Regulatory T cells control NK cells in an insulitic lesion by depriving them of IL-2

Jonathan Sitrin,[1] Aaron Ring,[2] K. Christopher Garcia,[2] Christophe Benoist,[1] and Diane Mathis[1]

[1]Division of Immunology, Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115
[2]Department of Molecular and Cellular Physiology, and Department of Structural Biology, Stanford University School of Medicine, Stanford, CA 94305

Regulatory T (T reg) cells control progression to autoimmune diabetes in the BDC2.5/NOD mouse model by reining in natural killer (NK) cells that infiltrate the pancreatic islets, inhibiting both their proliferation and production of diabetogenic interferon-γ. In this study, we have explored the molecular mechanisms underlying this NK–T reg cell axis, following leads from a kinetic exploration of gene expression changes early after punctual perturbation of T reg cells in BDC2.5/NOD mice. Results from gene signature analyses, quantification of STAT5 phosphorylation levels, cytokine neutralization experiments, cytokine supplementation studies, and evaluations of intracellular cytokine levels collectively argue for a scenario in which T reg cells regulate NK cell functions by controlling the bioavailability of limiting amounts of IL-2 in the islets, generated mainly by infiltrating CD4+ T cells. This scenario represents a previously unappreciated intertwining of the innate and adaptive immune systems: CD4+ T cells priming NK cells to provoke a destructive T effector cell response. Our findings highlight the need to consider potential effects on NK cells when designing therapeutic strategies based on manipulation of IL-2 levels or targets.

CORRESPONDENCE
Christophe Benoist
AND
Diane Mathis Email:
cbdm@hms.harvard.edu

Abbreviations used: DTR, diphtheria toxin receptor; NOD, nonobese diabetic; MCMV, murine cytomegalovirus; MFI, mean fluorescence intensity; STAT, signal transducer and activator of transcription; T1D, type 1 diabetes; T reg cell, regulatory T cell.

Regulatory T (T reg) cells , in particular those expressing the forkhead box transcription factor Foxp3, are primary controllers of immune responsiveness and peripheral immunological tolerance (Rudensky, 2011). These critical immunoregulatory cells have been implicated in the control of an assortment of immunological processes, ranging from autoimmunity to infection. In humans, loss-of-function mutations of Foxp3 lead to a severe multi-organ autoimmune and inflammatory disorder called IPEX (immune dysfunction, polyendocrinopathy, enteropathy, X-linked inheritance). *Scurfy* mice, carrying a frameshift mutation in Foxp3, show a similar fatal systemic disease. Moreover, conditional ablation of the T reg cell lineage demonstrated a lifelong requirement for Foxp3-expressing cells to contain highly aggressive, multi-organ autoimmunity, even after normal development of the immune system.

T reg cells also regulate several organ-specific autoimmune diseases, notably type-1 diabetes (T1D), characterized by autoimmune attack specifically on β cells in the pancreatic islets of Langerhans (Bluestone et al., 2008). Supplementation with T reg cells or enhancement of their function protected from T1D, whereas genetic deficiencies in or experimental reductions of T reg cells exacerbated disease in the nonobese diabetic (NOD) mouse model or its T cell receptor (TCR) transgenic derivatives.

Exactly how T reg cells exert their impact on immune responsiveness has been the subject of extensive exploration. To date, numerous protective mechanisms have been ascribed to them, reflecting their expression of several regulatory molecules, either displayed at the cell surface or secreted (Vignali et al., 2008; Josefowicz et al., 2012). It has become clear that the context in which T reg cells perform their regulatory function can shape the mechanisms of immune suppression they use, i.e., the tissular location or inflammatory "flavor" of the response they are participating in (Sojka et al., 2008; Josefowicz et al., 2012).

The behavior of T reg cells in the insulitic lesion of BDC2.5/NOD TCR transgenic mice

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(Katz et al., 1993) serves as an instructive example. This line carries the rearranged TCR genes of a diabetogenic T cell clone isolated from a NOD mouse and has been instrumental in the identification of a spectrum of immunoregulatory genes, molecules, and cells that control the frequency and aggressivity of diabetogenic T cells (André et al., 1996). When the BDC2.5 TCR transgenes are propagated on the NOD genetic background, T cells stereotypically invade the islets at 15–18 d of age and seed a massive infiltration therein; however, progression to diabetes occurs rarely (10–20%) and only months later, reflecting strong immunoregulation (Gonzalez et al., 1997). When a transgene expressing the diphtheria toxin receptor (DTR) under the dictates of the Foxp3 promoter/enhancer elements was crossed into this system (BDC2.5/NOD.Foxp3^{DTR} mice), conditional T reg lineage ablation provoked nearly 100% penetrance of diabetes within days (Feuerer et al., 2009), highlighting the requirement for T reg cells to guard against T1D. Analysis of the insulitic lesion revealed, surprisingly, that the earliest detectable responders to the loss of T reg cells were NK cells, which accumulated to a higher fraction of the infiltrating cells and began to produce IFN-γ within hours. Subsequently, there was increased activation of diabetogenic CD4$^+$ T cells, including their production of IFN-γ. Neutralizing IFN-γ or depleting NK cells dampened pancreatic CD4$^+$ T cell activation and substantially delayed the onset of diabetes. Thus, there seemed to be a direct and continual requirement for T reg cells to keep NK cells, and ultimately diabetes, in check.

Much of the T reg cell–centered research over the last decade has focused on their control of populations typically considered to be participants in adaptive immune responses, especially other T cells and antigen-presenting cells. Less emphasis has been placed on their impact on cells involved in innate immune responses, notably NK cells. This neglect is a bit surprising given that NK cells were long ago found to be hyperproliferative and functionally enhanced in *scurfy* mice (Ghiringhelli et al., 2005). Furthermore, the original report describing T reg ablation also documented a large increase in NK cell numbers (Kim et al., 2007). An exception is the growing body of work on mouse cancer models and human cancer patients that demonstrates a negative correlation between NK and T reg cells, as concerns both presence and function (Shimizu et al., 1999; Ghiringhelli et al., 2005, 2006, 2007; Smyth et al., 2006). The mechanism most commonly highlighted in these studies was T reg mobilization of TGF-β, often in surface-bound form, to directly inhibit NK cell function. In support of this scenario, blockade of TGF-β signaling in NK cells in a mutant TGF-β receptor transgenic model caused a dramatic increase in cell numbers and enhanced secretion of IFN-γ (Laouar et al., 2005).

Given that this axis is still relatively unexplored, particularly in the context of autoimmune disease, we sought to identify the molecular underpinnings of T reg cell/NK cell cross-talk in control of diabetes in the BDC2.5/NOD model. Our explorations were greatly facilitated by the rapidity and synchrony of the diabetogenic changes unleashed by punctual ablation of T reg cells in BDC2.5/NOD.Foxp3^{DTR} mice. This feature permitted us to perform an accurate, detailed inventory of molecular changes over time, and to test mechanistic hypotheses with short courses of inhibitory or enhancing treatments. Our findings need to be considered in future strategies to prevent or dampen T1D.

RESULTS

Acute T reg cell perturbation in BDC2.5/NOD mice rapidly induced signs of activation in pancreas-infiltrating NK cells

To identify molecular pathways underlying the response of pancreatic NK cells to a loss of T reg control, we performed microarray-based gene-expression profiling, as a comprehensive and unbiased approach. NK cells from pancreata of insulitic BDC2.5/NOD.Foxp3^{DTR} mice and control DTR-negative littermates were analyzed 24 h after DT treatment (Fig. 1 A). Even at this relatively early time point, there were clear transcriptional changes in the T reg cell–depleted mice: induction of 89 genes >2-fold (highlighted in red) and repression of 123 genes >2-fold (in blue) compared with 1 and 0 loci, respectively, in an analogous comparison of randomized datasets. The transcripts up-regulated in the absence of T reg cells included indicators of the three canonical activities of NK cells: proliferation (*zbtb32*, 5.9-fold [J.C. Sun, personal communication]), cytokine production (*ifng*, 2.5-fold), and cytotoxicity (*gzmb*, 2.6-fold). Such changes fit well with our previous demonstration that ablation of T reg cells in this model induced cytotoxic activity from, proliferation of, and IFN-γ production by islet-infiltrating NK cells (Feuerer et al., 2009).

