Synergistic Reversal of Type 1 Diabetes in NOD Mice With Anti-CD3 and Interleukin-1 Blockade

Evidence of Improved Immune Regulation

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Inflammatory cytokines are involved in autoimmune diabetes: among the most prominent is interleukin (IL)-1β. We postulated that blockade of IL-1β would modulate the effects of anti-CD3 monoclonal antibody (mAb) in treating diabetes in NOD mice. To test this, we treated hyperglycemic NOD mice with $F(ab')_2$ fragments of anti-CD3 mAb with or without IL-1 receptor antagonist (IL-1RA), or anti–IL-1β mAb. We studied the reversal of diabetes and effects of treatment on the immune system. Mice that received a combination of anti-CD3 mAb with IL-1RA showed a more rapid rate of remission of diabetes than mice treated with anti-CD3 mAb or IL-1RA alone. Combination-treated mice had increased IL-5, IL-4, and interferon (IFN)-γ levels in circulation. There were reduced pathogenic NOD-relevant V7 peptide-$V7^+$ T cells in the pancreatic lymph nodes. Their splenocytes secreted more IL-10, had increased arginase expression in macrophages and dendritic cells, and had delayed adoptive transfer of diabetes. After 1 month, there were increased concentrations of IgG1 isotype antibodies and reduced intrapancreatic expression of IFN-γ, IL-6, and IL-17 despite normal splenocyte cytokine secretion. These studies indicate that the combination of anti-CD3 mAb with IL-1RA is synergistic in reversal of diabetes through a combination of mechanisms. The combination causes persistent remission from islet inflammation. ***Diabetes*** **61:145–154, 2012**

Immunologics can reverse diabetes in the NOD model of type 1 diabetes (T1D), and they have shown efficacy in clinical trials (1–4). However, there is a substantial variability in the responses of patients to immune therapies and loss of efficacy with time. There are many reasons for this, such as the effects of different immune response genes or inflammatory mediators that are present at the time of drug administration. For example, interleukin (IL)-1β is one such factor that has direct toxic effects on β-cells and also modulates T-cell activation and differentiation (5–9).

IL-1β was shown to directly inhibit islet insulin secretion and synthesis and affect β-cell viability (5,6), particularly in combination with other cytokines (7,10). Its direct involvement in β-cell death resulting in clinical diabetes has been proposed (11). Macrophages, a likely source of IL-1β, were identified in the insulitis lesions of patients with new-onset T1D, and monocytes are a source of circulating IL-1β in patients with T1D (12,13). More recently, it was shown that pancreatic islets themselves can produce IL-1, particularly in response to high glucose (14,15).

IL-1β may cause the release of chemokines and immune adjuvants (16). Transduction of human islets with the naturally occurring antagonist of IL-1 receptor (IL-1RA) by adenovirus protected them from formation of IL-1–induced nitric oxide (NO), functional inhibition, and apoptosis (17,18). Delivery of IL-1RA to rat islets resulted in increased β-cell replication in vitro and in vivo after transplantation into rats made diabetic with streptozotocin (19,20). In T1D patients, short-term administration of human IL-1RA (Anakinra) that antagonizes binding of IL-1β and IL-1α (21,22) resulted in decreased levels of circulating IL-8, downregulation of CD11b on monocytes, and upregulation of IL-8 receptor CXCR1, suggesting that IL-1RA may influence trafficking of monocytes (23).

However, blockade of IL-1β signaling has not been sufficient to prevent or reverse diabetes in animal models. IL-1 receptor deficiency slowed, but did not prevent, progression to diabetes in NOD mice, although islets were protected from the damaging effects of tumor necrosis factor (TNF) and interferon (IFN)-γ in vitro (24). IL-1RA treatment prevented rapid rejection of syngeneic NOD islets transplanted into spontaneously diabetic NOD females, but hyperglycemia recurred after the termination of treatment (25,26).

In addition, IL-1β may subvert the actions of immunologics used to treat T1D such as anti-CD3 mAb, which is thought to reverse diabetes in NOD mice by induction of adaptive regulatory T cells (Tregs) (27). It is postulated that IL-1β affects the differentiation of these adaptive Tregs and expands antigen-specific $CD4^+$ T cells (28,29). It is possible that the loss of efficacy of anti-CD3 mAb or other immune therapeutics with time in the clinical setting is related to the effects of IL-1β or other inflammatory mediators.

