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The Presence and Preferential Activation of Regulatory T Cells Diminish Adoptive Transfer of Autoimmune Diabetes by Polyclonal Nonobese Diabetic (NOD) T Cell Effectors into NSG versus NOD-scid Mice

Maximiliano Presa,* Yi-Guang Chen,† Alexandra E. Grier,* Edward H. Leiter,* Michael A. Brehm,‡ Dale L. Greiner,‡ Leonard D. Shultz,* and David V. Serreze*

NOD-scid.II2rg<sup>null</sup> (NSG) mice are currently being used as recipients to screen for pathogenic autoreactive T cells in type 1 diabetes (T1D) patients. We questioned whether the restriction of IL-2R γ-chain (Il-2r<sup>g</sup>)–dependent cytokine signaling only to donor cells in NSG recipients differentially influenced the activities of transferred diabetogenic T cells when they were introduced as a monoclonal/oligoclonal population versus being part of a polyclonal repertoire. Unexpectedly, a significantly decreased T1D transfer by splenocytes from prediabetic NOD donors was observed in Il-2r<sup>g</sup>-NSG versus Il-2r<sup>g</sup>-intact standard NOD-scid recipients. In contrast, NOD-derived monoclonal/oligoclonal TCR transgenic T cells in either the CD8 (A14, NY8.3) or CD4 (BDC2.5) compartments transferred disease significantly more rapidly to NSG than to NOD-scid recipients. The reduced diabetes transfer efficiency by polyclonal T cells in NSG recipients was associated with enhanced activation of regulatory T cells (Tregs) mediated by NSG myeloid APC. This enhanced suppressor activity was associated with higher levels of Treg GITR expression in the presence of NSG than NOD-scid APC. These collective results indicate NSG recipients might be efficiently employed to test the activity of T1D patient–derived β cell–autoreactive T cell clones and lines, but, when screening for pathogenic effectors within polyclonal populations, Tregs should be removed from the transfer inoculum to avoid false-negative results.

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Type 1 diabetes (T1D) in both humans and NOD mice results from the autoimmune destruction of insulin-producing pancreatic β cells mediated by the combined activity of pathogenic CD4 and CD8 T cells (1, 2). Although NOD mice develop T1D through mechanisms that appear to be pathologically similar to the case in humans, this model is not perfect, as some disease interventions effective in these animals have not yet proven to be clinically translatable (3). These difficulties have prompted the development of multiple humanized mouse models that could potentially be used to assess human T cells for diabeticogenic activity and to screen interventions that might attenuate such pathogenic effectors (4).

The most promising humanized mouse models are those derived from the immunodeficient NOD.Cg-Pkdcs<sup>scid</sup> II2rg<sup>null</sup> (NSG) stock. This NOD bicongenic stock harbors the spontaneous <i>scid</i> mutation that eliminates mature T and B lymphocytes, and also an engineered null mutation in the Il2rg gene (IL-2 common γ-chain receptor) that ablates signaling through the IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 cytokine receptors (4). These combined mutations, which prevent the development of functional NK cells as well as lymphocytes, in conjunction with unique features of the NOD genetic background, enable NSG mice to support engraftment with human cells and tissues far more efficiently than other immunodeficient strains (4).

In both humans and NOD mice, the primary T1D genetic risk factor is provided by various combinations of MHC (designated HLA in humans)-encoded class I and II molecules (2). For this reason, NSG mice have also been further modified to transgenically express various human T1D-associated HLA class I and class II molecules (5). In recent years, there have been several studies testing whether such NSG-HLA transgenic mouse stocks can be used to assess human T cells for diabeticogenic activity. Adoptive transfer of PBMCs containing a polyclonal array of T cells from a human T1D patient carrying the HLA-A2.1 class I variant was reported to induce a leukocytic infiltration of pancreatic islets (insulitis) in NSG-HLA-A2.1 transgenic recipients (6). However, the specificity of this inflammatory response was unclear. There have been two other reports that a T1D patient–derived CD8 T cell clone or CD4 T cell lines recognizing β cell autoantigens can induce both insulitis and specific β cell death when engrafted into appropriate HLA transgenic NSG recipients (7, 8). It should be noted that, to date, transferred polyclonal or monoclonal T cells from T1D patient donors have not yet induced overt hyperglycemia in NSG recipients. Hence, whereas introduction of the inactivated Il2rg gene enables higher engraftment levels of human T cells in NSG mice compared with first-generation NOD-scid recipients, this mutation’s negative effects on cytokine receptor signaling in host APC may also limit the functional activation of potential diabeticogenic effectors in the...
transfer inoculum. Furthermore, in NSG recipients, IL-2rγ−dependent cytokine signaling is limited to donor cells. Consequently, different outcomes might ensue if the transferred diabetogenic T cells were monoclonal or oligoclonal in nature versus being a relatively small part of a polyclonal repertoire within a PBMC inoculum also containing donor APC.