The full list of genes whose expression was at least doubled or halved in response to T reg cell removal is presented in Table S1. A quick glance at the induced loci revealed many of them to be characteristic of activated NK cells. More precisely, we compared the response of pancreatic NK cells to T reg depletion with that of splenic NK cells challenged by general (cytokine) or more specific [murine cytomegalovirus (MCMV)] stimuli. The diagonal nature of the red cloud in the fold-change/fold-change (FC/FC) plot of Fig. 1 B denotes substantial overlap between the genes induced in pancreatic NK cells by removal of T reg cells and in splenic NK cells stimulated in culture with IL-12 + IL-18. The tilt toward the horizontal axis signifies that the cytokine stimulus was more potent under these particular experimental conditions. An analogous result was found for the blue-colored repressed transcripts, with an even more pronounced skewing. Overall, 79% of the transcripts that increased or decreased by >2-fold after T reg cell removal were similarly augmented or diminished, respectively, subsequent to cytokine stimulation in culture. Similar, although less striking, observations came from the comparison of pancreatic NK cells responding to a loss of T reg cells and splenic NK cells mobilized by MCMV infection (Fig. 1 C). In this case, 51% of the transcripts induced or repressed >2-fold by T reg cell ablation were enhanced or dampened, respectively. Notably, *ifng*, *zbtb32*, and *gzmb* were induced in both the cytokine-stimulated and MCMV-induced responses.

We also addressed how rapid and localized the transcriptome alterations were after punctual T reg cell ablation. Fig. 1 D

shows FC/FC plots comparing transcriptional changes in the pancreas and spleen with or without T reg cell perturbation at 8-, 15-, and 24-h time points. The bull's eye nature of the black cloud of expression values at 8 h indicates that the bulk of transcripts were only minimally changed in the two organs. Yet, values for transcripts destined to be up-regulated (red) or down-regulated (blue) in pancreas-infiltrating NK cells had already diverged in the pancreas, which is impressive given that there was no evident loss of T reg cells at this early time point (unpublished data). This divergence was further amplified at 15 h. At 24 h, slight tilts of both the induced and repressed transcript values away from the horizontal axis, toward the diagonal, suggest that the same set of genes was modulated in the spleen, but with delayed kinetics.

T reg control of pancreas-infiltrating NK cells did not operate acutely through TGF-β

Considering that TGF-β has repeatedly been implicated in the control of NK cell functions in other contexts (Uhl et al., 2004; Friese et al., 2004; Lee et al., 2004; Ghiringhelli et al., 2005,

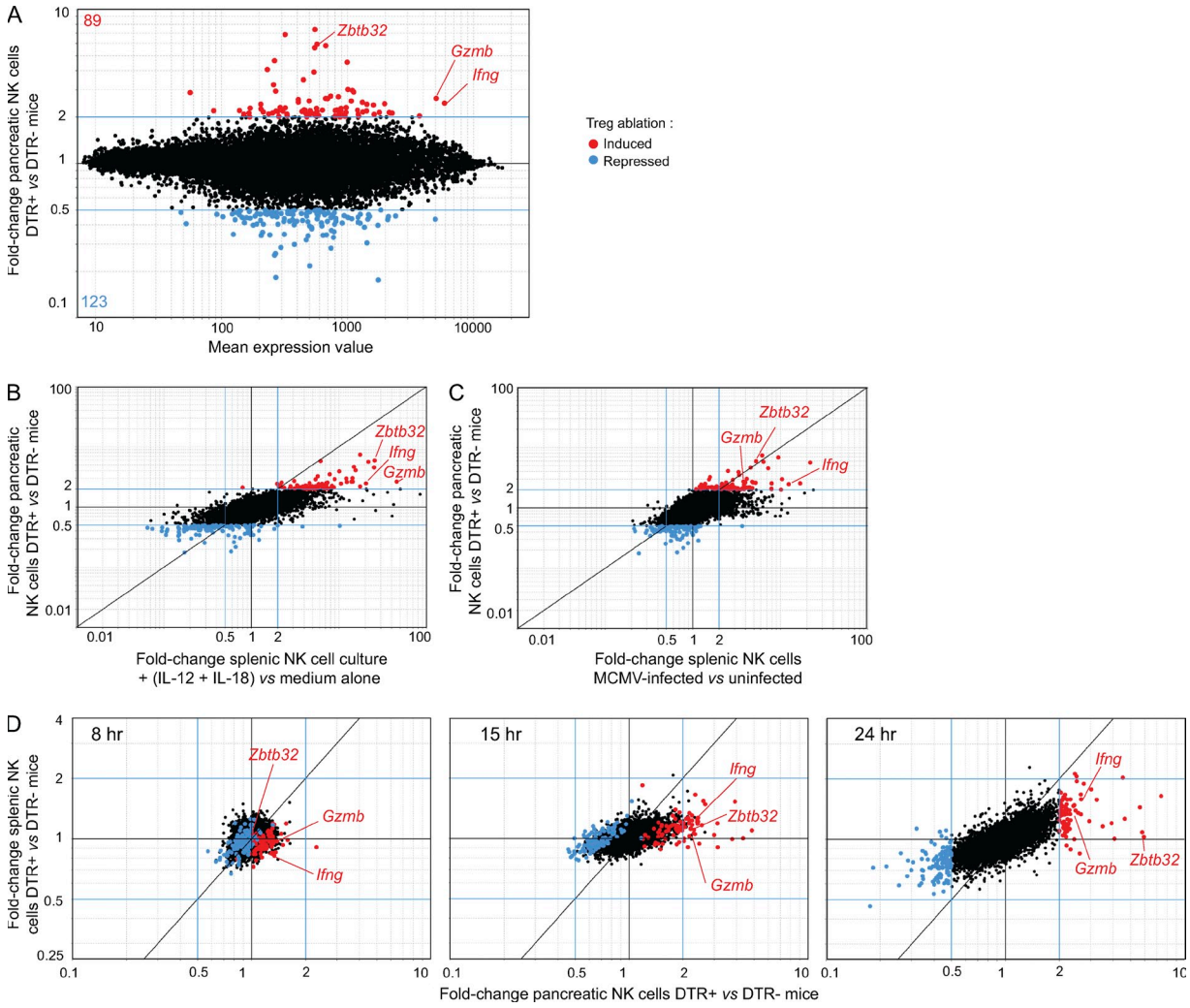

Figure 1. Gene expression changes in pancreatic NK cells soon after T reg cell perturbation. NK cells were sorted from the pancreatic infiltrate 24 h after DT injection into BDC2.5/NOD mice with or without DTR expressed in T reg cells, and were profiled by microarray. (A) Transcript changes. Differential gene-expression (FC) values for DTR$^+$ (n = 3) and DTR$^-$ mice (n = 6) (y axis) versus their two-class mean (x axis). Induced (>2-fold) genes are highlighted in red and repressed (>2-fold) genes are highlighted in blue. (B and C) Activation features. FC/FC plots of the same NK cell data as in A versus NK cells responding to different activation stimuli. Y axis, FC values for DTR$^+$ versus DTR$^-$ mice; x axis, FC values for splenic NK cells cultured with (n = 3) or without (n = 2) IL-12 + IL-18 (see Materials and methods for details; B) or splenic NK cells from C57/BL6 mice 24 h after being infected with MCMV or not (n = 3; C). (D) Time course of transcript changes. Carried out as in A, except additional time points at 8 and 15 h were examined. FC/FC plots comparing gene expression differentials for pancreatic NK cells from DTR$^+$ and DTR$^-$ BDC2.5/NOD mice (y axis) versus splenic NK cells from the same animals (x axis). Multiple replicates of cellular populations were collected (usually n = 3–6) and averaged. Highlighting in A–D represents the same set of genes.

