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Distinct Effector Mechanisms in the Development of Autoimmune Neuropathy versus Diabetes in Nonobese Diabetic Mice

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NOD mice deficient for the costimulatory molecule B7-2 (NOD-B7-2KO mice) are protected from autoimmune diabetes but develop a spontaneous autoimmune peripheral neuropathy that resembles human diseases Guillain-Barre syndrome and chronic inflammatory demyelinating polyradiculoneuropathy. Similar observations have now been made in conventional NOD mice. We have shown previously that this disease was mediated by autoreactive T cells inducing demyelination in the peripheral nervous system. In this study, we analyzed the molecular pathways involved in the disease. Our data showed that neuropathy developed in the absence of perforin or fas, suggesting that classic cytotoxicity pathways were dispensable for nerve damage in NOD-B7-2KO mice. In contrast, IFN-γ played an obligatory role in the development of neuropathy as demonstrated by the complete protection from disease and infiltration in the nerves in NOD-B7-2KO mice deficient for IFN-γ. This result was consistent with the inflammatory phenotype of T cells infiltrating the peripheral nerves. Importantly, the relative role of perforin, fas, and IFN-γ appears completely different in autoimmune diabetes vs neuropathy. Thus, there are sharp contrasts in the pathogenesis of autoimmune diseases targeting different tissues in the same NOD background.
TNFR (15–17). Evidence of Schwann cell apoptosis has been described in EAN (11, 18), and it has been suggested that fas-mediated cytotoxicity may be involved in nerve damage in some cases of diabetic neuropathy (19, 20). Finally, the effector mechanisms leading to islet cell destruction have been defined in NOD mice, particularly by using NOD mice deficient for molecules central to these different pathways. Indeed, it has been shown that whereas deficiency in fas (21) and perforin (22) substantially protected NOD mice from diabetes, the disease developed in NOD mice deficient for IFN-γ with normal incidence and only a slight delay in onset (23, 24). In this study, we examined the effector mechanisms central to the development of neuropathy in NOD-B7-2KO mice. Because T cells could transfer neuropathy in NOD-SCID recipients, we focused our analysis on cellular pathways that could result in nerve damage and that had been implicated previously in autoimmune diabetes in NOD mice. CD4+ and CD8+ T cells infiltrating the peripheral nerves displayed an inflammatory Th1/Tc1 phenotype. We demonstrated that neuropathy was abrogated in NOD-B7-2KO mice deficient for IFN-γ, whereas perforin and fas were dispensable for tissue damage and neuropathy. Together, these results suggest that diabetes and neuropathy depend on distinct pathogenic pathways.

Materials and Methods

Mice

NOD-B7-2KO mice have been described previously (3). NOD mice deficient in fas (21) and perforin (22) substantially protected NOD mice from diabetes, the disease developed in NOD mice deficient for IFN-γ (23). We performed the cumulative incidence of neuropathy in NOD-B7-2KO mice. NOD-B7-2KO-IFN-γ KO mice have been described previously (3). NOD mice deficient in IL-4 (NOD-IL-4KO) were generously provided by D. Serreze (The Jackson Laboratory, Bar Harbor, ME). NOD-lpr/lpr mice (deficient in fas) were generously provided by L. Wen (Yale University). NOD mice deficient in recombination-activating gene 1 (NOD-RAGKO) were purchased from The Jackson Laboratory and bred at our facility. NOD mice deficient in perforin (NOD-perforin (pore-forming protein) (pfp)KO) were purchased from The Jackson Laboratory. NOD-B7-2KO-IFN-γ KO mice. The same breeding strategy was used to prepare the cumulative incidence of neuropathy in NOD-B7-2KO mice deficient for B7-2 alone (NOD-B7-2KO) or for B7-2 and perforin (NOD-B7-2KO-pfpKO). We compared the cumulative incidence of neuropathy in NOD-B7-2KO (n = 5, ▲) and NOD-B7-2KO-pfpKO (n = 8, □) females. We performed histological analysis of the sciatic nerves in NOD-B7-2KO and NOD-B7-2KO-pfpKO mice. A representative section is shown for each strain.

Assessment of neuropathy and diabetes

Neuropathy was assessed weekly by phenotypic examination of the mice as described in Salomon et al. (3). Briefly, neuropathy was determined by two consecutive observations of hind leg weakness, twitching, and clumping, inability to grasp the wire bar lid of cages, and difficulty walking. Blood glucose levels were measured every week with an Accu-check Active glucose meter (Roche Diagnostics). Mice were considered diabetic after two consecutive measurements over 300 mg/dl. In some cases, insulin pellets (Lin Shin Canada) were introduced s.c. in diabetic mice to allow for continued scoring of neuropathy. For histological analysis, tissues were fixed in formalin and embedded in paraffin and multiple 5-μm sections were stained with H&E.