Because of these direct and indirect effects related to the development of T1D, we postulated that neutralizing IL-1β would improve the actions of anti-CD3 mAb in reversal of the disease. We tested the effects of IL-1RA in combination with non-Fc receptor (FcR) binding anti-CD3 mAb, which has been shown to preserve insulin production in patients with new-onset T1D (30–34). We report that combined administration of IL-1RA with anti-CD3 mAb to hyperglycemic mice improves the rate and frequency of reversal of diabetes compared with the mAb alone. Soon after drug administration, the insulin content is improved in

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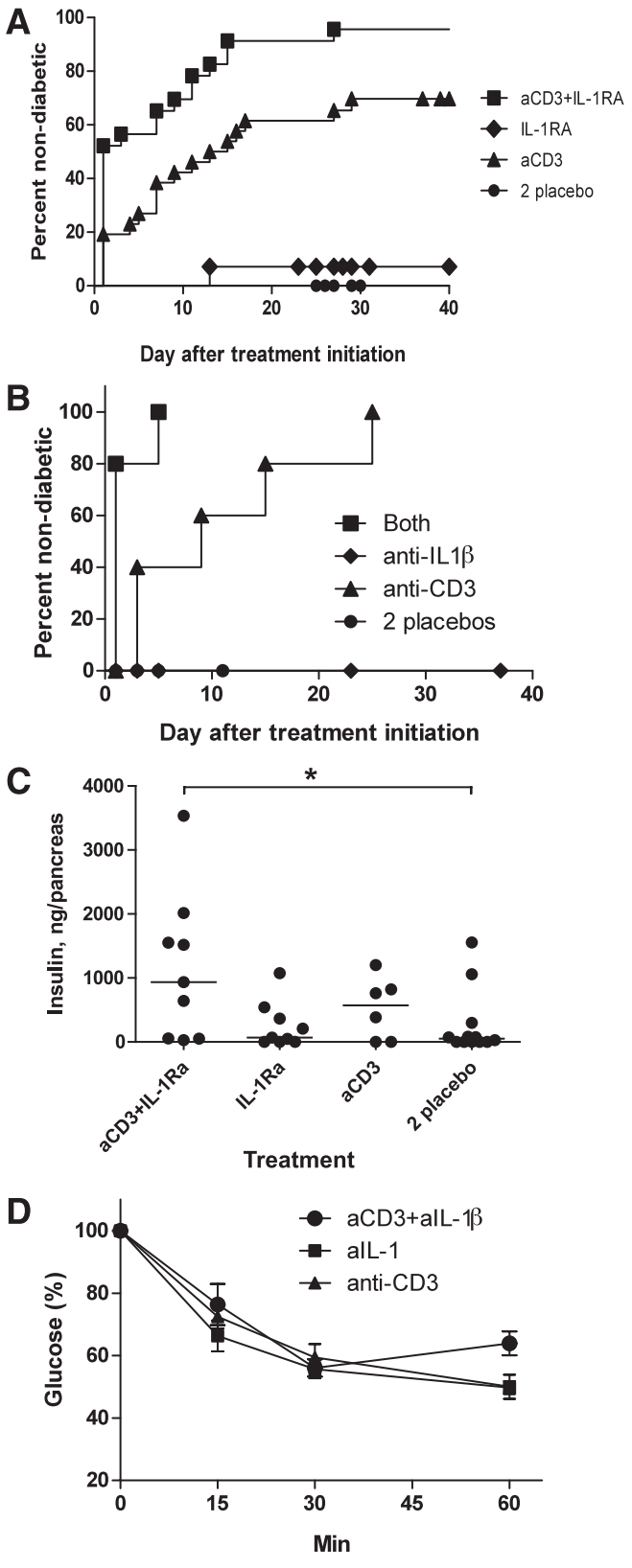

FIG. 1. Remission of hyperglycemia in diabetic NOD mice after treatment. *A*: NOD mice with hyperglycemia were randomized to treatment with anti-CD3 mAb + IL-1RA (n = 23), IL-1RA (n = 14), anti-CD3 mAb (n = 26), or two placebos (n = 12), and whole blood glucose levels were measured at the indicated times. Remission was declared when the glucose level was <200 mg/dL. Overall, there was a significant effect of treatment on disease reversal ($P < 0.01$). The rate of reversal of diabetes was greater for mice treated with anti-CD3 mAb ($P < 0.001$) and IL-1RA + anti-CD3 mAb ($P < 0.0001$) compared with placebo. There was also a significant increase in the proportion of mice with remission in the anti-CD3 mAb + IL-1RA–treated group compared with anti-CD3 mAb alone ($P < 0.01$) and a shorter median time to remission ($P = 0.003$). *B*: Diabetic NOD mice were randomly assigned to treatment with anti–IL-1β mAb + anti-CD3 mAb (n = 5), anti–IL-1β mAb alone (n = 5), anti-CD3 mAb alone (n = 5), or two placebos. There was a significant improvement in reversal of diabetes with the combination compared with anti-CD3 mAb alone ($P < 0.02$). The rate of reversal of diabetes was greater for mice treated with anti-CD3 mAb ($P < 0.01$) and anti–IL-1β mAb with anti-CD3 mAb ($P < 0.01$) compared with placebo. *C*: Insulin was extracted from the pancreata from mice in each of the indicated treatment groups, and the total insulin content was measured by ELISA. There was significantly greater pancreatic insulin in mice receiving combination treatment compared with placebo (*$P < 0.05$ by ANOVA). *D*: Glucose responses to insulin were compared. The percent drop in glucose was calculated for each mouse (n = 4 in each group) as 1− area under the curve of percent starting glucose at each time point.

