Stat4-null non-obese diabetic mice: protection from diabetes and experimental allergic encephalomyelitis, but with concomitant epitope spread

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Abstract

There is much interest in therapeutic manipulation of cytokine responses in autoimmunity, yet studies in mouse models have sometimes produced conflicting findings as to the role of particular mediators in disease. Examples include the contradictory findings regarding susceptibility to experimental allergic encephalomyelitis (EAE) or diabetes in knockout mice for various individual T\(_{h}1\) or T\(_{h}2\) cytokines or their receptors. An alternative approach to the analysis of T\(_{h}1\) and T\(_{h}2\) mechanisms in these diseases is to investigate strains carrying a null mutation for molecules involved in cytokine receptor signal transduction, signal transducer and activator of transcription (Stat4) and Stat6. Stat4 is pivotal in T\(_{h}1\) polarization, being activated when IL-12 binds the IL-12R and leading to the production of IFN\(_{\gamma}\). We here report disease susceptibility in non-obese diabetic mice carrying a Stat4-null mutation. Knockout mice were almost completely protected from diabetes, only rarely showing pancreatic peri-islet infiltrates. Furthermore, there was near complete protection from the induction of EAE by either of the two encephalitogenic myelin epitopes. Despite this protection, Stat4-null mice showed clear epitope spread compared with controls during myelin oligodendrocyte glycoprotein-induced EAE as judged by T cell proliferation, although this was not associated with a strong T\(_{h}1\) response to the initial or spread epitope and, furthermore, there was no evidence of a switch to T\(_{h}2\) cytokines.

Introduction

The non-obese diabetic (NOD) mouse spontaneously develops type I insulin dependent diabetes mellitus (IDDM), and can be induced to develop experimental allergic encephalomyelitis (EAE). NOD mouse diabetes and EAE, models for human multiple sclerosis and IDDM, respectively, are both diseases broadly believed to result from autoreactivity by pathogenic T\(_{h}1\) cells (1, 2). However, in both disease models, debate persists about the contribution of different T\(_{h}1\) and T\(_{h}2\) cytokines to disease progression or resolution, and conflicting findings have been obtained in different experimental systems.

In EAE, the relative contribution of different T\(_{h}1\) and T\(_{h}2\) cytokines to disease has been analyzed over several years by RNA or immunocytochemical analysis of central nervous system tissue from affected mice, by transfer of T cell clones with different cytokine profiles, by the use of cytokine neutralizing antibodies, by administration of exogenous cytokines and using mice carrying null mutations for various cytokines or...
their receptors (3–9). This wide range of complementary approaches has sometimes produced contradictory findings. Mice carrying a null mutation for IL-12, the cytokine at the head of the Th1 pathway, are protected from EAE (8). However, the role of IFNγ, the prototypic, IL-12-driven Th1 cytokine, has been confusing. We have previously reported that local production of a strong IFNγ response by CNS-infiltrating T cells is a good predictor of EAE relapse, and local IFNγ is associated with disease as predicted from the fact that most encephalitogenic T cell clones are strong secretors of IFNγ (1, 2, 10). However, treatment of mice with anti-IFNγ antibody paradoxically enhances disease (9). Furthermore, IFNγ-null mice show a similar time of onset and severity of EAE to controls with enhanced mortality, suggesting possible loss of IFNγ-dependent regulation (7). Indeed, transfer of Th1 clones to immunodeficient recipients can induce EAE, though this may not be representative of the typical pathological picture (11).