Because of the above possibilities, we assessed whether the well-known ability of total splenocytes or β-cell–autoantibody T cell clones derived from standard NOD donors to transfer T1D to NOD-scid recipients was recapitulated in NSG hosts.

Materials and Methods

Mice strains

NOD/ShiLtDvs, NOD-scid (NOD.Env30−/−; CB17-Prkdcscid), and NOD-NOD.scidPrkdcscid I2D-m2Smp/JicTac were maintained in our specific pathogen-free research colony at The Jackson Laboratory, NOD (NOD.Cg-Prkdcscid I2D-m2Smp/JicTac) mice were purchased from Taconic (Germantown, NY). A NOD stock transgenically expressing the TCR from the diabetogenic AH4 CD8 T cell clone, and also homozygous for an inactivated Rag1 allele (NOD.Rag1−/−A140Htg/Dvs/Dvs), in this work designated NOD-SCID (12), has been previously described (9). NOD stocks transgenically expressing diabetogenic TCRs derived from the NY8.3 CD8+ [NOD.Cg-Tg (TcraTcrbNY8.3.1)Pesa/DvsJ] (10) or BDC2.5 CD4+ [NOD.Cg-Tg (TcraBDC2.5, TcrrBDC2.5.2)D10s/D10j] T cell clones (11) were acquired from the type I diabetes resource (http://type1diabetes.jax.org/) operated at The Jackson Laboratory. A N18 backcross generation NOD stock congenically carrying the previously described Foxp3tm2(eGFP)-Foxp3 reporter construct (12) (formal designation NOD/L1DvS3.Eb6-Foxp3tm2(eGFP)Jigcs/pDvs/) and in this work noted as NOD-Foxp3-eGFP JAX stock 25097) was generated and also typed as homozygous for NOD alleles at markers delineating all known Idd genetic loci (2). The enhanced GFP (eGFP) reporter is a knockin downstream of the Foxp3 promoter and coding sequences designed to ensure independent and functional expression of both proteins (12).

Abs and flow cytometry analyses

Various fluorochrome-labeled Abs specific for CD8 (53-6.72), CD4 (RM4-5), CD3 (145-2C11), TCRαβ8.3 (B21.14), TCRβ8.1.2 (K166), TCRβ4 (KT4), CD44 (IM7.81), CD62L (MEL-14), CD25 (7D4), CD19 (ID3), CD11c (N418), CD11b (M170), PDCA-1 (9270), CD86 (16-10A1), CD86 (GL-1), CTLA-4 (IB8), GITR (YIGITR765), GITRIL (YGL386), and CD70 (FR70) were purchased from BD Biosciences (San Jose, CA) or BioLegend (San Diego, CA). Anti-mouse Foxp3 (FJK-16s) was purchased from eBioScience (San Diego, CA). NRP-V7-H-2Kk tetramer-KYKANVFL-PE was purchased from the National Institutes of Health tetramer core facility (Atlanta, GA). Anti-mouse CD25 (PC6.2.5) and CD4 (GK1.5) were purchased from Bioxcell (West Lebanon, NH). Dead cells were excluded by DAPI staining. Stained cells were acquired using a FACS LSRII instrument with sort gates set to exclude dead cells and debris.

Differential T1D induction in NSG recipients by polyclonal versus monoclonal autoreactive T cells

We compared the ability of total splenocytes from 6- to 8-wk-old NOD female donors to adoptively transfer T1D to NOD-scid and NSG recipients. As expected, NOD splenocytes efficiently transferred T1D to NOD-scid mice, but curiously induced a significantly lower level of disease in NSG recipients (Fig. 1A). In contrast, NOD-derived monoclonal CD8 T cells transgenically expressing the diabetogenic A4 TCR (15) transferred disease significantly more rapidly to NSG than NOD-scid recipients (Fig. 1B). Thus, normally diabetogenic T cells residing within a polyclonal repertoire had a limited ability to transfer disease to NSG recipients, but a monoclonal population of such effectors significantly did so.

