

# T-Cell Compartments of Prediabetic NOD Mice

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**Given the importance of the NOD mouse as a model of type 1 diabetes, there is a surprising lack of published information on the overall composition of the thymic and peripheral T-cell compartments. In this study, we revisited some earlier reports of T-cell abnormalities in this strain and examined a number of additional parameters to provide a global view of T-cells in prediabetic NOD mice. In some cases, we concur with past conclusions, but in other important areas, we find that NOD mice closely resemble nonautoimmune strains. Specifically, and contrary to published reports, the thymocyte subset distribution, the rate and composition of thymic export, and the composition of the peripheral T-cell pool, including the proportion of CD25<sup>+</sup>CD4<sup>+</sup> T-cells, are essentially normal in prediabetic NOD mice. These factors are therefore unlikely to be involved in the loss of tolerance that leads to autoimmunity within this strain. *Diabetes* 52:327–334, 2003**

**N**OD mice develop an autoimmune form of type 1 diabetes that is similar to the human disease (1). The model provides an opportunity to study diabetes in a genetically and environmentally controlled setting, yet surprisingly few studies offer a global view of lymphocytes. Of particular note, the characteristics of the NOD T-cell compartment remain poorly defined, despite the involvement of T-cells in type 1 diabetes being universally accepted. Among the articles that do exist are reports of problems within the thymus and peripheral T-cell compartments that may predispose, or otherwise directly contribute, to the autoimmune phenotype of the NOD strain (1–5).

In the context of autoimmune diabetes, abnormalities within the NOD thymus are especially noteworthy because of the strong association between disorganization of the thymus structure and defective central tolerance (6,7). Abnormalities ascribed to the NOD thymus include unusually large perivascular spaces (5), a poorly defined demarcation between the cortex and medulla (8), increased numbers of mature T- and B-cells (9), a relative deficiency of NKT cells (10), and defects in negative selection and T-cell export to the periphery (4,11,12). A variety of

peripheral defects have also been reported, some of which could stem from thymic abnormalities. For instance, one often-cited feature of NOD mice is an unusually high proportion (rather than number) of T-cells in peripheral lymphoid organs, possibly resulting from excessive thymic export (1,3,4,13,14). Other studies cite deficiencies within the peripheral immunoregulatory T-cell compartments of NOD mice more consistent with low rates of thymic export (5) or independent of thymic dysfunction (15,16). Unfortunately, causative links between the thymus and peripheral T-cell abnormalities in NOD mice remain speculative because thymus emigration has not been directly tested.

Abnormal thymocyte differentiation, thymic export, or T-cell pool composition could predicate autoimmune disease and are therefore deserving of careful analysis in NOD mice (6,17). Unfortunately, some studies cited in support of peculiarities appear rather unconvincing in hindsight. Sometimes, this is because of the unsophisticated analytical tools available at the time or because only a partial comparison, or comparison with a single purportedly normal strain, was reported. In other cases, mice were examined at an age likely to coincide with systemic autoimmunity, making it difficult to exclude the possibility that abnormalities were secondary to disease. The result is a vague characterization of T-cell compartments in the dominant animal model of type 1 diabetes and uncertainty about how much it really does differ from the norm. It is therefore imperative to reexamine the reported abnormalities and, more generally, to characterize the T-cell repertoire of NOD mice in the context of today's knowledge and tools.

The value of our study rests on three critical features. First, NOD mice were examined at 6 weeks of age, when the T-cell compartments were fully established, but before diabetes onset or even substantial insulinitis. Second, comparisons were made with age- and sex-matched mice of usually three other strains, enabling us to distinguish between common strain-to-strain variability and a real difference between NOD and nonautoimmune animals. Third, we exploited modern technology, including multiparameter flow cytometry and direct tracking of peripheral T-cells. The result is a global view of the NOD T-cell compartment that should frame future discussions of the anti- $\beta$ -cell T-cell reactivity characteristic of this strain.

## RESEARCH DESIGN AND METHODS

**Mice.** Female NOD, BALB/c, C57BL/6, and CBA mice obtained from The Jackson Laboratories were maintained in clean conditions in the Joslin animal facility. NOD mice congenic (>10 backcrosses) for major histocompatibility complex (MHC) class II deficiency ( $I-A_{\beta}^{0/0}$ ) were obtained from our colony.

