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# Homeostatic Expansion of T Cells during Immune Insufficiency Generates Autoimmunity

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## Summary

**During illness and stress, the immune system can suffer a considerable loss of T cells (lymphopenia). The remaining T cells undergo vigorous compensatory expansion, known as homeostatic proliferation, to reconstitute the immune system. Interestingly, human diseases of autoimmune etiology often present with immune deficiencies such as lymphopenia. In this study, we show that reduced T cell numbers and the resulting exaggerated homeostatic-type proliferation of T cells generate autoimmunity. The cycling T cell population is short lived, and the depleted memory compartment fuels the generation of new effector T cells. A catalyst for these phenomena is the increased responses to the cytokine IL-21, a mediator that regulates T cell turnover. We conclude that poor T cell survival and lymphopenia precipitate autoimmune disease.**

## Introduction

The same factors that maintain T cell numbers at a steady state in lymphoid organs also drive their expansion following T cell loss. This mechanism, called homeostatic proliferation, is a feature of both naive and antigen-experienced lymphocytes and is tightly regulated by both the available space in lymphoid organs and engagement of specific growth factors (Rocha et al., 1983; Tanchot et al., 1997; Sprent and Surh, 2003). Interestingly, not all lymphocytes can expand by this mechanism. Therefore, homeostatic proliferation may affect the nature of T cell populations, favoring the expansion of T cells that are specific for the body's own constituents (Ernst et al., 1999; La Gruta et al., 2000). Since autoimmunity is comprised of self-antigen-specific responses, such expansion could, in theory, promote its initiation.

Many fascinating studies have been performed that address the nature of immune system deficiencies that lead to autoimmunity. These studies generally focus on the lack of immune regulatory cells, inappropriate immune activation, and crossreactive responses to viral antigens. Indeed, the ultimate destruction of tissues at the end stage of autoimmunity requires true conventionally activated responses. However, the origins of autoimmunity remain mysterious. Of particular interest is the fact that many cases of autoimmunity present with reduced numbers of immune cells or lymphocytes (lymphopenia) (Gleeson et al., 1996; Schaller, 1975). Viruses that are associated with the triggering of autoimmunity in humans are known to induce transient lymphopenia (Bateman et al., 1999; Hernan et al., 2001; Peacock et al., 2003; Permar et al., 2003). Furthermore, animal models of autoimmunity often involve the induction of lymphopenia by genetic or experimental means (Fowell et al., 1991; Powrie et al., 1993; Twarog and Rose, 1970). This suggests that lymphopenia may facilitate destructive autoimmunity. Indeed, compensatory homeostatic expansion, with the ability to favor immune populations that respond to tissue constituents, may provide a basis for the development of autoimmunity.

This study tests the hypothesis that lymphopenia and compensatory homeostatic expansion create autoimmunity. To address this question, we used a commonly studied model of autoimmune diabetes, the nonobese diabetic (NOD) mouse. In this experimental mouse strain, the target pancreatic insulin-producing β cells are attacked and destroyed by activated T cells, leading to diabetes. Herein, we demonstrate that lymphopenia and compensatory homeostatic expansion drive autoimmunity in the NOD mouse. Furthermore, we show that responses to the cytokine IL-21 feed this insidious circuit, creating an unstable niche of effector T cells. Our results uncover a systemic immune failure that underlies the development of autoimmunity.

## Results

### Lymphopenia in NOD Mice

Conveniently, lymphopenia is measured by quantifying lymphoid cells. We counted lymphocyte numbers and found that female diabetes-prone NOD mice have reduced numbers of T cells compared to nonautoimmune strains such as wild-type BALB/c mice. We also compared NOD with two additional congenic strains to identify lymphopenia-related chromosomal regions. Neither the BALB/c strain nor the NOD MHC-matched B10 mice (B10H2$^{g7}$), which contain the NOD MHC on the B10 background and do not develop spontaneous autoimmune diabetes (Figure 1A), were lymphopenic. The lymphopenia in NOD mice was attributed to a reduced number of CD4+ T cells relative to other strains (Figure 1B). Congenic-B6.Idd3.NOD mice that contain a 0.35 centimorgan protective interval from B6 mice (Denny et al., 1997; Wicker et al., 1994) that includes the leukocyte growth factors IL-2 and IL-21 were not CD4+ T cell lymphopenic (Figure 1B). In addition, NOD mice exhibited decreased numbers of B lymphocytes relative to B10H2$^{g7}$ mice and congenic-B6.Idd3.NOD mice (Figure 1C).

### Increasing T Cell Numbers Prevent Diabetes

Previous studies (Sadelain et al., 1990) and our own observations (Figure 1D) show that immunization with

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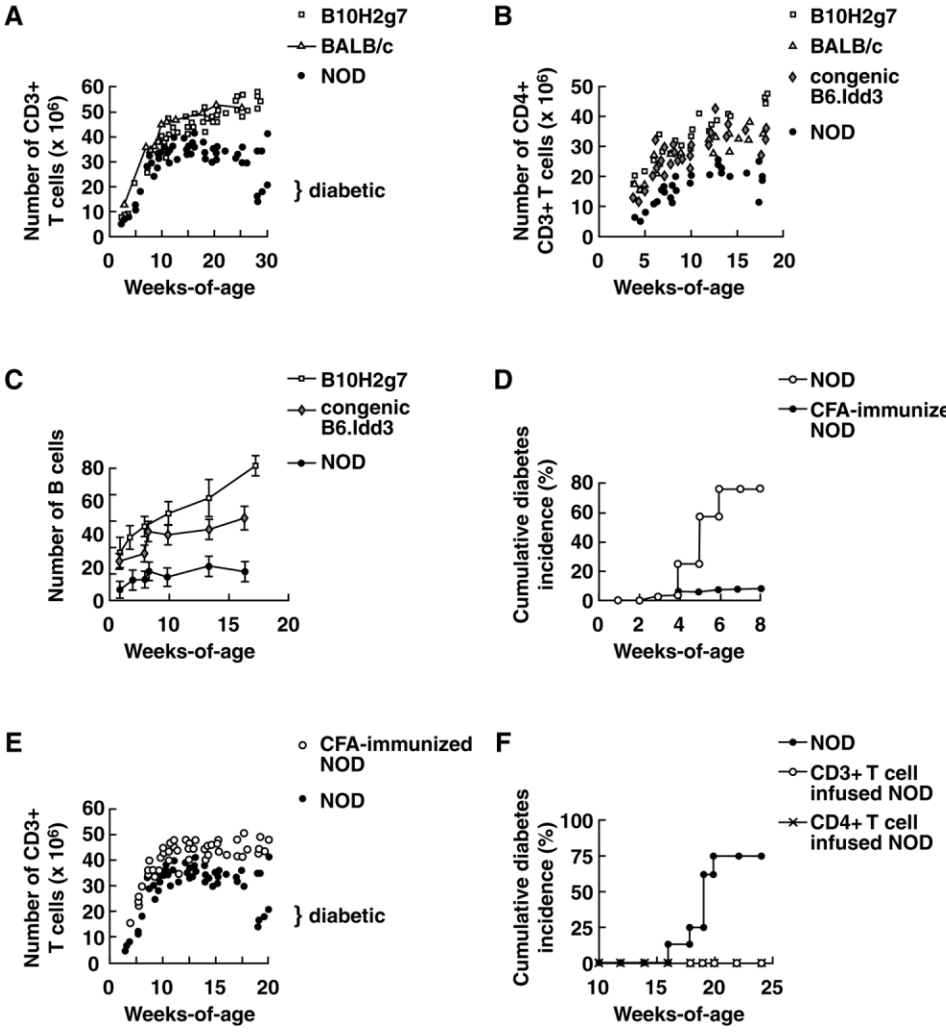


Figure 1. NOD Mice Exhibit Mild Lymphopenia

(A) Absolute T cell numbers are shown from the spleen of NOD (n = 59) compared to BALB/c (n = 36, p = 0.001) and B10H2$^{g7}$ (n = 56, p < 0.001) from 4 weeks of age to 30 weeks of age. (B) Absolute CD4+ T cell numbers are shown from the spleen of NOD compared to B10H2$^{g7}$ (p < 0.001) and congenic-B6.Idd3.NOD mice (n = 21, p < 0.001) and (C) absolute B cell numbers. (D) Immunization with CFA protects NOD mice from diabetes. (E) T cell numbers are increased following immunostimulation with CFA. Absolute T cell numbers shown for the spleen of NOD (n = 59) and NOD mice that had been immunized with CFA (n = 58, p = 0.002). (F) Cumulative incidence of diabetes showing the protective effect of syngeneic infusion of CD4+ T cells into NOD mice (n = 6).

