative decrease in the same cells in their pancreata, in comparison with the recipients of DCs from control mice (Fig. 8). Of note, in a series of control experiments akin to those shown in Fig. 3, neither group of DCs was able to stimulate BDC2.5.NOD CD4<sup>+</sup> T cells in vitro without the addition of soluble peptide Ag, in the presence of which both sets of DCs were equally effective in terms of T cell activation (data not shown). This suggests that DCs taken from prediabetic mice do not bear detectable Ag recognized by BDC2.5.NOD T cells that might otherwise contribute to an explanation of the observed distribution of transferred cells.

Discussion

In this study, we show that Salmonella infection alters DCs such that they are able to influence autoreactive T cell trafficking to the pancreas and inhibit onset of T1D in the NOD mouse. Using a highly aggressive, synchronized model of T1D enabled us to demonstrate the protective effects of DCs provided that they are taken from the spleens of NOD mice that have previously been infected with, but subsequently cleared, an attenuated strain of S. typhimurium. DCs taken from the spleens of their noninfected, littermate controls showed no such protective effects. There was no evidence in the transferred DC population of either the presence of live Salmonellae, or of the presentation of Salmonella Ags.

We believe this to be the first study to show the ability of DCs that have been exposed in vivo to a physiological stimulus, but not their unexposed counterparts to protect from an autoimmune disease. Because the original observations that DCs isolated from PLN, but not spleens, could modulate T1D (22, 23), a number of investigators have used a variety of in vitro manipulations to induce tolerogenic DCs capable of affording protection from different models of autoimmunity. These include the use of cultured, bone marrow-derived DCs to prevent T1D (14), genetic manipulation of DCs to prevent T1D or collagen-induced arthritis (16, 17), or exposure to immunomodulators including TNF-α (18) or TGF-β (24) to prevent experimental autoimmune encephalitis and experimental autoimmune myasthenia gravis, respectively. In particular, exposure ex vivo to IFN-γ has been shown to generate DCs that ameliorate experimental autoimmune encephalitis (25) or T1D (15). Given the sustained high levels of IFN-γ production we have shown in the spleen, PLN, and pancreata of NOD mice after S. typhimurium infection (4), our observations may represent an important in vivo correlate.

Observations suggest that only a small fraction of labeled DCs can be recovered from major organs 24 h after injection, presumably representing a high level of cell death after transfer (T. Raine, unpublished observations) (15). Overall, it seems that the number of injected DCs that traffic to the PLN is probably very small indeed. This not only points to the potency of these effects, but also argues against the possibility that the protective effects we observe might be due to the introduction of large numbers of cells, especially because the effects are specific to DCs taken from Salmonella, but not control mice. We sought to further characterize the nature of the effects of CY administration on the DC population in time course studies (Fig. 4). Because the ratio of T cells to DCs does not change after the administration of CY, and because the introduction of DCs from control mice is not protective, this argues against the possibility that the protective effects we observe might be due to the introduction of large numbers of cells, especially because the effects are specific to DCs taken from Salmonella, but not control mice. We sought to further characterize the nature of the effects of CY administration on the DC population in time course studies (Fig. 4). Because the ratio of T cells to DCs does not change after the administration of CY, and because the introduction of DCs from control mice is not protective, this argues against the simple explanation for the protective effects observed involving restorations of perturbations in the T:DC ratio. Furthermore, although the time course study revealed an increase in activation status of endogenous DCs after CY administration, we find that DCs taken from previously infected mice do not differ in terms of the expression of basic activation markers or cytokine responses.
to an inflammatory stimulus (Fig. 2). Again, this suggests that the protective effect is not due to the introduction of a large number of relatively immature DCs or DCs with a skewed cytokine expression pattern, as may have been the case in previous studies (14, 16, 17).

