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Resident macrophages of pancreatic islets have a seminal role in the initiation of autoimmune diabetes of NOD mice

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Treatment of C57BL/6 or NOD mice with a monoclonal antibody to the CSF-1 receptor resulted in depletion of the resident macrophages of pancreatic islets of Langerhans that lasted for several weeks. Depletion of macrophages in C57BL/6 mice did not affect multiple parameters of islet function, including glucose response, insulin content, and transcriptional profile. In NOD mice depleted of islet-resident macrophages starting at 3 wk of age, several changes occurred: (i) the early entrance of CD4 T cells and dendritic cells into pancreatic islets was reduced, (ii) presentation of insulin epitopes by dispersed islet cells to T cells was impaired, and (iii) the development of autoimmune diabetes was significantly reduced. Treatment of NOD mice starting at 10 wk of age, when the autoimmune process has progressed, also significantly reduced the incidence of diabetes. Despite the absence of diabetes, NOD mice treated with anti-CSF-1 receptor starting at 3 or 10 wk of age still contained variably elevated leukocytic infiltrates in their islets when examined at 20–40 wk of age. Diabetes occurred in the anti-CSF-1 receptor protected mice after treatment with a blocking antibody directed against PD-1. We conclude that treatment of NOD mice with an antibody against CSF-1 receptor reduced diabetes incidence and led to the development of a regulatory pathway that controlled autoimmune progression.

macrophage | islets of Langerhans | type 1 diabetes | nonobese diabetic mouse | regulation

Tissue-resident macrophages constitute a heterogeneous and multifunctional cell type that populates virtually every tissue (reviewed in refs. 1 and 2). In addition to their primary role as sentinels for invading pathogens and as initiators of inflammation, macrophages have important homeostatic roles in many tissue-specific processes such as development, metabolism, and tissue remodeling, providing trophic factors within the tissue microenvironment. Their homeostatic role is evidenced by the deleterious phenotype that arises when macrophage development is genetically constrained. The osteopetrotic mouse (*op/op*) harbors a severe paucity of macrophages due to a null mutation in the gene encoding the macrophage differentiation and survival factor, colony stimulating factor-1 (CSF-1) (3). These mice present with a number of developmental defects. Notably, the islets of Langerhans, which normally contain a population of CSF-1-dependent macrophages, are atrophic in the *op/op* mouse, suggesting an integral role for macrophages during the development of the endocrine pancreas (4, 5).

The pancreatic islets of all species contain a small number of myeloid cells represented by macrophages. These macrophages were first identified by immunohistochemical analysis using macrophage-specific markers. Although initially thought to be “passenger leukocytes,” subsequent studies using lineage tracing approaches identified them as resident cells (5). These self-replicate and are replaced minimally, if at all, by blood monocytes. Islet macrophages are highly activated with a complex gene transcriptome reflecting their interactions with beta cells and

with blood components (6). They express high levels of class II histocompatibility molecules (MHC-II) and a number of cytokines and chemokines including TNF- α and IL-1 β (5, 6). The islet macrophages are always next to blood vessels, in close contact with beta cells, capture insulin-containing granules, and present insulin peptides to autoreactive CD4 T cells (7). Moreover, macrophages extend filopodia into the vessel lumen and can respond to blood stimuli (6, 8).

Our laboratory focuses on the early events that initiate autoimmune diabetes as exemplified in the NOD mouse strain. Understanding the initiation of autoimmune diabetes is of considerable clinical interest. Along those lines, identifying the key cellular and molecular events that transition the immune-privileged islet to an immunologically reactive environment is paramount as it pertains to diabetes initiation. By 3 wk of age, islets show significant cellular and molecular changes compared with nondiabetic strains: an early entrance of CD4⁺ T cells reactive to insulin in contact with the macrophages, a heightened gene signature of activation in the macrophages, and the appearance of the XCR1⁺ subset of dendritic cells (DCs) (9, 10). The deficit of XCR1⁺ DCs in the NOD *Batf3*^{-/-} mice translates into a profound reduction in the autoimmune process (10).

Here, we examine the role of the resident macrophages in diabetogenesis. The islet macrophage expresses both inhibitory and stimulatory ligands and receptors, as well as chemokines and

Significance

Our studies indicate that the resident macrophages of the pancreatic islets of Langerhans have a seminal role in the initiation and progression of autoimmune diabetes in NOD mice. In this study, islet macrophages were depleted by administration of a monoclonal antibody to the CSF-1 receptor. Macrophage depletion, either at the start of the autoimmune process or when diabetogenesis is active, leads to a significant reduction in diabetes incidence. Depletion of the islet macrophages reduces the entrance of T cells into islets and results in the absence of antigen presentation. Concordantly, a regulatory pathway develops that controls diabetes progression. We conclude that treatments that target the islet macrophages may have important clinical relevance for the control of autoimmune type 1 diabetes.

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chemokine receptors (6). They are the sole hematopoietic cell within the islets before 3 wk of age and are able to present unconventional insulin epitopes to autoreactive T cells (6). Altogether, this posits the intraislet macrophage as a key regulator of lymphocyte entry into the islet. We have eliminated them using a monoclonal antibody to the CSF-1 receptor (CSF-1R), AFS98 (11). This monoclonal antibody, and the M279 antibody against CSF-1R, when administered *in vivo* resulted in the loss of macrophages from many tissues (12–14). The extent of depletion depended on the amount and frequency of administration (12). Although treatment with AFS98 resulted in the loss of resident macrophages, blood monocyte counts and inflammatory responses were not affected (13). We present evidence that the anti-CSF-1R antibody AFS98 depleted the islet resident mac-

rophages in two strains of mice: the normal C57BL/6 (B6) and the diabetogenic NOD. The depletion of macrophages did not affect the metabolism or islet transcriptome of the mouse. However, diabetes progression was severely blunted.

Results

Evaluation of C57BL/6 Mice. First, we evaluated the nondiabetic B6 strain, following treatment with the monoclonal antibody against CSF-1R. Injecting B6 mice with 0.25, 0.50, and 2.0 mg of AFS98 led to a dose-dependent elimination of the intraislet macrophages (Fig. 1*A*). This reduction was observed as early as 7 d after treatment and was equally evident at day 14. The depletion was long lived. A 2.0-mg dose of AFS98 resulted in elimination of the islet macrophage lasting for at least 6 wk (Fig.