2006; Laouar et al., 2005; Smyth et al., 2006), we wondered whether this cytokine is involved in the ability of T reg cells in the pancreas of BDC2.5/NOD mice to keep the insulitic lesion in check. The slight underrepresentation of TGF-β–dependent genes in the pancreatic $CD4^+$ T cell transcriptome after T reg ablation fit this hypothesis (Feuerer et al., 2009). First, we confirmed that TGF-β could regulate IFN-γ production by NOD-genotype NK cells in an in vitro culture system. Indeed, splenic NK cells stimulated in culture with IL-12+IL-18 produced less IFN-γ in the presence of TGF-β (Fig. 2 A; mean fluorescence intensity [MFI] reduced 77 ± 11%). Moreover, microarray analysis revealed a clear negative correlation between transcript changes provoked by addition of TGF-β to cultures of activated splenic NK cells and by interference with in vivo T reg control of pancreatic NK cells; i.e., the majority of genes up-regulated in the absence of T reg

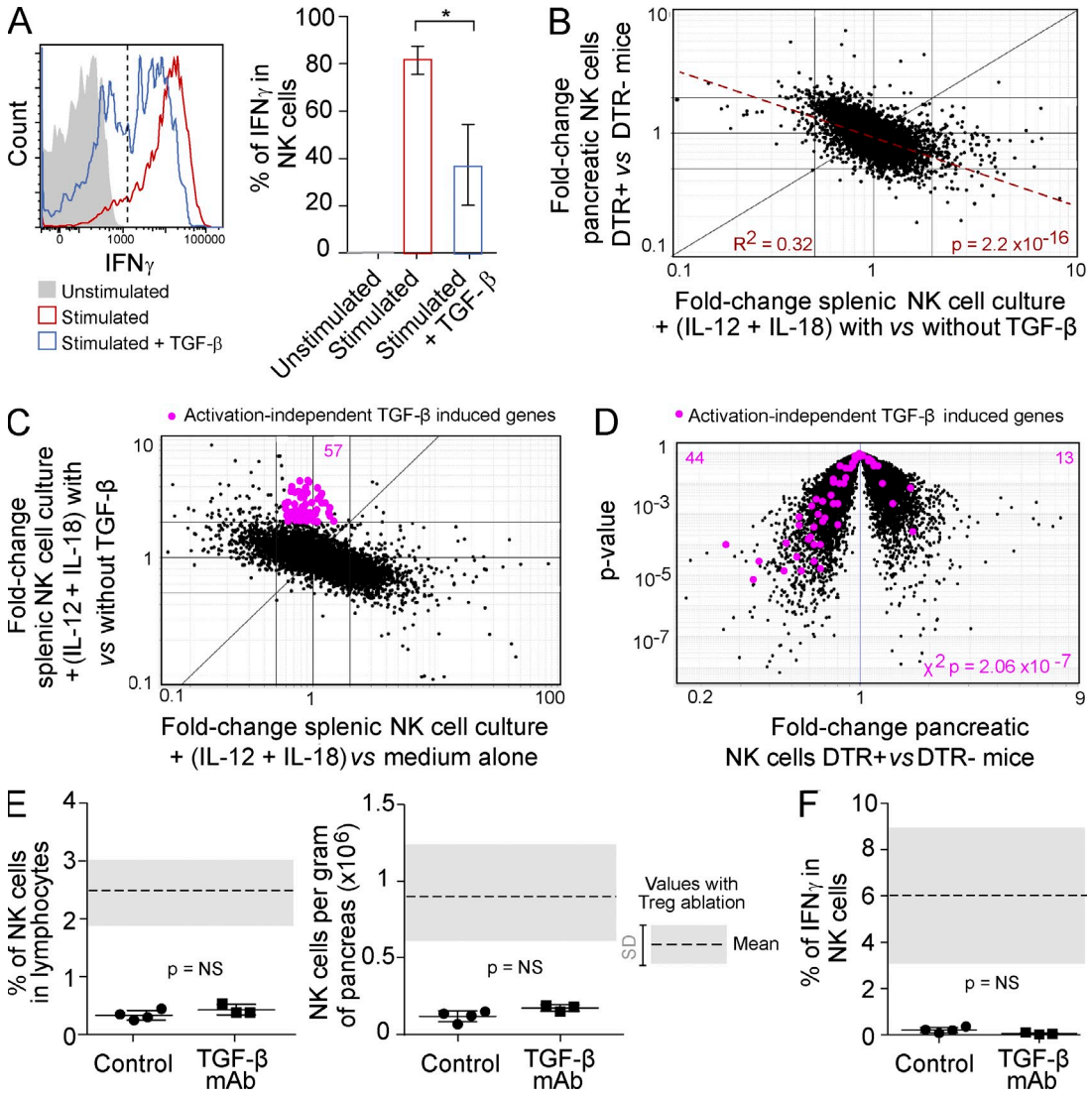

Figure 2. The role of TGF-β signaling. (A, left) Representative flow cytometry profiles of IFN-γ expression in NOD-derived splenic NK cells cultured with (red) or without (gray) IL-12 + IL-18 in the absence of TGF-β, or with both cytokines and TGF-β (blue). (right) Summary data from three independent experiments. Mean ± SD. The p-value was calculated using the two-tailed unpaired Students' *t* test. (B) Reciprocal transcript changes promoted by the loss of TGF-β and T reg cells. FC/FC plot comparing cytokine-activated, cultured, splenic NK cells ± TGF-β (x axis, $n = 3$) versus pancreatic NK cells from BDC2.5/NOD mice ± T reg ablation (y axis, same data as in Fig. 1 A). Dashed red line, linear regression. (C) FC/FC plot comparing transcriptional profiles of cytokine-activated, cultured, splenic NK cells ± TGF-β (y axis, as in Fig. 2 B) versus NK cells ± cytokine-activation (x axis, as in Fig. 1 B). Activation-independent TGF-β–induced genes were highlighted in pink and were superimposed (D in pink) on a volcano plot (p-value vs. fold change) of NK cell transcripts from BDC2.5/NOD mice depleted or not of T reg cells (same data as in Fig. 1). The number of signature genes up-regulated (right) or down-regulated (left) 24 h after T reg cell perturbation are indicated. P-value from the χ^2 test. (E and F) Summary flow cytometry data for BDC2.5/NOD mice treated for 24 h with anti–TGF-β versus either an isotype-control mAb or PBS (combined). Percentage of NK cells of lymphocytes (E) and percentage of IFN-γ^+ NK cells (F) for two or three independent experiments. Mean ± SD. Dotted line (mean) and gray shading (SD) represents values achieved after T reg cell ablation (a composite of multiple independent experiments).