the pancreas, and there is evidence for reduced numbers of pathogenic effector cells and increased immune regulatory mechanisms. Long-term combination treatment causes sustained reduction in mediators of islet inflammation.

RESEARCH DESIGN AND METHODS

Mice. Animal experiments were approved by the Yale Institutional Animal Care and Use Committee. Female NOD/ShiLtJ or NOD/*scid* mice were purchased from The Jackson Laboratory and kept in a specific pathogen-free environment. Nonfasting blood glucose was measured every other day with an Easy Check monitor (Home Aide Diagnostics, Deerfield Beach, FL) beginning at 12 weeks of age. Mice were considered diabetic when blood glucose exceeded 250 mg/dL on at least one of two consecutive determinations. Treatment was started on the day of the second high glucose reading, designated as day 0.

Reagents for animal treatment. F(ab′)$_2$ fragments of hamster anti-mouse CD3 mAb (clone145–2C11; ATCC, Manassas, VA) and hamster IgG, as a control for the anti-CD3 mAb (BioXCell, West Lebanon, NH), were prepared by Rockland Immunochemicals (Gilbertsville, PA). The fragments were administered to diabetic mice at a dose of 50 μg/mouse i.p. daily for 5 days (a dose that achieved remission in 50–70% of mice) (35–37). IL-1RA (Anakinra; Amgen, Thousand Oaks, CA) was administered, 10 mg/mouse i.p. daily, for 5 days mixed with the respective F(ab′)$_2$ before injection. The mice were randomly assigned to one of four treatment groups: *1*) anti-CD3 F(ab′)$_2$ plus placebo (control protein; a gift from Dr. C. Dinarello; *2*) IL-1RA + placebo control F(ab′)$_2$; *3*) anti-CD3 mAb + IL-1RA; and *4*) placebo + control F(ab′)$_2$. Glucose levels were measured every other day, and the mice were considered to be in remission if blood glucose levels were <200 mg/dL. In other experiments, an anti-mouse IL-1β mAb (Novartis, Switzerland) was used at a dose of 75 μg/mouse on days 0, 2, and 4.

Immune monitoring. Circulating cytokines were measured on day 2 after commencement of treatment in plasma by a Milliplex Map kit (Millipore, Billerica, MA) and Bioplex analyzer (Bio-Rad, Hercules, CA). Some mice were killed on days 3 or 7 after diagnosis and others were studied at day 30. Cells from spleens or pancreatic lymph nodes were counted and analyzed for expression of cell surface markers (CD4, CD8, CD19, CD11b, CD11c, CD25, and ICOS) and intracellular molecules (FoxP3) using mAbs from BD Pharmingen (Franklin Lakes, NJ) by flow cytometry (FACSCalibur, BD Immunocytometry Systems, San Jose, CA). Flow data were analyzed with FlowJo software (TreeStar, Ashland, OR). Splenocytes from some mice were also harvested aseptically and activated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) and ionomycin (500 ng/mL) (Sigma, St. Louis, MO) for 24 h. Supernatants were collected and cytokines were measured as above. Pancreatic lymph nodes were isolated during collagenase digest, and NRP-specific CD8$^+$ lymphocytes were identified by staining with phycoerythrin-conjugated H-2K^d tetramer with NRP-V7 peptide KYNKANVFL, a mimotope of islet-specific glucose-6-phosphatase catalytic subunit–related protein (IGRP) (38). TUM (KYQAVTTTL)/H-2K^d tetramer was used as a negative control (39,40). In separate experiments, day 7 splenocytes were positively sorted using anti-CD11b, or anti-CD11c magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), and lysed for measurement of arginase 1 (*Arg1*) and inducible nitric oxide synthase (iNOS) gene expression by quantitative PCR (qPCR).