CFA, which is composed of immune-activating mycobacterial cell wall constituents, protects NOD mice from developing diabetes. We tested the idea that such immunization may lead to an increase in lymphoid cell numbers, thereby curtailing disease development. Indeed, our results show that CFA-mediated protection is associated with both acute and long-lasting increases in T cell numbers in NOD mice (Figure 1E). This result shows a correlation between immunostimulation, increased T cell numbers, and disease protection. To ask whether simply increasing T cell numbers would protect from diabetes in NOD mice, we injected NOD mice with $2 \times 10^7$ T cells or $1 \times 10^7$ purified CD4+ T cells from their NOD littermates. We found that infusion with excess T cells prevented diabetes development (Figure 1F). This experiment demonstrates that enhanced T cell numbers, per se, inhibit the development of autoimmunity.

### Lymphocytes Expand in the Available Space

To test whether the lymphopenic environment in NOD mice promotes homeostatic proliferation, we probed the expansion and cell surface characteristics of experimentally marked T cells in NOD. Pancreatic β cell-specific TCR Tg NOD T cells were labeled with 5- and 6-carboxy-fluorescien succinimidyl ester (CFSE), which halves in intensity at each cell division, and then transferred into NOD, congenic-B6.Idd3.NOD, CFA-immunized NOD, and NOD mice that had been experimentally "filled" with syngeneic T cells. The TCR transgenic T cells (Verdaguer et al., 1997) proliferated in the lymph nodes of NOD mice as shown by multiple peaks of CFSE (Figure 2A). In contrast, the TCR transgenic T cells proliferated poorly in the lymph nodes of the CFA-immunized NOD mice, experimentally filled NOD mice, or congenic-B6.Idd3 NOD mice. This demonstrated that the expansion only occurs in lymphopenic mice (Figure 2A). Fur-

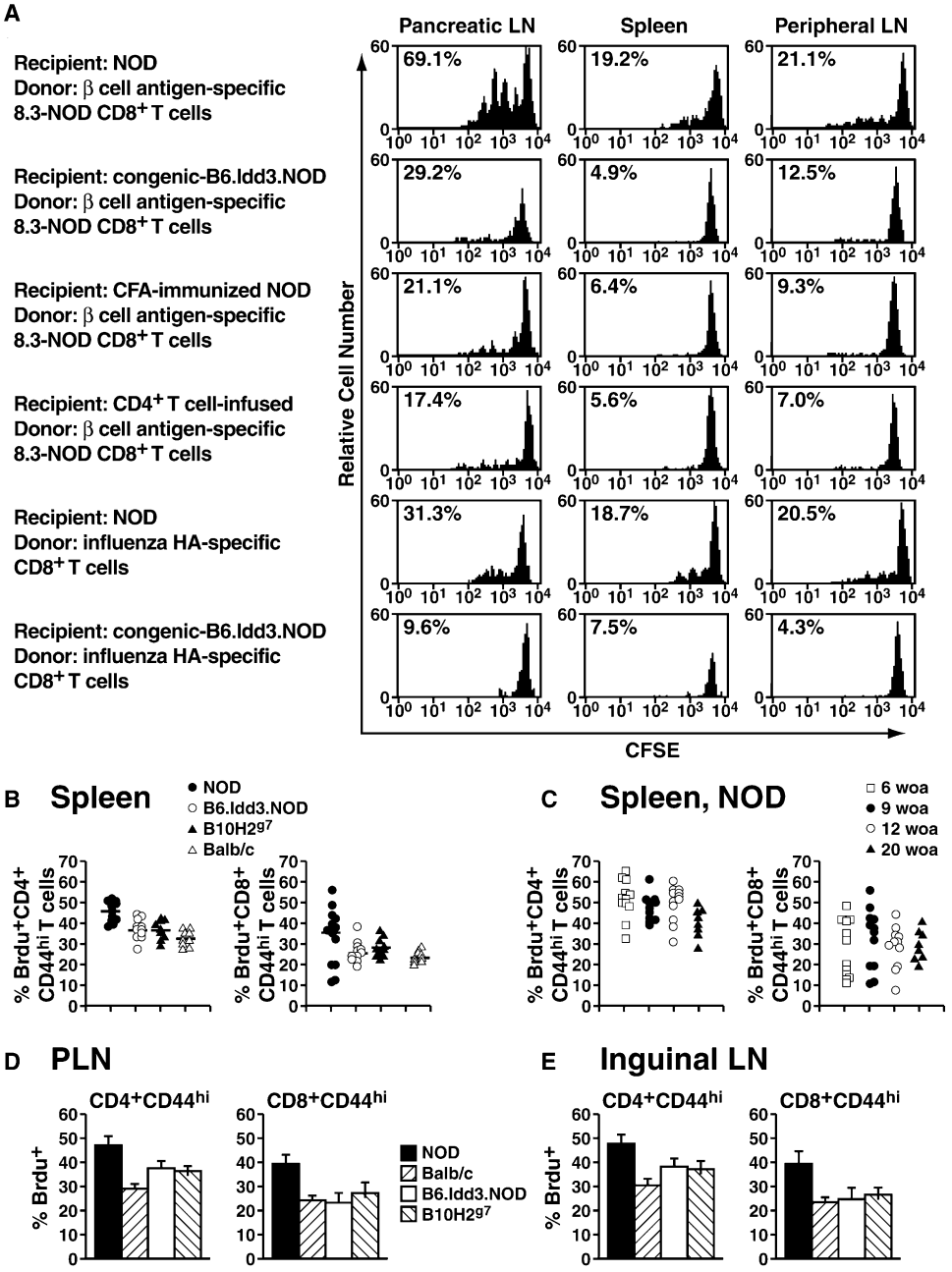


Figure 2. TCR Tg CD8+ T Cells Exhibit Increased Proliferation in NOD Hosts

(A) Proliferation profiles of CFSE-labeled β cell antigen-specific 8.3-NOD TCR Tg CD8+ T cells and TCR Tg NOD.HA CD8+ T cells recovered 3 days after transfer from the pancreatic lymph nodes, peripheral lymph nodes, and spleen of 11- to 12-week-old unmanipulated NOD, congenic-B6.Idd3.NOD, CFA-immunized NOD, or NOD hosts infused with syngeneic CD4+ T cells. CD8+CFSE+ populations are plotted in histogram format, and percentages of CFSE-labeled CD8+ T cells that have undergone one or more divisions are shown. Representative plots from three separate experiments are shown, n = 4–6 mice per group. Increased T cell proliferation in the secondary lymphoid organs of NOD mice. Flow cytometric analyses showing the percentages of BrdU+CD4+CD44hi and BrdU+CD8+CD44hi T cells from the spleen (B and C), pancreatic lymph nodes (PLN) (D), and inguinal lymph nodes (inguinal LN) (E) of 8- to 10-week-old mice (n = 10–14 mice/group from five separate experiments) after 8 days on BrdU water. NOD mice have a greater percentage of CD4+CD44hi T cells and CD8+CD44hi T cells proliferating in secondary lymphoid organs compared with congenic-B6.Idd3.NOD ($p = 0.005$, $p = 0.028$) or B10H2$^{g7}$ ($p = 0.018$, $p = 0.03$) or BALB/c mice ($p = 0.008$, $p = 0.024$, respectively). For the lymph nodes analyses, data is presented as the means plus standard deviations from five separate experiments.

thermore, influenza hemagglutinin-specific (HA) TCR transgenic T cells also underwent division in NOD mice (Figure 2A). The increased proliferation in NOD mice compared with the B6.Idd3.NOD shows that genes within the Idd3 locus of the nonautoimmune-prone B6 mice regulate this expansion.