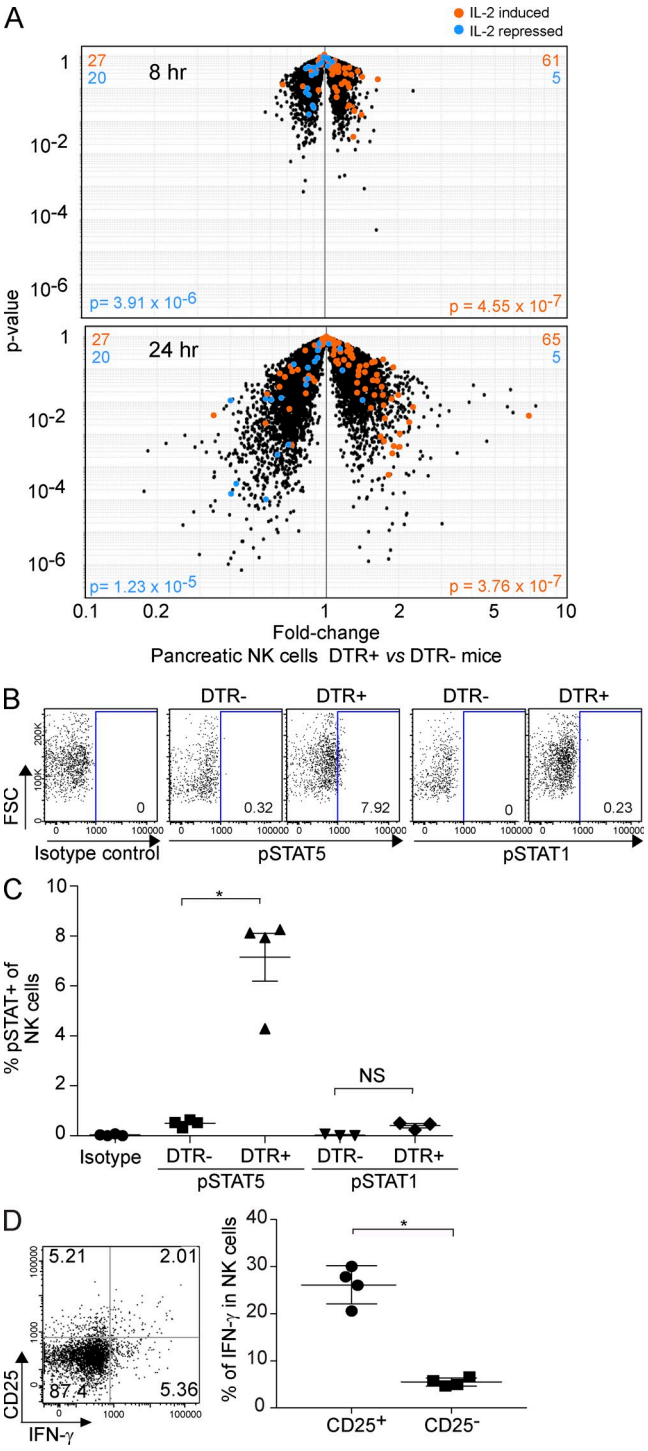

Figure 3. An IL-2 footprint is induced by T reg ablation. (A) IL-2–induced (orange) and –repressed (blue) gene transcripts (Marzec et al., 2008) are superimposed on volcano plots (p-value versus FC) of pancreatic NK cell transcripts up-regulated (to the right) or down-regulated (to the left) by perturbation of T reg cells (data from Fig. 1) at 8 h (top) and 24 h (bottom). P-values calculated using the χ^2 test. (B) Representative flow cytometry plots for NK cells isolated from the pancreas of BDC2.5/NOD mice 24 h after T reg ablation or not. (C) Summary data for three to four independent experiments with mean ± SD. (D, left) Representative flow cytometry plots for pancreas-infiltrating NK cells after T reg cell ablation; (right) summary for the percentage of CD25-expressing versus CD25-negative NK cells simultaneously expressing IFN-γ from at least three independent experiments.

cells were down-regulated by TGF-β, and vice versa (Fig. 2 B). Lastly, we generated a signature for TGF-β's impact on NK cells independent of general activation by comparing transcriptional profiles of NK cells that were cytokine-activated or not and stimulated with TGF-β or not (Fig. 2 C; genes listed in Table S2), and followed its distribution in pancreas-infiltrating NK cells unleashed in the absence of T reg cells (Fig. 2 D). The TGF-β–induced genes were repressed upon removal of T reg cells (i.e., they fall to the left of unity in the FC versus p-value volcano plot of Fig. 2 D). These data were all consistent with the notion that T reg cells might operate through TGF-β signaling to control NK cells in the insulitic lesion.

To directly test this hypothesis, we attempted to mimic punctual T reg cell ablation by injecting a mAb recognizing TGF-β into BDC2.5/NOD mice. This intervention was unable to recapitulate the effects of T reg depletion as neither the fraction/number of pancreatic NK cells (Fig. 2 E) nor their production of IFN-γ (Fig. 2 F) was induced anywhere near the levels observed with T reg ablation (shaded gray). The mAb was bioactive, however, as it substantially reduced the fraction of CD103$^+$ T reg cells, in particular in the mesenteric lymph nodes (not shown), a population known to be TGF-β dependent (Feuerer et al., 2010; Reynolds and Maizels, 2012). Thus, TGF-β may not play an important role in T reg control of NK cell activation in this context, at least not acutely. It is also possible that the insulitic lesion provides an environment that is unusually resistant to neutralization of TGF-β.

Punctual ablation of T reg cells elicited an IL-2 response signature in pancreas-infiltrating NK cells

For several reasons, we wondered whether IL-2 might be a driver of the pancreatic NK cell transcriptome changes provoked by loss of T reg cells in BDC2.5/NOD mice: first, a rapid loss of cells, like T reg cells, that express the high-affinity IL-2R component IL-2Rα (CD25) stands to substantially increase IL-2 bioavailability; second, sequestration of IL-2 by T reg cells is known to be one of their mechanisms of controlling T cells (Barthlott et al., 2005; Scheffold et al., 2005; Pandiyan et al., 2007); third, IL-2 can prime NK cells, both in vitro and in vivo, for proliferation and IFN-γ production (Fehniger et al., 2003; Granucci et al., 2004; Lee et al., 2012); and, finally, previous studies on the NOD mouse model of T1D suggested a limited IL-2 availability in the islet infiltrate (Tang et al., 2008b). To address this possibility, we overlaid an IL-2–responsive gene signature derived from a human CD4$^+$ T cell lymphoma (Marzec et al., 2008) onto a volcano plot depicting the transcriptional response of pancreatic NK cells to punctual T reg ablation (Fig. 3 A). As early as 8 h and continuing through 24 h after DT treatment, there was a significant skewing of IL-2–induced genes within the set of loci up-regulated in pancreatic NK cells and an analogous enrichment of signature IL-2–repressed genes among the down-regulated loci,

suggesting an increased signaling by the cytokine. Such enrichment was also seen with two other IL-2 responsive gene signatures: one derived from murine $CD8^+$ T cells (Verdeil et al., 2006) and the other from human NK cells (Dybkaer et al., 2007; not depicted).

In addition, flow cytometric analysis revealed a substantial increase in the phosphorylation of STAT5, an event downstream of IL-2R engagement and required for signal transduction, in pancreatic NK cells from T reg cell–depleted versus control mice. STAT1, not a member of the signaling cascade downstream of IL-2R, showed minimal additional phosphorylation, which was not statistically significant (Fig. 3, B and C). When we gated on IFN-γ^+ NK cells, the MFI of pSTAT5 was 2.2-fold (±0.7) higher. Furthermore, a higher fraction of CD25-expressing, as opposed to CD25-nonexpressing, NK cells in the pancreatic infiltrate made IFN-γ (Fig. 3 D), suggesting that the former might have a competitive advantage. However, the fact that a clear $CD25^-$ IFN-γ^+ population was detectable indicated that expression of CD25 was not required for IFN-γ production. Collectively, these data indicate that, upon removal of T reg cells, pancreatic NK cells experienced heightened signaling through the IL-2R, which could potentially be responsible for their activation.

IL-2 was required for the activation of pancreas-infiltrating NK cells provoked by loss of T reg cells

To evaluate the importance of IL-2 in the pancreatic NK cell response to acute T reg depletion in BDC2.5/NOD mice, we tested the effect of neutralizing this cytokine with a mAb. Complicating such an experiment was the fact that the mAb could potentially stabilize IL-2 and direct its binding to the high- and/or low-affinity receptor (Boyman et al., 2006). Therefore, we used the mAb JES6-1, as the complexes it forms with IL-2 bind highly preferentially to the α chain of the high-affinity IL-2R (expressed mostly on T reg cells) rather than to the low-affinity IL-2Rβ chain (expressed on NK cells; Boyman et al., 2006). In the context of T reg cell ablation, then, any such in vivo–generated IL-2–JES6-1 complexes should be fairly innocuous.

Treatment of BDC2.5/NOD mice with JES6-1 in conjunction with T reg cell ablation blocked NK cell accumulation, whereas analogous administration of an isotype-control mAb had no detectable effect (Fig. 4 A). Importantly, no significant drop in NK cell number or fraction occurred when JES6-1 was administered to DTR-negative littermate controls, indicating that simple IL-2 starvation did not provoke a wave of NK cell death. T reg cells remained at low levels after DT plus anti–IL-2 injection, demonstrating that IL-2–JES6-1 complexes did not trigger "bounce-back" of the T reg cell population in the pancreas (Fig. 4 B).

IL-2 neutralization also blocked the NK cell production of IFN-γ elicited by punctual T reg depletion, as measured by the percentage of IFN-γ–expressing NK cells (Fig. 4 C) and a reduction in the MFI of IFN-γ in NK cells (55 ± 19%). This is a critical point because we have previously documented that experimental blockade of IFN-γ signaling in this model sufficed in and of itself to halt the characteristic extremely rapid induction of T1D (Feuerer et al., 2009). Thus, IL-2 appears to be a crucial element in the unleashing of pancreatic NK cells when relieved of T reg cell control.

A potential caveat to the use of a mAb that forms IL-2 complexes preferentially capable of engaging CD25 is that NK cells often increase CD25 expression in response to activating stimuli. Therefore, we examined to what extent T reg cell ablation induced expression of this receptor and if mAb blockade altered that effect. Levels of CD25 did not increase on NK cells in the absence of T reg cells; however, T reg ablation combined with mAb treatment did enhance expression of CD25 (Fig. 4 D).

