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Autoimmunity to Both Proinsulin and IGRP Is Required for Diabetes in Nonobese Diabetic 8.3 TCR Transgenic Mice

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T cells specific for proinsulin and islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP) induce diabetes in nonobese diabetic (NOD) mice. TCR transgenic mice with CD8+ T cells specific for IGRP_{206–214} (NOD8.3 mice) develop accelerated diabetes that requires CD4+ T cell help. We previously showed that immune responses against proinsulin are necessary for IGRP_{206–214}-specific CD8+ T cells to expand. In this study, we show that diabetes development is dramatically reduced in NOD8.3 mice crossed to NOD mice tolerant to proinsulin (NOD-PI mice). This indicates that immunity to proinsulin is even required in the great majority of NOD8.3 mice that have a pre-existing repertoire of IGRP_{206–214}-specific cells. However, protection from diabetes could be overcome by inducing islet inflammation either by a single dose of streptozotocin or anti-CD40 agonist Ab treatment. This suggests that islet inflammation can substitute for proinsulin-specific CD4+ T cell help to activate IGRP_{206–214}-specific T cells. The Journal of Immunology, 2008, 180: 4458–4464.

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3 Abbreviations used in this paper: T1D, type 1 diabetes; NOD, nonobese diabetic; IGRP, islet-specific glucose-6-phosphatase catalytic subunit related protein; DC, dendritic cell; NOD-PI, NOD mice tolerant to proinsulin; STZ, streptozotocin; IAA, insulin autoantibody assay; Treg, regulatory T cell.

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proinsulin is even present in the majority of NOD8.3 mice with a pre-existing repertoire of IGRP206–214-specific cells.

Materials and Methods

Mice

NOD mice expressing mouse proinsulin 2 under the control of an MHC class II (I-E) promoter (NOD-PI) and NOD8.3 mice expressing the TCRβ rearrangements of the H-2Kd-restricted CD8+ T cell clone NY8.3 have been described (3, 5, 6). Perforin knockout NOD mice were obtained from The Jackson Laboratory T1D repository. All animal studies were conducted at St. Vincent’s Institute (Melbourne, Australia) and were approved by the institutional animal ethics committee.

Peptides and Abs

The peptides IGRP$_{206-214}$ (VYLKTNVFL) and TUM (KYQAVTTTL) were synthesized by Auspep. H-2Kd tetramers were made by ImmunoID. Tetramer function was validated by staining NOD8.3 splenocytes.

Tetramer staining

Islet infiltrating T cells were stained with IGRP$_{206-214}$ or TUM H-2Kd tetramer as previously described (6).


CFSE labeling and adoptive transfer

CD8+ T cells from NOD8.3 mouse were labeled with CFSE as previously described (6). In cotransfer experiments, mice receiving CFSE-labeled 8.3 CD8+ T cells were injected i.v. (a) on day 0 with 100 μg agonistic anti-CD40 mAb FGK45 or 100 μg isotype control Ab (GL121). The dose was 1 mg followed by 0.5 mg every week until the mouse became diabetic or for 8 wk. Mice were monitored for success of depletion (Fig. 1, A and B). IGRP206–214-specific naive T cells from the spleens of NOD-PI/NOD8.3 mice proliferated in response to the peptide to a similar extent, and splenic T cells from NOD-PI/NOD8.3 and NOD8.3 mice were identified based on their high autofluorescence (14).

Anti-CD4 Ab treatment

Five 7-wk-old NOD-PI/NOD8.3 mice were injected with anti-CD4 mAb (GK1.5) or isotype control (GL121). The dose was 1 mg followed by 0.5 mg after 3 days. Thereafter 0.5 mg was injected every week until the mouse became diabetic or for 8 wk. Mice were monitored for success of depletion using noncompeting anti-CD4 (clone RM4-4; BD Pharmingen).

Histological analysis

Immunohistochemical staining and scoring of frozen pancreas sections was performed as described (15, 16). Mice were monitored for diabetes as described (6).

Insulin autoantibody assay (IAA)

IAA were measured with a 96-well filtration plate micro IAA assay as described (6). We have participated in all the Diabetes Autoantibody Standardization Program workshops. In the murine IAA workshop (2002), the sensitivity and specificity for IAA were 69 and 83%, respectively.

Statistics

Insulin autoantibody levels and insulitis scores were analyzed by Student’s t test. Survival curves were analyzed with the log-rank test. Statistical tests used PRISM software (version 3.02; GraphPad). Values of p < 0.05 was considered significant.

Results

NOD-PI/NOD8.3 mice are protected from diabetes

We have shown that tolerance to proinsulin prevents expansion of IGRP206–214-specific T cells (6). To study the effect of tolerance to insulin on an expanded pool of autoreactive T cells, we crossed NOD8.3 TCR transgenic mice to NOD-PI mice.