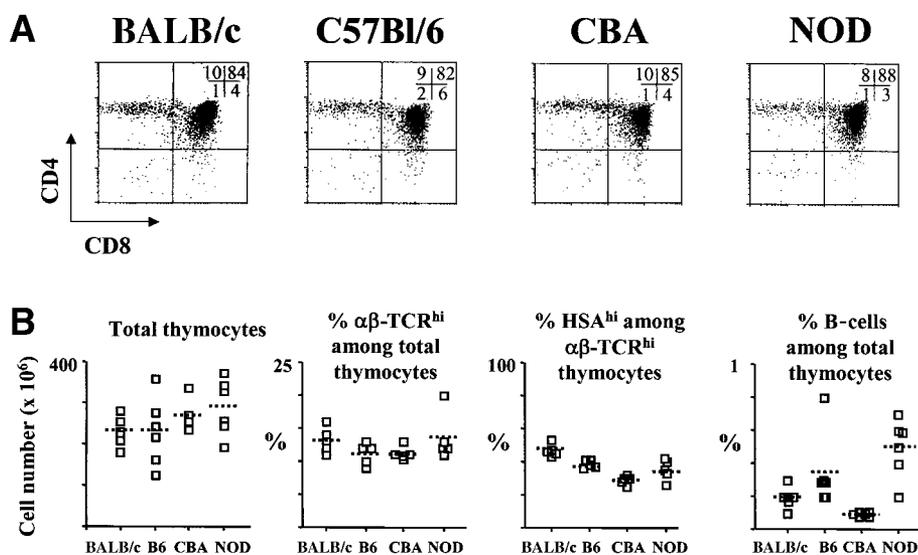
**Flow cytometry.** Lymphocyte suspensions were prepared and stained according to standard protocols using Pharmingen or Caltag antibodies. Cells

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BM, bone marrow; BrdU, bromodeoxyuridine; CFSE, carboxyfluorescein diacetate succinimidyl ester; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; MHC, major histocompatibility complex; RTE, recent thymic emigrant.



**FIG. 1.** T-cell differentiation. Thymocytes from NOD mice were compared with three non-autoimmune-prone strains on the basis of subset distribution and cell number. The distribution of subsets defined by the differential expression of CD4 and CD8 was normal for all mice (A). With the exception of B-cell levels in the NOD thymus, other markers of maturity (e.g.,  $\alpha\beta$ -TCR and HSA) also revealed no differences between strains (B). A minimum of five age-matched female mice were assessed for each strain.

were analyzed using Coulter instrumentation, and data were analyzed using EXPO or WinMDI software.

**Bone marrow chimeras.** Chimeras were prepared as described previously (18). Briefly, lethally irradiated mice reconstituted with  $1.5 \times 10^7$  bone marrow (BM) cells derived from the femurs of donor mice were analyzed by fluorescence-activated cell sorting (FACS) 8 weeks later.

**Analysis of thymic export.** Details of this technique are described elsewhere (19). Briefly, thymic lobes were injected with  $\sim 10 \mu\text{l}$  of 350  $\mu\text{g/ml}$  fluorescein isothiocyanate (FITC), randomly labeling 30–60% of thymocytes. Mice were killed 24 h later, and lymphoid organs were analyzed by FACS. Emigrants were identified as live-gated FITC<sup>+</sup> cells expressing CD4 or CD8 (omitting autofluorescing cells and doublets) and quantified by considering the size of the peripheral pool to be equal to the number of spleen cells plus twice the number of cells pooled from mesenteric, inguinal, brachial, and axillary lymph nodes (20). Cell counts were performed in duplicate using a hemocytometer and trypan blue.

**Homeostatic proliferation.** Cell suspensions from pooled lymph nodes of B6 and NOD mice were resuspended to  $1 \times 10^7$  cells/ml in 1  $\mu\text{mol/l}$  carboxyfluorescein diacetate succinimidyl ester (CFSE) in Dulbecco's modified Eagle's medium 10, incubated for 10 min at 37°, and washed. The  $5 \times 10^6$  cells were intravenously injected into age-matched syngeneic recipients, sublethally irradiated 2 days prior. Recipients were analyzed by FACS on days 2, 5, 8, and 13.

**Statistical significance.** For normally distributed data, Student's unpaired *t* tests were used to compare two groups; ANOVA tests were used for three or more groups. Mann-Whitney tests were used when normal distribution could not be demonstrated.

## RESULTS AND DISCUSSION

### Thymus

**T-cell differentiation.** Thymic abnormalities reported to affect NOD mice include structural irregularities (9), accumulations and deficiencies of particular thymocyte subsets (10), and export dysregulation (4), yet the size and distribution of NOD thymocyte subsets is generally observed to be normal (21). In light of this apparent inconsistency and the recent suggestion of defective negative selection (12), we compared the size and relative proportions of different thymocyte subsets in prediabetic NOD mice versus control (nonautoimmune) strains.

The distribution and size of the CD4<sup>-</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, and CD4<sup>-</sup>CD8<sup>+</sup> subpopulations (collectively spanning all stages of thymic T-cell differentiation) were similar for all strains (Fig. 1A and B). Within these compartments, the expression of other maturity markers (such as  $\alpha\beta$ -T-cell receptor, heat stable antigen, or peanut agglutinin and CD25, CD44, and CD69 (Fig. 1B; data not

shown) on NOD thymocytes (and other strains) was within the boundaries of strain-to-strain variation and was therefore consistent with normal T-cell differentiation. NOD mice were unusual only in their thymic B-cell compartment (which is two to five times larger than that in other strains [Fig. 1B]) and  $\alpha\beta$ -TCR<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> subset, which contains regulatory NKT cells. This latter deficiency has been reported previously (10,21) and will be discussed further.