IL-2 supplementation overcame T reg cell control of pancreas-infiltrating NK cells, inducing their activation

We explored to what extent the addition of IL-2 to BDC2.5/NOD mice could surmount T reg cell control of NK cell activities within the insulitic lesion. Given the relatively short in vivo half-life of IL-2 (Donohue and Rosenberg, 1983), we chose to administer IL-2–anti–IL-2 mAb complexes, which stabilize the cytokine and target it preferentially to one or the other of the IL-2 receptors. In this case, S4B6 was the most appropriate mAb, as IL-2–S4B6 complexes bind well to IL-2Rβ–expressing cells, notably NK cells, and poorly to cells displaying IL-2Rα, such as T reg cells (Létourneau et al., 2010). Although treatment of BDC2.5/NOD mice with IL-2–S4B6 complexes did not result in the anticipated expansion of the pancreatic NK cell population (Fig. 5 A), it did elicit IFN-γ synthesis (Fig. 5 B; also true for splenic NK cells; not depicted). The induction of IFN-γ, measured as an increase in IFN-γ^+ cells, was similar to what was seen after ablation of T reg cells (Fig. 2 E and Fig. 4 C). However, we realized that this experiment had an unexpected complication that might explain the lack of NK cell proliferation: although previous studies claimed no effect on T reg cells after treatment of mice with IL-2–S4B6 complexes, we observed an approximate doubling of T reg cells in terms of both fraction of T cells and total cell numbers (unpublished data).

To circumvent this issue, we treated BDC2.5/NOD mice with a mutant form of IL-2 (Super 2), which structurally relieves the dependence of IL-2 on engaging the IL-2Rα chain, thereby shifting the competitive advantage for binding of IL-2 from T reg cells to IL-2Rβ chain–expressing NK cells (Levin et al., 2012). This intervention also induced IFN-γ synthesis by pancreatic NK cells in the absence of population expansion (Fig. 5, A and B), with a less pronounced influence on T reg cells than found in IL-2–S4B6 complex treatment (not depicted).

Although disjunctions between readouts of NK cell proliferation and production of IFN-γ have been described (Cooper et al., 2009), we were surprised to find a divergence in this context because past studies have documented both activities after treatment of mice with IL-2–S4B6 complexes (Jin et al., 2008). However, we noticed that this study and others (Boyman et al., 2006; Mostböck et al., 2008; Létourneau et al., 2010), focused on expansion of NK cells quantified at later time

points. Indeed, when we administered IL-2–S4B6 complexes to BDC2.5/NOD mice and assayed at day 4 (rather than the usual 24 h), there was a clear induction of both proliferation of and IFN-γ synthesis by pancreatic NK cells (Fig. 5 A and B). Thus, supplementation with IL-2 was able to overcome the restraint on NK cells imposed by T reg cells in the insulitic lesion of BDC2.5/NOD mice. Most important was the augmentation of IFN-γ synthesis 24 h after IL-2 administration, as this cytokine is an important and required element in the rapid induction of T1D after T reg ablation. NK cell proliferation was also evident, but was delayed in comaparison with the T reg ablation model, perhaps reflecting a requirement to surpass a higher signaling threshold or the need for an additional cofactor.

To determine whether clinical diabetes could be rescued by supplementing BDC2.5/NOD mice with IL-2, we injected them for 3 d with IL-2–S4B6 complexes. Most complex-injected mice rapidly succumbed to diabetes, starting 6 d after the beginning of treatment. In contrast, none of the control mice developed diabetes during this time frame (Fig. 5 C).

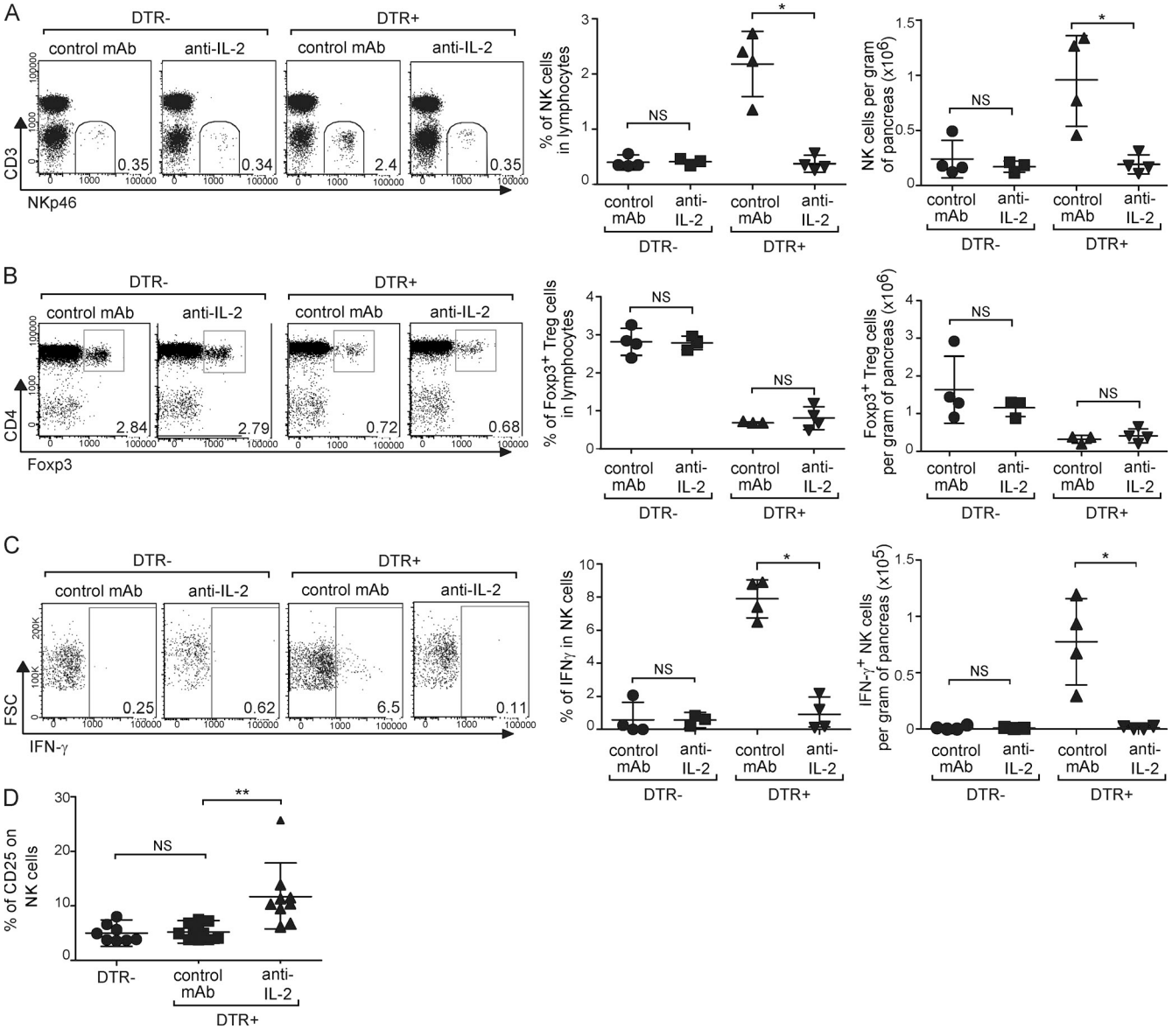

Figure 4. Neutralization of IL-2 prevents the activation of pancreatic NK cells in response to T reg cell ablation. (A–D) The pancreatic infiltrate from BDC2.5/NOD mice (DTR$^+$ or DTR$^-$ control littermates) was analyzed 24 h after DT injection ± anti–IL-2 mAb JES6-1 (or isotype control) co-injection. (left) Representative flow cytometry plots. (right) Summary data for fraction and numbers with mean ± SD from three to four independent experiments. (A) NK cells (NKp46$^+$CD3$^-$CD19$^-$) and (B) Foxp3$^+$ T reg cells (Foxp3$^+$CD4$^+$CD3$^+$CD19$^-$) in the pancreatic infiltrate (FSC/SSC lymphocyte gated). (C) IFN-γ–producing NK cells among total NK cells in the pancreatic infiltrate. (D) CD25 expression on pancreatic NK cells.