Fortuitously, homeostatically expanding T cells do not bear the same cell surface markers as conventionally

activated T cells, which express high levels of CD25 (IL-2 receptor) and CD69 but have low levels of CD62L, which is cleaved upon conventional activation. Therefore, to distinguish between these two types of T cell expansion, we performed phenotyping experiments. Interestingly, our results show that NOD mice exhibited few T cells undergoing conventional activation. This phenotype was evident for T cells from all of the lymph nodes of NOD mice, with the exception of the lymph nodes that drain the pancreatic site of autoimmune destruction (pancreatic LN), where the proportion of conventionally activated CD4+ T cells was the same as control mice. In contrast, the strains exhibiting no diabetes had higher numbers of conventionally activated T cells (see Supplemental Table S1 at http://www.cell.com/cgi/content/full/117/2/265/DC1). These results suggest that a significant fraction of the T cells in the NOD autoimmune strain expand homeostatically.

Since we have observed lymphopenia in NOD mice, we next asked whether this influenced the turnover of endogenous T cells in the NOD mice. Groups of BALB/c, NOD, B10H2$^{g7}$, and congenic-B6.Idd3.NOD mice ranging from 6 weeks of age up to 5 months of age were given the nucleotide analog BrdU (Tough and Sprent, 1994). Indeed, we found a much greater percentage of T cells proliferating in NOD mice compared with BALB/c, B10H2$^{g7}$, or congenic-B6.Idd3.NOD mice (Figures 2B–2E). This increased percentage of proliferating T cells was observed in NOD mice from 6 to 20 weeks of age (Figure 2C).

### Homeostatic Expansion Correlates with Disease

We next determined whether increased T cell expansion correlated with lymphocyte infiltration into the pancreatic islets. Our analysis revealed that NOD mice with the greatest fraction of T cells proliferating in lymphoid organs exhibited the greatest fraction of islets with insulitis (Figures 3A and 3C), and those mice with infiltration around the islets (peri-insulitis) had the greatest fraction of CD4+ T cells proliferating locally in the pancreatic lymph nodes (Figure 3B). The degree of most severe destructive inflammation, obscuring the entire islet, correlated with the fraction of rapidly dividing CD8+ T cells (Figure 3D).

We then asked whether exposure to CFA could block the vigorous T cell expansion we observed in NOD mice. Indeed, we found that CFA decreased the fraction of proliferating CD8+ T cells and CD4+ T cells relative to control littermates (Figure 3E). Interestingly, in conjunction with this finding, there was a significant reduction in the percentage of islets infiltrated with lymphocytes (Figure 3F and data not shown), accompanied by an increased fraction of islets surrounded by benign inflammation (Figure 4F). Our data supports the notion that increased T cell numbers buffer the expansion of self-reactive T cells.

### Rapidly Proliferating T Cells Are Pathogenic

Homeostatically dividing T cells can eventually acquire functional features of pathogenic T cells after multiple rounds of replication (Surh and Sprent, 2000). We asked whether the proliferating T cell population in NOD mice could become tissue destructive. Interestingly, the rapidly proliferating CD44+ T cells, concentrated in the S-G2/M fraction (Figure 4A), induced lysis of purified islet cells from NOD mice (Figure 4B) but did not lyse control MHC-mismatched islet cells (data not shown). In contrast, quiescent T cells did not induce lysis of islet cells from either strain (Figure 4B and data not shown). More importantly, the actively proliferating (S-G2/M) T cell population rapidly induced diabetes in vivo (Figure 4C). Clearly, the T cells undergoing rapid turnover demonstrated specific self-tissue-destructive capability.

### Increased Expression of IL-21 and Its Receptor in NOD Mice

Residual T cells in lymphopenic environments may be confronted with excess quantities of T cell cytokines. Of particular importance are the shared common $\gamma$ chain binding cytokines that mediate T cell expansion and survival, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (Asao et al., 2001; Leonard et al., 1995). To define the proliferative milieu of the autoimmune-prone T cells and thus understand the forces governing their regulation, we examined the steady-state expression of the common $\gamma$ chain (gc) (CD132), the $\beta$ chain of the IL-2 and IL-15 receptors (CD122), and the receptor for interleukin-21 (IL-21R) on T cells from NOD mice and the congenic-B6.Idd3 (Table 1). IL-21 is a recently identified $\gamma$c-signaling cytokine (Parrish-Novak et al., 2000) and is a putative susceptibility gene in the Idd3 locus (Todd and Wicker, 2001). IL-21 is produced by activated CD4+ T cells and costimulates T cell proliferation (Parrish-Novak et al., 2000).

Interestingly, we found that a greater percentage of T cells from NOD mice express the IL-21R as compared with congenic-B6.Idd3.NOD mice (Table 1). In addition, the IL-21R was upregulated on T cells from NOD mice as compared with the other control strains and CFA-treated mice (Figure 5A). This was particularly striking in the memory T cell population (Figure 5A). Indeed, 2- to 3-fold more memory-phenotype T cells expressed the IL-21R in NOD mice as compared with the congenic-B6.Idd3.NOD strain (Table 1). In contrast, the percentages of T cells expressing CD132 and CD122 were decreased as compared with the congenic- B6.Idd3 mice (Table 1). These findings indicate that the protective B6.Idd3 locus, or immunization with CFA, alters the expression of receptors for the common $\gamma$ chain binding cytokines in NOD mice. Differential receptor expression would be expected to modify the responsiveness of T cells to survival-promoting cytokines.

The presence of the gene for IL-21 within the Idd3 susceptibility locus might implicate this cytokine in the disease process. Indeed, increased expression of the receptor for IL-21 on NOD T cells suggests differences in exposure to IL-21. To analyze expression of IL-21, we used interval-specific RT-PCR TaqMan to determine relative IL-21 expression levels. Our results show that unmanipulated lymphocytes from 60% of NOD mice tested ($n = 16$) expressed levels of IL-21 mRNA that were 4- to 7-fold greater than the congenic-B6.Idd3.NOD and C57BL/6 mice (Figure 5B). Assessment of IL-7 mRNA levels, done in parallel, showed an upregulation of IL-7

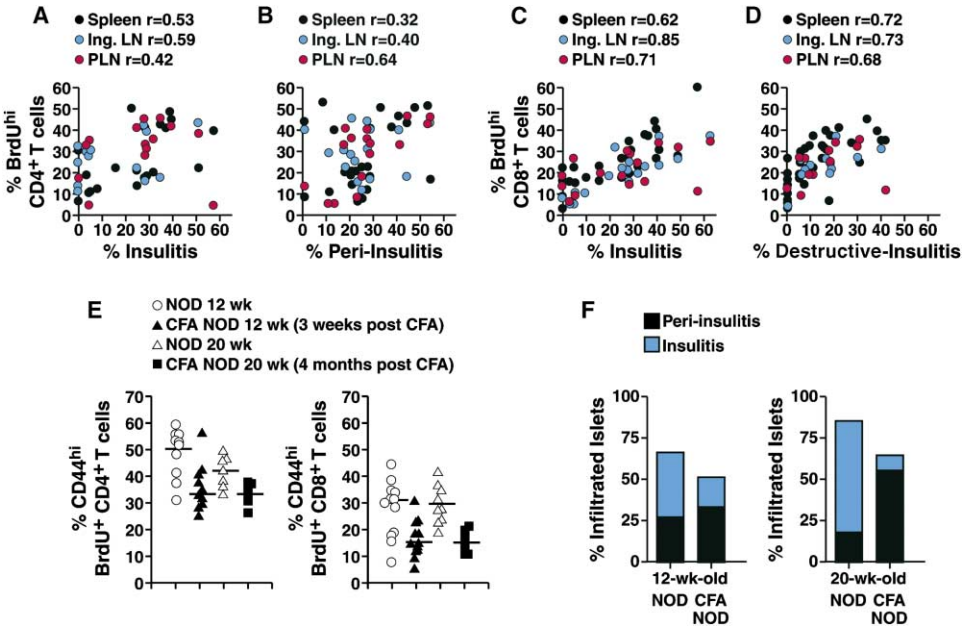


Figure 3. Proliferation of CD44hi T Cells in Secondary Lymphoid Organs of NOD Mice Correlates with the Type and Severity of Islet Infiltration