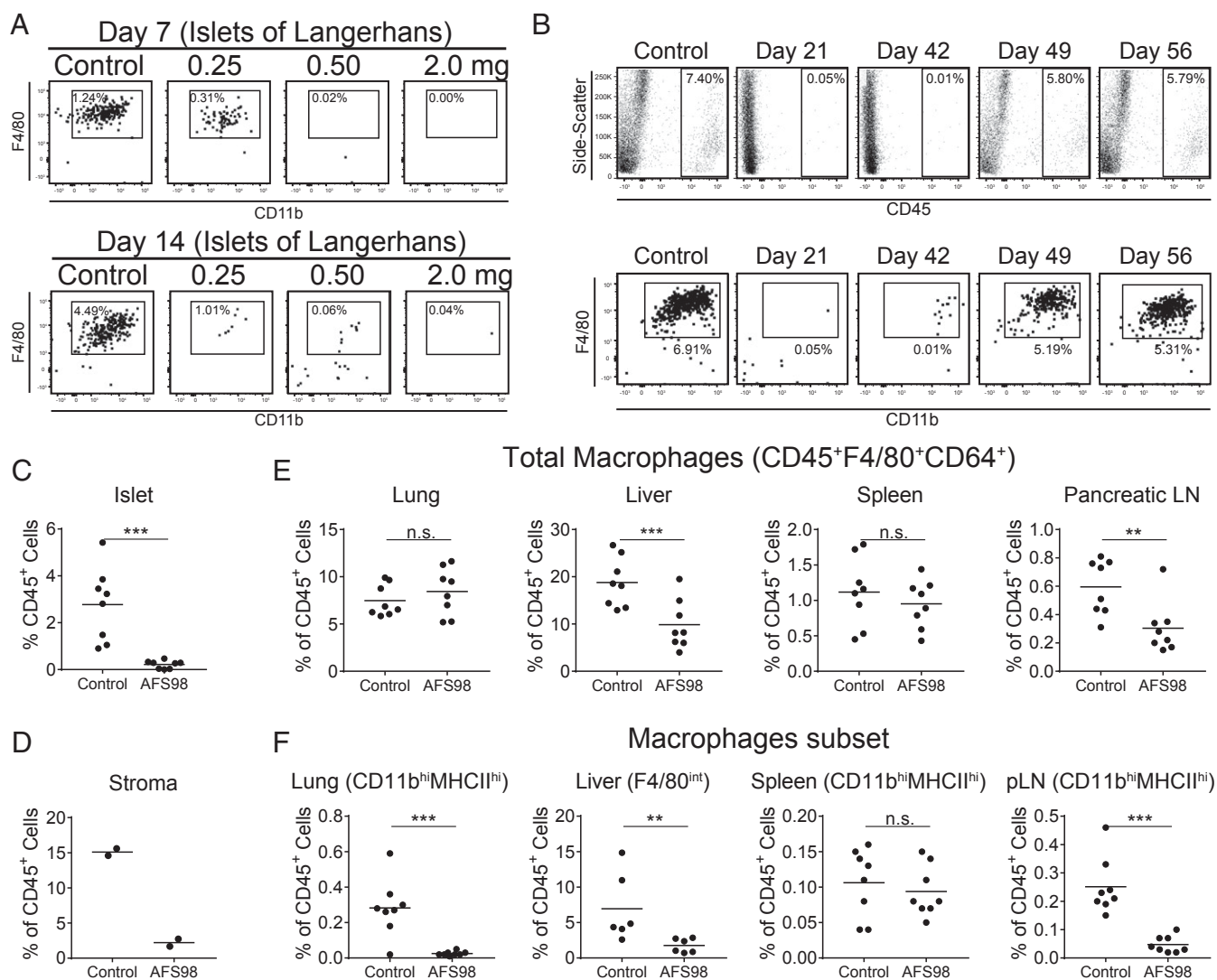


Fig. 1. Treatment of C57BL/6 mice with AFS98 antibody depleted their macrophages. (*A*) Female B6 mice aged 6–8 wk were administered 0.25, 0.50, or 2.0 mg of AFS98 antibody *i.p.* Islets were examined 7 and 14 d after injection for the presence of macrophages. Box indicates CD45⁺CD11b⁺MHCII⁺F4/80⁺CD11b⁺ cells as a percent of total islet cellularity. (*B*) Female B6 mice aged 6–8 wk were treated with 2.0 mg of AFS98, and their islets were examined at the time points indicated for the presence of macrophages. *Top* show the CD45⁺ cells, and *Bottom* show the CD45⁺CD11b⁺MHCII⁺F4/80⁺CD11b⁺ cells as a percent of total islet cellularity. (*C*) Graph of the CD45⁺ cells found in mice 1–2 wk after treatment with 2.0 mg of AFS98. (*D*) Graph of CD45⁺F4/80⁺CD64⁺ islet stromal macrophage populations as a percent of CD45⁺ cells in control and AFS98-treated mice 2 wk after treatment. (*E*) Mice were treated for 1–2 wk with AFS98 antibody, and the percent of total macrophages in lung, liver, spleen, and pancreatic lymph nodes were determined. (*F*) Macrophages were examined as in *E* except plots show the percent of the indicated subpopulation of macrophages. For all graphs, controls were untreated age-matched mice. Flow cytometry plots in *A* and *B* are representative of individual islet, lung, liver, spleen, and lymph nodes, from two to three experiments with two to four mice per treatment group. Scatter plots in *E* and *F* were calculated from three independent experiments with two to three mice per group. *P* values were calculated using Mann–Whitney *U* test with the following style: not significant (n.s., >0.1234), **P* = 0.0332, ***P* = 0.0021, ****P* = 0.0002, *****P* < 0.0001.

Evaluation of NOD Mice.

Islets. Similar to the results observed in B6 mice, Fig. 3A shows that treatment with 0.5 or 2.0 mg of AFS98 depleted the islet macrophages in 4- to 5-wk-old NOD mice. Comparable results were obtained following treatment of NOD.*Rag1*^{-/-} mice with the AFS98 antibody (Fig. S3). An analysis of NOD mice at 3–4 wk of age showed that the initial islet infiltrating T cells were all CD4⁺ and mostly in contact with the intraislet macrophage (9). The islet macrophages express CD11b, CD11c, and MHC-II highly on their surface. At this 3–4 wk of age period, there are very few XCR1⁺ DCs in islets (10).