Flow cytometric analyses of PBL from both recipient types of total NOD splenocytes showed a progressive increase in CD8 T cells posttransfer, but with significantly higher proportions in NSG than NOD scid mice (Fig. 1C). The proportion of CD4 T cells increased over time to significantly higher levels in NOD-scid than NSG recipients (Fig. 1D). These data indicated the lack of IL-2rγ expression in NSG host cells resulted in differential engraftment of donor polyclonal CD8 and CD4 T cells compared with NOD-scid recipients. However, these data also indicated that a gross engraftment deficiency of adoptively transferred NOD polyclonal T cells did not account for their suppressed induction of T1D in NSG recipients. Furthermore, both NSG and NOD-scid recipients of total NOD splenocytes exhibited equivalent levels of donor B lymphocyte engraftment (data not shown).

Flow cytometric analyses of PBL from recipients of monoclonal A4 CD8 T cells showed a higher proportion of such effectors in NSG than NOD-scid mice (Fig. 1E). As expected, PBL from either NOD-scid or NSG recipients of monoclonal A4 cells did not contain CD4 T cells (Fig. 1F). Similar to the case for PBL, at 2 wk postransfer significantly higher numbers of transferred monoclonal A4 CD8 T cells were detected in the spleens of NSG than NOD-scid recipients (Fig. 1G). In addition, analyses of activation markers on the same samples showed the proportion of effector-memory type
AI4 CD8 T cells in pancreatic lymph nodes (PLN) and mesenteric lymph nodes was significantly higher in NSG than NOD-scid recipients (Fig. 1H). This greater expansion and conversion to an effector memory phenotype of transferred monoclonal AI4 T cells in NSG than NOD-scid mice most likely account for why these effectors induced T1D more aggressively in the former recipients.

Diabetogenic activity of monoclonal CD8 T cells in NSG recipients is suppressed in the presence of polyclonal NOD splenocytes

The inactivated Il2rg gene in NSG host cells apparently contributes to the heightened ability of monoclonal AI4 CD8 T cells to engrift and induce T1D in this strain compared with NOD-scid recipients. Conversely, in contrast to standard NOD-scid recipients, polyclonal splenic NOD T cells could not efficiently induce T1D in NSG mice. We hypothesized these results could be explained by IL-2γ-dependent cytokine-signaling events only taking place in donor-derived cells in NSG recipients. To initially test this possibility, we compared the in vitro ability of residual myeloid splenocytes from NSG or NOD-scid mice to capture and internalize IL-2 as a model of a common γ-chain cytokine. This assay (Fig. 2A) clearly showed an impaired ability of NSG-derived splenocytes to respond to the IL-2 common γ-chain cytokine. Based on this finding, we next tested whether the NSG environment allows some cell population present in standard NOD, but not NOD-AI4 donor splenocytes, to

**FIGURE 1.** T1D transfer to NOD-scid and NSG recipients by polyclonal versus monoclonal autoreactive T cells. (A) Adoptive transfer of T1D by total NOD splenocytes (5 × 10^6) from prediabetic 6- to 8-wk-old female donors to NOD-scid (n = 20) and NSG (n = 20) recipients. (B) Adoptive transfer of T1D by monoclonal AI4 CD8 T cells (2.5 × 10^6 NOD-AI4 splenocytes) to NOD-scid (n = 27) and NSG (n = 31) mice. In both (A) and (B), T1D development rates compared by log-rank (Mantel–Cox) test (**p < 0.0001). (C and D) Frequency over time following engraftment with total NOD splenocytes of CD8 and CD4 T cells among PBL in NOD-scid and NSG recipients. (E and F) Frequency over time following engraftment with NOD-AI4 splenocytes of CD8 and CD4 T cells among PBL in NOD-scid and NSG recipients. (G) Total AI4 T cell numbers (CD8^+TCRVα8.3^+) in secondary lymphoid tissues: spleen (SPL), PLN, and mesenteric lymph nodes (MLN). (H) Proportion of AI4 CD8 T cells with effector memory phenotype (CD44^{high}CD62L^{low}), (G and H) Data from three independent experiments with a total n = 9 in each strain. In (C)–(H), *p < 0.05, **p < 0.001, ***p < 0.0001; ns, p > 0.05, two-way ANOVA with Bonferroni posttest correction.