Analyses of pancreatic sections stained for insulin (n = 120 islets/group) showing the fraction of islets with peri-insulitis, insulitis (total), and destructive insulitis (loss of insulin staining). Correlation of the proliferation of CD4+ CD44hi T cells in the secondary lymphoid organs of NOD mice with (A) insulitis and (B) peri-insulitis of the pancreatic islets of Langerhans. Proliferation of CD8+ CD44hi T cells in the periphery directly correlates with (C) insulitis and (D) destructive insulitis. (E) Immunization with CFA causes a significant reduction in the proliferation of CD4+ CD44hi T cells ($p = 0.05$) and CD8+CD44hi T cells ($p = 0.026$) in the periphery of NOD mice. Percent BrdU+CD4+CD44hi and BrdU+CD8+CD44hi T cells are shown from the spleens of 12-week-old NOD mice (n = 12) versus 12-week-old CFA-immunized NOD mice (n = 12) and 20-week-old NOD mice (n = 10) versus 20-week-old CFA-immunized NOD mice. (F) Insulitis indices from sections of pancreata from NOD mice and CFA-immunized NOD mice, stained for insulin and glucagon, showing percent islets exhibiting peri-insulitis ($p = 0.016$) or insulitis ($p = 0.008$) (n = 112 islets per group).

in only 30% of NOD mice tested (Figure 5B). Importantly, we found that IL-21 exposure leads to upregulation of its own receptor (Figure 5C). In this regard, excess IL-21 in NOD mice would lead to an (autocrine) increase in the number of IL-21 receptor-expressing T cells, enhancing the responsive population. In support of this,

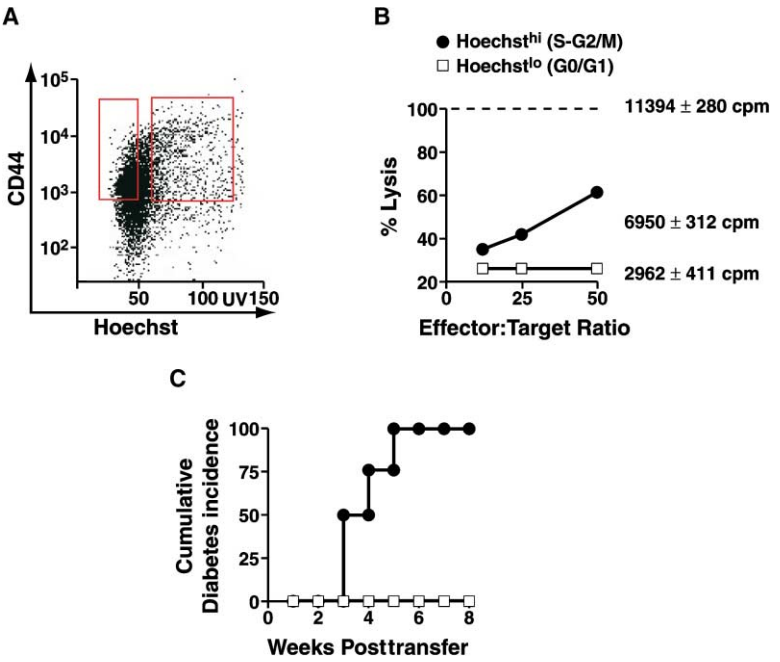


Figure 4. Actively Proliferating T Cells Cause Diabetes

CD44+ T cells were sorted into two populations based upon the linear staining intensity of a cell-permeable DNA (Hoechst 33342) dye (Sitnicka et al., 1996). (A) Dot plot showing CD44-PE immunofluorescence on the y axis and Hoechst 33342-UV fluorescence on the x axis. Gates are shown for quiescent (G0/G1) T cells and actively proliferating (S-G2/M) T cells. (B) Percent lysis of islet cell targets labeled with chromium. Shown is lysis of islet cell targets above a background of 26.3% spontaneous lysis. Results are the mean values from two separate experiments, in which n = 5 mice. (C) Adoptive transfer of CD44int-hi T cells in G0/G1 and S-G2/M phases ($p < 0.001$) from NOD mice (n = 8 mice) into NOD/scid recipients (n = 5 mice) from three separate experiments.

Table 1. Percentages of CD122, CD132, and IL-21R-Expressing T Cells

| | | CD122 | CD132 | IL-21R |
|---|---|---|---|---|
| Spleen | | | | |
| NOD | CD4+CD44hi | 10.2 + 2.0 | 53.7 + 5.7* | 23.3 + 2.6* |
| | CD4+CD44lo | 2.1 + 0.1 | 40.3 + 2.8* | 10.1 + 2.1 |
| Congenic-B6.Idd3 | CD4+CD44hi | 11.3 + 1.2 | 62.7 + 3.3* | 12.2 + 1.4* |
| | CD4+CD44lo | 1.6 + 0.08 | 57.1 + 8.2* | 6.6 + 1.2 |
| NOD | CD8+CD44hi | 26.0 + 3.8* | 58.5 + 5.3* | 30.1 + 4.0* |
| | CD8+CD44lo | 5.7 + 1.1* | 51.3 + 4.4* | 7.1 + 2.9 |
| Congenic-B6.Idd3 | CD8+CD44hi | 36.2 + 2.7* | 70.3 + 6.7* | 11.5 + 2.1* |
| | CD8+CD44lo | 10.5 + 0.9* | 69.6 + 8.1* | 4.0 + 0.5 |
| PLN | | | | |
| NOD | CD4+CD44hi | 1.4 + 0.3* | 45.0 + 4.8* | 16.8 + 3.3* |
| | CD4+CD44lo | 0.3 + 0.02 | 34.1 + 2.6* | 7.2 + 1.7* |
| Congenic-B6.Idd3 | CD4+CD44hi | 8.1 + 1.1* | 60.9 + 4.9* | 8.8 + 1.7* |
| | CD4+CD44lo | 2.6 + 0.4 | 52.2 + 3.8* | 2.4 + 1.1* |
| NOD | CD8+CD44hi | 32.8 + 1.5* | 64.3 + 5.9 | 4.7 + 1.8* |
| | CD8+CD44lo | 7.8 + 1.2* | 60.2 + 5.5* | 2.2 + 1.4* |
| Congenic-B6.Idd3 | CD8+CD44hi | 24.7 + 1.7* | 58.6 + 7.2 | 11.6 + 1.2* |
| | CD8+CD44lo | 3.6 + 0.8* | 45.3 + 4.0* | 4.0 + 0.3* |

Percentages of CD122, CD132, and IL-21R-expressing T cells are shown from the spleen and PLN of 9-week-old NOD and congenic-B6.Idd3 (n = 8 mice per group). Results are the mean values from two separate experiments ± SEM, rounded to the nearest integer.
*Values are significantly different between NOD and the congenic B6.Idd3.NOD (Anova).

we observed that a significantly greater percentage of T cells had proliferated in NOD mice in response to intravenously administered IL-21 than in CFA-immunized NOD or the congenic-B6.Idd3.NOD mice (Figure 5D).

### Reduced Expression of Prosurvival Proteins

Regulation of T cell survival is a critical axis for the maintenance of cell numbers, most notably for long-lived memory T cells. Indeed, lymphopenia-inducing conditions and subsequent homeostatic proliferation result in a biased and permanent loss of long-lived virus-specific memory CD8+ T cells (Peacock et al., 2003). The mitochondrial protooncogenes Bcl-2 and Bcl-X(L) delay entry into the cell cycle (O'Reilly et al., 1996), and their expression is thought to contribute to the slow turnover of memory T cells (Zhang et al., 2002). The levels of the prosurvival proteins Bcl-2 and Bcl-X(L) are increased upon exposure to several cytokines that signal through the γc (such as IL-7 and IL-15) and are maximally increased in memory CD8+ and CD4+ T cells, respectively (Akbar et al., 1996; Grayson et al., 2000). In support of this, we found that in disease-resistant B10H2$^{g7}$, B6.Idd3.NOD, and CFA-immunized NOD, the levels of Bcl-2 and Bcl-X(L) in memory T cell populations were greater than those levels observed in naive T cells (Figures 6A and 6B). In contrast, NOD mice exhibited only a negligible increase in Bcl-2 or Bcl-XL in their memory T cells (Figures 6A and 6B, respectively). These results demonstrate a deficit of prosurvival molecules in memory T cells from NOD mice that would lead to a paucity of long-lived memory T cells.