Abs and other reagents

Purified anti-CD3e (145-2C11), anti-β7-1 (16.10A1), and anti-CD28 (PV-1) mAbs were prepared in our laboratory. Anti-CD25 mAb (7D4) was obtained from Southern Biotechnology Associates. Anti-CD8, anti-CD4, anti-IL-2, anti-IL-4, anti-IL-10, anti-IFN-γ, and isotype control mAbs were purchased from BD Pharmingen.

Flow cytometry

To isolate T cells infiltrating the PNS, sciatic nerves from indicated mice were digested with collagenase IV (Sigma-Aldrich) and DNase I (Boehringer Mannheim) as described (3). For intracellular cytokine staining, cells were labeled with anti-CD4 and anti-CD8 mAbs, fixed, lightly permeabilized, and stained with anti-cytokine or isotype control mAbs. Flow cytometric analyses were performed on a FACScalibur flow cytometer with CellQuest software (BD Pharmingen).

In vitro cytokine production

Single-cell suspensions were prepared from spleen and lymph nodes (LN) of indicated mice, DMEM-Glutamax medium (Invitrogen Life Technologies), supplemented with 5% heat-inactivated FCS (Summit Biotechnology), 100 U/ml penicillin, 100 U/ml streptomycin, nonessential amino acids, 10 mM HEPES, and 50 μM 2-ME (all from Invitrogen Life Technologies), was used for cell culture. Spleen and LN cells (2 × 106) were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) mAbs. Supernatant was harvested from triplicate cultures after 1 (IL-2) or 2 (IL-4, IL-10, and IFN-γ) days. Levels of cytokine were measured by commercial ELISA kits according to the manufacturer’s recommendations (BD Pharmingen).

Adoptive transfer experiments

Single-cell suspensions were prepared from spleen and LN T cells from indicated mice and stimulated for 4 days with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) mAbs, the last day in the presence of 20 U/ml recombinant human IL-2 (Chiron), and 10 × 106 cells were transferred via retro-orbital injection into 5- to 7-wk-old NOD-RAGKO recipients. Retro-orbital injection is a well-characterized route to transfer diabetes as well as neuropathy (3, 4).

Results

Perforin is dispensable for the development of neuropathy in NOD-B7-2KO mice

Because perforin has been proposed to participate in islet damage in NOD mice (22), we analyzed whether this cytotoxic pathway contributed to neuropathy in NOD-B7-2KO mice by crossing these mice to NOD-pfpKO mice (deficient for perforin). We followed the development of neuropathy in NOD-B7-2KO-pfpKO mice. Peripheral neuropathy developed with normal kinetics in the absence
of perforin (Fig. 1A) and was associated with an intense mononuclear infiltration in the PNS (Fig. 1B), suggesting that perforin was dispensable for tissue damage and neuropathy in NOD-B7-2KO mice.

**Tissue damage can occur independently of the fas pathway in NOD-B7-2KO mice**

The fas-fas ligand pathway has been shown to be important for islet damage in NOD mice (21, 26). To examine whether this pathway was involved in pathogenic mechanisms leading to nerve destruction in NOD-B7-2KO, we crossed NOD-B7-2KO mice to NOD-Ipr/lpr mice (deficient for fas). Initial analyses suggested that fas controlled disease progression as NOD-B7-2KO-Ipr/lpr mice demonstrated reduced neuropathy (data not shown). However, historically, the interpretation of the protection from autoimmunity in NOD-Ipr/lpr mice has been complicated by the abnormal immune phenotype resulting from fas deficiency (27, 28). Similarly, NOD-B7-2KO-Ipr/lpr mice developed severe lymphadenopathy resulting in 2- to 5-fold increases in spleen and LN size and total cell numbers and other abnormalities in the immune system (data not shown). Thus, we took advantage of an accelerated model of neuropathy allowing us to study the role of fas in tissue damage before the lymphoproliferative disease was evident. Treatment of NOD-B7-2KO mice with anti-B7-1 mAbs between 2 and 4 wk of age accelerated neuropathy because of enhanced T cell activation resulting from reducing the number of circulating regulatory T cells (Tregs) and/or abrogating CTLA-4 signaling (Fig. 2A, top panel). Thus, we used this approach to analyze whether fas deficiency would differentially affect autoimmune diabetes and neuropathy. NOD-B7-2KO-Ipr/lpr mice were treated with anti-B7-1 mAbs between 2 and 4 wk of age. As previously described (4), anti-B7-1 treatment dramatically reduced the level of Tregs to <1% of CD4+ T cells in NOD-B7-2KO and NOD-B7-2KO-Ipr/lpr mice (data not shown). Anti-B7-1-treated NOD-B7-2KO-Ipr/lpr mice developed neuropathy with kinetics similar to NOD-B7-2KO mice (Fig. 2A, top panel). Histological examination of neuropathic NOD-B7-2KO-Ipr/lpr mice treated with anti-B7-1 demonstrated an intense mononuclear infiltrate in the sciatic nerves (Fig. 2B). The autoimmune basis of the neuropathy in NOD-B7-2KO-Ipr/lpr mice was further confirmed by adoptive transfer experiments. Transfer of spleen and LN cells from neuropathic anti-B7-1-treated NOD-B7-2KO-Ipr/lpr induced neuropathy in NOD-RAGKO recipients within 4–6 wk after transfer (Fig. 2C). Together, these results suggested that fas expression was not essential for the development of disease and particularly nerve damage in NOD-B7-2KO mice.