We confirmed the colocalization of CD4 T cells with intraislet macrophages by examining islets using two-photon microscopy. NOD mouse islets were examined at 3–4 wk of age, the earliest age where one can identify the initial infiltrating T cells. Indeed, 27% of NOD islets had CD4 T cells, 70% of which were in contact with the F4/80⁺ macrophages, confirming our initial studies. In the mice that were injected with 0.5 mg of anti-CSF-1R at 2 wk of age, the islets did not harbor any myeloid cells at 4 wk of age. In these mice, only 3% of islets had a detectable CD4 T cell.

Next, we examined insulin peptide presentation by isolating the islets, dispersing the cells, and culturing them with insulin-reactive CD4 T cell hybridomas. Depletion of islet macrophages resulted in marked reduction of presentation to two different CD4 T cell hybridomas recognizing different MHC-II epitopes of insulin (17). The addition of the cognate peptides reflects the availability of MHC-II⁺ presenting cells. As noted in Fig. 3B, this addition did not lead to presentation in the islets of the treated mice, reflecting the paucity of presenting cells and the inability of any other islet cell to express MHC-II or present peptide. The same findings were reproduced in B6.g7 mice (Fig. S4).

Lymph node responses. While the islets from the macrophage-depleted mice were impaired in antigen presentation, this was not the case in the peripheral lymph nodes. This was determined using two approaches: carboxyfluorescein succinimidyl ester (CFSE) dilution of transferred T cells and recall response by ELISpot. For CFSE dilution experiments, we transferred CD4 (BDC2.5) or CD8 (NY8.3) T cell clones into control or AFS98-treated NOD. BDC2.5 responds to a chromogranin peptide in the context of I-A^{g7} (18), while the NY8.3 T cell divides in response to a peptide derived from IGRP, the islet-specific glucose-6-phosphatase-dependent catalytic subunit-related protein,

in the context of H-2Kd (19, 20). Fig. 4A and B shows that both T cell clones proliferated in the draining pancreatic lymph node, but not in the distant inguinal lymph node. Next, to determine if depletion of the islet macrophage affected the trafficking of diabetogenic cells to the islets, we examine the entrance of TCR transgenic T cells into them. Both BDC2.5 and NY8.3 T cells entered islets of control mice but neither entered the islets from AFS98-treated mice (Fig. 4C). While the T cells entered and reacted to antigen presented in the pancreatic lymph node, this was not the case in the islets.

In the second approach, AFS-treated mice were immunized with various autoreactive peptides in the footpads and the T cell response was tested a week later; there was no impairment of the response (Fig. 5). Immunization with the insulin B:9–23 peptide elicited an IL-2 and IFN- γ response to the peptide but not to the insulin protein, as reported before (17). Immunization with peptides from IGRP known to elicit CD4 or CD8 T cell responses was also unaffected by AFS98 treatment. The CD4 or CD8 T cell responses to the foreign protein hen egg lysozyme (HEL) were also unaffected.

In brief, examination by flow cytometry, direct microscopy of islets, antigen presentation assays, and T cell migration assays shows that the lack of macrophages translates into an absence of early CD4 T cell infiltration and antigen presentation capability of the islets. In contrast, the lymph nodes in the AFS-treated mice were active in antigen presentation.

Diabetes. Female NOD mice were followed for diabetes after treatment with AFS98 or control rat IgG_{2a} antibody. In two experiments, mice were treated at 2–3 wk of age, a time when the diabetogenic process is starting in limited islets. The treatment was continued for several weeks using two different concentrations of antibody. In a third experiment, NOD mice were treated starting at 10 wk of age, a time when the diabetogenic process is active and most islets are already infiltrated by both CD4 and CD8 T cells. Both treatments led to a marked reduction in diabetes incidence when the mice were followed for 40 wk. The pooled results are shown in Fig. 6A. Fig. 6C shows the results of the individual experiments. Combining the three experiments, 4 of 40 AFS98-treated mice and 24 of 39 of the control mice became diabetic.

At the end of the 40–42 wk of observation, two manipulations were performed on the AFS98-treated mice. First, the mice were administered an anti-PD-1 monoclonal antibody and this led to

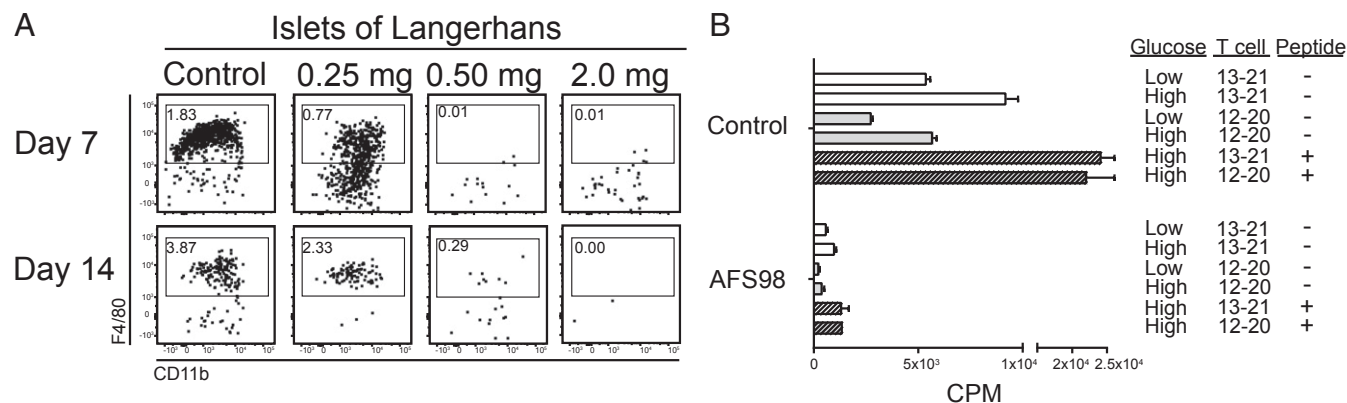


Fig. 3. Effect of AFS98 treatment on NOD mice. (A) Male NOD mice at 4–5 wk of age were administered 0.25, 0.50, or 2.0 mg of AFS98 i.p., and their islets were examined by flow cytometry. Plots show the CD45⁺CD11c⁺MHCII⁺ gate at either 7 or 14 d after treatment. Results are representative of two experiments performed in duplicate. Values in the box represent the percent of cells as a function of total islet cellularity. (B) Two-week-old male NOD mice were left untreated (Control) or injected i.p. with 0.5 mg of AFS98 at 2 wk of age and 2.0 mg of AFS98 at 4 wk of age (AFS98). At 6 wk of age, the islets were harvested, dispersed, and tested for their MHCII-peptide presentation to two T cell hybridomas that recognize insulin. High (25.0 mM) or low (5.0 mM) glucose and two different insulin peptides (Ins B:12–20 and Ins B:13–21) were evaluated. Bars represent the mean \pm SD of ³H incorporation by the IL-2-dependent cell line CTLL-2.