**FIGURE 2.** The inability of NSG recipients to respond to common γ-chain cytokines is associated with an ability of NOD donor polyclonal T cells to impair T1D transfer by AI4 CD8 T cells. (A) Total splenocytes from NOD-scid or NSG mice (2.5 × 10^5) were cultured in vitro in the presence of 10 pg/ml IL-2 and with or without 0.1% NaN3 acting as inhibitor of cytokine translocation. After 3 d in culture, the amount of IL-2 remaining in the supranatant was quantified by ELISA. (B) NSG and NOD-scid mice were injected i.v. with a mixture of 2.5 × 10^6 NOD-AI4 and 5 × 10^6 standard NOD splenocytes. Data indicate T1D incidence from two independent experiments, total n = 20, for each strain. Disease incidence compared by log-rank (Mantel–Cox) test (**p < 0.0001). (C and D) At 2 wk posttransfer, the total number and activation phenotype of AI4 CD8^+ T cells in each type of recipient (n = 3 and 4, respectively, for NOD-scid and NSG recipients) were analyzed by flow cytometry in spleen (SPL), PLN, and mesenteric lymph node (MLN). In (A), (C), and (D), bars represent mean ± SEM. *p < 0.05, **p < 0.001; ns, p > 0.05; two-way ANOVA with Bonferroni posttest correction.
exert enhanced T1D-protective effects not elicited in NOD-scid recipients. This was done by determining whether the ability of monoclonal AI4 T cells to aggressively mediate T1D development in NSG recipients was attenuated by cotransfer of NOD splenocytes. This was indeed the case (Fig. 2B). We next evaluated whether the presence of some NOD splenocyte-derived cell population(s) blocked engraftment of diabetogenic AI4 CD8 T cells in NSG recipients. When cotransferred with NOD splenocytes, AI4 T cells continued to engraft at significantly higher levels in the spleens of NSG than NOD-scid recipients (Fig. 2C). However, in contrast to when they were transferred alone, following coinfusion with NOD splenocytes, AI4 T cells underwent significantly lower conversion levels to an effector memory phenotype in the PLNs of NSG than NOD-scid recipients (Fig. 2D). These collective findings indicate that when in a NSG, but not NOD-scid recipient environment, a NOD leukocyte population(s) acquires an enhanced ability to limit the cytopathic activation of diabetogenic T cells.

IL-2γr–dependent receptors that can bind, but not induce, cytokine signaling in host cells alter the engraftment of diabetogenic T cells without blocking their activation

The results described above indicated that when transferred alone into NSG, but not in NOD-scid recipients, common γ-chain cytokines produced by donor monoclonal AI4 T cells would only be able to feed back to these pathogenic effectors. We reasoned such lessened receptor-mediated competition for IL-2γr–dependent cytokines in NSG than NOD-scid recipients could explain why, when engrafted alone, monoclonal AI4 CD8 T cells elicit a more aggressive T1D onset in the former host environment. To further test this possibility, we analyzed another NOD-scid–related strain, NOG mice encoding an alternatively mutated version of the IL-2γ subunit that is expressed on the cell surface, but lacking a cytoplasmic domain needed to initiate cytokine signaling (5, 16). As a result, leukocytes in NOG mice can bind, but not respond, to IL-2γr–dependent cytokines. Thus, we tested whether a possible ability of host cells to limit availability of IL-2γr–dependent cytokines to pathogenic effectors resulted in less aggressive T1D development in NOG than NSG recipients of only monoclonal AI4 CD8 T cells. Splenic engraftment levels of adoptively transferred monoclonal AI4 T cells were significantly less in NOG than NSG recipients, but higher than in NOD-scid mice (Fig. 3A). This result might suggest that NSG-based stocks are a better choice if needed as recipients to support high expansion levels of T cell clones. However, despite this engraftment difference, IL-4-mediated T1D development was comparable in both NSG and NOG and equally more aggressive than in NOD-scid recipients (Fig. 3B). Similar results were observed in an independent experiment when 10-fold lower numbers (2 × 10^6) of NOD-AI4 splenocytes were transferred into NSG and NOG recipients (data not shown). It should be noted that, when observed for longer times posttransfer than in the experiment shown in Fig. 3B, most NOD-scid recipients of monoclonal AI4 T cells also eventually developed T1D (see Fig. 1B). These findings utilizing NSG versus NOG recipients indicate variability in host cell–mediated competition for binding of IL-2γr–dependent cytokines may alter the engraftment levels of monoclonal diabetogenic T cells, but does not result in differential pathogenic activation of such effectors.