In the inflammatory events leading to diabetes in the NOD mouse, the contribution of Th1 and Th2 cytokines has been similarly unclear, not least because the NOD mouse appears to have inherent abnormalities in a number of cytokines (12). In general, the ability to transfer disease to NOD mice is a property of Th1 rather than Th2 cells (13). Analysis of cytokine mRNA from diabetic pancreata has shown either a correlation with local IFNγ or a mixture of Th1 and Th2 cytokines (14, 15). Administration of IL-12 causes accelerated diabetes with increased production of Th1 cytokines by pancreas-infiltrating T cells (16). However, in IL-12-deficient mice it is found that, despite the predicted, systemic defect in activation of Th1 responses, diabetes occurs at a normal frequency and is associated with recruitment to the islets of IFNγ-producing cells (17). With respect to IFNγ, it was initially found that IFNγ-null NOD mice succumb to diabetes at the normal rate (18). However, it was subsequently reported that disruption of the IFNγ-R pathway leads to the generation of mice showing resistance to diabetes (19). A third strain of knockout mice has more recently been reported, those carrying a null mutation for the other chain of the receptor, IFNγ-Rβ (20). Interestingly, these mice show the predicted lack of Th1 responses with greatly enhanced Th2 responses, yet remain highly susceptible to diabetes.

In light of the problems in trying to attribute an unequivocal pathogenic role to particular cytokines, presumably due to redundancy between overlapping pathways (21), a complementary approach is to analyze the consequences of disrupting cytokine receptor signaling molecules (22). The Janus kinases/signal transducer and activator of transcription (Stat) pathway constitutes a central route of intracellular cytokine receptor signaling. Specifically, Stat6 is thought to be absolutely required for IL-4R signal transduction and subsequent Th2 polarization (22). Stat4 is central to Th1 polarization, being activated when IL-12 binds the IL-12R and leading to the production of IFNγ. T cells from mice lacking Stat4 mount no IFNγ response following stimulation by anti-CD3 antibody or heat-killed Listeria monocytogenes, and do not proliferate in response to IL-12 (23). In many experimental systems, this reduction in Th1 cytokine responses is accompanied by much enhanced release of IL-4. Stat4 also operates at other points in the development of the Th1 response, for example controlling induction of both the IL-12 and IL-18Rs (24). However, the roles of Stat6 in Th2 polarization and of Stat4 in Th1 polarization are not precisely equivalent: whereas Stat6 appears to be absolutely required for IL-4 transcription, there is a Stat4-independent pathway for the development of Th1 cells (25). Thus, the phenotype of Stat4−/− mice with respect to loss of the IFNγ response is partly attributable to a secondary effect of Th1 inhibition by enhanced IL-4 release since double knockout Stat4/Stat6−/− mice show Th1 responsiveness to be partially rescued.

The impact of Stat4 disruption on type 1 diabetes was first studied using the transgenic islet antigen-targeted lymphochoriomeningitis virus (LCMV) RIP-LCMV-NP model (26). In that system, crossing onto a Stat4−/− background caused a reduction in the incidence of diabetes from 85 to 30%, although, interestingly, this was accompanied by a lack of shift to Th2 cytokines, a reduction of IFNγ production to about one-half of the normal and generation of a normal CD8 Tc1 response. Another group analyzed the susceptibility of Stat4-null mice on a C57B6 background to EAE induced by the myelin oligodendrocyte glycoprotein (MOG) epitope 35–55 (27). Both the incidence and severity of disease were significantly reduced. We set out to investigate the role of Stat4 signaling in autoimmunity by crossing the knockout for several generations onto the NOD mouse background. This strain develops a number of autoimmune diseases spontaneously, notably diabetes, thyroiditis and sialitis, and can also be induced to develop EAE after injection of spinal cord homogenate or specific myelin epitopes (28, 10). We report that Stat4-null NOD mice are largely protected from diabetes and suffer much reduced insulinitis. They are resistant to EAE induction by MOG or proteolipoprotein (PLP) epitopes, although this protection is accompanied by clear epitope spread. However, this spread is essentially silent with respect to cytokine activation, presumably explaining why it is not associated with disease.