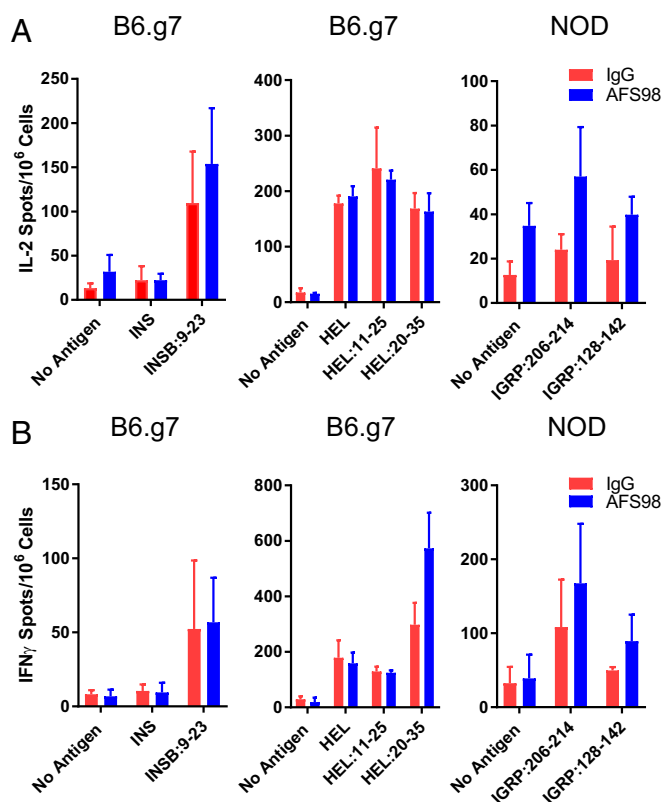


Fig. 5. Lymph node priming is not reduced by AFS98 treatment. B6.g7 or NOD mice were treated with 2 mg of AFS98 antibody for 1 wk and then injected with 10 nmols INS:9–23, HEL, or IGRP peptides in complete Freund's adjuvant. The draining popliteal lymph nodes were isolated and tested by ELISPOT for IL-2 (A) and IFN- γ (B) production. Recall antigens for the ELISPOT are shown in the figure and include the following: insulin protein (INS) and INS:9–23, HEL protein and peptides that elicit CD4 (11–25) and CD8 (20–35) responses, and the IGRP peptides that elicit CD4 (128–142) and CD8 (206–214) responses. Results are taken from two individual mice tested in duplicate or triplicate.

the rapid development of diabetes in 7 of 7 of the mice evaluated (Fig. 6C). Others have found a profound regulatory effect of the PD-1/PD-L1 pathways in regular diabetogenesis, and at least this pathway is operational following AFS98 treatment (21–23). Anatomically, insulin-reactive T cells express PD-1, while in islets the beta cells, vascular endothelium, macrophages, and inflammatory DCs express high levels of PD-L1 (21, 24, 25).

Second, splenocytes from the AFS98-treated mice were transferred into NOD.*Rag1*^{-/-} mice. NOD splenocytes transfer disease to normally nondiabetic NOD.*Rag1*^{-/-} mice and is used as a measure of the diabetogenic potential of the T cells in the NOD mouse following genetic or pharmaceutical manipulations. The mice first treated with AFS98 at the 2- to 3-wk period transferred diabetes poorly; only 1 of 14 became diabetic 20 wk after transfer (Fig. 6 B and C). In contrast, splenocytes from 10-wk, AFS98-treated NOD mice transferred diabetes to 8 of 8 recipients.

We also examined the islets for their leukocyte content at various time points after their treatment with AFS98. Fig. 7 shows results from mice treated at 2–3 wk of age. AFS98 treatment resulted in a reduction of total leukocytes (CD45⁺) from ~10% in controls to ~1% in the treated mice by 8 wk after treatment (Fig. 7A). There was also a reduction of F4/80⁺ macrophages from ~4% of total islet cellularity to undetectable levels. Over time, the control NOD mice showed progressive increase in the CD45⁺ cells (Fig. 7B). After the end of the observation period—at 44 wk—a number of the islets from AFS-treated mice were infiltrated. We

observed two phenotypes: 4 of 8 mice contained ~20–25% CD45⁺ cells in the islets, and 4 of 8 mice contained 2–5% CD45⁺ cells in the islets (Fig. 7 C and D). (The IgG-treated control NOD mice contained ~60–70% CD45⁺ cells in islets.) Corroborating the flow cytometry ~50% of the pancreata examined had a degree of perinsulinitis (Fig. 7E). However, the other 50% of pancreata examined showed little to no insulinitis (Fig. 7F). Therefore, macrophage depletion is protective in NOD mice long term, despite the infiltration or expansion of leukocytes in ~50% of the mice.

In mice treated with AFS98 starting at 10 wk of age, there also was a reduction in the number of leukocytes in islets at 22 wk of age. However, by 40 wk of age, all of the mice had comparable infiltrates to a 22-wk-old NOD mouse (Fig. 8). Thus, late macrophage depletion does not stop the infiltration of islets, despite preventing the development of hyperglycemia.

These results suggest that macrophage depletion can act before and after the diabetogenic T cell pool in the NOD has fully developed. Early AFS98 treatment blocks diabetes by eliminating a key antigen-presenting cell in islets, preventing early entry of activated T cells, and limiting the expansion of the diabetogenic T cells. In contrast, in late-treated NOD mice, there are active diabetogenic T cells but disease is controlled by the development of a regulatory condition through at least one mechanism, PD-1/PD-L1 interaction. In both situations, we find islet infiltration with preservation of islet function.

Discussion

Injection of NOD mice with antibodies directed against CSF-1R depleted the islet resident macrophages while having a variable effect on macrophages of secondary lymphoid tissues and various other organs. The treatment had a profound effect in the development of diabetes. The manner in which the islet macrophage was eliminated by the antibody treatment is not clear, although it does not involve an acute inflammatory reaction (14). The islet transcriptome analysis displayed no up-regulation of inflammatory transcripts following macrophage depletion (Fig. 2C). CSF-1R signaling is crucial for sustaining macrophage viability, which is most likely the reason for its loss following the antibody treatment (11).