The curiously high level of thymic B-cells was consistent with previous reports of mature T- and B-cells accumulating in the perivascular regions of the thymus from 12 weeks of age (5,22). We found no evidence of additional mature T-cells, but our finding of a similar trend in 6-week-old mice demonstrates that this trait develops before disease onset. Small numbers of B-cells are found normally in the thymus (23), but accumulation is associated with thymic abnormalities in other autoimmune models (24), making the higher incidence in NOD mice worthy of further investigation. B-cells play an important role in the development of diabetes in NOD mice (25–27), but in the thymus, they represent an extremely minor population of cells (<1%), and their impact on T-cell development is generally regarded as insignificant.

**Dynamics of T-cell differentiation.** Before export, mature thymocytes undergo a highly regulated cascade of differentiation and proliferative events. The kinetics of development are as predictable as the phenotypic changes that take place, so abnormal kinetics can indicate aberrant maturation. The unusual kinetics of T-cell differentiation in diabetes-prone BB rats, for example, coincide with thymic defects linked to spontaneous T-cell autoreactivity in that strain (28).

In vivo pulse-labeling with bromodeoxyuridine (BrdU) was used to examine the dynamics of T-cell differentiation in NOD mice. BrdU is incorporated by dividing cells and retained until passed to daughter cells, thereby allowing the progression and kinetics of differentiation to be tracked. The initial incorporation of BrdU by thymocytes was similar for NOD and control strains for the proportion and phenotype of cells stained, resulting in comparable profiles of BrdU staining 24 h after BrdU administration.

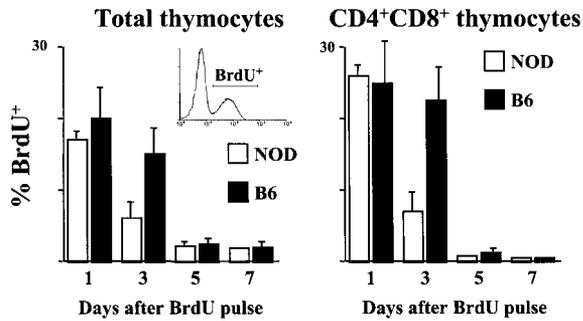


FIG. 2. Kinetics of T-cell differentiation. After two injections of 1 mg/ml BrdU (4 h apart), the thymi of mice were harvested and the incorporation of BrdU was determined by FACS. Levels were comparable for NOD and B6 mice at days 1, 5, and 7, but at day 3 (and day 2 not shown), two- to threefold fewer NOD thymocytes were positive for BrdU. The pooled results from one of three separate experiments are shown.

Interestingly, a significantly lower proportion of BrdU<sup>+</sup> cells was observed among NOD thymocytes at day 3 (Fig. 2) (and to a lesser extent, day 2 [not shown]). The phenomenon was most dramatic among CD4<sup>+</sup>CD8<sup>+</sup> cells where the proportion of BrdU<sup>+</sup> cells in NOD mice was two to three times lower than that for B6 mice. This could have been caused by the loss of BrdU<sup>+</sup> cells or may reflect the dilution of BrdU to background levels due to increased turnover. The former is more likely because levels of incorporation at 24 h were similar in all compartments. However, this is such an early defect, it seems difficult to attribute great significance to it in the context of tolerance induction.

**Thymocyte deletion mediated by BM-derived antigen-presenting cells.** Thymic tolerance is mediated by the deletion of thymocytes with high affinity for self-peptides. Defects in the stringency of negative selection can lead to the release of autoreactive T-cells and an increased risk of autoimmune disease. The presence of overtly autoreactive T-cells in NOD mice has led to suggestions of defective negative selection. Supporting evidence has been largely circumstantial (8,29), but Kishimoto and Sprent (12) recently reported impaired negative selection among semi-mature NOD thymocytes. Stimuli of signaling pathways associated with negative selection induced significantly less apoptosis among NOD thymocytes compared with other strains. No direct link to peripheral autoimmunity was demonstrated, and non-specific stimulation was used in preference to physiological signals such as MHC-peptide complexes, but the data were consistent with a central tolerance defect.

We assessed the global extent of negative selection in NOD mice using a classic strategy developed by van Meerwijk et al. (18). Since BM-derived antigen-presenting cells are important mediators of negative selection (30), one can grossly measure the extent of negative selection by constructing BM chimeras that express MHC molecules on radio-resistant stromal cells but not on radio-sensitive BM-derived cells. For example, it is well established that B6 mice expressing MHC class II molecules on stromal, but not hemopoietic, cells have ~50% more CD4<sup>+</sup>single-positive thymocytes than control animals, indicating that about one-third of positively selected class II-restricted cells are negatively selected by BM-derived cells in the thymus (18).