CD4+ T cells were by far the major producers of IL-2 in the pancreatic lesion in the presence or absence of T reg cells

These findings raised a pair of important questions: what cells produced IL-2 in the pancreatic infiltrate of BDC2.5/NOD mice? And how did IL-2 levels change with T reg ablation? To address the first issue, we performed intracellular staining of IL-2 in pancreatic cells from unmanipulated BDC2.5/NOD mice. CD45+ cells showed a clear IL-2 signal, easily distinguishable from the isotype control background staining. The vast majority of IL-2-expressing cells (>95%) were CD4+ T cells (Fig. 6 A). The remaining signal (~3%) came from CD3+CD4− cells, which further analysis revealed to be CD8+ (unpublished data), and double-negative cells (<1%). Next, we compared levels of IL-2–expressing cells in the BDC2.5/NOD pancreatic infiltrate, with or without T reg cell ablation, at 8 h after DT treatment (when the increased transcriptional activity of IL-2 response genes is clearly detectable; Fig. 1 D). This analysis included the entire CD45+ population to ensure a broad coverage. No difference was found in the fraction or number of IL-2–expressing cells, or in the MFI of IL-2–expressing cells (Fig. 6 B). Although it remains possible that at later time points an expansion of CD4+ T cells entails higher IL-2 levels, this scenario is unlikely within the first few hours, as the fraction and total number of IL-2–producing CD4+ T cells remained constant (Fig. 6 C). Thus, T reg cells seem to acutely regulate NK cells by sequestering local IL-2 rather than dampening its synthesis.

DISCUSSION

Foxp3+CD4+ T reg cells are known to regulate the progression of T1D in several mouse models, and are thought to exert an analogous influence in human T1D patients (Bluestone et al., 2008). Nonetheless, our understanding of the role of T reg cells during the human disease remains very limiting owing to a dearth of preclinical samples, when T reg cells likely exert the most important influences. Mouse models such as the BDC2.5/NOD line, which have facilitated the study of

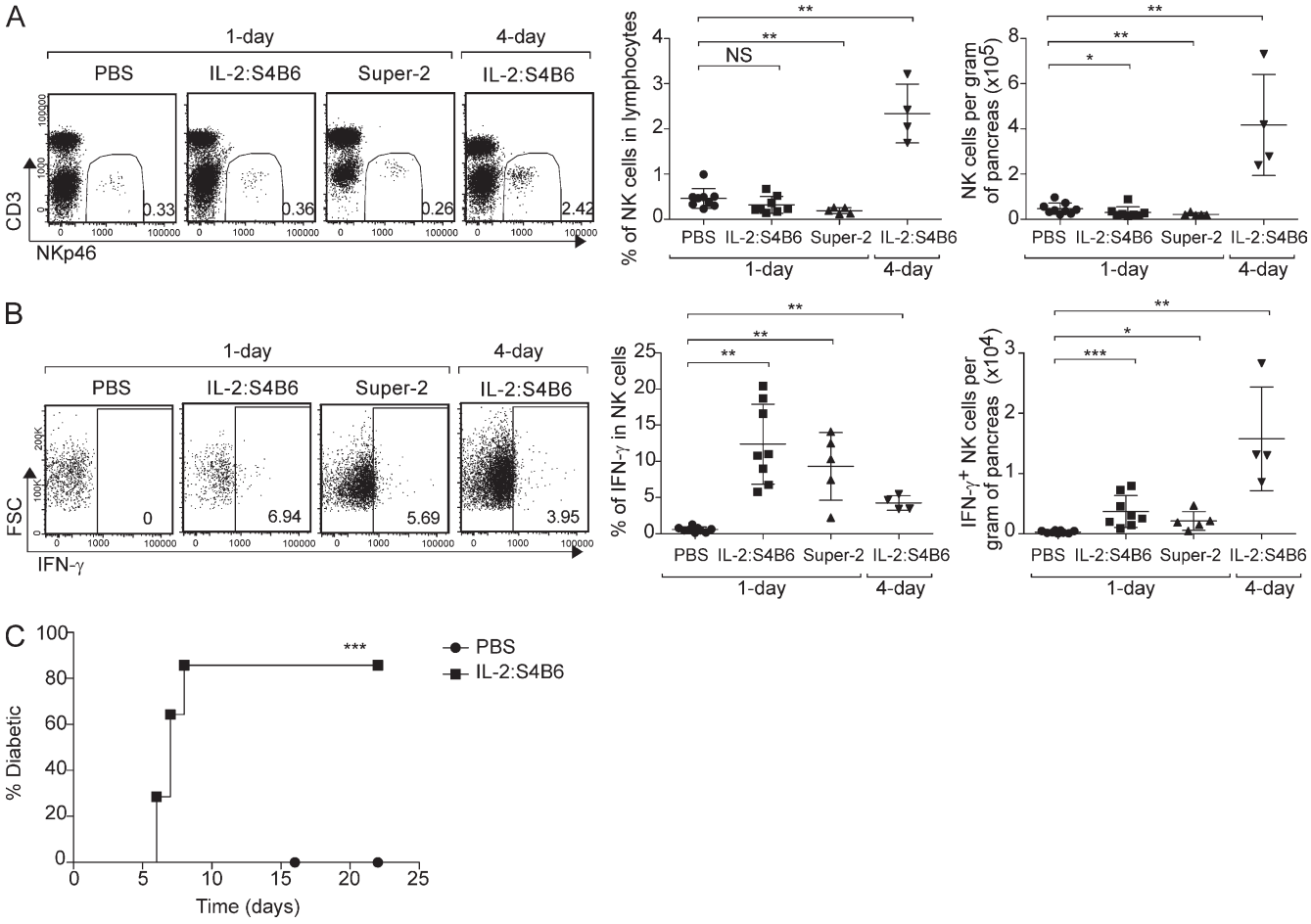

Figure 5. Supplementation with IL-2 induces early IFN-γ production and eventual accumulation of NK cells in the pancreatic lesion, as well as clinical diabetes. Pancreatic infiltrate from BDC2.5/NOD mice was analyzed 24 h after treatment with control PBS, IL-2–S4B6 complexes, or mutant IL-2 analogue Super-2, or 4 d after injection of IL-2–S4B6 complexes. (A, left) Representative flow cytometry data. (middle) Summary data for fraction of NK cells in the FSC/SSC lymphocyte gate. (right) Cell number summary data. (B) Analogous data for IFN-γ–producing NK cells. Mean ± SD, at least three independent experiments for both panels. (C) Summary diabetes data for 2 cohorts after 3 consecutive treatments with IL-2–S4B6 complexes (n = 14 IL-2–S4B6 complex; n = 9 PBS).

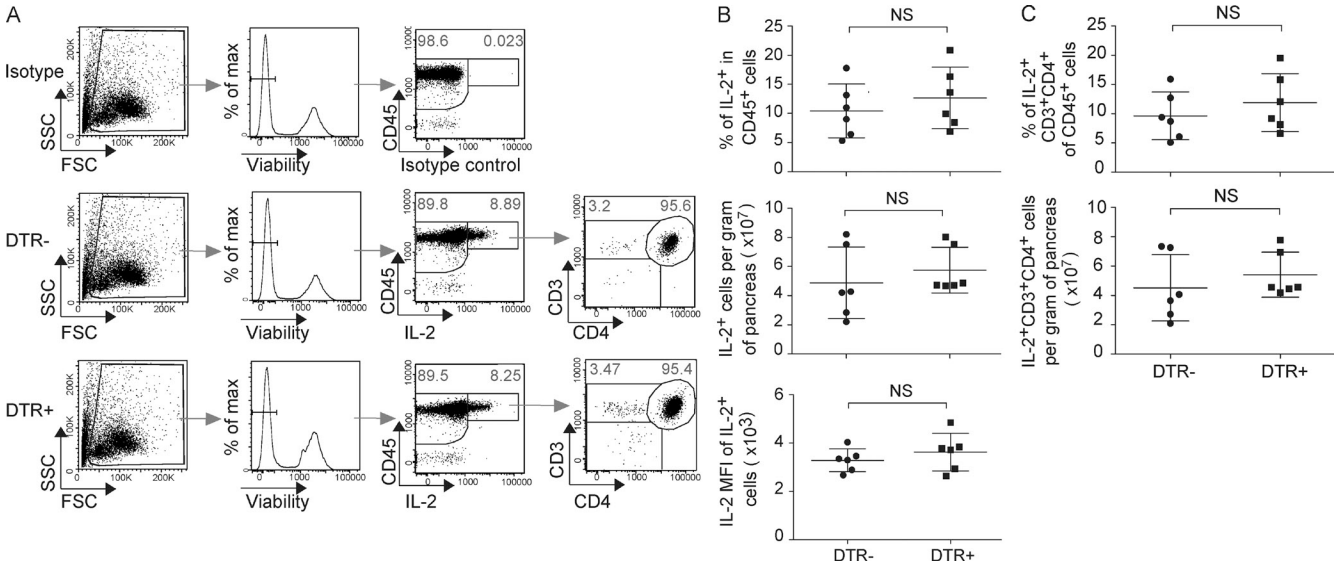

Figure 6. IL-2 production in the pancreas before and after T reg ablation. (A) Representative flow cytometry plots for pancreatic infiltrate from BDC2.5/NOD mice stained and analyzed for IL-2 by intracellular flow cytometry. An extended FSC/SSC gate was taken to include both lymphocytes and leukocytes along with a live/dead stain to exclude dead cells. $CD45^+IL\text{-}2^+$ cells were analyzed for CD3 and CD4 expression. (B) Summary data for pancreatic infiltrate from mice depleted of T reg cells (8 h with DT, DTR^+) or not (8 h with DT, DTR^-). Mean ± SD, at least three independent experiments. (C) Summary data, as in B, gated on $CD4^+$ T cells.