There are two distinct scenarios that likely contributed to the attenuation of the active autoimmune process: (i) the impairment of islet antigen presenting function and (ii) the development of a regulatory process. Concerning the islets, these studies confirm the centrality of the resident macrophage in the pathophysiology of pancreatic islets. The islet macrophage arises during embryonal development. It is required for the early development of islets and during postnatal growth as is evident in the op/op mouse that lack CSF-1 (4, 5). However, in adult mice, islet function is not impaired when the macrophage is absent (our studies in B6 mice). We have not probed other possible roles for the islet macrophage such as maintaining vessel permeability, protecting against infectious agents, or modulating the physiology of beta cells. The islet macrophage is highly activated, as evidenced by their expression of TNF and IL-1 transcript and protein, and high expression levels of MHC-II (5, 10). In the context of autoimmunity, such as in NOD mice, autoreactive T cells escape thymic control and enter islets whereupon they establish long-lived contact with the macrophage (26).

The macrophage-depleted islet per se was impaired in its capacity to receive diabetogenic T cells. In their absence, lymphocyte entry into islets was impaired. The immunofluorescence and two-photon imaging of isolated islets showed the majority of CD4 T cells in islets in contact with the macrophage. Indeed, previous analyses showed macrophage filopodia extending into the blood vessel lumens (8). I.v. injecting 0.5- μ m latex beads coated with antibodies to I-A^{B7} localized the beads to the macrophage/blood vessel interface. These findings point to the macrophage filopodia as the anatomical element that captures

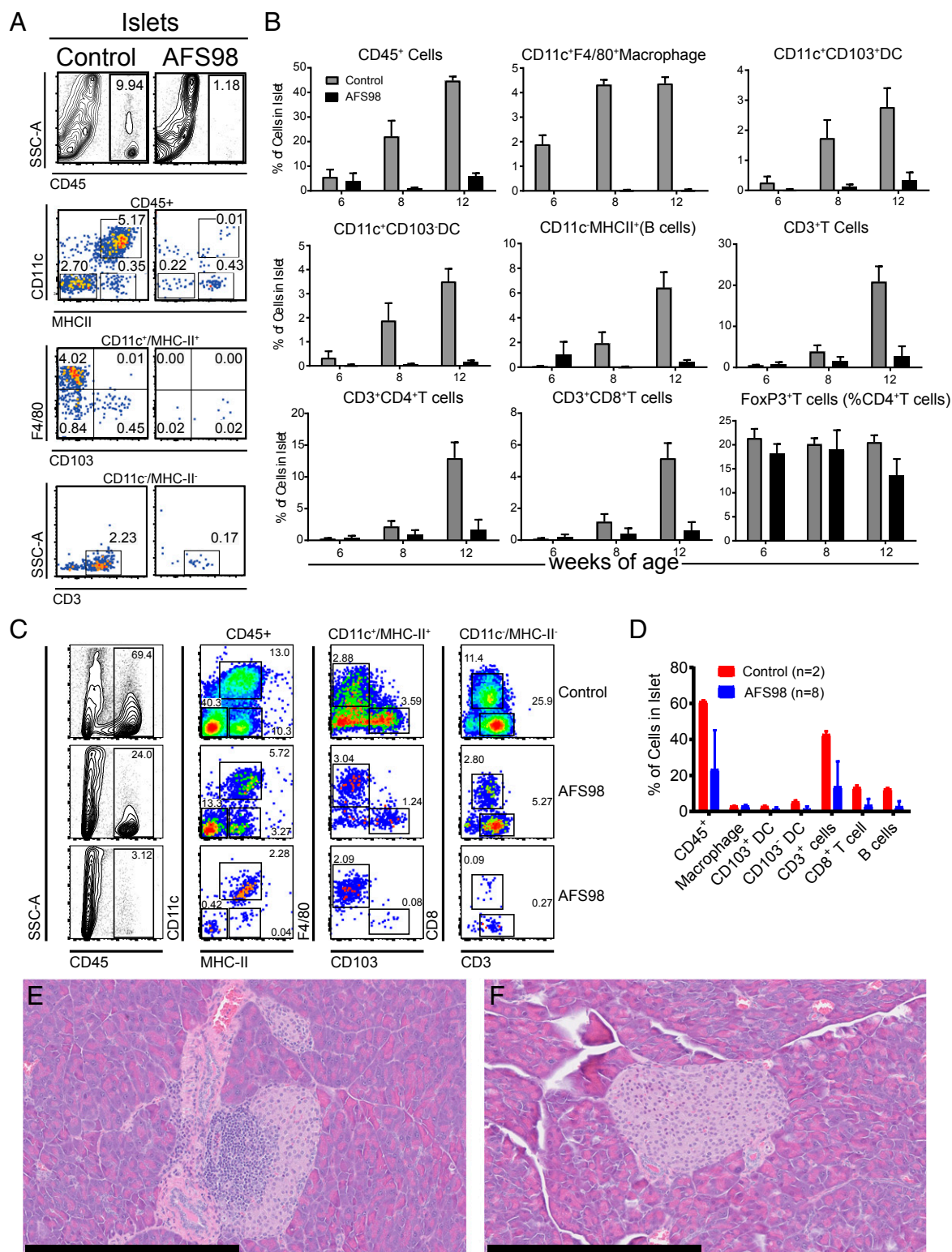


Fig. 7. Early treatment with AFS98 reduces infiltrating leukocytes in NOD mice up to 12 wk of age. (**A** and **B**) Female NOD mice were left untreated or injected with 0.5 mg of AFS98 i.p. at 2 wk of age and 2.0 mg of AFS98 i.p. at 4, 7, and 10 wk of age. The islets of mice at 6 (**B**), 8 (**A** and **B**), and 12 (**A**) wk of age were isolated and analyzed by flow cytometry. (**A**) Shows the flow cytometry plots of myeloid and T cell compartments. Gating is indicated over the plots. Flow cytometry plots are representative of individual islet preparation from three control or four AFS98-treated mice. (**B**) Summarizes the flow cytometry data for all time course experiments (three to four mice per group). Bars represent the mean \pm SD for two independent experiments with two to three replicates per group. (**C**) Flow cytometry plots of immune cell populations in islets isolated from nondiabetic control or AFS98-treated mice taken from Fig. 6 **A** and **C** early treatment examined at 40–44 wk of age. Plots were generated from individual mice. Gates are indicated on the *Top* of each column of plots. Flow cytometry plots are representative of individual islets preparations isolated from two control and eight AFS98-treated mice. (**D**) Summary of the data shown in **C**. (**E** and **F**) Hematoxylin/eosin staining of pancreatic sections isolated from nondiabetic AFS98-treated mice taken from Fig. 6**A**: The mice were treated early, and their islets were examined at 40–44 wk of age. (Scale bars, 400 μ m.)