**Methods**

**Generation of animals and screening**

Stat4-null mice were originally obtained from W. Thie菲尔德 and J. Ihle (St Jude’s Hospital, Memphis, TN, USA) (29). The mice were crossed onto the NOD strain over a period of >4 years and 16 generations. Mice carrying the disrupted allele were identified by PCR of tail biopsy DNA; using the oligonucleotide primers, Stat4 wild-type (WT) sense, GAGG- TAGGTTTCTAACTAGTA, anti-sense, ATGTGAAATCAATTAGCATG and hygromycin, CAGGGGTACTTACTGGA. The Stat4 primers were used to amplify the WT band using an annealing temperature of 52°C for 30 cycles, and the knockout band was amplified using the Stat4 sense primer and the hygromycin primer at an annealing temperature of 55°C for 30 cycles. For the control back-cross (129/Sv WT crossed with NOD and then with NOD at each subsequent generation), offspring were selected at each generation for the 129/Sv allele of the Stat4-flanking R51 microsatellite marker, sense CTACTATCTACCTCTCTTAATC and anti-sense ATACTGTGTTGATACG-TATGTAATG.
Assessment of diabetes

Female NOD mice that were either Stat4+/+ or −/− were observed clinically for the development of diabetes by twice a week screening of urine glucose using Diastix (Miles Ltd, Slough, UK). All mice were sacrificed at 26 weeks of age, at which time blood glucose was measured using Glucostix on a Glucometer (Miles Ltd). Blood glucose of >11 mmol l⁻¹ was defined as diabetic, this being the mean plus 2.5 SD from readings in our colony of H2-E transgenic mice, which show complete protection from diabetes. Pancreata were fixed for paraffin embedding, sectioning and staining with hematoxylin and eosin staining. Islets were scored as follows: 0 = no infiltrate, 1 = peri-islet infiltration, 3 = severe intra-islet infiltration, 4 = loss of islet architecture. Each islet was sampled at two levels, taking the mean score from a total of six islets.

Induction of EAE

EAE was induced by injection of the peptides, mouse PLP 56–70 or mouse MOG 35–55 (10) (synthesized by Haemostasis Group, MRC Clinical Sciences Centre, London, UK). Briefly, peptide was emulsified with adjuvant containing heat-killed Mycobacterium butyricum peptide was emulsified with adjuvant containing heat-killed Mycobacterium butyricum and Mycobacterium tuberculosis, and mice received two injections separated by 1 week of 200 µg peptide in the flank. Immediately after each immunization and 48 h later, mice were given 200 ng Bordetella pertussis toxin (ICN, Hampshire, UK). Mice were weighed daily and observed for signs of paralysis, which were scored as follows: 0 = normal, 1 = limp tail, 2 = impaired righting reflex, 3 = partial hindlimb paralysis, 4 = complete hindlimb paralysis, 5 = moribund.

Accelerated diabetes

Diabetes was actively induced by transfer of splenocytes from diabetic NOD mice or by acceleration with cyclophosphamide. In the former case, a single-cell splenocyte suspension was prepared from diabetic NOD mice and 5 × 10⁶ cells were administered to female, young adult recipients intravenously. For cyclophosphamide-accelerated diabetes, female, young adult mice received an intra-peritoneal injection of 200 mg kg⁻¹ cyclophosphamide (Pharmacia, Milton Keynes, UK) at days 0 and 14.

T cell proliferation

Spleen cells were disaggregated using fine gauge needles, re-suspended in HL-1 serum-free medium (Hycor Biomedical, Irvine, CA, USA) supplemented with l-glutamine (2 mM), 2-mercaptoethanol (5 × 10⁻⁵ M), 30 IU penicillin and 30 µg ml⁻¹ streptomycin, and 4 × 10⁵ cells per well were aliquoted in triplicate in flat-bottom microculture plates. Peptides were added to wells at a final concentration of 50 µg ml⁻¹. After 48 h, 100 µl of tissue culture supernatant was collected for measurement of cytokotns by ELISA. Wells were supplemented with 100 µl additional medium and, for the final 6 h of culture with 1 µCi per well of [³H]thymidine. Cultures were harvested at 72 h for counting in a beta scintillation counter.

Measurement of cytokines

Cytokine analysis was conducted on tissue culture supernatants collected after 48 h of splenocyte culture with peptide at 50 µg ml⁻¹. IFNγ was assessed in tissue culture supernatants using a sandwich ELISA assay with an anti-IFNγ and biotinylated anti-IFNγ mAb pair (R&D Systems, Abingdon, UK). The IL-4 ELISA used an anti-IL-4 and biotinylated anti-IL-4 mAb pair (R&D Systems). In some cases supernatants were screened for tumor necrosis factor (TNF)α, IFNγ, IL-2, IL-4 and IL-5 using the T₅₁/T₅₂ Luminex Cytokine Bead Array system (Pharmingen, Oxford, UK).