The proportions of CD4+ or CD8+ T cells in NOD-PI/NOD8.3 mice were similar to NOD8.3 mice, and there was no difference in numbers of IGRP206–214-specific T cells, suggesting normal development of CD8+ T cells in NOD-PI/NOD8.3 mice (Fig. 1, A and B). IGRP206–214-specific naive T cells from the spleens of NOD-PI/NOD8.3 mice proliferated in response to the peptide both in vitro and in vivo similarly to IGRP206–214-specific T cells from NOD8.3 mice (Fig. 1, C and D). Moreover, IGRP206–214-specific splenic T cells from NOD-PI/NOD8.3 and NOD8.3 mice secreted IFN-γ in response to the peptide to a similar extent, and splenocytes from these mice transfer diabetes into irradiated NOD mice to a similar extent, suggesting normal cytotoxic potential (Fig. 1, E and F).

Despite normal number and function of IGRP206–214-specific T cells, NOD-PI/NOD8.3 mice have reduced insulitis and are markedly protected from diabetes (Fig. 2, A and B). Most of the CD8+ T cells infiltrating islets of NOD-PI/NOD8.3 mice are specific for IGRP (Fig. 2E). The important difference between NOD-PI/NOD8.3 mice and NOD8.3 mice is the ability to mount an immune response against insulin. A total of 40% of female NOD8.3 mice (4/10) had high titer insulin. A total of 40% of female NOD8.3 mice (4/10) had high titer insulin autoantibodies, whereas only 9.4% NOD-PI/NOD8.3 mice (3/32) had a borderline positive IAA (Fig. 2C). Recently, it has been shown that insulin primed CD4+ T cells are required for expression of IAA (16). IGRP206–214-specific T cells therefore depend on proinsulin-specific...
immune responses for their full activation and ability to mediate β cell destruction.

**Dominant tolerance does not account for protection from diabetes in NOD-PI/NOD8.3 mice**

Regulatory T cells (Tregs) could account for diabetes protection in NOD-PI/NOD8.3 mice but we previously did not find evidence for this mechanism in NOD-PI mice (6). Also, there was no difference in number of Foxp3⁺ CD4⁺ T cells between NOD and NOD-PI mice and between NOD8.3 and NOD-PI/NOD8.3 mice (Fig. 3A). However, Tregs could have different suppressive potential. No difference in suppressive activity of CD4⁺CD25⁺ from NOD or NOD-PI mice on proliferation of IGRP-specific CD8⁺ T cells from NOD or NOD-PI mice. D, Diabetes incidence in NOD-PI/NOD8.3 mice treated with GK1.5 (anti-CD4) mAb (n = 5) or isotype control Ab (n = 5). Arrows indicate the time of administration of Ab. P = ns. E, Islets from anti-CD4 mAb or isotype control Ab-treated NOD-PI/NOD8.3 or untreated NOD8.3 mice were analyzed for expression of class I MHC expression using anti-mouse H-2Dβ.

**Stimulating Ag presentation bypasses tolerance to proinsulin leading to proliferation of IGRP-specific T cells and diabetes**

It is surprising that despite being present in such a large number, IGRP-specific T cells are tolerant to IGRP in the absence of immune responses to insulin. IGRP206-214-specific T cells remain ignorant of their Ag in NOD-PI hosts (6). We therefore reasoned that IGRP206-214-specific T cells, despite being present in large numbers, do not mediate protection from diabetes in NOD-PI/NOD8.3 mice.
numbers, might remain ignorant of their Ag, meaning that tolerance should be broken by increasing T-cell activation signals. Indeed, FACS analysis of β cells from NOD-PI/NOD8.3 mice showed decreased MHC class I expression, which could reduce their targeting by CD8⁺ T cells (Fig. 2D).

We questioned whether decreased cross-priming of IGRP206–214-specific T cells in NOD-PI hosts could be overcome in response to β-cell death or islet inflammation. In the absence of infection, DC maturation occurs following uptake of Ags from apoptotic cells or by ligation of CD40 by CD154 on CD4⁺ T cells (18–21). We assessed proliferation of transferred IGRP206–214-specific T cells following CD40 agonist Ab treatment or induction of β-cell apoptosis by low dose STZ. A single dose of 80 mg/kg of STZ or 100 μg of CD40 agonist Ab was used based on published reports (22–24). Each of these increased proliferation of NOD8.3 T cells (Fig. 4, A–C).

In addition, we also analyzed IGRP206–214-specific T cells transferred into perforin knockout NOD mice (NOD perforin−/−). These mice have insulitis but very little β-cell destruction and reduced and delayed diabetes development (25). Transferred IGRP206–214-specific T cells proliferated to a similar extent as in NOD mice (Fig. 4D), indicating that the reduced β-cell destruction in these mice does not decrease Ag presentation. Together, these experiments suggested that protection by induction of tolerance to proinsulin could be bypassed.