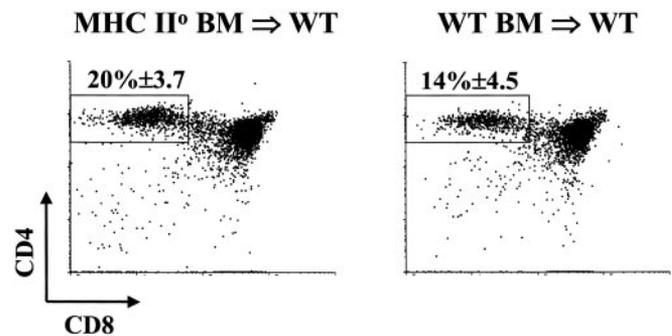
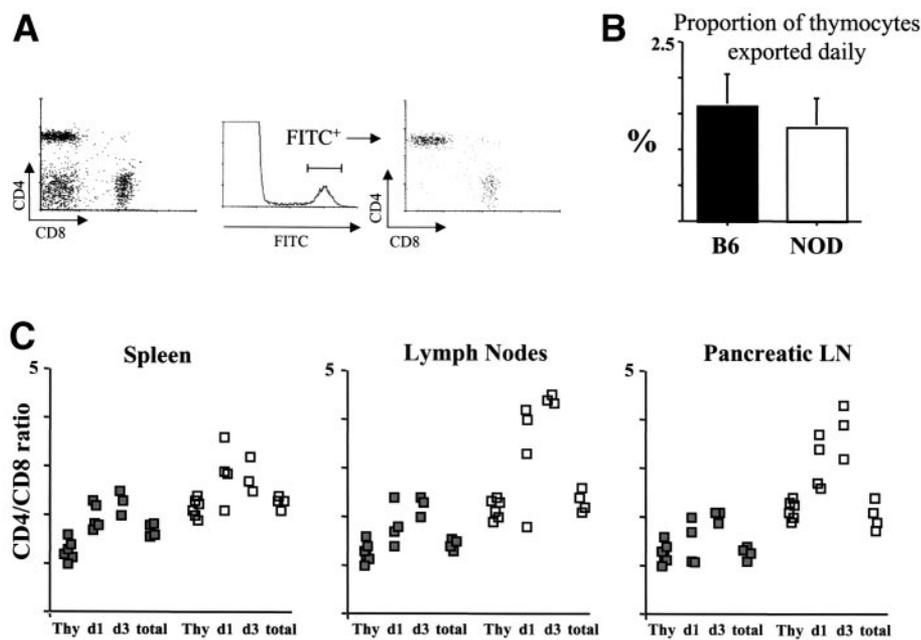


FIG. 3. MHC class II-mediated negative selection. NOD mice were lethally irradiated and then reconstituted with BM from congenic donors that were either MHC class II positive (i.e., wild-type [WT]) or deficient (MHC II<sup>o</sup>). Six weeks after reconstitution, the thymi were assessed for the presence of CD4<sup>+</sup>CD8<sup>-</sup> T-cells, with the difference between the two groups a reflection of deletion mediated by MHC class II-positive BM-derived antigen presenting cells.

When lethally irradiated NOD recipients were reconstituted with BM from MHC class II-deficient NOD donors, the proportion of CD4<sup>+</sup>CD8<sup>-</sup> cells in the thymus was augmented by ~50%, consistent with the level of deletion in the nonautoimmune B6 strain (Fig. 3). This does not exclude the possibility of more subtle defects, but at a gross level, the extent of deletion mediated by MHC class II-positive cells in the NOD thymus appears similar to that of B6 mice and does not support the concept of a general failure of negative selection. This conclusion is supported by the consistently similar distribution of CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, and CD4<sup>-</sup>CD8<sup>+</sup> thymocytes in NOD mice and other strains (Fig. 1) and the similar rate of conversion from CD4<sup>+</sup>CD8<sup>+</sup> to CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>+</sup> thymocytes revealed using BrdU pulse-labeling (data not shown). A significant failure of negative selection in the NOD thymus would presumably permit higher-than-normal proportions of single-positive NOD thymocytes to survive, and the absence of this phenomenon argues for comparable levels of negative selection.

#### T-cell export from the thymus

Abnormal thymic emigration is causally linked to a variety of autoimmune diseases (31,32), and reports alluding to defective export in NOD mice make it a logical target for investigation (4,5,11). Although a confirmed defect in the emigration of T-cells from the NOD thymus would provide important clues about the initiation of diabetes, to date, assessments of NOD thymic export have been based on indirect histological studies. The abnormal congregation of mature lymphocytes in the perivascular spaces of the NOD thymus led to suggestions that emigration of mature thymocytes, or certain regulatory subsets, was retarded (5). Reduced export can induce peripheral lymphopenia and homeostatic expansion of peripheral T-cells, which can perturb normal subset distribution and is associated with the development of autoimmunity in clinical and experimental settings (32–35). Other groups proposed a contradictory defect in which thymocyte export might occur at higher-than-normal levels, thereby contributing to increased peripheral T-cell levels (4). A third possibility is that immature thymocytes were erroneously released from the NOD thymus, exposing the host to cells that would normally be negatively selected (36). Unfortunately, the confusion surrounding the possible dysregulation of the