A CD4 cell population diminishes the diabetogenic activity of NOD polyclonal T cells in NSG recipients

We hypothesized that the restriction in NSG recipients of IL-2γr–dependent cytokine-signaling events to donor cells might preferentially boost the numbers or functional activity of a Treg-like subset(s) present in a transferred polyclonal, but not monoclonal effector population, to levels inhibiting T1D development. This hypothesis was based on previous findings that the survival and immunosuppressive activity of the CD4–Foxp3^* subset of Tregs are highly IL-2 dependent (17). The AI4 CD8 T cell clonotype does not require interactions with CD4 helper populations to efficiently induce T1D (15). Thus, we determined whether the capacity of total NOD splenocytes to inhibit the diabetogenic activity of cotransferred AI4 CD8 T cells in NSG recipients required CD4 T cells. Flow cytometric analyses of PBL indicated only cotransfer of total and not CD4 T cell–depleted NOD splenocytes suppressed the engraftment levels of AI4 T cells in NSG recipients (Fig. 4). Furthermore, total, but not CD4 T cell–depleted NOD splenocytes inhibited the ability of AI4 T cells to transfer T1D to NSG recipients (Fig. 5A). These results demonstrated a CD4-expressing population(s) is responsible for the ability of total NOD splenocytes to inhibit disease induced by monoclonal AI4 CD8 T cells in NSG recipients.

Polyclonal repertoire-derived CD4^* Foxp3^* Tregs inhibit diabetogenic T cells in NSG recipients

Foxp3^* Tregs are a crucial CD4-coexpressing immunological suppressor subset (17). We used a newly developed NOD mouse stock in which an eGFP reporter is specifically expressed in Tregs (NOD.Foxp3^*eGFP) to determine whether this immunosuppressive population differentially engrafts NOD-scid and NSG recipients. The NOD.Foxp3^*eGFP stock develops T1D at a rate similar to standard NOD/LtDvs mice and expression of Foxp3 correlated with that of eGFP (Fig. 5B, 5C). Flow cytometric analyses of CD4^*Foxp3^* Treg levels at different time points postengraftment with NOD.Foxp3^*eGFP splenocytes showed no numerical strain differences between NOD-scid and NSG recipients (data not shown). This indicated the lower level of T1D development in NSG versus NOD-scid recipients of polyclonal NOD T cells is not due to a greater numerical engraftment of Tregs in the former strain. However, we hypothesized that enhanced Treg function may contribute to the limited
diabetogenic activity of polyclonal NOD T cells in NSG recipients. NSG recipients were infused with AI4 T cells alone or admixed with either total or reporter cell-depleted NOD Foxp3-eGFP splenocytes.

To avoid the generation of newly differentiated Foxp3+ Tregs (Fig. 5D), NSG mice engrafted with reporter cell–depleted NOD.Foxp3-eGFP splenocytes were treated with a CD25-specific Ab to maintain Treg ablation. Total, but not Treg-depleted splenocytes from NOD.Foxp3-eGFP donors blocked TID development in NSG recipients coinfused with AI4 T cells (Fig. 5E). Furthermore, after being depleted of marked Tregs, NOD.Foxp3-eGFP splenocytes containing polyclonal T cell populations acquired an ability when transferred on their own to efficiently induce T1D in NSG recipients (Fig. 5F). Importantly, we also found the residual myeloid APC population in NSG mice supported Treg suppressive activity to a significantly greater extent than those of NOD-scid origin (Fig. 6A, 6B). In at least some circumstances, increased expression and activity of the GITR costimulatory molecule can enhance Treg function (18). Thus, we tested whether GITR expression by Tregs resident among NOD splenocytes differed when these cells were transferred alone or in combination with AI4 effectors into NSG and NOD-scid recipients. Under both transfer situations, GITR expression by NOD donor splenocyte-derived Tregs, but not conventional T cells, was significantly higher in the PLNs of NSG than NOD-scid recipients (Fig. 6C, 6D). No differences were observed for GITR ligand expression (data not shown). Thus, the induction of a higher level of GITR expression may at least partially explain why Foxp33 Tregs exert greater suppressive activity when interacting with myeloid APC in NSG than NOD-scid recipients.
indicated ratios for 2 d in the presence of 1 
670 dye. The conventional CD4 and Treg populations were cocultured at the 
expression. The conventional CD4 T cells were then labeled with the eFlour 
*Foxp3+ Treg-mediated suppression of conventional CD4 T cell proliferative 
portions of conventional CD4 T cells remaining in an undivided state.