### IL-21R-Expressing T Cells Are Short Lived

Since IL-21 is augmented in the NOD strain, we determined whether IL-21 directly influenced the survival of T cells and whether the IL-21-responsive T cells were phenotypically distinct in terms of their rate of expansion and expression of genes involved in cell survival. To directly compare the survival functions of IL-21 with a known T cell survival-promoting γ chain cytokine, namely IL-7, we performed in vitro studies. IL-21 did not support the survival of T cells at any of the concentrations used (Figure 6C). In contrast, IL-7 strongly promoted the survival of CD8+ T cells over the 3 day culture period but had a lesser effect on CD4+ T cells (Figure 6C).

IL-21R+ T cells are a rapidly proliferating population, as shown by incorporation of BrdU into the majority (>80%) of these cells over a 2 day pulse (Figure 6D, Table 2). Their propensity to enter the cell cycle was matched by a reduction in the levels of Bcl-2 and Bcl-XL and increased staining for a preapoptotic marker (Annexin-V), compared to T cells that did not express the IL-21R (Table 2). In addition, a significantly increased percentage of IL-21R+ T cells expressed IFN-γ, the cytokine that defines autoimmune effector function (Table 2). This effector phenotype was more pronounced in IL-21R+ T cells from NOD mice, which may reflect increased exposure to IL-21. Taken together, our results show that the IL-21R+ T cells in NOD mice are a highly unstable effector population and the source of the phenotypic differences that we observed in T cells from NOD mice.

IL-21 has been reported to signal distinctly through the common γ chain compared with other strong prosurvival cytokines such as IL-7 and IL-15, activating Stat1 and Stat3 but not Stat5 (Bennett et al., 2003). We found that phosphorylated Stat3 (P-Stat3) was significantly (4- to 8-fold) augmented in the IL-21R-expressing T cell population compared with T cells that did not express IL-21R in NOD mice (Table 2). This was most striking in the CD8+ T cells in the PLN of NOD mice (Table 2). However, we did not observe a corresponding increase in the percentage of T cells containing phosphorylated Stat5 in NOD mice (data not shown). In contrast, P-Stat3 was not dependent on IL-21 receptor expression in the congenic-B6.Idd3.NOD strain (Table 2). Therefore, the

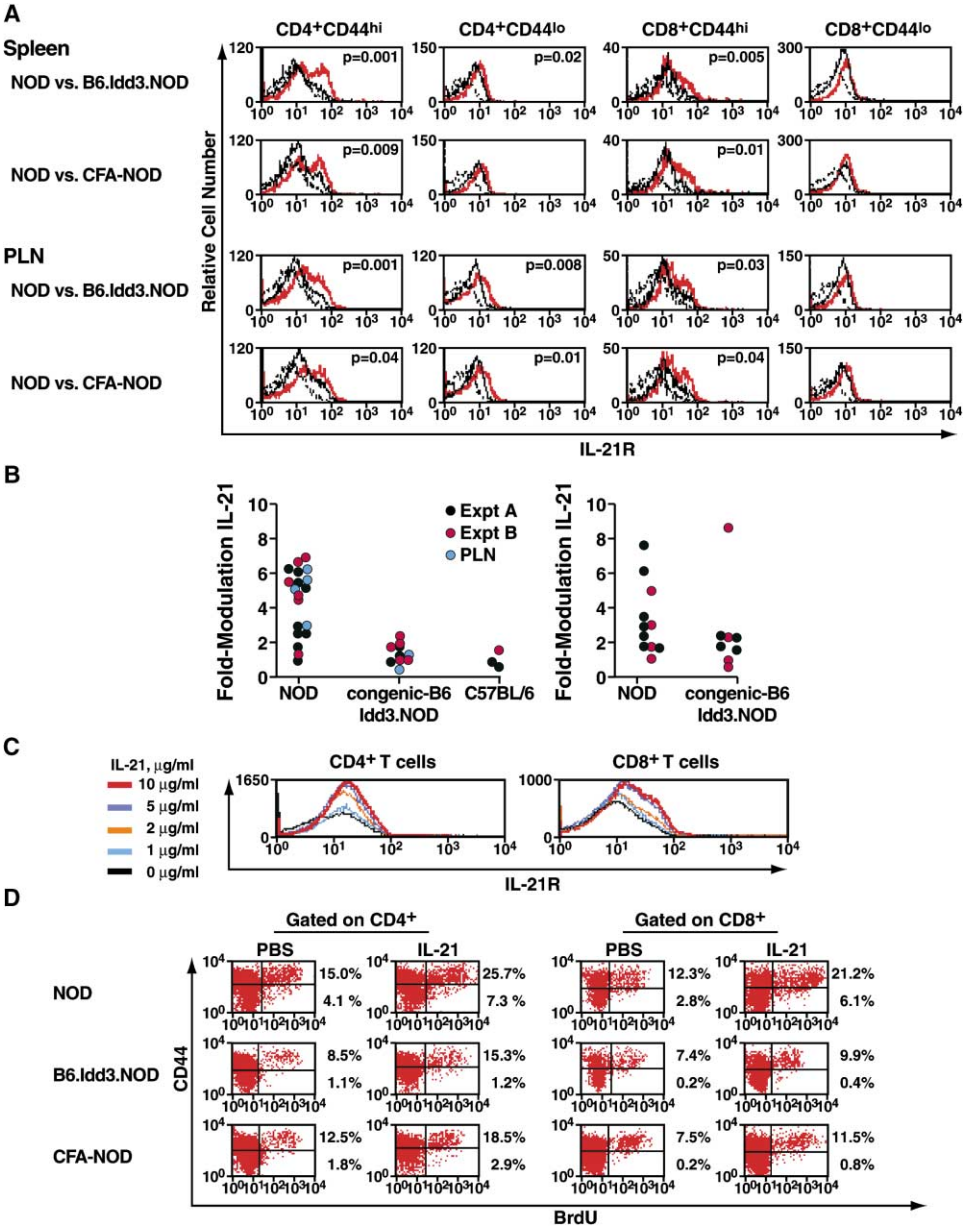


Figure 5. Increased Expression of IL-21 and Its Receptor in NOD Mice

Histogram plot overlays showing expression of (A) the receptor for IL-21 on naive (CD44lo) and activated/memory (CD44hi) CD4+ and CD8+ T cells from the spleen and pancreatic lymph nodes of 8-week-old NOD (red line), compared to age-matched congenic-B6.Idd3.NOD (black line) or age-matched CFA-immunized NOD mice (black line). Dotted line shows background staining from specificity control in which cells were pretreated with excess unlabeled IL-21. Representative plots from four separate experiments in which n = 12 mice per group. p values indicate a significant difference (in which $p < 0.05$) in the percentage of T cells expressing the IL-21 receptor. (B) Real-time quantitative PCR analyses showing fold modulation of cytokine mRNA from three separate experiments: two from whole spleen RNA (black and red circles) and one from the pancreatic lymph nodes (blue circles) of individual NOD (n = 18), congenic-B6.Idd3.NOD (n = 11), and C57BL/6 (n = 3) mice. (C) Induction of the IL-21 receptor by its ligand. Histogram plots showing the induction of the IL-21 receptor on CD4+ and CD8+ T cells from C57BL/6 mice stimulated for 2 days with anti-CD3 monoclonal antibody and increasing concentrations of rmIL-21. Data are representative plots from two separate experiments. (D) Flow cytometric analyses of NOD, congenic-B6.Idd3.NOD, and NOD mice that had been neonatally immunized with CFA (n = 8 mice per group). Representative plots from three separate experiments show percentages of CD4+ and CD8+ T cells that had incorporated BrdU after intravenous administration of 100 ng of rmIL-21 and BrdU water for 2 days plotted against the activation/memory marker CD44.

IL-21R+ T cells bear the signature hallmarks of exposure to their ligand. These studies indicate that the signal of distinction in the NOD mouse is IL-21, the culprit creating the unstable lymphoid microenvironment in NOD mice.

## Discussion

Our findings indicate that the state of lymphopenia is a factor that drives the development of autoimmunity.