Previous studies showed that anti-B7-1-treated NOD-B7-2KO mice also developed diabetes (4), reflecting the total breakdown in immune regulation allowing an opportunity to examine the fas and perforin pathways in the same animals. Perforin and fas deficiency had no effect on neuropathy after anti-B7-1 treatment (Fig. 2A, top panel). More importantly, although autoimmune diabetes was restored in anti-B7-1-treated NOD-B7-2KO mice, it did not occur in NOD-B7-2KO-Ipr/lpr and NOD-B7-2KO-pfpKO mice (Fig. 2A, bottom panel). Taken together, these results demonstrate that both the fas and perforin cytotoxic pathways are critical for islet destruction in autoimmune diabetes but not for PNS damage in neuropathy in the NOD background even under conditions of minimal immune regulation.

**Peripheral neuropathy is associated with inflammatory Th1 cells in the PNS**

We analyzed cytokine production by T cells from NOD-B7-2KO mice after in vitro stimulation with anti-CD3 and anti-CD28 mAbs. Similarly to what has been described in NOD mice, NOD-B7-2KO T cells from spleen and LNs produced high levels of IL-2 and IFN-γ in vitro, low levels of IL-10, and undetectable levels of IL-4 (Fig. 3A). More importantly, we analyzed cytokine production by CD4+ T cells infiltrating the peripheral nerves of neuropathic NOD-B7-2KO mice by intracellular staining. As shown in Fig. 3B, CD4+ T cells displayed an inflammatory Th1 phenotype in the nerves, characterized by the production of IL-2 and IFN-γ, but no or little IL-4 and IL-10. A similar T cytotoxic 1 phenotype was observed in CD8+ T cells infiltrating the nerves (data not shown).

**IFN-γ production is necessary for the development of the disease**

NOD-B7-2KO mice were crossed to NOD mice deficient for IFN-γ (NOD-Ifn−/−) to create mice doubly deficient in B7-2 and IFN-γ (NOD-B7-2KO-Ifn−/−). We then followed the development of neuropathy in these mice. As shown in Fig. 4A,
deficiency in IFN-γ completely abrogated the development of neuropathy in NOD-B7-2KO-IFN-KO mice. Indeed, 0 of 12 NOD-B7-2KO-IFN-KO female mice developed neuropathy by 40 wk of age, compared with 100% of NOD-B7-2KO females. In addition, histological analysis of sciatic nerves isolated from age-matched NOD-B7-2KO or NOD-B7-2KO-IFN-KO mice over 30 wk of age demonstrated that, whereas peripheral nerves of NOD-B7-2KO mice were heavily infiltrated, the mononuclear infiltrate was greatly reduced or absent in the PNS of NOD-B7-2KO-IFN-KO mice (Fig. 4B). Finally, although IFN-γ played a major role in the development of neuropathy, IL-4 did not dramatically affect the disease since NOD-B7-2-IL-4KO mice developed neuropathy with similar incidence and kinetics compared with NOD-B7-2KO mice (Fig. 4C).