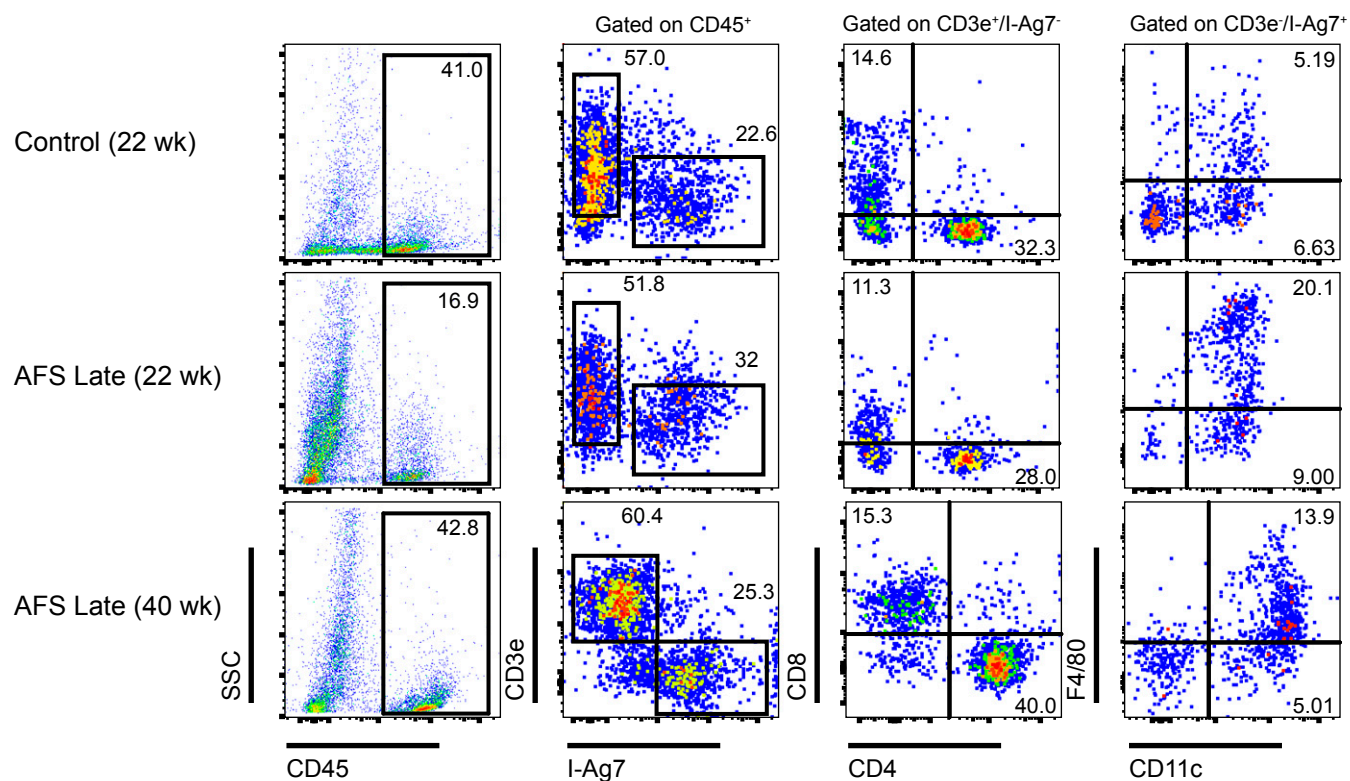


Fig. 8. Leukocyte infiltrates are reduced following macrophage depletion at 10 wk of age. NOD mice were either treated with AFS98 or control Rat IgG2a starting at 10 wk of age. At 22 or 40 wk of age, the islet cells of control or AFS-treated mice were isolated and analyzed by flow cytometry as indicated. Values in the CD45 by SSC plot represent the percent of leukocytes in islets. Values in the CD3e by I-Ag7 plot represent the percent CD3e⁺ or I-Ag7⁺ cells as a percent of CD45⁺ cells. Values in the T cell (CD3e⁺, I-Ag7⁻) and APC (CD3e⁻, I-Ag7⁺) plots represent the percent of cells in each quadrant as a function of total islet cellularity. Gating is indicated over the top for each column of plots. Results are representative of two mice per group.

Materials and Methods

Mice. Female C57BL/6 mice (B6), female NOD/ShiLtJ (NOD), NOD.129S7(B6)-*Rag1*^{tm1Mom/J} (NOD.*Rag1*^{-/-}), NOD.Cg-Tg(Tcr α BDC2.5,Tcr β BDC2.5)1Doi/DoiJ (BDC2.5), and NOD.Cg-Tg(Tcr α Tcr β NY8.3)1Pesa/DvsJ (NY8.3) mice were purchased from the Jackson Laboratories and bred in house. NY8.3 mice were backcrossed onto the congenic NOD.B6-*Ptprc*^{b/6908MrkTacJ} (NOD.CD45.2) background for T cell transfer experiments (18). All mouse experiments were performed in accordance with the Division of Comparative Medicine of Washington University School of Medicine [Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accreditation no. A3381-01].