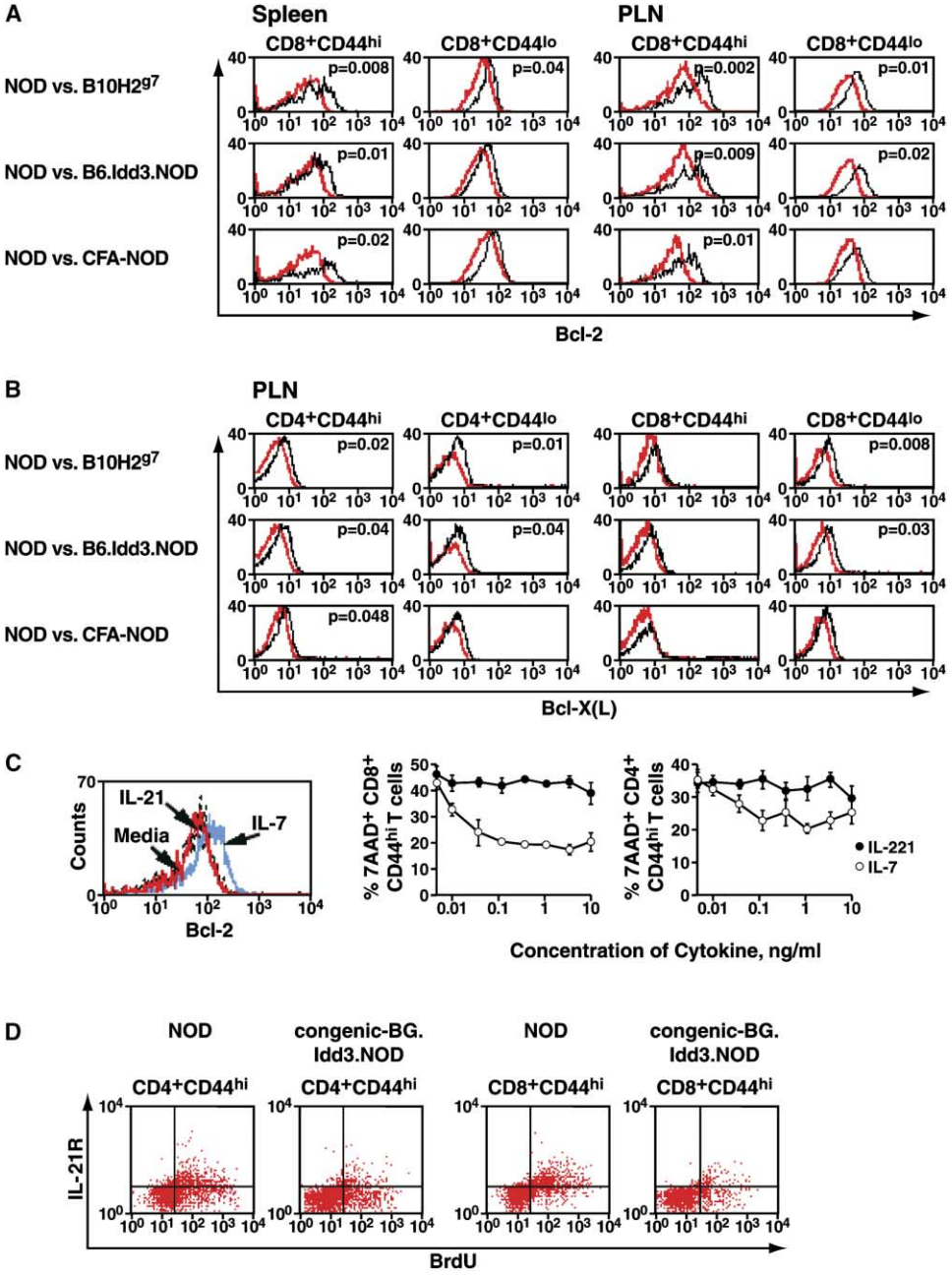


Figure 6. Decreased Survival Signals in NOD T Cells

Levels of (A) Bcl-2 and (B) Bcl-X(L) in naive (CD44lo) and activated/memory (CD44hi) CD4+ and CD8+ T cells from the spleen and pancreatic lymph nodes of 7-week-old NOD mice (red line), compared with age-matched B10H2$^{g7}$ mice, congenic-B6.Idd3.NOD strain, or CFA-immunized NOD mice (black lines). Representative plots from four separate experiments are shown (n = 14 mice per group). p values are shown where there is a significant difference (where $p < 0.05$) from NOD. (C) Histogram overlays for Bcl-2 levels in CD3+ T cells from C57BL/6 mice cultured for 3 days with either 1 ng/ml of IL-21, 1 ng/ml of IL-7, or media alone. Shown are the percentages of CD4+ and CD8+ T cells that incorporated the vital dye 7-AAD after 3 days with or without increasing concentrations of rmIL-7 or IL-21, determined from flow cytometric analyses of live cells. Representative plots from triplicate determinations showing mean ± SEM from three separate experiments. (D) The IL-21 receptor distinguishes the population of actively proliferating T cells in NOD mice. Representative plots from four separate experiments show percentages of CD4+ CD44hi and CD8+ CD44hi T cells that express the receptor for IL-21 and are actively proliferating as determined by incorporation of BrdU over a 2 day pulse.

NOD mice harbor a labile population of actively expanding T cells within the depleted memory niche. They are distinguished from typical memory T cells by a more rapid turnover and by reduced levels of prosurvival proteins. Indeed, this population of actively proliferating T cells in NOD mice is distinguished by their expression of the receptor for the costimulatory cytokine IL-21, indicating that increased expression of IL-21 drives this

Table 2. Percentages of IL-21 Receptor-Expressing or Nonexpressing Memory Phenotype CD4+ and CD8+ T Cells

| CD4+CD44hi T Cells | NOD | B6.Idd3.NOD | CD8+CD44hi T Cells | NOD | B6.Idd3.NOD |
|---|---|---|---|---|---|
| IL-21R+BrdU+ | 84.1 ± 8.8 | 78.3 ± 4.1 | IL-21R+BrdU+ | 81.3 ± 9.2 | 78.3 ± 12.8 |
| BrdU+IL-21R+ | 34.3 ± 4.1* | 18.1 ± 1.1* | BrdU+IL-21R+ | 54.5 ± 6.2* | 26.1 ± 3.3* |
| IL-21R+IFN-g+ | 10.8 ± 1.4* | 3.6 ± 0.8* | IL-21R+IFN-g+ | 21.5 ± 2.8* | 8.1 ± 0.7* |
| IL-21R−IFN-g+ | 0.2 ± 0.06 | 0.6 ± 0.1 | IL-21R−IFN-g+ | 1.9 ± 0.7 | 1.3 ± 0.3 |
| IL-21R+Annexin+ | 48.3 ± 2.9* | 25.7 ± 4.2* | IL-21R+Annexin+ | 52.6 ± 11.6* | 10.2 ± 1.1* |
| IL-21R−Annexin+ | 4.8 ± 0.9 | 5.0 ± 0.4 | IL-21R−Annexin+ | 1.5 ± 0.6 | 0.4 ± 0.2 |
| IL-21R+Bcl-XL+ | 13.3 ± 1.6* | 21.4 ± 3.2* | IL-21R+Bcl-2+ | 19.9 ± 2.4* | 31.1 ± 3.0* |
| IL-21R+P-Stat3+ | 7.7 ± 0.7* | 12.3 ± 2.1* | IL-21R+P-Stat3+ | 18.6 ± 1.4 | 18.9 ± 1.5 |
| IL-21R−P-Stat3+ | 0.9 ± 0.6* | 6.7 ± 0.5* | IL-21R−P-Stat3+ | 3.0 ± 0.6* | 9.3 ± 1.6* |
| IL-21R+P-Stat3+ (PLN) | 11.4 ± 1.9 | 11.1 ± 2.1 | IL-21R+P-Stat3+ (PLN) | 21.6 ± 1.9* | 9.2 ± 1.7* |
| IL-21R−P-Stat3+ (PLN) | 1.1 ± 0.1* | 5.7 ± 0.1* | IL-21R−P-Stat3+ (PLN) | 1.6 ± 1.2 | 2.6 ± 0.3 |

The percentages of IL-21R-expressing (IL-21R+) or nonexpressing (IL-21R−) memory phenotype (CD44hi) CD4+ and CD8+ T cells that are proliferating (BrdU+), apoptotic (Annexin-V), or express interferon-gamma (IFN-g), the prosurvival molecules Bcl-2 or Bcl-XL, or the phosphorylated form of Stat3 (P-Stat3) are shown. T cells were analyzed from the spleen and pancreatic lymph nodes of 8-week-old NOD, CFA-immunized NOD (5 weeks after CFA administration), and congenic-B6.Idd3 (n = 14 mice per group). Results are the mean values ± SEM from three separate experiments (n = 11 mice/group).