**FIG. 4.** T-cell export from the thymus. T-cell export from the thymus was directly assayed by injecting FITC (0.3 mg/ml) into the thymic lobes. Mice were killed 24 h later, and emigrant cells were identified by virtue of fluorescence (A). B6 and NOD mice exported similar levels of T-cells (B), but the variability of the CD4/CD8 ratio among emigrants at both 24 and 72 h after export from the thymus was far greater in the NOD group (C). The CD4/CD8 ratio was similar for B6 (■) and NOD mice (□) among medullary thymocytes (Thy) and the overall peripheral pool (total). Pooled results from one representative experiment (of four) involving four to six mice per group are shown. d, day; LN, lymph node.

thymic export in NOD mice has remained unresolved. Before this study, export from the NOD thymus had not been directly tested and there was a complete lack of data relating to the maturity of recent thymic emigrants (RTEs).

FITC was intrathymically injected to randomly label thymocytes, thereby allowing identification of emigrant cells in the periphery (19). The number and proportion of FITC<sup>+</sup> T-cells within the periphery were determined according to methods established in our laboratory (20) and other laboratories (36), and markers of maturity (e.g., HSA) were used to assess whether immature thymocytes were inappropriately released.

For 6-week-old mice, there was no difference in the rate of T-cell export from the NOD and B6 thymus (Fig. 4A and B). Previous reports, including our own, report the rate of thymic export for B6 and other nonautoimmune mice to be 1–2% of total thymocytes per day (19,20). Our results for 6-week-old B6 and NOD mice fall within this range (Fig. 4), as do preliminary results from 15-week-old mice (data not shown). Importantly, emigrants from the NOD thymus were almost exclusively CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup> and expressed low levels of HSA (data not shown), indicating that thymocytes were not prematurely released.

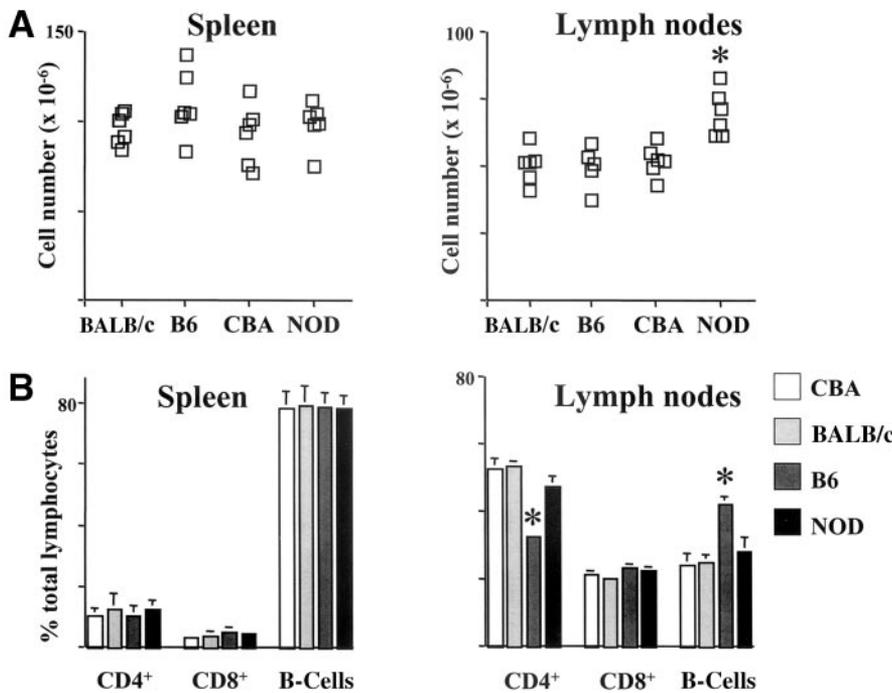
To ensure that the initial seeding of the T-cell pool was normal, we assessed thymus export in 3-day-old mice. NOD and B6 neonates had similar T-cell subset distributions in the thymus and spleen, and the phenotype of thymic emigrants resembled that of adult mice, with NOD RTEs almost entirely CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup> and HSA<sup>10</sup>, consistent with the exclusive release of mature T-cells (data not shown). It is noteworthy, however, that the distribution of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells within NOD RTEs was different from B6 mice (Fig. 4C). The CD4/CD8 ratio among RTEs, and in the periphery, is normally remarkably stable in mice and humans, reflecting the tight regulation of thymocyte differentiation and genetic controls on the relative sizes of the subsets (20,37,38). Typically, the CD4/CD8 ratio of RTE is slightly higher than that of mature thymocytes and peripheral T-cells, perhaps

indicative of the independent regulation of the export process (20,39) and the expansion phase undertaken by many RTEs immediately before export (40). Although the number of RTEs released from the NOD thymus remained both normal and stable, the CD4/CD8 ratio was variable and typically contained higher proportions of CD4<sup>+</sup> cells, suggesting that this aspect of thymic export was not under the tight control seen with other strains.