Adoptive transfer studies. A total of 10 million spleen cells from nontreated diabetic NOD mice (effector cells) were cotransferred intravenously with 5 × 10^6 spleen cells from mice that received either anti-CD3 mAb + IL-1RA or anti-CD3 mAb alone or placebo into immunodeficient NOD/*scid* recipients. The mice were monitored twice a week for development of hyperglycemia and declared diabetic when the glucose level was >250 mg/dL.

Insulitis score, pancreatic insulin content, and gene expression. Pancreata were removed on days 7 or 30. They were fixed in formalin and stained with hematoxylin and eosin. An insulitis score for each islet was determined (0 = no insulitis; 1 = periinsulitis; 2 = invasive insulitis with <50% islet area affected; 3 = invasive insulitis with >50% islet area affected). Between 36 and 59 islets were scored from three to six mice in each treatment group. Other pancreata were snap-frozen and minced in precooled acid-ethanol for 48 h. Mouse insulin was measured by ELISA h (Crystal Chem, Downers Grove, IL) (41). Pancreatic proinsulin content was measured in acid-ethanol extracts using a rat/mouse proinsulin ELISA kit (Mercordia AB, Uppsala, Sweden). In other mice, pancreata and spleens were homogenized in lysis buffer, and RNA was isolated using the RNeasy Mini Kit (Qiagen Sciences, Germantown, MD). cDNA was prepared, and gene expression was measured by RT-PCR using the primers shown in Supplementary Table 1. Expression of genes of interest was normalized to actin.

Statistical analysis. Statistical analysis was performed using GraphPad Prizm, Version 5 (GraphPad Software, San Diego, CA). One-way ANOVA or Kruskal-Wallis test with Dunnett or Dunn multiple comparison posttests were used to evaluate differences between the groups, with $P < 0.05$ considered significant. For survival curves, a log-rank test was used. The displayed data were pooled from a minimum of three replicate experiments. Each data point presented in scatter plots was from a single mouse.

RESULTS

Combination of IL-1RA and anti-CD3 mAb causes rapid reversal of diabetes. To identify the effects of IL-1 blockade on the reversal of diabetes in NOD mice, we administered IL-1RA with and without $F(ab')_2$ fragments of anti-CD3 mAb 145–2C11. Figure 1*A* shows that diabetic NOD mice treated with this combination had significantly faster restoration of euglycemia (i.e., glucose <250 mg/dL) with median reversal time of 1 day, compared with mice treated with anti-CD3 alone (median reversal time of 14 days; $P = 0.003$). In addition, a larger proportion of animals (96 vs. 70%, $P < 0.01$) reached euglycemia. We followed eight of the anti-CD3 mAb–treated and eight of the combination-treated mice for up to 60 days and found no recurrence of T1D at a later time in the mice that had reversal at day 30 (not shown). To confirm that the synergy observed was due to a specific blockade of IL-1 action rather than a nonspecific effect of the protein, we used another anti–IL-1 reagent, a neutralizing anti–IL-1β mAb. We again saw rapid reversal of diabetes when anti-CD3 mAb was combined with anti–IL-1β mAb ($P < 0.02$, Fig. 1*B*). In these experiments, all of the mice treated with anti-CD3 mAb and placebo eventually did show reversal of diabetes.

The rapid reversal of clinical diabetes suggested an acute effect of the combination therapy on either β-cell secretory function or improved sensitivity to insulin. We extracted total insulin from the pancreata of the mice on day 7 and found that the pancreatic insulin content of the combination-treated mice was higher than that of placebo-treated mice ($P < 0.05$), whereas that of mice treated with either anti-CD3 mAb, or IL-1RA alone, was not (Fig. 1*C*). In addition, we performed insulin tolerance tests on the treated mice on day 2, but found that the decline in glucose levels were similar in mice that received the combination treatment (a decrease of 31.4 ± 3.5%) compared with mice treated with anti-CD3 mAb alone (a decrease of 38 ± 2.4%; NS) or IL-1RA alone (a decrease of 34.6 ± 2.28%; NS), indicating that improved insulin sensitivity was not the basis for the improved response (Fig. 1*D*).