*Value is significantly different ($p < 0.05$) between NOD and the congenic-B6.Idd3.NOD strains.

population of unstable, self-tissue-destructive T cells. We hypothesize that a consequence of these anomalies is that the constricted NOD T cell pool is unable to buffer the expansion of self-tissue-responsive T cells nurtured by robust homeostatic factors. This concept is compatible with the idea that regulation of self-tissue-destructive T cells occurs via competition for growth-inducing resources (Ernst et al., 1999; Kedl et al., 2000). Indeed, the cytokines and endogenous protein antigens that govern homeostatic expansion may drive the development of tissue-destructive T cells.

Importantly, we show that immune stimulation causes an increase in T cell numbers and creates a population of long-lived memory T cells that are otherwise absent from the NOD strain. Indeed, an extensive list of stimulatory factors is reported to reduce the incidence of diabetes in NOD mice, including the following: persistent viral infection, mycobacterial infection, complete (heat-killed mycobacterium-containing) Freund's adjuvant (CFA), and other bacterial antigens (Rabinovitch, 1994). The "clean" domestic environments of Western societies resemble the specific pathogen-free conditions under which NOD mice are housed. This concept of hygiene developed from the original ideas of Oliver Wendell-Holmes, Sr., and Ignaz Philipp Semmelweis in the 1840s (Greenhill, 1966) and was subsequently popularized by Louis Pasteur with his "germ theory of disease" (Mendelsohn, 2002). However, the increase in autoimmunity in Western societies in recent years (Kukreja and Maclaren, 1999; Kwoh, 1992) may be compounded by the zealous use of disinfectants and antibiotics during the 20th century. Indeed, protection from diabetes by immunostimulation with bacterial antigen or infection parallels the state of non-Western societal contexts and reflects the fact that microinoculations can beneficially instruct the maturation of the immune system in infancy (Holt, 1998).

A variety of autoimmmune diseases in humans, including IDDM (Kaaba and Al-Harbi, 1995), rheumatoid arthritis, systemic lupus erythematosus, Sjogren's syndrome, ankylosing spondylitis, and Coeliac disease (Di Sabatino et al., 2001), can present with lymphopenia (Sleasman, 1996). Furthermore, autoimmune diseases in humans are precipitated following infection with T cell-depleting viruses (Bateman et al., 1999; Hernan et al., 2001; Schaller, 1975) or irradiation and other immunosuppressive therapies (Issacs et al., 2001). One broad conclusion of this study is that the NOD mouse may be included in the unifying theme of lymphopenia-associated autoimmune disease (Gleeson et al., 1996; Theofilopoulos et al., 2001). Previous studies collectively demonstrate that T cell depletion and its associated expansion and repopulation of the peripheral lymphoid compartments lead to increased severity and incidence of IDDM (Dardenne et al., 1989; Sai et al., 1994). In our T cell transfer studies, we show that a relatively small number of syngeneic T cells can prevent diabetes in recipient mice. However, we cannot rule out a possible effect from the cotransfer of environmental antigens in these experiments. In addition to diabetes, other manifestations of autoimmunity in the NOD mouse and its genetically manipulated counterparts include mononuclear cell infiltration of the submandibular and lacrimal glands and the presence of circulating autoantibodies (Wicker et al., 1992) as well as autoimmune neuropathy and arthritis (Kouskoff et al., 1996; Salomon et al., 2001). Therefore, the lymphopenic NOD genetic background confers diverse self reactivity.

Increased expression of IL-21 mRNA suggests an inherent differential expression of this cytokine in autoimmune NOD mice, but it is equally possible that other genes in the Idd3 locus may contribute to increased expression of IL-21. A number of previous studies support the idea that increased responsiveness to IL-21 could play a causal role in generating the disturbed lymphoid compartments of NOD mice. In this regard, IL-21 has also been shown to promote apoptosis with an associated decrease in Bcl-2 and Bcl-XL levels in B cells (Mehta et al., 2003). IL-21 has been reported to antagonize the effects of the prosurvival cytokine IL-15 (Parrish-Novak et al., 2002). CD122 signaling is absolutely critical for memory CD8+ T cells, since it also confers responsiveness to IL-15, their required growth signal. Indeed, we observed that NOD memory CD8+ T cells have reduced expression of CD122 relative to CFA-immunized and congenic-B6.Idd3 mice. In addi-

tion, the finding that IL-21 and IL-4 bind overlapping epitopes on the common $\gamma$ chain (Zhang et al., 2003) suggests that increased levels of IL-21 may hinder IL-4 access, impairing lymphocyte survival (Marrack et al., 1998) and favoring a Th-1-type cytokine environment.

IL-21 signals through the common $\gamma$ chain, activating Stat1 and Stat3 in T cells. However, in contrast to both IL-7 and IL-15, IL-21 does not activate Stat5 (Bennett et al., 2003). In support of this, we observed an IL-21R-dependent increase in T cells containing P-Stat3 but not P-Stat5 in NOD mice. IL-7 and IL-15 mediate some of their effects on cell survival gene expression through the positive effect of Stat5 on Bcl-2 and Bcl-XL expression (Silva et al., 1999; Snow et al., 2003). These differences in intracellular signaling may contribute to our observation that, in direct contrast to the effects of IL-7, IL-21 had no effect on the survival of T cells in culture nor did it support Bcl-2 levels in T cells in vitro. IL-21 has been shown to costimulate T cell proliferation (Parrish-Novak et al., 2002) and thus may contribute to the high rate of proliferation of endogenous T cells in NOD mice. In this regard, short-term neutralization with polyclonal anti-mIL-21 substantially reduced the proliferation of $\beta$ cell antigen-specific CD8+ T cells in NOD hosts (data not shown). Taken together, these data suggest that IL-21 costimulation of T cell proliferation may occur without the promotion of survival or rescue signals, eventually leading to replicative senescence. IL-21 therefore becomes the dominant voice limiting access to critical survival signals and promoting the expansion of self-tissue-destructive T cells.

Our study complements an interesting previous report revealing expansion of autoreactive T cells from a reduced precursor pool in NOD mice (Serreze et al., 2001). We showed that actively dividing (memory-type) T cells of NOD mice are diabetogenic, a finding in contrast to some models in which autoimmune disease following homeostatic expansion of naive T cells is prevented by cotransfer of memory-phenotype T cells (Fowell et al., 1991; Powrie et al., 1993; Twarog and Rose, 1970). However, the T cells of NOD mice are distinguished from typical memory T cells by reduced levels of the prosurvival proteins Bcl-2 and Bcl-XL and increased proliferation. These diabetogenic T cells are rapidly cycling and short lived, a finding supported by another recent study showing that Bcl-2 overexpression in T cells prevents insulitis in NOD mice (Rietz et al., 2003).

Previous studies report reduced T cell numbers in secondary lymphoid organs of NOD mice (Kataoka et al., 1983; Satoh et al., 1988). However, our findings are in contrast to a recent report by Berzins et al. showing similar or increased T cells numbers in secondary lymphoid organs of NOD mice compared to a number of other nonautoimmune strains (Berzins et al., 2003). Further, that report showed that NOD mice had lymph nodes that were 20% larger than the other strains, and their T cells had an activated phenotype early in life (Berzins et al., 2003) that is not commonly observed (Green et al., 2002; Salomon et al., 2000; Szanya et al., 2002). One plausible explanation for differences between these studies is that the T cell numbers and their activation status reflect stimulation by environmental pathogens present in the colony. This is most important in the case of the NOD mouse, which has well-documented immune deficiencies (Kataoka et al., 1983). In support of this notion, young NOD mice from colonies with a low incidence of diabetes harbor T cells with a more activated/memory phenotype than do NOD mice with a high incidence of diabetes (Berzins et al., 2003; Green et al., 2002; Salomon et al., 2000). Indeed, delayed disease kinetics may reflect the presence of space-filling immunostimulatory pathogens that inhibit homeostatic expansion.