The effects we observed in the CY model led us to compare the Ag presentation occurring in control and previously infected mice. We set out to examine the outcome of the transfer in vivo of labeled autoreactive splenocytes from the BDC2.5NOD, which are known to be first primed with Ag in the PLN, before entering the pancreas (21). Our previous studies had shown that for at least the first 72 h, these cells will proliferate in the PLN, but not the spleen or mesenteric lymph nodes, of both the control and previously infected mice, suggesting that they were being presented with a relevant Ag in this location only (4). There were no significant differences between the proliferative capacities of cells transferred into previously infected mice compared with the noninfected littermate controls (Fig. 5 and data not shown). However, a more detailed analysis using multicolor flow cytometry revealed that, in the S. typhimurium-exposed group, a significantly larger population of autoreactive T cells retained a naive phenotype (CD4\(^{+}\)CD62L\(^{hi}\)CD44\(^{int}\)CD25\(^{-}\)). Further analysis revealed that these differences were attributable to expression of CD62L among the CFSE\(^{hi}\) population, suggesting that undivided cells were less prone to cleavage of this cell surface marker in mice previously infected with S. typhimurium. These observations are in keeping with previous reports that cell division and changes in surface activation marker expression, including CD62L cleavage, are not coupled events (26).

These observations suggest alterations in autoantigen presenta-
tion in the PLN of mice previously infected with S. typhimurium. The consequences of such differences are reflected in our data showing the failure of autoreactive T cells to enter the pancreatic infiltrates of the previously infected mice (Fig. 6). This failure might represent a number of factors, including preferential retention in the PLN, failure to up-regulate relevant chemokine receptors or adhesion molecules, altered patterns of vascular adhesion molecule expression in the pancreata of previously infected mice, or altered patterns of chemokine expression within the infiltrates of the previously infected mice.

Consequently, the ability of transferred T cells to respond to chemokine cues was investigated by flow cytometry using Abs specific for three key chemokine receptors known to be expressed on T cells and identified as being of interest based upon reports in the literature. Ab blockade of CCR5 prevents development of TID in NOD mice (27), and treatments known to protect from TID have been associated with down-regulation of CCR5 expression (28). CXCR3 is the receptor for CXCL10, which has been implicated in controlling T cell trafficking between the PLN and pancreas (29, 30). CCR7 is the common receptor for two key chemokines, CCL19 and CCL21, known to be important for recruitment of T cells and DCs to secondary lymphoid tissue (31, 32). In our experiments, higher levels of expression of CCR5 and CCR7 were seen on T cells extracted from pancreatic infiltrates than on T cells in the PLN, underlining the relevance of these receptors on infiltrating T cells (data not shown). However, patterns of chemokine receptor expression were similar among infiltrating T cells in control and previously infected mice. Furthermore, expression of CCR5, CCR7, and CXCR3 on transferred autoreactive T cells in the PLN was not altered between cells introduced into previously infected mice or into their noninfected littermate controls (Fig. 7, A–C).

Because no differences were observed in the expression patterns of the chemokine receptors examined that might account for the differences in autoreactive cell trafficking in noninfected control and S. typhimurium-exposed NOD mice, the expression patterns of chemokines between the organs of the two groups of mice were next investigated. In S. typhimurium-exposed mice, the PLN to pancreatic gradient for CCL21a and CXCL10 expression was much less, in the case of CCL21a being reversed so that pancreatic expression levels of between 0.15 and 0.3 of those seen in the PLN were observed (Fig. 7, D and E).

Expression of CXCL10 within the pancreas has been linked to the recruitment of lymphocytes into the pancreas by a diabetogenic CD8 T cell clone, while expression within the PLN appears to retain autoreactive T cells and prevent pancreatic infiltration (30, 33). Raised CXCL10 levels have been demonstrated after infection of mice with a number of pancreas-tropic viruses capable of triggering TID in a mouse model, while neutralization of CXCL10 prevented TID in this model (34). Likewise, CCL21a is able to drive the pancreatic recruitment of lymphocytes (35, 36) and is required for islet transplant rejection (37). CCL21a appears to increase the binding of T cells to vascular endothelium and has been shown in blocking studies to be required for the entry of a diabetogenic islet-reactive CD8 T cell clone into the pancreas of NOD mice (38). In the NOD mice protected from TID by exposure to CFA, the expression of CCL21a on the vascular endothelium of the pancreas is reduced (39). Furthermore, CCL21a expression has been demonstrated on a range of inflamed nonlymphoid tissues in a variety of human autoimmune pathologies, including ulcerative colitis (40), rheumatoid arthritis (40, 41), primary sclerosing cholangitis (42), and infiltrative skin pathologies (43). The receptor for CCL21a is CCR7, the expression of which was up-regulated on pancreatic infiltrating T cells, indicating that CCL21a/CCR7 interactions may be of particular importance in the recruitment of BDC2.5.NOD T cells to the pancreas. Taken together, these results suggest a role for decreased expression of T cell attractant chemokines, including CCL21a and CXCL10, in the pancreas relative to the PLN in the phenomena observed in this study.