We measured the levels of cytokines in the serum after the second dose of drugs and also characterized cytokine production by splenocytes that were activated with PMA/ionomycin at the conclusion of the treatments (Fig. 2). There were increased levels of IL-5 ($P < 0.02$) and IFN-γ ($P < 0.02$) in serum in mice that received anti-CD3 mAb alone or in combination with IL-1RA. The increased levels of these cytokines were most likely due to the activation of T cells in vivo, since they were seen in both groups that received anti-CD3 mAb. However, the level of IL-4 was also significantly greater in the combination-treated versus anti-CD3 mAb–treated mice ($P < 0.05$, Fig. 2) but was not significantly increased in the group that received anti-CD3 mAb alone, since 6 of 10 mice had undetectable serum levels. In the culture supernatants from splenocytes that were activated at the conclusion of treatment (day 7), we found significantly increased levels of IL-10 in mice treated with the combination compared with anti-CD3 mAb alone. The levels of IL-4, IL-5, and IFN-γ were not significantly different in the supernatants from splenocytes in the treatment groups (not shown).

The combination affected pathogenic T cells. We enumerated autoantigen-specific NRP-V7$^+$ T cells in the spleen and pancreatic lymph nodes on day 7 with class I major histocompatibility complex tetramers (39,40). This analysis failed to show a difference in the number of the tetramer$^+$ CD8$^+$ T cells between the groups

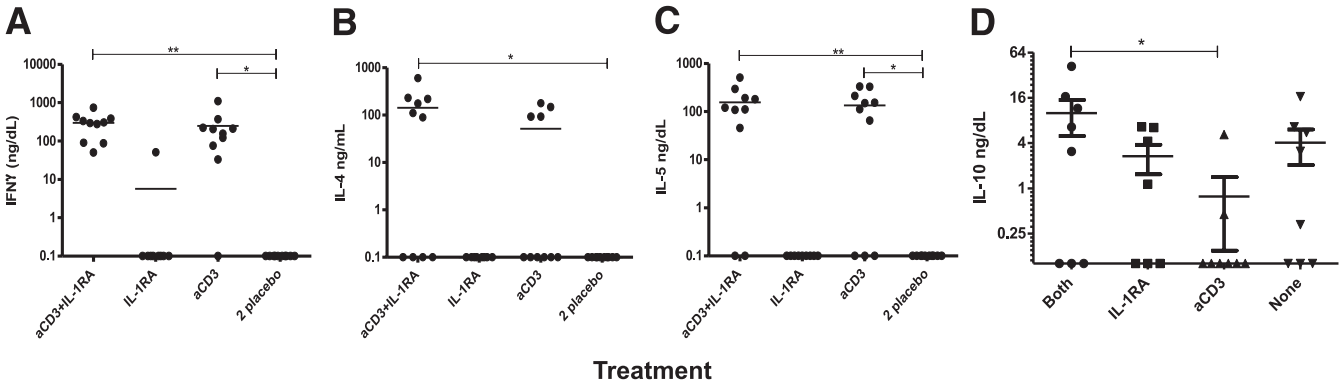

FIG. 2. Levels of cytokines in serum and from activated splenocytes. *A–C*: After serum was isolated from mice in each of the treatment groups after treatment on day 2, the levels of cytokines were measured. Each dot represents a single mouse. The levels of IFN-γ and IL-5 are significantly increased in combination-treated or anti-CD3 mAb–treated mice versus placebos (*$P < 0.05$, **$P < 0.02$), but IL-4 is significantly increased in combination-treated mice. The levels of TNF, IL-2, IL-6, or IL-12 were increased in mice treated with anti-CD3 mAb, but the differences from placebo were not statistically significant (not shown). *D*: Splenocytes were harvested on day 7 and stimulated with PMA/ionomycin for 24 h, as described in RESEARCH DESIGN AND METHODS. The level of IL-10 was measured in the culture supernatants ($P < 0.05$, combination vs. anti-CD3 mAb, by ANOVA with Dunn correction for multiple comparisons). The levels of other cytokines (IFN-γ, IL-4, IL-5, IL-2, IL-17, TNF, and IL-6) were not significantly different between the groups (not shown).