We propose that organ-specific autoimmunity is initiated by homeostatic proliferation of T cells driven by lymphoid space and costimulated by IL-21. Human autoimmune diseases are often associated with viral infections that disrupt T cell survival mechanisms, resulting in lymphopenia. By extension, the pool of host tissue constituents would determine which T cells repopulate the peripheral lymphoid organs (La Gruta et al., 2000). These straightforward parameters explain tissue-specific autoimmunity. In the context of the inefficient thymic function of NOD mice (Kishimoto and Sprent, 2001), the exaggerated proliferation of T cells represents unbridled self reactivity. We concur that a number of factors, such as local inflammation and lack of immune regulation, are likely to play a role in the end stages of T cell activation that precede clinical autoimmunity. However, in this study, autoimmune disease can be explained by the space-induced expansion of self-reactive lymphocytes, fueled by their own demise.

## Experimental Procedures

### Mice

Transgenic mice expressing a TCR specific for an epitope of HA that is restricted by MHC class I H-2Kd (Clone-4 TCR) (TCR Tg NOD.HA mice) (Morgan et al., 1996) were crossed onto NOD/shi for 14 generations and were a kind gift from Dr. Linda Sherman. 8.3-TCR transgenic NOD mice (8.3-NOD mice) (Verdaguer et al., 1997) were bred to NOD/scid and maintained in our colony. NOD Ltj mice were purchased from Jackson Laboratories, the congenic-C57BL/6.Idd3(R450).NOD mice were purchased from Taconic Farms, and B10H2$^{g7}$ mice were bred and housed in our colony. Blood glucose values (BGV) were determined using Glucofilm blood glucose strips (Miles Diagnostic, Elkhart, IN). All mice were housed together under specific pathogen-free (SPF) housing conditions at the Scripps rodent colony.

### Immunohistochemical Staining

Paraffin-embedded tissue sections were conventionally stained with H&E for histological evaluation or prepared for immunocytochemical techniques as previously described (Sarvetnick et al., 1990). For destructive insulitis (loss of insulin staining), insulin-stained sections of pancreata were matched to serial sections stained for glucagon.

### Immunostaining for Flow Cytometric Analyses

To determine absolute cell numbers, lymphocyte cell suspensions were prepared from spleen and lymph nodes, red blood cells (RBC) were removed by hypotonic lysis, and cells were counted on a Coulter counter. T cells were identified with monoclonal antibodies directed against surface markers (eBiosciences, BD PharMingen, La Jolla, CA). Intracellular staining for BrdU incorporation was performed according to the manufacturer's instructions (BrdU labeling-Kit BD PharMingen). Intracellular levels of Bcl-2 (BD PharMingen), Bcl-XL (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated Stat-3, phosphorylated Stat-5 (Upstate, Lake Placid, NY), and IFN-g (homegrown biotinylated XMG) were determined using standard procedures (King et al., 2001). Positively stained populations in each instance were determined by gating against isotype control antibodies.

### BrdU Labeling Experiments

Mice were given 0.8 mg/ml BrdU in sterile water each day for either 2 days or 8 days total (Tough and Sprent, 1994). For proliferation in response to IL-21, mice were injected intravenously with 100 ng of IL-21.

### Immunization with Complete Freund's Adjuvant

NOD mice were immunized subcutaneously at 3 weeks of age with 50 μg of complete Freund's adjuvant at the base of the tail.

### Syngeneic Infusion of T Cells

Five- to six-week-old NOD mice were injected intravenously with $2 \times 10^7$ CD3+ T cells or CD4+ T cells that had been sorted the previous day on a flow cytometer (FACS DIVA) from the spleen of NOD littermates.

### Adoptive Transfer of T Cells

Donor NOD.HA or 8.3-NOD CD8+ T cells were purified from the spleen and lymph nodes of 8-week-old TCR transgenic mice. Red cells were depleted by hypotonic lysis, and cells were washed and enriched for naive CD8+ T cells by complement depletion with anti-mouse CD4+ (YTS 191.1.2), followed by 2 hr adherence on plastic petri dishes and 2 hr adsorption on nylon wool columns (Poly-Sciences, Inc., Warrington, PA).

### 5- and 6-Carboxy-Fluorescien Succinimidyl Ester Labeling

Cells were incubated with 5 μM CFSE (Molecular Probes) in HBSS for 10 min at 37°C. Twelve-week-old normoglycemic NOD female hosts were injected intravenously with $5 \times 10^6$ CFSE-labeled CD8+ T cells/200 μl of sterile PBS.

### Hoechst Labeling of T Cells

CD3+ CD44int-hi T cells were positively sorted from pooled lymph node and splenocyte cell suspensions from individual 12- to 14-week-old normoglycemic NOD mice rested overnight and then incubated with 10 μM Hoechst 33342 (Molecular Probes) in complete RPMI for 20 min at 37°C. Cells were sorted in FACs buffer containing Hoechst 33342 on a UV laser-supported flow cytometer. $3 \times 10^6$ of either CD3+ CD44int-hi T cells in G0/G1 or S/G2/M phase were transferred intravenously into 8-week-old NOD/scid recipients.

### Cytotoxicity Assays

Islet cell lysis was determined in a 5 hr in vitro $^{51}Cr$ assay. Briefly, islets were hand sorted from the collagenase P-dispersed pancreata. After 5 days of culture in complete RPMI changed daily, islets were incubated in $Mg^{2+}$- and $Ca^{2+}$-free media and then a further 2 days in complete RPMI. Effectors were in the form of proliferating (S-G2/M) and quiescent (G0/G1) CD44int-hi T cells from the spleen of 12- to 14-week-old NOD mice.

### T Cell Survival Assay

To assess the effect of IL-21 on T cell survival, $1 \times 10^6$ T cells/ml from C57BL/6 mice were cultured alone or with 0–10 ng/ml murine rIL-21 (R & D Systems, Minneapolis, MN) or IL-7 (eBiosciences). Cell viability was assessed at the indicated times by staining with Annexin V-FITC and propidium iodide together with CD8-APC, CD4-APC, and CD44-PE mAbs.

### Detection of IL-21 Receptor

The cell surface receptor for IL-21 was detected with the use of biotinylated IL-21. rmIL-21 (200 μg) (R & D Systems, Minneapolis, MN) was biotinylated at a 1:12 ratio (IL-21:biotin-DNP) as previously described (Armitage et al., 1990). Biotinylated IL-21 was used at 3 μg/ml.

### IL-21R Induction

Splenocytes from C57BL/6 mice were cultured ($5 \times 10^6$/ml) for 2 days in the presence of 0.1 μg/ml of anti-CD3 monoclonal antibody and either with or without increasing concentrations of recombinant murine IL-21 (R & D Systems, Minneapolis, MN).

### Reverse Transcription and Real-Time Quantitative PCR

Total RNA was extracted using the Qiagen RNAeasy procedure (Qiagen, Valencia, CA). Complementary DNA (cDNA) was generated from total RNA using a random hexamer primer, Superscript II (Invitrogen, Carlsbad, CA). Real-time quantitative PCR analyses were performed with TaqMan chemistry (Applied Biosystems, Foster City, CA) and the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Duplicate PCR reactions were cycled at 50°C for 2 min and 95°C for 10 min, then at 95°C for 15 s and 60°C for 1 min in 45 cycles. Real-time PCR primers were designed with Primer Express 1.5 (PE Biosystems) software. Values for IL-7 and IL-21 were normalized to GAPDH expression in each sample. Modulation of IL-7 and IL-21 in the experimental samples was calculated by employing a comparative CT method (reference sample, 1). mIL-7-35-F, 5′-TCC CTC CAC TGA TCC TTG TTC TG-3′; mIL-7-115-R, 5′-TCT CAT ATG CTT TAC CTT CTT TGT CTT T-3′; mIL-7-59T, 5′-/56-FAM/TGC CTG TCA CAT CAT CTG AGT GCC AC/3BHQ-1/-3′. mIL-21-154F, 5′-GAA CTT CTA TCA GCT CCA CAA GAT GT-3′; mIL-21-235R, 5′-GCT TGA GTT TGG CCT TCT GAA-3′; mIL-21-187T, 5′-/56-FAM/CAC TGT GAG CAT GCA GCT TTT GCC TG/3BHQ-1/-3′. Mu GAPDH-247F, 5′-CAA CGG GAA GCC CAT CAC-3′; Mu GAPDH-311R, 5′-CGG CCT CAC CCC ATT TG-3′; Mu GAPDH-268T, 5′-/TET/CTT CCA GGA GCG AGA CCC CAC TAA CA-3′.

### Statistical Analyses

Statistical analyses were performed by using ANOVA T test (Stat View), and Excel was used to determine Gaussian distributions and line of best fit (R values).

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