Results

Stat4+/− NOD mice are protected from diabetes

Stat4+/− NOD mice were compared with Stat4+/+ WT NOD mice for the incidence of diabetes (Fig. 1A). Only a minority of knockout mice showed any glucosuria, and this was considerably delayed compared with WT mice. A cohort of WT and knockout mice was sacrificed at 6 months for the analysis of islet histology and blood glucose. Only a majority of the knockout mice showed any lymphocytic infiltration of islets, this being limited to the peri-islet region (Fig. 1B), while all WT mice showed extensive islet infiltrates. Blood glucose in these knockout mice was normal while the majority of WT mice was diabetic (Fig. 1C): mean blood glucose ± SE in WT mice was 17.7 ± 5.1 mmol l⁻¹ compared with 5.7 ± 0.6 mmol l⁻¹ in knockouts (P < 0.03 by analysis of variance).

Stat4+/− NOD and WT controls both show spontaneous T cell responses to glutamic acid decarboxylase 65

Others and we have previously correlated the onset of diabetes with the appearance of spontaneous T cell proliferative responses to peptides from various islet antigens (30–33). Among these are the glutamic acid decarboxylase 65 (GAD₆₅) epitopes 505–519 and 524–543. We found no significant difference in the proliferative response of splenic T cells to these epitopes from WT and knockout mice analyzed at 26 weeks of age (Fig. 2).

Active induction of diabetes in Stat4+/− NOD and WT controls

In an effort to gain further insights into whether the nature of disease resistance in Stat4+/− NOD mice relates simply to a lack of T₅₁ effector cells or might alternatively involve default to some form of active regulation, we turned to protocols for the active induction of diabetes (Table 1). Using cyclophosphamide to induce accelerated disease, diabetes was observed in all NOD controls, and in zero out of five of the knockout mice. Knockout mice in fact died at 10–12 weeks after receiving cyclophosphamide, but the cause of death was unclear and did not involve elevated blood glucose. Cyclophosphamide has been assumed to act in NOD diabetogenesis by removing a regulatory or suppressor population. However, recent array analysis suggests a more complex course of events, in which the major group of down-regulated transcripts is B cell derived, and chemokine genes and IFNγ-related genes are induced (34). This accords with an earlier observation that T₅₁ IFN-producing cells may be particularly resistant to cyclophosphamide (35). The lack of disease induction in Stat4+/− mice may indicate a paucity of
competent effector cells for disease (with the caveat that we saw a small proportion of mice developing spontaneous diabetes at >20 weeks). However, when diabetogenic spleen cells were then transferred to knockout mice, still no disease could be induced (Table 1). This suggests that, while there may be a lack of endogenous effector cells in these mice, disease resistance must also entail additional immune regulation processes. This will require further investigation.

**Stat4−/− mice are protected from EAE**

We then compared the susceptibility of knockout and WT mice to EAE. The PLP epitope, 56–70, induces only mild EAE in NOD mice; in the experiment shown, four out of six mice in the control group were affected, disease starting from day 20 (Fig. 3A). None of the mice in the Stat4−/− group showed any sign of disease. The MOG 35–55 epitope induced disease in all control mice (six out of six in the experiment shown), starting from day 15 (Fig. 3B). Again, Stat4−/− mice were largely protected, two out of seven mice in the experiment were shown to develop very mild disease with a time lag of 10 days.