FIGURE 6. Residual myeloid APC from NSG mice support higher levels of Treg activity than those of NOD-scid origin. CD4 T cells within splenocytes from NOD,Foxp3-eGFP mice were sorted into conventional or Treg populations based on the respective absence or presence of eGFP marker expression. The conventional CD4 T cells were then labeled with the eFlour 670 dye. The conventional CD4 and Treg populations were cocultured at the indicated ratios for 2 d in the presence of 1 µg/ml anti-CD3 (1452C11) and 2 x 10^5 NSG or NOD-scid splenic leukocytes as a source of APC. The percentages of proliferating CD4 T cells were determined by flow cytometric analyses of eFlour 670 dilution. (A) Representative flow cytometric profiles of conventional CD4 T cell proliferation (indicated by eFlour 670 dye dilution) in the presence or absence of the indicated ratios of Foxp3+ Tregs and either NOD-scid or NSG APC. Numbers shown in each panel depict the mean percentage ± SEM of conventional CD4 T cells that remained in an undivided state (within the 0 dilution peak) under the indicated culture condition (n = 3 for each). (B) Summary of the percent suppression ± SEM (n = 3 for each culture condition) of conventional CD4 T cell responses in the presence versus the absence of Foxp3+ Tregs and either NOD-scid or NSG APC. Suppression was defined by the proportions of conventional CD4 T cells remaining in an undivided state. "Foxp3+ Treg-mediated suppression of conventional CD4 T cell proliferative responses significantly greater (p < 0.05, Student t test) in the presence of NSG than NOD-scid APC. (C) NSG or NOD-scid mice (n = 3 per group) were infused with 5 x 10^6 NOD,Foxp3-eGFP splenocytes alone or (D) in combination with 2.5 x 10^5 NOD-AH splenocytes. At 10 d posttransfer, the mean fluorescence intensity (MFI) of GITR-specific Ab staining by Foxp3+ Tregs as well as CD4 and CD8 conventional T cells within the PLN of each recipient type was determined. Data represent mean ± SEM. *p < 0.05; ***p < 0.0001; two-way ANOVA and Bonferroni posttest.

We also evaluated whether the greater suppressor activity of Tregs detected in the presence of NSG than NOD-scid–derived myeloid APC could be related to a differing distribution of dendritic cell (DC) subsets in these two strains. Total numbers of splenic myeloid cells were significantly less in NSG than NOD-scid mice (Fig. 7A). After normalizing the data to 4 x 10^7 total splenocytes (approximate mean level in NSG mice), analyses of the most common DC subsets and macrophages (19) by flow cytometry did not detect any significant proportional differences between the stocks (Fig. 7B). Furthermore, residual myeloid cells in NSG and NOD-scid mice did not differ in baseline expression levels of T cell costimulatory (CD80, CD86) or inhibitory (PD-L1, CD70) molecules (data not shown). There were also no differences in expression of these molecules on APC from the two stocks cultured with activated T cells in vitro. These collective data indicate the greater induction of Treg activity in the presence of NSG than NOD-scid–derived myeloid cells cannot be attributed to the dominance in the former strain of any specific APC subset or to their variable expression of the analyzed T cell costimulatory or inhibitory molecules.

Oligoclonal β cell–autoimmune MHC class I– and II–restricted T cells more readily induce T1D in NSG than NOD-scid recipients

To analyze whether the accelerated T1D development in NSG mice mediated by adoptively transferred monoclonal AI4 CD8 T cells was a phenomenon restricted to this clone, we tested the capacity of other NOD-derived MHC class I– and class II–restricted β cell–autoimmune active TCR transgenic T cells to induce disease in such recipients. NOD-NY8.3 mice transgenically express the TCR of a potent diabetogenic CD8 T cell clone specific for a peptide derived from islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP206–214) (20). Because the used NOD-NY8.3 stock does not also carry the scid or a rag gene knockout mutation, it produces NY8.3 CD8 T cells as an oligoclonal rather than a monoclonal population. Indeed, the residual CD4 T cells present in NOD-NY8.3 mice have been reported to enhance the diabetogenic activity of the NY8.3 effectors (10). Similar to the case with monoclonal AI4 effectors, oligoclonal NY8.3 CD8 T cells transferred T1D...
significantly more rapidly to NSG than NOD-scid recipients (Fig. 8A). Analyses of PBL at 3 wk posttransfer in mice receiving NOD-NY8.3 splenocytes showed a greater proportion of total and tetramer-specific NY8.3 CD8 T cells in NSG than NOD-scid recipients (Fig. 8B). Conversely, the proportion of NOD-NY8.3 donor-derived residual CD4 T cells was higher in NOD-scid than NSG recipients (Fig. 8B). Although CD4 Foxp3+ Tregs are phenotypically present in NOD-NY8.3 mice (∼20% of total residual CD4 T cells in spleen), their function is clearly not sufficient to suppress the diabetogenic activity of NY8.3 effectors in the donors. The residual phenotypic Tregs present in the NY8.3 donor cell inoculum engrafted at higher levels in NOD-scid than NSG recipients (Fig. 8C). The potential importance of this engraftment difference is questionable because we also found that, when depleted of Tregs, the NY8.3 donor cell inoculum continued to more readily induce T1D in NSG than NOD-scid recipients (data not shown). However, similar to the case for monoclonal AI4 cells, the higher engraftment levels of oligoclonal NY8.3 effectors in NSG than NOD-scid recipients are most likely an important factor for the more aggressive T1D development in the former host environment.