NOD-B7-2KO-IFN-KO mice were treated with anti-B7-1 mAbs between 2 and 4 wk of age. As shown in Fig. 4D, autoimmune diabetes developed in the absence of IFN-γ in anti-B7-1-treated NOD-B7-2KO-IFN-KO mice, in agreement with previous reports that IFN-γ deficiency had little effect on the development of diabetes in NOD mice (23, 24). In contrast, anti-B7-1 treatment did not restore neuropathy in NOD-B7-2KO-IFN-KO mice (Fig. 4D), suggesting that IFN-γ deficiency prevented the development of autoimmune neuropathy in NOD-B7-2KO mice even in conditions of increased T cell activation. Interestingly, one-third of NOD-B7-2KO-IFN-KO mice treated with anti-B7-1 developed asymmetrical extreme stiffness and paralysis affecting only one hind limb. This disease is reminiscent of the paralytic autoimmune

FIGURE 3. Neuropathy is mediated by inflammatory Th1 T cells. A. Spleen and LN cells from NOD-B7-2KO females were stimulated with anti-CD3 and anti-CD28 mAbs. Culture supernatants were analyzed by ELISA for IL-2, IFN-γ, IL-10 and IL-4. Each circle represents an individual mouse. B. Cell suspensions were prepared from sciatic nerves of a neuropathic NOD-B7-2KO female, stimulated with PMA and ionomycin, and labeled with anti-CD4 mAbs on the cell surface (x-axis) and anti-cytokine mAbs intracellularly (y-axis). The percentage of cytokine-positive cells within the CD4+ population is indicated.

FIGURE 4. Deficiency in IFN-γ prevents the development of neuropathy. A. We generated NOD mice deficient for B7-2 and IFN-γ (NOD-B7-2KO-IFN-γKO). We compared the cumulative incidence of neuropathy in NOD-B7-2KO (n = 7, ▲) and NOD-B7-2KO-IFN-γKO (n = 9, ●) females. B. We performed histological analysis of the sciatic nerves in NOD-B7-2KO and NOD-B7-2KO-IFN-γKO mice. A representative section is shown for each strain. C. We generated NOD mice deficient for B7-2 and IL-4 (NOD-B7-2KO-IL-4KO). We compared the cumulative incidence of neuropathy in NOD-B7-2KO (n = 5, ▲) and NOD-B7-2KO-IL-4KO (n = 16, ×) females. D. We treated NOD-B7-2KO (n = 7, triangles) and NOD-B7-2KO-IFN-γKO (n = 6, diamonds) mice with anti-B7-1 mAbs between 2 and 4 wk of age. We followed the incidence of neuropathy (closed symbols) and diabetes (open symbols) after 8 wk of age. Similar results were observed in males and females and were pooled.
myositis that develops in NOD mice deficient in IFN-γ production secondary to constitutive expression of an IFN-γ receptor β-chain transgene in T cells (29).

Discussion

The NOD mouse is a unique model of multiorgan autoimmunity where distinct autoimmune syndromes can be observed depending on subtle differences in environment and genetic make-up. One such syndrome is the development of spontaneous autoimmune peripheral polyneuropathy observed in B7-2-deficient NOD mice (3). Diseases targeting the nervous system have also been observed in other settings including the treatment of NOD mice with pertussis toxin (7) or anti-IL-2 therapy (9). In our study, we chose to analyze the development of autoimmune neuropathy in NOD-B7-2KO mice because of high disease penetrance (100% of females develop the disease by 30–35 wk of age) and the unique possibility to induce diabetes and neuropathy concomitantly in this model (4), which allowed us to ask two questions. First, what are the effector mechanisms leading to nerve destruction in a spontaneous model of autoimmune peripheral neuropathy? Second, how does this compare with other autoimmune syndromes in the same mouse strain, particularly, diabetes? The relevance to therapeutics is that if there are clearly different pathways to tissue destruction in individual autoimmune syndromes in this one inbred mouse strain, approaches to the treatment of humans with multiorgan autoimmunity may be complex. In the present study, we observed that CD4+ and CD8+ T cells infiltrating the peripheral nerves produced IFN-γ and IL-2 but no IL-4, consistent with the etiology of an inflammatory autoimmune disease. A central role for IFN-γ was demonstrated by the abrogation of neuropathy and T cell infiltration in the PNS of NOD-B7-2KO-IFN-γKO mice. Furthermore, neuropathy was not restored following treatment with anti-B7-1 between 2 and 4 wk of age, although this treatment has been shown to enhance autoimmune responses by affecting Tregs and/or CTLA-4 signaling (4). In contrast, the results clearly showed that perforin deficiency in NOD-B7-2KO mice had no effect on the development of neuropathy. In addition, the fas pathway was dispensable for CNS damage induced by autoreactive T cells. It is noteworthy that the prominent role of IFN-γ compared with the nonessential function of perforin and fas in the development of neuropathy is in concordance with our previous finding that CD4+ T cells were necessary and sufficient to transfer the disease, whereas CD8+ T cells, usually associated with cytotoxic pathways of tissue damage, were not (3).