Importantly, we show in this study that the failure of autoreactive T cells to enter the pancreas of previously infected mice may be replicated in mice given CY simply by the transfer of a small number of DCs from the previously infected mice (Fig. 8). This potent effect immediately suggests a mechanism whereby the same dose of DCs from previously infected mice could prevent the development of TID in CY-treated recipient mice, as seen in Fig. 1. Because all the CY recipients will have similar patterns of pancreatic infiltration before the DC transfer, Salmonella-modulated DCs must play a central role in this system for directing the T cell movements, and, potentially, for excluding autoreactive T cells from the site to which they must traffic to cause autoimmune pathology. However, our investigations are ongoing both into the comparative distribution of DCs transferred from control and previously infected mice, and into whether these DCs might influence local patterns of chemokine production either directly or through interaction with other cell types.

Control of autoreactive T cell trafficking may play an important, but underrecognized role in the prevention of autoimmunity. The expression pattern of chemokines has been shown to control autoreactive T cell migration in the initiation and prevention of TID (28, 30, 33). Prevention of TID in NOD mice exposed to the NKT cell ligand α-galactosylceramide has been linked to alterations in chemokine expression patterns and the preferential retention of transferred autoreactive CD8\(^{+}\) T cells in the PLN (44). Likewise, transferred autoreactive CD4\(^{+}\) T cells will accumulate within granulomas outside of the CNS in the protection from experimental autoimmune encephalitis associated with bacillus Calmette-Guerin infection (45). Furthermore, the importance of
cell trafficking in governing autoimmune responses does not appear to be limited to T cells, because the control of migration into lymphoid follicles is an important point of regulation for autoreactive B cells (46). In this study, we show that changes in autoreactive T cell trafficking are induced after a prior bacterial infection and may relate to changes in the Ag presentation environment and DC behavior.

In conclusion, we show in this study that *S. typhimurium* infections in the NOD mouse, which we know to halt the development of T1D, induce lasting changes in the DC phenotype of these mice, including the capacity to prevent diabetes in an accelerated model. These same DCS will alter the trafficking of autoreactive T cells in this model. This is similar to the effects that we observe in intact mice, in which the autoreactive cells are unable to enter the pancreas and show different outcomes on autoantigen-driven proliferation. Given the relatively short *t*1/2 of DCS in vivo (47) and our observations of bacterial clearance within 6 wk, it seems unlikely that the DCS we are transferring would have been in contact with any *Salmonella*. Indeed, we could find no evidence for carriage of either live bacteria or bacterial Ags in the DC inoculum. Therefore, it seems that the effects of the prior infection must have been to induce long lasting alterations in immune homeostasis within these animals. However, the legacy of the infection is not seen solely among DCs, because there are also lasting disturbances among NKT cells (4). Thus, it seems that a relatively brief exposure to a potent immune stimulus alters a wide variety of immune functions, forcing the immune system to a new set point, which, in the NOD mouse, prevents the subsequent development of T1D. It is possible that a similar immune adjustment is diminished in the modern environment, with the rising incidence of autoimmunity apparently mirroring a decreasing incidence of infectious disease (1). Future studies into what underlies the effects reported in this work, and how they are related to the prevention of T1D by *S. typhimurium* infection, should yield valuable lessons by which to understand the growing problem of autoimmunity in the developed world.

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Disclosures

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References