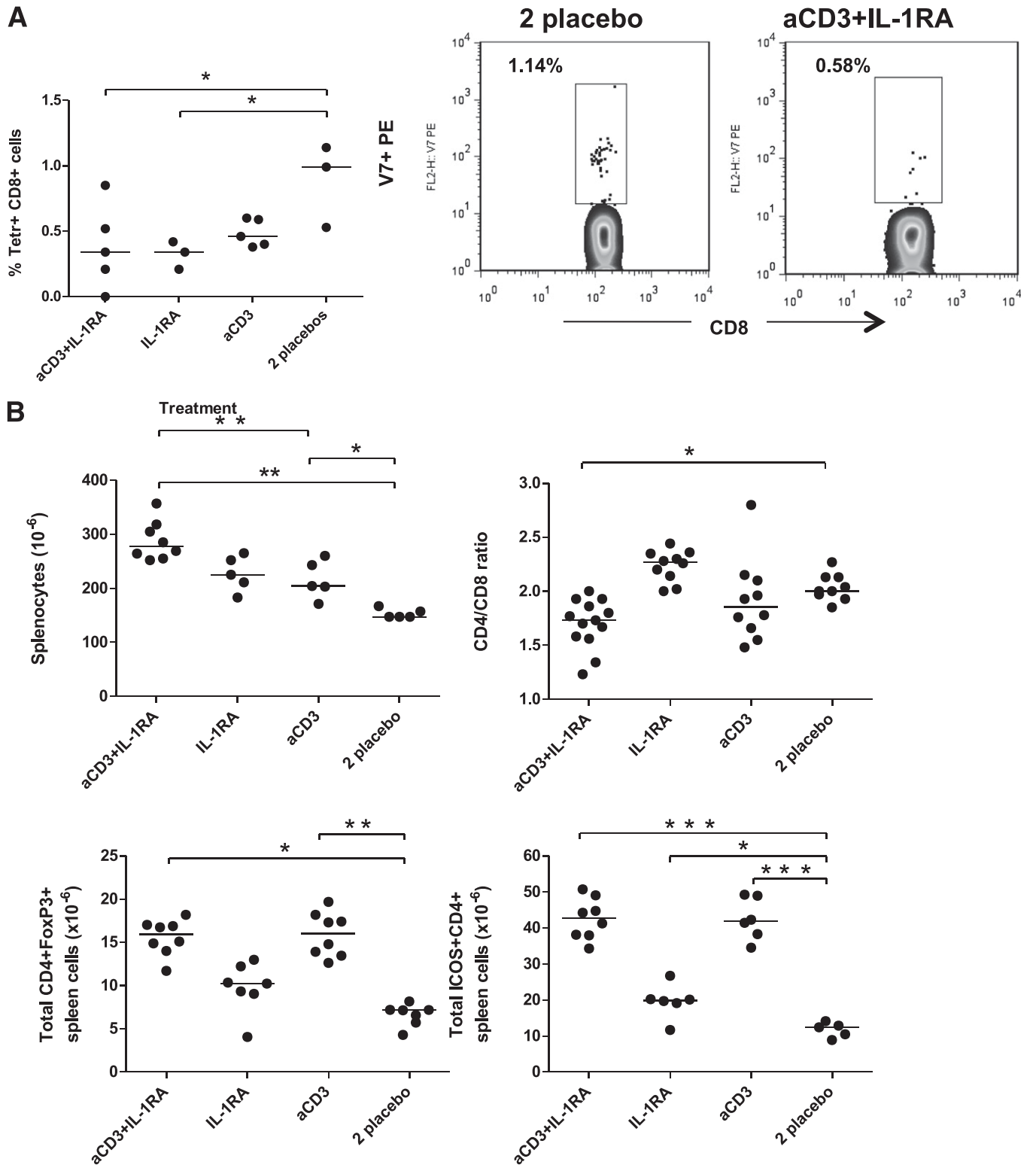

FIG. 3. Analysis of T cells at day 7. *A*: The percentage of NRP-V7$^+$ CD8$^+$ T cells in pancreatic lymph nodes is shown for each of the treatment groups (*$P < 0.05$). Two additional panels show representative staining of CD8$^+$ lymph node T cells with tetramer (on the *y*-axis vs. CD8 on the *x*-axis) in double-placebo or combination-treated mice. *B*: There was a significant increase in the number of splenocytes in all treatment groups compared with placebo treatment (*$P < 0.05$, **$P < 0.01$). The ratio of CD4$^+$/CD8$^+$ T cells was decreased in mice that received anti-CD3 mAb compared with placebo or IL-1RA. The absolute numbers of Foxp3$^+$CD4$^+$ T cells were also increased in mice that received the combination or only anti-CD3 mAb compared with placebo or IL-1RA ($P < 0.01$). The absolute numbers of ICOS$^+$CD4$^+$ T cells were increased in mice that received anti-CD3 mAb compared with placebo or IL-1RA (*$P < 0.01$).**