**Epitope spread during EAE in Stat4-null mice**

In several models of EAE, disease progression (and in the context of relapsing and remitting models, relapse) can be associated with epitope spread to additional myelin epitopes. However, we have previously reported that in the EAE of the NOD mouse, T cell responses remain remarkably focused on the original disease-inducing epitope (10). This was reiterated in our WT mice: following disease induction with MOG 35–55, we observed no spread of the T cell response from the one, myelin-derived, H2-A^q^−presented epitope to the other (Fig. 4). However, when the T cell responses of the Stat4-null mice were analyzed, we found clear evidence, in most mice analyzed, for
epitope spread from the MOG epitope to PLP 56–70. We then analyzed the pattern of epitope spread following EAE induction by PLP 56–70 (Fig. 5). Again, we observed differences in the pattern of epitope spread between knockout and WT mice: in WT NOD mice there was spread of the response from PLP 56–70 to one of the other epitopes tested, MOG 35–55. The disease-resistant knockout mice did not show the spread response to MOG 35–55, but instead showed a response to the epitope overlapping PLP 56–70 and previously described as an H2-Ag7-restricted, non-encephalitogenic epitope, PLP 60–74 (36).

We then analyzed the cytokine profile of responses to myelin antigens in the two strains after disease induction with PLP 56–70 (Fig. 6). The central finding was that disease in NOD WT mice is associated with a strong IFN\(\gamma\) and TNF\(\alpha\) response to the disease-inducing epitope. The ‘spread response’ to MOG 35–55 was essentially functionally silent in terms of responses by any of the cytokines measured (IFN\(\gamma\), TNF\(\alpha\), IL-4, IL-5, IL-2). Cytokine responses to either the disease-inducing epitope or the ‘spread epitope’ in the NOD Stat4\(^{-/-}\) mice were marginal, with the exception of one mouse that mounted an IFN\(\gamma\) response to PLP 56–70, albeit lower than WT mice (Fig. 6a). Contrary to some expectations on the nature of Th1/Th2 polarization in Stat4 knockout mice, IL-5 responses (Fig. 6f) and IL-4 responses (data not shown) gave no clear evidence of switch to a default Th2 response.

**Discussion**

The generality that, in many autoimmune diseases, Th1 cytokine responses are pathogenic and Th2 responses neutral or protective has largely withstood the test of time although studies with knockout mouse strains and specific cytokine antagonists have severely dented this model. The details of Th1 and Th2 cytokine interactions in many autoimmune diseases appear far more complex than once envisaged. In terms of the development of new therapeutic strategies, there is much interest in the possibility of modulating cytokine responses at the level of differential cell signaling or transcriptional control (37). The lymphocyte signaling molecules, Stat4 and Stat6, are obvious targets in this respect, in view of their pivotal roles resulting from recruitment for IL-12 and IL-4 signaling, respectively (22).

The role of Stat4 signaling has previously been examined in a model of diabetes by Holz et al. (26) who investigated the effect of a Stat4-null mutation in the RIP-LCMV transgenic mouse model. The impact of this mutation on diabetogenesis would not necessarily be predictable from cytokine/cytokine

### Table 1. Susceptibility of NOD WT and Stat4 knockout mice to accelerated diabetes after cyclophosphamide or cell transfer

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<td>Diabetes following transfer of splenocytes from diabetic donor</td>
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**Fig. 3.** Stat4\(^{-/-}\) NOD mice are protected from EAE. EAE was induced in WT mice (filled symbols) or knockout mice (open symbols), six–seven mice per group, using (A) PLP 56–70 or (B) MOG 35–55.

**Fig. 4.** Protection from MOG-induced EAE in Stat4\(^{-/-}\) mice coincides with epitope spread. WT mice (A) and Stat4\(^{-/-}\) mice (B) were sacrificed at day 50 after encephalitogenic EAE immunization with MOG 35–55. Splenocytes were analyzed for proliferation to the immunizing epitope and for spread to the alternate PLP 56–70 epitope. Background counts per minute in wells containing cells with medium were 1410 ± 391.
responses identifiable during disease (30, 31). Here we were able to dissociate proliferative responses to GAD 524–543 per se from the disease process since NOD Stat4−/− mice showed normal, spontaneous splenic T cell responses to this epitope. How can the observation of more or less complete protection from diabetes in Stat4−/− NOD mice be reconciled with the observation from Trembleau et al. (17), showing no reduction in diabetes in IL-12-deficient NOD mice? In that study, it was proposed that IL-18, together with other factors, could substitute for IL-12 in driving the pathogenic Tn1 response and that, in the knockout mice, some IL-12-dependent regulatory population may additionally be impaired. From our results, this regulatory population is likely to be Stat4 independent.