T Cell Transfer Assay; ELISPOT; Anti-PD-1 Treatment. 8F10, BDC2.5, and NY8.3 TCR transgenic mouse spleens and lymph nodes were harvested and dispersed into single-cell suspensions. CD4- or CD8-positive cells were selected via magnetic microbead cell separation (Miltenyi Biotec) according to the manufacturer's protocol. The cells were then stained with 1 μ M carboxy-fluorescein diacetate succinimidyl ester (Life Technologies) for 15 min at 37 °C and immediately quenched with 4 °C DMEM supplemented with 10% FCS. CFSE-labeled cells (3.0×10^6) were then transferred into recipient mice via tail vein injections. Islets, draining pancreatic lymph nodes, and inguinal lymph nodes were harvested, dispersed into single-cell populations, and stained with fluorescent antibodies for analyses by flow cytometry. For experiments dealing with anti-PD-1, AFS98-treated or control NOD mice were injected with 200 μ g of antibody three times over 6 d (days 0, 3, and 6; Anti-PD-1 clone RMP1-4; Leinco Technologies). Mice were then followed for diabetes incidence. For experiments examining the lymph node response by ELISPOT, B6.g7 or NOD mice (7–10 wk old) were treated with a single 2.0-mg dose of AFS98 or control rat IgG2a. Seven days later, the mice were immunized in the footpad with 10 nmol INS B:9–23 peptide, HEL protein (Sigma-Aldrich), or IGRP:206–214 peptide. After 7 d, the animals were killed and the popliteal lymph nodes were removed and made into single-cell suspensions. Lymph nodes were assayed for reactivity by eliciting a recall response on either IL-2 or IFN- γ -coated 96-well multiscreen plates (MilliporeSigma) for

ELISPOT. One million popliteal lymph node cells were plated per well with 10 μ M antigen, and reactive cells were counted using Immunospot Software (C.T.L.). Immune responses in mice challenged with INS B:9–23 peptide were recalled with either insulin or INS B:9–23. Immune responses in mice challenged with HEL protein were recalled with either HEL protein or HEL peptides 11–25 (CD4 epitope) or 20–35 (CD8 epitope). Finally, responses in mice challenged with IGRP:206–214 peptide were recalled with either IGRP:128–142 (CD4 epitope) or IGRP:206–214 (CD8 epitope).

Glucose Tolerance Assay and Insulin Quantitation. For glucose tolerance experiments, female B6 mice were maintained on a normal diet and then fasted overnight for 12 h. Fasting blood glucose levels were tested before a challenge with 1.5 g/kg glucose in PBS (Life Technologies) administered by i.p. injection. Blood glucose was measured at the intervals indicated in the experiments. For insulin quantitation, control or α CSF-1R-treated mice were maintained on 20% sucrose water for 7 d, returned to regular water for an additional 2 d, fasted overnight, and then their pancreata were removed and weighed. Pancreata was minced and homogenized. The supernatant was collected after pelleting the homogenate, and insulin was quantified using the Rat Insulin Radioimmune Assay (MilliporeSigma), as per the manufacturer's instruction.

Antigen Presentation Assay. Islets were dispersed nonenzymatically, and 4.0×10^4 islet cells per well were plated together in a 96-well plate with 5.0×10^4 T cell hybridoma cells per well, in DMEM containing 10% FCS and 5 mM or 25 mM glucose (low or high glucose media, respectively). After 24 h, the supernatants were removed and the production of IL-2 was measured by culturing supernatants in the presence of the IL-2-dependent cell line, CTLL-2. Proliferation of CTLL-2 cells was measured by the incorporation of [³H] thymidine. For the antigen presentation assays, the 8F10 and IIT3 T cell hybridomas directed to the insulin B:12–20, and B:13–21 epitopes, respectively, were used (7).

Histology and Imaging. Microscopy imaging was performed using an Eclipse E800 microscope (Nikon) equipped with CFI Plan Apo Lambda DM 20 \times air objective, X-Cite 120PC light source (Excelitas Technologies), EXi blue fluorescence microscopy camera, and QCapture 64-bit v2.9.13 acquisition software (QImaging).

Islets, Pancreatic Stroma, and Lymph Node Isolation. Pancreata were perfused through the common bile duct with 5.0 mL of calcium-free HBSS supplemented with 400.0 μ g/mL of collagenase. Pancreata were then removed, and digested in a 37 $^{\circ}$ C water bath followed by vigorous shaking for 90 s, washed three times in HBSS, and then passed through a 70.0- μ m strainer to retain the islets. Cells that passed through the strainer represented the pancreatic stroma and were filtered a second time through a 40.0- μ m filter to prepare a single-cell suspension. Islets retained on the 70.0- μ m filter were then flushed into a Petri dish for hand-picking using a zinc-chelating dye, Dithizone (200 μ g/mL 10% DMSO PBS; Sigma), to identify the islets. Hand-picked islets were then dispersed using Cell Dissociation Solution Non-Enzymatic (Sigma) for 10 min at 37 $^{\circ}$ C. Lymph nodes and spleens were digested by incubation at 37 $^{\circ}$ C in DMEM supplemented with 10% fetal calf serum, 125 μ g/mL Liberase TL (Roche Life Science), and 50 μ g/mL DNase I (Roche Life Science). All single-cell suspensions were then incubated with 2.4G2 conditioned media (PBS, 1% BSA, and 50% 2.4G2 in DMEM) at 4 $^{\circ}$ C for

15 min to block FC receptors. Samples were then stained with fluorescent antibodies for flow cytometry or sorting.

Antibodies for Flow Cytometry and Sorting. Flow cytometry data were acquired on a FACSCanto II (BD Biosciences) and analyzed on FlowJo v10.2 software (FlowJo, LLC). Cell sorting was performed using a FACSARIA II (BD Biosciences). The following antibodies were purchased from BioLegend: BV510 anti-CD45 (30-F11), Pacific Blue (PB) anti-I-A/I-E (pan MHC-II), FITC anti-CD3 (2C11), FITC and APC anti-F4/80 (BM8), PE-Cy7 anti-CD11b (M1/70), PE anti-CD103 (2E7), PerCP-Cy5.5 anti-Ly6C (AL-21), PerCP-Cy5.5 anti-B220 (RA3-6B2), and APC anti-CD64 (X54-5/7/1). APC-eFluor780 anti-CD11c (N418), PerCP-eFluor710 anti-CD8 (53-6.7), eFluor450 anti-Ki-67 (SolA15), and APC FoxP3 (FJK-16) were purchased from eBioscience. For intracellular staining, the FoxP3 Fix/Perm kit (eBioscience) was used according to the manufacturer's instructions.