We also tested whether an oligoclonal population of diabetogenic CD4 T cells differed in their capacity to transfer disease to NSG and NOD-scid recipients. Indeed, when transferred as an oligoclonal population, autoreactive BDC2.5 TCR transgenic CD4 T cells induced T1D more efficiently in NSG than NOD-scid recipients (Fig. 8D). It should be noted that NOD-BDC2.5 mice producing such CD4 effectors as an oligoclonal population have a very low T1D incidence (21, 22). By contrast, when residual nontransgenic CD4 T cells or a monoclonal TCR transgenic CD8 T cell clonotype (AI4) to transfer T1D to NOD-scid recipients was recapitulated in NSG hosts. As expected, adoptively transferred β cell–autoreactive AI4 monoclonal CD8 T cells elicited T1D development in NOD-scid controls, but interestingly engrafted at higher levels and induced more aggressive disease onset in NSG recipients. Unlike NSG mice, the NOD-scid–modified NOG stock expresses receptors that can bind IL-2γ-dependent cytokines, but Tregs in the former host environment (Fig. 8E). Thus, we tested whether such differential engraftment and/or functional activity of cotransferred residual donor Tregs could account for the varying ability of BDC2.5 T cells introduced as an oligoclonal population to induce T1D in NSG and NOD-scid recipients. This was found to be the case because purified CD4 CD25 T cells (hence lacking Tregs) from NOD-BDC2.5 donors rapidly transferred T1D to both NSG and NOD-scid recipients (Fig. 8F). In this experiment, we did not find in either recipient type any donor CD4 T cells expressing the BDC2.5 TCR that had converted to a Foxp3 Treg phenotype by 2 wk posttransfer (data not shown).

**FIGURE 8.** Oligoclonal β cell–autoreactive CD8 and CD4 T cells can induce T1D in NSG mice. Total splenocytes from recently diabetic NOD-NY8.3 females were adjusted to inject i.v. 2.5 × 10^7 NY8.3 CD8 T cells into 8- to 10-wk-old NOD-scid or NSG females. (A) Diabetes incidence study showing representative results from three experiments. PBL from mice receiving NY8.3 T cells were analyzed by FACS at 3 wk posttransfer, for presence of (B) CD4, CD8, tetramer-specific NY8.3 CD8 T cells and (C) for presence of CD4 Foxp3 T cells. A total of 1.5 × 10^6 purified splenic CD4 T cells from NOD-BDC2.5 mice was injected i.v. into NOD-scid or NSG mice. (D) Mice were monitored for diabetes development. (E) At 2 wk posttransfer, the proportion of circulating Foxp3 Tregs was assessed by FACS in PBL isolated from mice receiving CD4 T cells from NOD-BDC2.5 donors. (F) A total of 2 × 10^6 purified CD4 CD25 T cells from NOD-BDC2.5 donors was injected i.v. into NOD-scid (n = 10) or NSG (n = 10) mice; diabetes incidence is shown. Data represent mean ± SEM. **p < 0.001, ***p < 0.0001, ****p < 0.00001, nonparametric t test (Mann–Whitney).
does not enable them to induce signaling responses. Nonetheless, despite possible differences in host cell–mediated competition for binding of IL-2γ–dependent cytokines, T1D transfer kinetics by monoclonal NY8.3 T cells in NOD and NSG were comparable. Thus, diminished receptor-mediated competition by host cells for IL-2γ–dependent cytokines that we demonstrated would occur in NSG versus NOD-scid mice is unlikely to solely account for why adoptively transferred monoclonal NY8.3 CD8 T cells induce a more aggressive T1D onset in the former recipients.