The question of susceptibility to EAE in Stat4−/− mice has previously been addressed by Chitnis et al. (27), using the model of disease induction against MOG 35–55 in C57Bl/6 mice. In that model disruption of Stat4 caused a substantial decrease in the incidence and severity of EAE. This was associated with decreased CNS lymphocytic infiltrates, leading the authors to propose that protection may be associated with a block in the chemokines required for migration to this site. The IFNγ response to MOG peptide was greatly reduced and the IL-5 response enhanced although, interestingly, the IL-4 response was unaltered. We here analyzed disease induced by each of the two epitopes, PLP 56–70 and MOG 35–55. In each case we found more or less complete protection in Stat4−/− mice. This is compatible with the view that in the absence of Tn1 cytokine release, cells migrate poorly across the blood–brain barrier into the CNS (27). However, the data must be reconciled with the observation from Trembleau et al. (17), showing no evidence of more suppressive Stat4−/− mice develop EAE normally (7). Other T cell products believed to be involved in EAE, TNFα and lymphotoxin α, can also be removed by homologous recombination without any effect on disease (38). These findings raise the possibility either that the developmental deficiency of particular cytokines in knockout strains leads to compensatory action by related mediators performing the same role or that a major effect of pathogenic Tn1 cells is mediated by one of the other inflammatory products highlighted by recent DNA array studies in mice and humans (21, 39).

One of the most surprising observations in the Stat4-null NOD mice was the detection of enhanced epitope spread following induction of EAE. In Stat4−/− NOD mice immunized with MOG 35–55, the majority of mice developed strong T cell responses to another H2-A	extsuperscript{K7}-restricted myelin epitope, PLP 56–70, despite the fact that they showed protection from EAE. WT mice, on the other hand, showed no evidence of spread in this disease model. Clearly, the fact that it is possible to detect enhanced spread in the face of considerably diminished susceptibility is of interest in relation to the argument that epitope spread may not necessarily be causally related to autoimmune pathogenesis (40–42). However, we also obtained no clear evidence to suggest that these responses may be regulatory in nature. Though detectable with respect to T cell proliferation, the responses appeared to be more or less functionally silent in terms of all the cytokines analyzed. The concept of regulatory responses to spreading epitopes has previously been proposed in the context of the response to self–heat shock protein 60 in adjuvant arthritis (43, 44).
Interestingly, the PLP 56–70 model of EAE again showed that WT and knockout mice have different patterns of epitope spread: both groups showed spread, WT mice to MOG 35–55 and knockout mice to PLP 60–74. While this is in some respects difficult to reconcile with the central paradigm of spread as a phenomenon driven by and in turn driving tissue destruction, one caveat is that we did not in this study conduct exhaustive analysis to check histologically for subclinical disease in Stat4 knockouts (45). The very different pattern of spread obtained in the two experimental groups is most readily explained by an impact of IFN-γ on antigen processing and the hierarchy of presented self-peptides. Furthermore, we cannot exclude the possibility that the apparent increase in the appearance of spread-specific T cells in the periphery of these mice is a result simply of their inability to cross the blood–brain barrier in the absence of IFN-γ release (27).

In summary, we have found by analysis of susceptibility to EAE and IDDM in Stat4-null NOD mice that the protection observed is more profound than might have been predicted from previous studies using mice lacking particular T<sub>h</sub>1 cytokines or cytokine receptors. This raises the possibility that other inflammatory mediators downstream of the initial Stat4-mediated T<sub>h</sub>1 signaling event deserve further attention. Furthermore, the observation that protection from EAE in knockout mice is accompanied by enhanced epitope spread argues against the view that the phenomenon must always be associated with pathology.

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Abbreviations
EAE experimental allergic encephalomyelitis
GAD<sub>65</sub> glutamic acid decarboxylase 65
IDDM insulin dependant diabetes mellitus
LCMV lymphocchoriomeningitis virus
Autoimmunity in Stat4-null non-obese diabetic mice

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