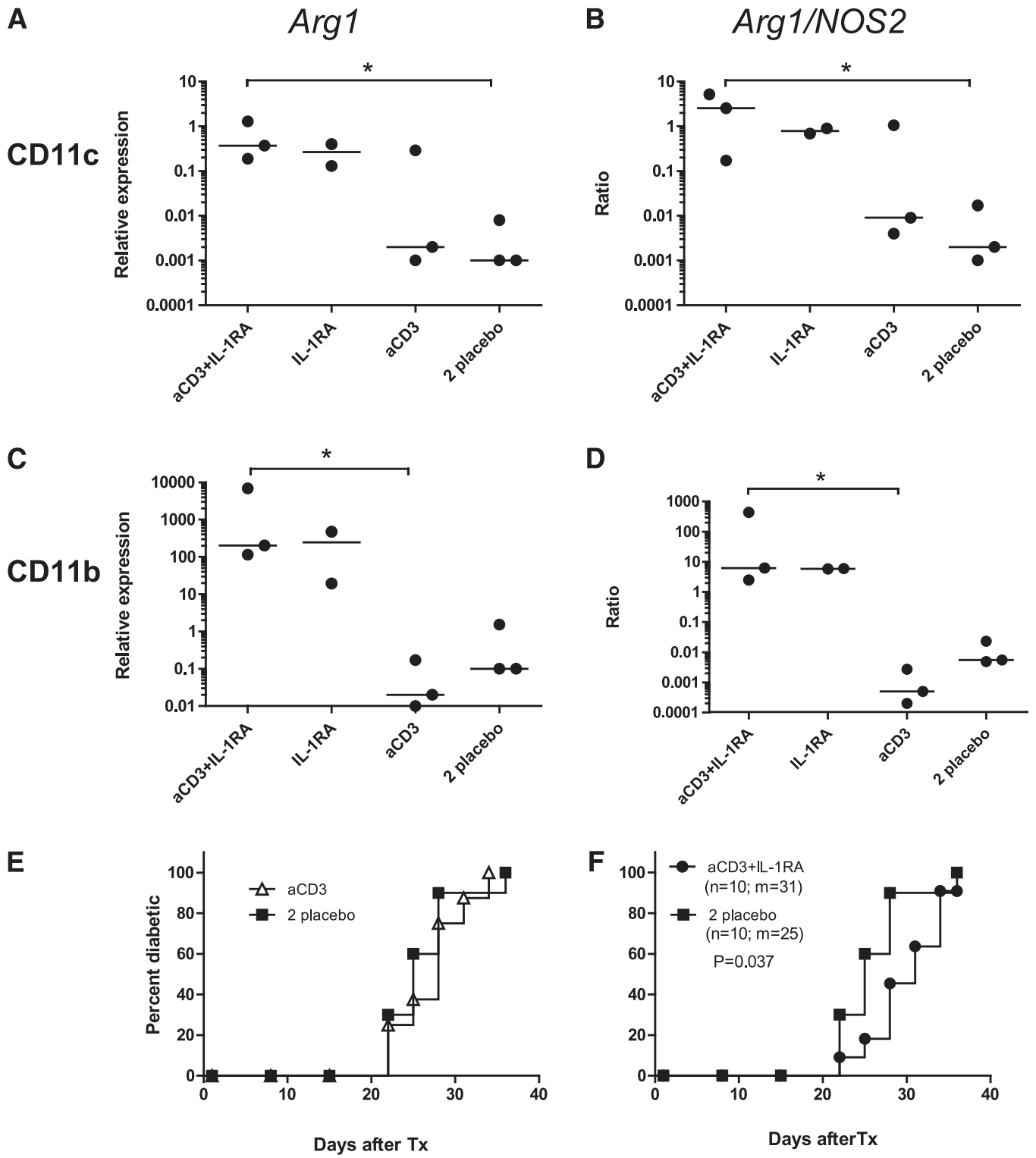

FIG. 4. ***Arg1*** **expression in magnetically sorted CD11c$^+$ or CD11b spleen cells and the ability of spleen cells to delay adoptive transfer of diabetes.** ***Arg1*** **gene expression in day 7 splenocytes was measured by RT-PCR in CD11c$^+$ (*A*) and CD11b$^+$ (*C*) sorted cells, and the** ***Arg1*** **to** ***NOS2*** **ratios were determined (*B* and *D*). The expression of** ***Arg1*** **is higher in the groups that received IL-1RA with anti-CD3 mAb compared with anti-CD3 mAb alone (*$P < 0.05$). *E* and *F*: 5×10^6 splenocytes from mice treated with anti-CD3 mAb or placebos (*E*) or the combination or placebos (*F*) were mixed with 10^7 splenocytes from diabetic NOD mice and administered to NOD/*scid* recipients. There was a significant delay in the onset of diabetes in mice that received splenocytes from the combination-treated mice ($P = 0.037$). Tx, treatment.**

in the spleen (not shown), but the combination- and IL-1RA–treated groups had lower frequencies of auto-antigen-specific CD8$^+$ cells in pancreatic lymph nodes (Fig. 3*A*).