prediabetic pathogenic processes, particularly those that play out within the insulitic lesion, are an important resource. We recently reported that T reg cells control the conversion of insulitis to diabetes in this model primarily by reining in the activities of islet-infiltrating NK cells, notably their production of IFN-γ (Feuerer et al., 2009). Neutralization of this cytokine during ablation of T reg cells inhibited the mobilization of T effector (T eff) cells within the islets and drastically reduced the incidence of hyper-acute T1D that typically develops in this model in the absence of T reg cells. Here, we have explored the molecular underpinnings of the NK cell−T reg cell axis, exploiting the robust, rapid, and synchronous phenotypic changes characteristic of the model. Our studies uncovered a previously undocumented scenario whereby T reg cells control NK cell activation in the islet infiltrate by limiting their exposure to IL-2. This mechanism was indicated by the induction of an IL-2–dependent gene signature in NK cells upon T reg cell ablation, a parallel increase in NK cell pSTAT5 levels, reduction in NK cell accumulation and IFN-γ production after treatment of T reg cell–depleted BDC2.5/NOD mice with anti–IL-2 mAb, and a corresponding enhancement of these parameters in BDC2.5/NOD mice supplemented with IL-2.

More precisely, the scenario we propose is that in the insulitic lesion of prediabetic BDC2.5/NOD mice: (a) $CD4^+$ T eff cells (primarily) produce limiting amounts of IL-2; (b) T reg cells in the vicinity efficiently consume most of this cytokine, reflecting their elevated expression of the high-affinity IL-2R, composed of the γ (CD132), β (CD122), and α (CD25) chains; (c) expressing mostly the low-affinity IL-2Rβγ complex, NK cells are deprived of IL-2, dampening their activation; (d) punctual ablation of T reg cells liberates IL-2 at a sufficient concentration to permit NK cell (and eventual T cell) activation, unleashing their proliferation and production of IFN-γ; and (e) IFN-γ, likely in concert with the liberated IL-2, drives the activity of T eff cells, provoking conversion of an innocuous to a pathogenic insulitic lesion and the development of T1D. This sequence of events is consistent with the belief that IL-2 can prime NK cells, both in vitro and in vivo, for proliferation and IFN-γ production (Fehniger et al., 2003; Granucci et al., 2004; Lee et al., 2012). This represents an interesting intertwining of innate and adaptive immunity, wherein the adaptive ($CD4^+$ T cells) primes the innate (NK cells) to promote the adaptive ($CD4^+$ and, eventually, $CD8^+$ T eff cells) immune response.

The primary means reported for T reg control of NK cells thus far is production and cell-surface display of inhibitory TGF-β (Ghiringhelli et al., 2005), a mechanism that seems less critical in the present context. However, a scenario conceptually similar to ours has been proposed for the impact of T reg cells on T cell responses in several experimental models; i.e., T reg and T eff cell competition for limiting IL-2 (Barthlott et al., 2005; Scheffold et al., 2005; Pandiyan et al., 2007). Other studies (see Gasteiger et al. and Gasteiger et al. in this issue) that were carried out on the basis of punctual T reg cell ablation experiments have recently found that T reg control of IL-2 availability is an important systemic control on NK cell homeostasis and activation (A. Rudensky, personal communication).

Our findings should be viewed in the context of an extensive body of work weighing the role of IL-2 in human and mouse T1D (Hulme et al., 2012; Shevach, 2012). For example, *Il2* and *IL2RA* have shown a genetic association with disease in NOD mice and human diabetes patients, respectively. The effects of IL-2–IL-2R signaling on T reg cell homeostasis and function were routinely cited in interpretation of these associations. Indeed, mice devoid of IL-2, IL-2Rα, IL-2Rβ, or STAT5 all succumb to lymphoproliferative disease caused by T reg cell reduction or dysfunction, which can be reversed by administration of exogenous IL-2 or wild-type T reg cells. Nonetheless, given the data presented herein, it seems plausible that allelic variation in IL-2–IL-2R signaling could, instead or in addition, result in aberrant NK cell function and thereby exacerbate disease in a manner not currently appreciated. Consistent with this possibility, the mouse *Klr* and human *killer immunoglobulin-like receptor* (*KIR*) families that modulate NK cell activation have also been associated with diabetes in numerous human studies (van der Slik et al., 2003; Nikitina-Zake et al., 2004; Rodacki et al., 2007; Ramos-Lopez et al., 2009), as well as in the NOD mouse (Rogner et al., 2001). More information on the effects of mutant alleles of elements of the IL-2–IL-2R signaling pathway on NK cells is imperative.

In humans, modulation of the IL-2–IL-2R axis has been achieved through treatment with daclizumab, a mAb targeting IL-2Rα. In multiple sclerosis, where autoreactive T cells recognizing antigens from the central nervous system promote inflammation and demyelination, daclizumab induced a population of $CD56^{bright}$ NK cells that can target and kill $CD4^+$ T cells. Expansion of this NK cell population was associated with enhanced disease outcomes (Rose, 2012). Although multiple mechanisms of action for this mAb have been proposed, increased bioavailability of IL-2 as a result of IL-2Rα blockade would mirror the findings and interpretation reported here.

The role of IL-2–IL-2R signaling in diabetes progression has been experimentally dissected in NOD mice (Hulme et al., 2012; Shevach, 2012). Alone, or in combination with agents such as rapamycin, IL-2 supplementation had disease-modulating effects, in both preventive and curative protocols. Interestingly, one set of studies reported that although low-dose IL-2 treatment suppressed diabetes, high-dose administration actually triggered disease (Tang et al., 2008b). The diabetes-suppressive effect of low-dose IL-2 was interpreted as correction of an imbalance between T reg and T eff cells downstream of a genetic deficiency in IL-2 signaling. The relatively low expression of CD25 on islet-infiltrating T reg cells was taken as evidence of this notion, although it later became apparent that dampened CD25 expression is a characteristic of T reg cells at inflammatory sites in general (Lazarski et al., 2008; Tang et al., 2008a). The diabetogenic effect of high-dose IL-2 was explained as an enhancement of the activities of pathogenic T eff cells (although there was also a striking systemic expansion of NK cells). The results presented here emphasize that the response of islet-infiltrating NK cells to manipulation of IL-2–IL-2R signaling is not to be ignored in interpreting outcomes.

Given a strong rationale from studies on both mice and humans, there has been substantial interest in developing protocols for treating T1D patients with IL-2, likely in combination with other agents (Hulme et al., 2012; Shevach, 2012). The results from a phase I clinical trial wherein IL-2 and rapamycin were administered to 9 T1D patients within 4 yr of diagnosis were recently published, and proved disappointing (Long et al., 2012). There was, as hoped for, a transient augmentation in T reg cell fraction and numbers, accompanied by a more persistent enhancement of their STAT5 phosphorylation levels. However, these changes were accompanied by an unanticipated transient impairment in islet β-cell function, in concert with increases in the NK and eosinophil populations, ultimately resulting in trial closure. As cogently argued by Bonifacio (2012), the detrimental impact on β cells could reflect rapamycin effects and/or influences of the expanded populations of innate immune system cells. Certainly, rapamycin has been reported to inhibit β-cell regeneration and normalization of blood-glucose levels in mice (Nir et al., 2007). However, our findings, especially the rapid induction of diabetes after IL-2 supplementation, highlight the potentially destructive effects of IL-2–mediated unleashing of NK cells. Activation of islet-infiltrating NK cells could be directly cytotoxic or, through production of IFN-γ, could promote the activities of pathogenic T eff cells. This represents another example of the predictive value of murine models of T1D, though only if the available mouse data are reviewed and translation to humans is performed in a precise and critical manner (Shoda et al., 2005).