#### Peripheral T-cell compartment

The CD4/CD8 ratio among RTEs might be expected to reasonably determine the ratio within the periphery. However, NOD mice displayed similar CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proportions to other strains (Fig. 5). This result indicates that a greater correction of the RTE CD4/CD8 ratio (through preferential division of CD8<sup>+</sup> cells or loss of CD4<sup>+</sup> cells) must occur for NOD mice to produce the homeostatically controlled peripheral CD4/CD8 ratio of ~2. However, high CD4/CD8 ratios among NOD RTEs may offer an explanation for previous reports of a steadily increasing peripheral CD4/CD8 ratio during the onset of insulinitis in NOD mice (41). Coupled with the variability within the export ratio of different NOD mice, further analysis is warranted to explicitly exclude the possibility that thymic export irregularities contribute to the onset of autoimmunity.

In addition to the normal distribution of CD4<sup>+</sup> and CD8<sup>+</sup> cells within the periphery of NOD mice, the relative proportions of T- and B-cells were also similar to other strains. This finding conflicts with previous reports that T-cell “hyper-accumulation” or “T-accumulation” characterizes the NOD strain, reflecting persistent T-cell hyperplasia. Abnormal proportions or numbers of T-cells in the peripheral pool could indicate dysregulation within the repertoire, and previous reports have speculated on whether T-lymphoaccumulation was caused by problems with apoptosis, proliferation, or thymic export (1,4,14). Each might conceivably predispose to autoimmunity or at the very least indicate problems associated with tolerance breakdown. Our findings, however, argue that no such problems exist in the periphery of NOD mice before



**FIG. 5.** Peripheral lymphocyte pool. Peripheral T- and B-cell numbers and proportions were assessed for NOD mice and compared with three other strains. The spleen of all strains contained similar numbers and proportions of T- and B-cells. The lymph nodes of NOD mice were typically 20% larger than those of other strains, although this reflected increased numbers of both T- and B-cells, because these made up similar proportions of the pool in all strains. A minimum of five age-matched female mice were assessed per group. \*A characteristic significantly different from the other three strains ( $P < 0.001$ ).

disease onset. The number and relative proportion of CD4<sup>+</sup> and CD8<sup>+</sup> T- and B-cells in the NOD spleen were consistently similar to other strains. Higher lymphocyte numbers were observed in the NOD lymph nodes (Fig. 5A), but this is not indicative of T-cell hyperplasia as previously described because the increase applied equally to the T- and B-cell compartments. The reason for the increase in lymph node cell numbers was not immediately evident, but given that it was not observed in the spleen and was relatively minor, it seems likely to reflect simple strain-to-strain variation.

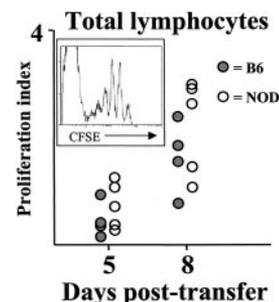
We also investigated the capacity for homeostatic expansion of T-cells in NOD mice. When T-cells are transferred into a lymphopenic recipient, the availability of space provokes the T-cells to proliferate (42). It is a recurring theme that T-cell homeostasis may be disrupted in NOD mice, perhaps leading to too many or too few T-cells within the overall pool or within particular T-cell subsets. To test homeostatic proliferation, we transferred CFSE-labeled lymphocytes from NOD or control mice into irradiated age-matched syngeneic recipients. Recipients were killed on day 2, 5, 8, or 13, with the extent of cell division revealed by CFSE dilution. On day 2, transferred cells had not proliferated significantly in either strain, but on day 5 and 8, extensive, but similar, levels of proliferation had occurred (Fig. 6). After 13 days, most cells had divided to the extent that peaks of CFSE staining were no longer apparent. Although the possibility of changes arising after day 13 cannot be excluded, we found no significant difference in the extent or rate of CD4<sup>+</sup> or CD8<sup>+</sup> T-cell proliferation between NOD and B6 mice up to this time point. This indicates that in addition to the largely normal number and distribution of T-cells within the peripheral pool, the homeostatic response to lymphopenia was also normal.

### Regulatory T-cells

There are many reports of defects affecting the regulatory T-cell compartment of NOD mice. Some describe func-

tional deficiencies, whereas others report abnormally low numbers of particular regulatory subsets (43). Most prominent are reported deficiencies of NKT cells (well represented within the  $\alpha\beta$ -TCR<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> compartment) (15) and CD4<sup>+</sup>CD25<sup>+</sup> T-cells (16). Both subsets are thought to dampen autoreactivity. Reports of deficiencies within this compartment are especially interesting because they offer a credible, yet simple, explanation of how autoimmunity might develop in NOD mice and suggest strategies for resolving the dysfunction.