Although the exact mechanism underlying nerve damage initiated by inflammatory cytokines is not fully known, it is believed to be part of an inflammatory response involving numerous mediators related to these cytokines, including nitric oxides, matrix metalloproteinases, macrophage activation, up-regulation of the NF-κB pathway, and increased level of MHC molecules on APCs (10). In this regard, in vivo treatments affecting these pathways have been effective at preventing and/or reversing EAN and the clinical amelioration correlated with decreased levels of Th1 differentiation and IFN-γ production (14, 30, 31). In addition, major molecular pathways involved in Th1 cell differentiation and cytokine production, such as the IL-12 and IFN-γ signaling pathways, have been shown to be critical to the development of the animal model EAN (15–17, 32).

IFN-γ and other proinflammatory cytokines such as TNF-α and IL-1 have been proposed to play a critical role in the process leading to nerve damage in patients with GBS or CIDP (33–38). Analysis of serum levels of different cytokines suggested that the relative amount of Th1 vs Th2 cytokines generally correlates with the clinical stage of disease in GBS and CIDP (39–41). Furthermore, current therapeutic options for GBS or CIDP include steroids and immunosuppressant drugs, in agreement with a central role of inflammatory T cells in the disease, as well as plasmapheresis or i.v. Ig, in which mechanisms of action are not completely understood (6, 42). Interestingly, Goto et al. (43) reported that plasmapheresis could induce changes in the Th1-Th2 ratio in two patients suffering from CIDP, suggesting that a reduction in inflammatory cytokines could participate in the beneficial effect of plasmapheresis. Similarly, i.v. Ig treatment can reduce the serum levels of proinflammatory markers in peripheral neuropathies, although it is unclear whether this decrease is causing the clinical amelioration or merely reflecting it (44). Finally, it is noteworthy that although proinflammatory cytokines and mediators are clearly associated with tissue damage in autoimmune neuropathies, some of them have been linked to normal function of the nervous system and even neuroprotection in some cases (45). Particularly, TNF-α is produced by astrocytes, microglia, and neurons and has been associated both with CNS damage and neuroprotection against excitotoxicity following ischemia or stroke (45–49). In contrast, IFN-γ is produced only by infiltrating leukocytes but not by resident cells in the CNS, thus making this cytokine a better target for immunotherapy than TNF-α. Although future studies will be needed to define the downstream mediators of tissue damage in the PNS, this study clearly identifies IFN-γ as an important therapeutic target in the human diseases GBS and CIDP.

The two cytotoxic pathways involving fas-fas ligand interaction and perforin release have been proposed to be involved in autoimmune diabetes in NOD mice (21, 22). Although the analysis of the fas pathway in NOD mice was complicated by the abnormal immune phenotype observed in Ifnγ−/− mice, alternative models convincingly demonstrated a role for the fas-fas ligand pathway in autoimmune diabetes (26, 50). Thus, it was surprising that perforin and fas deficiency were not necessary for tissue damage in NOD-B7-2KO mice. Dosch and colleagues (8) recently demonstrated autoreactivity against Schwann cells which colocalized with islets in the pancreas and proposed that the reactivity against the Schwann cells may be a precursor to the development of diabetes. Our demonstration of a distinct mechanistic basis for the two diseases has important implications for the interpretation of these observations. Finally, Schwann cell-induced apoptosis of T cells has been proposed to be a mechanism of protection against immune attacks on the PNS and it has been associated with remission of disease in relapsing-remitting models of EAN (51–53). It is notable that neuropathy was not accelerated in the absence of either perforin or fas, implying that T cell apoptosis induced by resident PNS cells through one of these mechanisms does not occur in the PNS in our model of neuropathy. This result is compatible with the progressive course of disease that does not include periods of remission as seen in EAN or EAE.

It is intriguing that the molecular and cellular pathways involved in autoimmune neuropathy in NOD-B7-2KO mice appear clearly distinct from the mechanisms responsible for autoimmune diabetes in NOD mice. Indeed, although blocking IFN-γ by mAbs reduced the incidence of diabetes in NOD mice (54), NOD-IFN-γKO mice develop diabetes with incidence similar to NOD mice and only slightly delayed kinetics (23, 24). In contrast, whereas fas and perforin are involved in the development of autoimmune diabetes and islet destruction in NOD mice (21, 22, 26, 50), both pathways were dispensable for autoimmune neuropathy. These results suggest that although both diabetes and neuropathy are T cell-mediated inflammatory autoimmune diseases occurring on the autoimmune-prone NOD background, the immune mechanisms
underlying these diseases are distinct. Thus, autoimmunity targeting different tissues can exploit distinct effector pathways in susceptible individuals.

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Disclosures
The authors have no financial conflict of interest.

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