We next examined whether breaking tolerance by STZ or CD40 Ab treatment in NOD-PI/NOD8.3 could induce diabetes. Administration of a single dose of STZ or CD40 Ab treatment increased MHC class I expression on β cells within 3 days (Fig. 5A) and resulted in diabetes within 1–4 wk in NOD-PI/NOD8.3 mice (Fig. 5, C and D). The islet infiltrating cells were predominantly IGRP206–214 CD8⁺ cells in these mice (Fig. 5B). This suggests that even with many Ag-specific T cells, immune responses to insulin are required for full diabetes development. However, this requirement can be bypassed by stimuli that induce β-cell apoptosis or promote inflammation. In contrast, this treatment had no effect on the incidence of diabetes in NOD or NOD-PI mice indicating (a) the treatment is stimulating T cells to mediate diabetes and not directly inducing β-cell death and (b) mere stimulation of β-cell apoptosis or promotion of islet inflammation is not sufficient, without a large number of Ag-specific T cells.

Discussion
This study showed that immune responses against proinsulin, that could be eliminated by expression of proinsulin in APCs, are required in the majority of NOD8.3 TCR transgenic mice for their IGRP-specific T cells to become activated and kill β cells. Diabetest was reduced from nearly 100 to 20%, indicating that little T-cell activation and progression occurred in mice with proinsulin immune tolerance (confirmed by absent IAA). That an immune response to one Ag is required for an effective immune response by TCR transgenic T cells specific for a different Ag has not previously been reported. However, aspects of previous studies are consistent with the current finding.

Some previous CD8⁺ TCR transgenic mice specific for certain Ags that have been transgenically expressed in the β-cell have not shown immune reactivity against that Ag (26). An activation step is required, either infection with virus, for example LCMV when LCMV glycoprotein is expressed in β cells (26), or cotransfer of Ag-specific CD4⁺ T cells when a model pathogen or nonpathogen-derived Ag, such as influenza hemagglutinin or OVA, is expressed in β cells (27, 28). Therefore exposure to pathogens with activation of the innate immune system or “help” from CD4⁺ T cells can promote progression to diabetes. Further, it was already
known that NOD8.3 CD8+ T cells need CD4+ "help" for activation since NOD8.3 mice on a recombination activating genes knockout (Rag1−/−/−) background also reduced frequency of diabetes (13). NOD8.3 mice have high titer IAA, indicating that the mice can generate immune responses against islet Ags other than IGRP despite their biased repertoire. Lastly, we previously showed that IGRP responses in nontransgenic NOD mice are dependent on responses to proinsulin (6).

In contrast to NOD-PI mice, the protection from diabetes is not complete in NOD-PI/NOD8.3 mice. Approximately 20% of the mice develop delayed diabetes. Reduced and delayed diabetes also occurs in NOD8.3 mice on Rag1−/− background, in which insulin-specific T cells are not expected to be present (13). Because of the unnaturally high frequency of β cell-specific T cells in NOD8.3 mice, some may undergo activation without insulin-specific T cells either in response to cross-reactive Ags or IGRP occasionally shed from β cells and presented in the pancreatic lymph node even in the absence of any autoimmune-mediated β cell damage (13).

The current study shows that requirements for T cell help, suggested by artificial transgenic models of diabetes, apply also to NOD8.3 mice with T cells that recognize IGRP, a natural β cell Ag and, in the NOD mouse, a spontaneous model of diabetes. Surprisingly, it appears that this help can come from T cells specific for proinsulin, spontaneously generated as a result of the NOD MHC and other NOD genes, rather than a more general requirement for CD4+ T cells of broad specificity. Although there is evidence that induction of tolerance to insulin can induce dominant tolerance to other Ags (29), to our knowledge, this is the first study to show recessive tolerance to insulin can induce tolerance to other Ags in a TCR transgenic mouse. We could reproduce the effect of insulin-specific T cells in vivo by activating APCs with agonistic anti-CD40 Ab or by STZ-induced cell apoptosis, that may act through TLR-2 (30). Therefore, IGRP-specific CD8+ T cells, even when present in large numbers, need an insulin-specific response, or something to replace it, to become fully active effector T cells and cause diabetes.

There may be significant clinical implications for this study. Subjects with preclinical diabetes enrolled in autoantigen intervention studies usually have responses to several autoantigens, indicating existing expanded T and B cell autoreactive populations. Our study suggests that depletion of proinsulin (primary autoantigen)-specific T cells may have a beneficial effect on other autoreactive T cells despite their prior expansion. There may be indirect evidence in humans for this. There was a significant effect of oral insulin in individuals who began the DPT-1 trial with high levels of insulin autoantibodies, indicating insulin autoimmunity. Tolerance induction with proinsulin in...
such subjects might, however, be bypassed if stimuli that enhance Ag presentation occur. Expansion of Ag-specific T cells in NOD8.3 mice is a result of expression of the TCR transgene in T cell precursors and thymic positive selection and is independent of T cell help. This has allowed us to show that NOD8.3 T cells need T cell help for effector function independent of proliferation. In a nontransgenic T cell repertoire, expansion and activation of Ag-specific T cells are not separated in this way. The current study suggests there might be beneficial effects of terminating insulin-specific CD4+ T cell help in NOD mice or humans with established preclinical diabetes and autoimmunity to multiple β cell Ags.

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Disclosures
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