Diametrically opposite results were obtained when NOD diabeticogenic T cells were introduced into NOD-scid and NSG recipients as a broad polyclonal repertoire. In this case, polyclonal NOD splenocytes induced a significantly lower level of T1D in NSG than NOD-scid recipients. Interestingly, cotransfer of total NOD splenocytes inhibited the ability of monoclonal NY8.3 T cells to induce T1D in NSG recipients. This inhibitory activity was ultimately traced to enhanced activity, but not numbers, of CD4+ Foxp3+ Tregs present in polyclonal NOD splenocytes upon transfer into NSG recipients. Indeed, when first depleted of Tregs, NOD splenocytes on their own efficiently transferred T1D to NSG recipients. The question then becomes why the functional activity of Tregs resident among NOD polyclonal T cells populations only reaches a T1D-protective level when transferred into NSG recipients. Again, the most likely explanation lies in the fact that, in NSG recipients, IL-2γ–dependent cytokine-signaling events only occur in donor cells. The survival and activities of Tregs are highly dependent on IL-2 (17). Thus, the lack of host cell–mediated receptor competition in NSG recipients, demonstrated in part by our current studies, may enhance IL-2 availability to Tregs resident among transferred NOD splenocytes and increase their functional activity to T1D inhibitory levels. This possibility is further supported by previous reports that IL-2 treatment inhibits T1D development in NOD mice by boosting Treg activity (26, 27). The lack of host cell–mediated receptor competition may also enable other IL-2γ–dependent cytokines to contribute to enhanced donor Treg activity in NSG recipients. Indeed, a recent study indicated that, at least under some circumstances, IL-15 contributes to the accumulation of Tregs (28). Furthermore, it has been reported that IL-21–dependent signaling events are required for the normal ability of NOD APC to exert diabetogenic activity (29). Regardless of the particular cytokine(s) involved, our current results indicate that NOD APC in which IL-2γ–dependent signaling responses do not occur may be preferentially skewed to a state promoting the maintenance of T1D-protective immune tolerance through an enhanced capacity to elicit Treg activity. Our studies did not find significant recipient strain differences in the proportions of common DC and macrophage subsets or their expression patterns of analyzed T cell costimulatory or inhibitory molecules that could account for the greater ability of NSG-derived APC to support Treg activity. However, we did find that GITR was expressed at significantly higher levels by NOD donor Tregs, but not conventional T cells, when interacting with myeloid APC in NSG than NOD-scid recipients. Previous studies (18) found at least under some conditions that increases in GITR expression and activity can enhance the suppressive activity of Tregs.

Our results utilizing the A4H system can be extended to other diabetogenic TCR transgenic models such as NOD-NG8.3 and NOD-BDC2.5. We are aware that our results indicating oligoclonal NY8.3 CD8 T cells efficiently transfer T1D to NSG mice contradict an earlier report that such donor cells poorly induce disease in the same recipients, which was attributed to a lack of IL-15 signaling in host APC (30). Although the true basis for this discrepancy remains unknown, it might be attributable to a varying dose of transferred NY8.3 splenocytes that was not detailed in this previous study, or differing environmental conditions. Regardless, our results indicate that in order for a range of diabetogenic T cells to induce disease in NSG recipients, they must either be introduced as monoclonal or oligoclonal populations or, if part of a polyclonal repertoire, the transfer inoculum must be first purged of Tregs. Another consideration is that, whereas NK cells remain present in NOD-scid mice, they are absent in the NSG strain (5). Hence, the augmented ability of monoclonal or oligoclonal diabetogenic T cells to induce disease onset in NSG recipients is a NK cell–independent process.

In conclusion, our findings reveal some important considerations that may be applicable when contemplating the possible use of NSG mice as recipients to screen for the presence of diabetogenic T cells resident within various human donor cell inocula, or as a resource to develop means to attenuate such effectors. Our results indicate that NSG-based models may be good recipients for studies entailing the transfer of β cell–autoimmune T cell clones or lines from human donors. Other results indicate that if the goal is to maximize expansion of a transferred clonal population of diabetogenic T cells in murine recipients, then NSG may be preferable to NOG mice for this purpose. Finally, the current results indicate that, when considering the use of NSG recipients to test for the presence of diabetogenic effectors among polyclonal or even some oligoclonal populations of human T cells, Tregs should first be removed from the transfer inoculum to eliminate false-negative results.

Disclosures
The authors have no financial conflicts of interest.

References