**$\alpha\beta$ -TCR<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> T-cells.** One well-documented defect in the NOD mouse is a relative paucity of NKT cells (10). The deficiency was first identified as a significantly reduced proportion of  $\alpha\beta$ -TCR<sup>+</sup> cells within the CD4<sup>-</sup>CD8<sup>-</sup> compartment, with subsequent studies confirming that a large proportion of  $\alpha\beta$ -TCR<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> T-cells are NKT cells (15). The importance of NKT cells to the diabetes process remains controversial, particularly because their numbers appear to normalize with age, but the deficiency has been implicated as a contributing factor



**FIG. 6.** Homeostatic proliferation. After the adoptive transfer of syngeneic CFSE-labeled lymphocytes to sublethally irradiated NOD and B6 recipient mice, mice were harvested at different time points to assess the extent of homeostatic proliferation. The similar levels of division occurring within the periphery of NOD and B6 mice are shown. The proliferation index represents the proportion of proliferating cells divided by the proportion of quiescent cells.

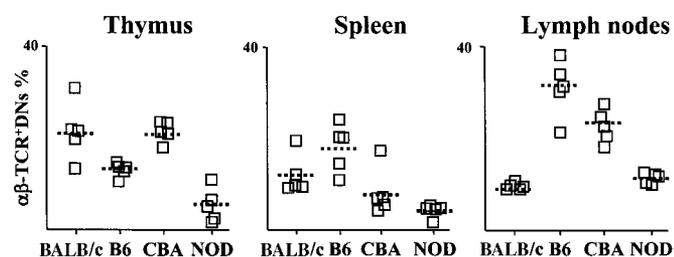


FIG. 7. Assessment of the  $\alpha\beta$ -TCR<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> population and the prevalence of  $\alpha\beta$ -TCR<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> cells. Levels of  $\alpha\beta$ -TCR<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> T-cells in NOD mice were measured and compared with three nonautoimmune strains. The proportions were lowest in the NOD strain, particularly in the thymus. NOD levels were similar to that of CBA mice in the spleen and to BALB/c in the lymph nodes. DN, double negatives.

in NOD autoreactivity (44–46). Our analysis of NOD mice confirms the widely reported comparative deficiency of  $\alpha\beta$ -TCR<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> T-cells in the spleen, lymph nodes, and, most prominently, the thymus (Fig. 7). The deficiency was least obvious in the periphery and was not statistically significant when compared with the CBA spleen or BALB/c lymph node. Although beyond the scope of this study, analysis of subsets within the broader NKT cell population in different tissues is likely to be informative.

**CD25<sup>+</sup>CD4<sup>+</sup> T-cells.** The CD25<sup>+</sup>CD4<sup>+</sup> T-cell compartment appears to contain cells with an important role in maintaining self-tolerance in the periphery (47). Significantly, some recent reports describe NOD mice as deficient for CD25<sup>+</sup>CD4<sup>+</sup> T-cells (16,48,49). Given the thymic origin of these cells, a peripheral deficit could potentially arise from maturational defects in the thymus, during export to the peripheral pool, or in the periphery itself.

In the NOD thymus, ~5% of CD4<sup>+</sup>CD8<sup>-</sup> T-cells expressed CD25, a fraction falling within the same range as

nonautoimmune strains (Fig. 8A). The proportion fell to ~3% among RTEs (at 24 and 72 h after export), but this also occurred in the control B6 strain, indicating no defect in the production or export of these cells in NOD mice (Fig. 8A).

The spleen, lymph nodes, and pancreatic lymph node were examined for evidence of a systemic or localized peripheral deficiency. Surprisingly, the proportion of CD25<sup>+</sup>CD4<sup>+</sup> T-cells in NOD mice was no different from that of control strains at any site (Fig. 8B). Consistent with reported levels in nonautoimmune strains (50,51), ~7% of CD4<sup>+</sup> T-cells in the lymph nodes and spleen of CBA, BALB/c, and B6 mice expressed CD25. We observed the same incidence for NOD mice. Subtle differences sometimes occurred, but were rarely significant, and often took place between control strains.

Lastly, no differences were observed in the thymus, RTE (24 h after export), or spleen of 3-day-old NOD mice, arguing against any transient deficiency during the establishment of the NOD pool (data not shown). Although functional differences within the compartment cannot be ruled out, quantitative deficiencies in the CD25<sup>+</sup>CD4<sup>+</sup> compartment do not appear to explain the loss of tolerance in the NOD mouse.

It is difficult to be certain why our results differ from others who report a CD4<sup>+</sup>CD25<sup>+</sup> T-cell deficiency in NOD mice. At present, it is not possible to isolate the regulatory component of the CD4<sup>+</sup>CD25<sup>+</sup> T-cell compartment, so activated peripheral cells provide a feasible source of variability. However, our stringent comparison of age-matched female mice found no indication of a CD4<sup>+</sup>CD25<sup>+</sup> T-cell deficiency in the thymus or thymic emigrants of NOD mice, nor was an indication found in the spleen, lymph nodes, or pancreatic lymph nodes. It should