As recognized early on (Tang et al., 2008b; Grinberg-Bleyer et al., 2010; Hulme et al., 2012; Shevach, 2012), harnessing the tremendous potential of IL-2–IL-2R-based therapies while avoiding the detrimental side-effects is a great challenge. Novel approaches, such as the engineering of designer IL-2 derivatives (Levin et al., 2012), are promising in this regard. Even so, we need to understand much better how the various regulatory and effector populations in the insulitic lesions are orchestrated, and perspicaciously apply this knowledge to the development of therapeutic protocols.

MATERIALS AND METHODS

Mice. NOD/ShiltJ (NOD), BDC2.5/NOD TCR transgenic (Katz et al., 1993), and BDC2.5/NOD.$Foxp3^{DTR}$ double-transgenic (Feuerer et al., 2009) mice were bred in our colony at The Jackson Laboratory, and were genotyped and maintained at Harvard Medical School (under specific pathogen–free conditions). Females between 7 and 10 wk of age were generally used. Protocols were approved by Harvard Medical School's Institutional Animal Care and Use Committee.

In vivo treatments. All in vivo treatments were via intraperitoneal injection. For T reg cell ablation, BDC2.5/NOD.$Foxp3^{DTR}$ mice were administered 1 μg DT (Sigma-Aldrich) in sterile PBS, and samples were taken at the indicated time points. Control mice were littermates lacking the DTR transgene. For blockade of TGF-β, anti–TGF-β mAb (clone 1D11.11) was produced in the laboratory, and 1 mg was injected in sterile PBS 24 h before experimentation or at day 0, 3, and 5 for the long-term experiments. Control mice received an equal amount of isotype-control mAb (MOPC-21;

BioLegend). For neutralization of IL-2, 100 µg anti–IL-2 mAb (JES6-1A12; BioLegend) was injected along with DT, and analysis was done at 24 h. Control mice were treated with 100 µg isotype-control mAb (RTK2758; BioLegend). For IL-2 treatments, IL-2–anti–IL-2 complexes were prepared by adding 5 µg rIL-2 (PeproTech) to 50 µg of an IL-2 mAb (S4B6; BD) in 200 µl sterile PBS before injection, as previously described (Tang et al., 2008b), and analysis was done at 24 h. For treatments longer than 24 h, IL-2 complexes were injected daily, including 2 h before organ harvest on the final day. Mutant cytokine "Super-2" was prepared as previously described (Levin et al., 2012). 100 µg was injected at the outset and at 12 h, and analysis was at 24 h. Control mice were treated with an equal volume of PBS.

Disease assays. For diabetes incidence studies, BDC2.5/NOD mice were injected with IL-2–S4B6 complexes or PBS for 3 consecutive days, and diabetes was assayed by measuring blood glucose levels for up to 3 wk. Obtaining two consecutive draws of >250 mg/dl was considered diabetic.

Cell sorting and flow cytometry. For the initial NK cell microarray experiments, cells were isolated from the pancreas and spleen by mechanical separation with scissors. The pancreas was bathed in a shaking water bath at 37°C in digestion buffer (1 mg/ml collagenase IV [Sigma-Aldrich], 10 U/ml DNaseI [Sigma-Aldrich], and 1% [Thermo Fischer Scientific] in DMEM [Invitrogen]). For all other experiments, postmortem intracardial perfusion was performed with 30 ml room temperature PBS (or cold PBS for the intracellular phospho-STAT stains). After surgical removal of organs, cells were isolated from the pancreas and spleen by mechanically separating with scissors before passing through a 40-µm filter into DMEM supplemented with 2% FBS (Omega Scientific). Bloody samples were treated with ACK Lysing Buffer (Lonza) for 5 min on ice. Cells were Fc blocked (2G42, prepared in-laboratory) before surface or intracellular stains were performed using mAbs against CD3 (145-2C11 and 17A2; BioLegend), CD19 (6D5; BioLegend), NKp46 (29A1.4; BioLegend), CD4 (RM4-5; BioLegend), CD25 (PC61; BioLegend), and CD103 (2E7; BioLegend). For Foxp3 (FJK-16s; eBioscience) and IFN-γ (XMG1.2; BioLegend) stains, fixation/permeabilization were performed according to the manufacturer's instructions (eBioscience). For phospho-STAT staining, including pSTAT5 (C71E5; Cell Signaling Technology), pSTAT1 (58D6; Cell Signaling Technology), and isotype-control (DA1E; Cell Signaling Technology) mAbs, fixation/permeabilization/stains were done according to the manufacturer's instructions. For intracellular IL-2 staining of the pancreas (JES6-5H4 and isotype-control RTK4530; both obtained from BioLegend), cells were purified after PBS perfusion and digestion, using a Percoll gradient (GE Healthcare) according to manufacturer's instructions, and were stimulated with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (1 nM; EMD Millipore) for 4 h. GolgiStop (BD) was added to the culture during the last 3 h. Dead cells were discriminated using a live/dead fixable near-IR dead cell stain kit (L10119; Invitrogen), and then stained with fluorescent mAb followed by fixing and permeabilization, all according to the manufacturer's instructions (BD). Flow cytometry was performed using an LSRII (BD), and data were analyzed using FlowJo (Tree Star) software.

Microarrays. Cells were double-sorted to high purity (>99%) on a MoFlo Cell Sorter (Beckman Coulter) directly into TRIzol (Invitrogen). RNA was prepared as described by the Immunological Genome Project (www.immgen.org) and underwent the GeneChip Whole Transcript Sense Target Labeling Assay using the Ambion WT Expression kit and Affymetrix GeneChip WT Terminal Labeling and Controls kit (Affymetrix). The resulting ssDNAs were hybridized to the GeneChip Mouse Gene 1.0 ST Array (Affymetrix). Image reads were processed through Affymetrix software to obtain raw .cel files, and were background corrected and normalized using the RMA algorithm via Affymetrix Power Tools. Multiple replicates of cellular populations were collected (usually $n = 3–6$) and averaged. Data were analyzed with the Multiplot module from GenePattern. Randomized data were generated using the MultiplotPreprocess module from Genepattern. Data outside an acceptable range of replicate variation was filtered out using a coefficient of variation (CV) filter and when the represented gene was not expressed in any condition. Microarray data are available from the National Center for Biotechnology Information/GEO repository under accession no. GSE39197.

In vitro cultures. Before cell sorting, splenocytes were depleted of B and $CD8^+$ T cells using biotin-labeled mAbs to CD8 (2.43; prepared in-laboratory) and CD19 (6D5; BioLegend), followed by depletion using the CELLection Biotin Binder kit (Invitrogen) according to the manufacturer's instructions. NK cells ($CD19^-CD3^-NKp46^+$) were sorted, and then cultured in sterile complete RPMI (10% FBS, 1X PenStrep [Invitrogen], 2 mM L-glutamine, 55 µM 2-mercaptoethanol (Sigma-Aldrich), RPMI [Invitrogen]), and were activated with rIL-12 (0.5 ng/ml; PeproTech) and rIL-18 (0.5 ng/ml; MBL) for 15–20 h with or without rTGF-β (1 ng/ml; PeproTech). After culture, cells were either stained for intracellular IFN-γ or resorted to high purity for microarray analysis as described above.

Statistical analysis. All nonmicroarray statistical analyses for cytometry experiments were performed with GraphPad Prism software. For in vivo mouse readouts, p-values were calculated using the Mann-Whitney test. For diabetes incidence experiments, p-values were calculated using the Log-rank test. P-values were considered significant at $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***). χ^2 p-value analysis for microarray expression data (volcano plots) was calculated using Microsoft Excel based on the number of genes dropping to the left or right side of the fold-change distribution.

Online supplemental material. Table S1 lists transcripts differentially expressed in pancreatic NK cells in the presence or absence of T reg cells as described in Fig. 1. Table S2 shows the gene list of an activation-independent TGF-β-responsive signature used in Fig. 2. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20122248/DC1.

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J. Sitrin, C. Benoist, and D. Mathis declare no competing financial interests. K.C. Garcia, and A. Ring declare competing financial interests due to submission of a pending patent application describing the IL-2 superkine (Super-2).

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