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Supporting Information

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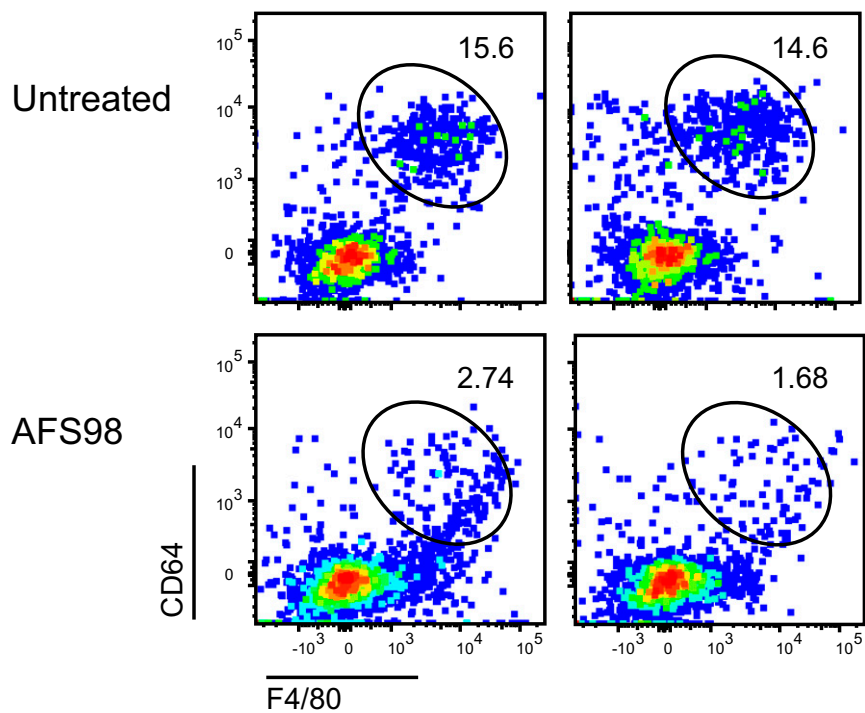


Fig. S1. Pancreatic stromal macrophages are reduced following AFS98 treatment. B6 mice were treated as in Fig. 1 and analyzed for the presence of CD45⁺F4/80⁺CD64⁺ macrophages. Numbers indicate the percentage of macrophages in the pancreatic stroma as a percent of the CD45⁺ cells. Cells were gated on FSC/SSC/CD45⁺. Data are taken from two independent mice per group and is representative of two independent experiments.

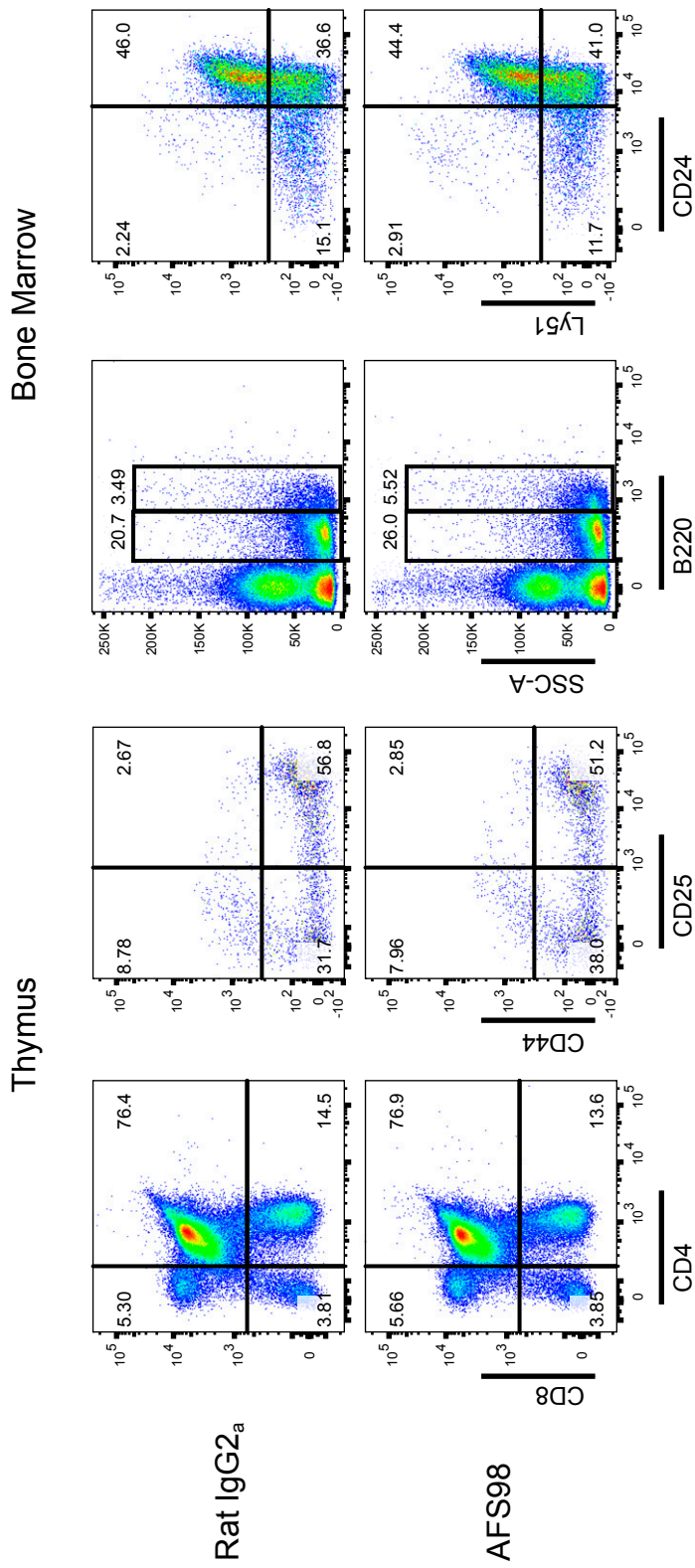


Fig. S2. Thymic T cells and bone marrow B cells are grossly normal following AFS98 treatment. Male NOD mice were injected with 2 mg of either AFS98 or Rat IgG_{2a} at 4 wk of age and then sampled at 6 wk of age. Thymus and bone marrow were harvested, and single-cell suspensions were generated. Cells were evaluated by flow cytometry. For thymus, cells were first gated on forward and side scatter, and CD45⁺ then CD4 by CD8 was plotted. Next, the CD4/CD8 double negative population was gated and examined for CD44 and CD25 as plotted on the *Right*. For bone marrow, cells were gated by forward and side scatter, and then plotted for B220 expression. The B220 intermediate cells were then gated and plotted for CD24 and Ly51 staining as shown on the *Right*. Numbers indicate the percentage of cells in each region as a function of the total cells in the plot. Results are representative of three individual mice per group.