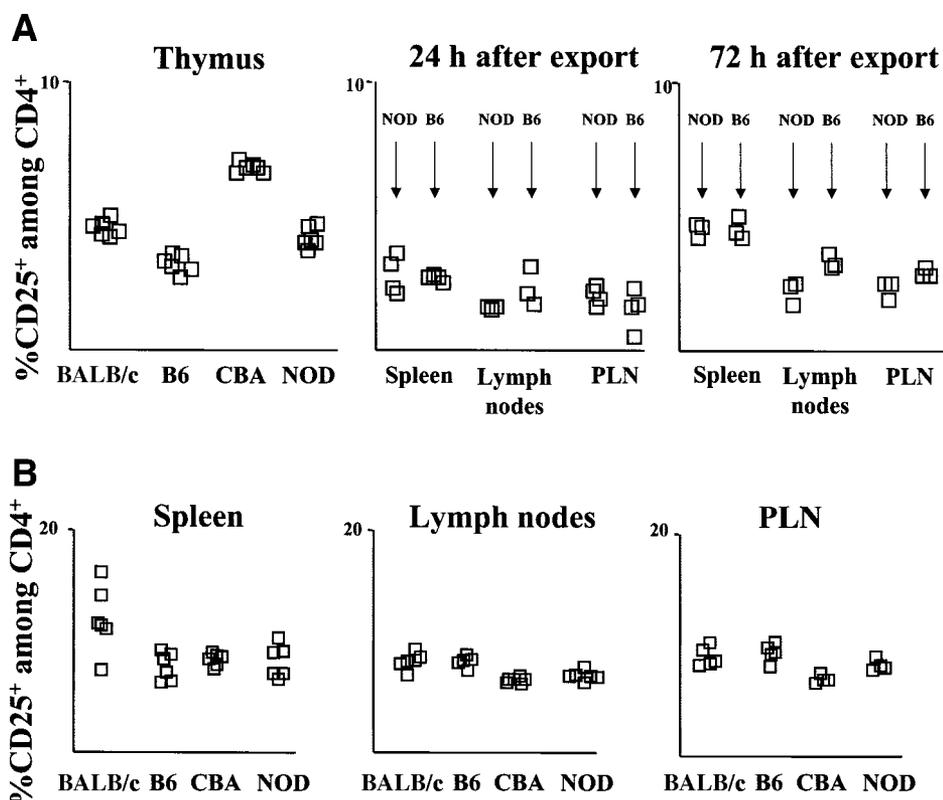


FIG. 8. Proportion of CD25<sup>+</sup>CD4<sup>+</sup> T-cells. **A:** Assessment of the thymic CD25<sup>+</sup>CD4<sup>+</sup> T-cell compartment. Levels of CD25<sup>+</sup>CD4<sup>+</sup> T-cells in the thymus of NOD mice were measured and compared with three nonautoimmune strains. No deficiencies were noted in any lymphoid compartment. Levels for the RTE population were also determined to be similar for NOD and the control B6 strain. Each symbol represents one mouse. **B:** Assessment of the peripheral CD25<sup>+</sup>CD4<sup>+</sup> T-cell compartment. Levels of CD25<sup>+</sup>CD4<sup>+</sup> T-cells in the spleen and lymph nodes of NOD mice were measured and compared with three other strains. The pancreatic lymph node (PLN) was assessed separately. No deficiencies were noted. Each symbol represents one mouse.

be noted that an analysis of the NOD CD4<sup>+</sup>CD25<sup>+</sup> compartment was not the primary focus of the earlier studies, and this aspect of the data was typically presented as not shown, or in a limited comparison with one control strain. **DX5<sup>+</sup> and CD45RB<sup>lo</sup> cells.** In addition to CD4<sup>+</sup>CD25<sup>+</sup> and NKT cells, other subsets of the T-cell repertoire proposed to have regulatory properties are DX5<sup>+</sup> and CD45RB<sup>lo</sup> T-cells (45,52). We again detected no obvious deficiencies in these compartments of the NOD T-cell pool compared with control mice (data not shown).

In conclusion, our analysis of the NOD T-cell compartment examined subpopulations and locations where abnormalities could potentially predispose to autoimmunity. With some exceptions, the size and composition of thymic, RTE, and peripheral T-cell compartments in NOD mice were similar to other strains. This finding indicates that no broad quantitative defect precedes the onset of autoimmunity. At the level of regulatory T-cells, we confirmed previous reports of a deficiency within the NKT cell compartment of NOD mice, but found no evidence to support recent suggestions that a deficiency in the CD25<sup>+</sup>CD4<sup>+</sup> compartment may also contribute to autoimmunity.

The use of prediabetic mice in our study was an important consideration because age (17) and autoimmunity (39) can induce significant changes within the T-cell repertoire that could collectively mask characteristics predisposing to autoimmunity. This issue of timing may be one reason why our global analysis found no evidence of abnormalities. In addition, in some instances, we were able to apply techniques unavailable to the original investigators and this is likely to be another reason why our findings disagree with some conclusions drawn from earlier work. In summary, NOD mice are clearly distinct from most other strains in their susceptibility to autoimmune attack, but the loss of tolerance does not appear to originate in general deficiencies within the T-cell compartment.

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