Combination treatment improves regulatory function of splenocytes. Previous studies by our group and others have suggested that anti-CD3 mAb induces adaptive Tregs that mediate the reversal and prevent progression of

diabetes in NOD mice (27,42). To determine the effects of the drug combination on Tregs, we first enumerated the T-cell subsets in the spleens of the treated mice on day 7. The total number of splenocytes was increased in mice that received anti-CD3 mAb with or without IL-1RA ($P < 0.001$), and the ratio of CD4:CD8$^+$ T cells was decreased by anti-CD3 mAb in combination with IL-1RA ($P < 0.001$, Fig. 3*B*). In addition, the percent and total number of Foxp3$^+$CD4$^+$ T cells was increased in the spleens of both the anti-CD3 mAb and combination-treated mice ($P < 0.001$, Fig. 3*B*). We also found an increase in the absolute numbers of ICOS$^+$CD4$^+$ T cells compared with mice that received placebo or IL-1RA alone (Fig. 3*B*). Upregulated FoxP3 and ICOS was due to the anti-CD3 mAb, since there were no detectable differences in cell numbers in mice that received the combination versus anti-CD3 mAb alone and we did not detect significant differences with IL-1RA alone.

Because IL-1RA and IL-10 are also products of monocytes/macrophages with anti-inflammatory properties (M2), we hypothesized that combination treatment may affect macrophage differentiation. We sorted macrophages and dendritic cells with CD11b$^+$ and CD11c$^+$ mAbs from day 7 spleens and analyzed expression of *Arg1* and *NOS2* genes, which encode arginase 1 (M2 marker) and iNOS (M1 marker), respectively, on these subpopulations. In both CD11b$^+$ and CD11c$^+$ cells, the relative expression of *Arg1* was increased in IL-1RA–treated mice (Fig. 4*A* and *C*). The levels of *NOS2* expression were not different between the groups (data not shown), but the *Arg1* to *NOS2* ratio was higher in IL-1RA–treated animals (Fig. 4*B* and *D*). Expression of other genes tested, which discriminate between classically and alternatively activated monocytes/macrophages or dendritic cells (*IL-1RN*, *CCR3*, *IL12*, *IL10*, *IL18*, and *Ym1*), were not significantly different (not shown).

This result suggested that IL-1RA with anti-CD3 mAb induced adaptive and innate immune cells that may have enhanced immune regulatory function. To test this directly, we performed adoptive transfer experiments mixing spleen cells from diabetic NOD mice with day 7 spleen cells from treated mice. There was a modest but significant delay (31 vs. 25 days, $P < 0.05$) in the time of onset of diabetes in NOD/*scid* recipient mice that received splenocytes from combination-treated mice but not anti-CD3 mAb–treated (Fig. 4*E* and *F*) or IL-1RA–treated (not shown) mice (Fig. 4*E* and *F*).

Combination therapy induces lasting remission of autoimmune diabetes. In addition to a more rapid reversal of diabetes, the combination treatment also had long-term effects on islet inflammation. We compared the immunologic effects and the pancreata from treated mice 30 days after treatment initiation. We measured the isotypes of immunoglobulins in the serum 25 days after completion of treatment and found that the relative concentrations of IgG1 were increased in mice that received the combination treatment compared with mice treated with anti-CD3mAb alone ($P < 0.05$, Fig. 5). This result is consistent with the effect of the combination on cytokine production.

We extracted and measured insulin and proinsulin in pancreata from mice at day 30 (Fig. 6*A* and *B*). Similar to observations on day 7, the increase in total insulin and proinsulin content persisted at day 30 in mice that received the combination of drugs compared with placebo, or IL-1RA (Fig. 6*A* and *B*; $P < 0.001$ and $P < 0.01$, respectively).

We compared islet inflammation and inflammatory gene expression in the pancreata from day 30 mice. The relative expression of CD45 in the pancreas was not distinguishable between the groups by qPCR, and the histological insulitis score was similar between the treatment groups (Fig. 6*C* and *D*). However, there were marked differences in the expression of immune response genes in the pancreas. The expression of IL-17 ($P < 0.02$), IL-6 ($P < 0.05$), and IFN-γ ($P < 0.05$) were all reduced in the pancreata from combination-treated mice compared with mice treated with placebo (Fig. 7*A*). The reduced expression of these cytokine genes was not significantly different in the mice treated with anti-CD3 mAb alone or IL-1RA alone compared with placebo. The absence of immune responses observed in the pancreas was not a global feature. When splenocytes from day 30 mice were activated with anti-CD3 mAb, we did not detect significant differences in levels of the six measured cytokines in culture supernatants (Fig. 7*B*).

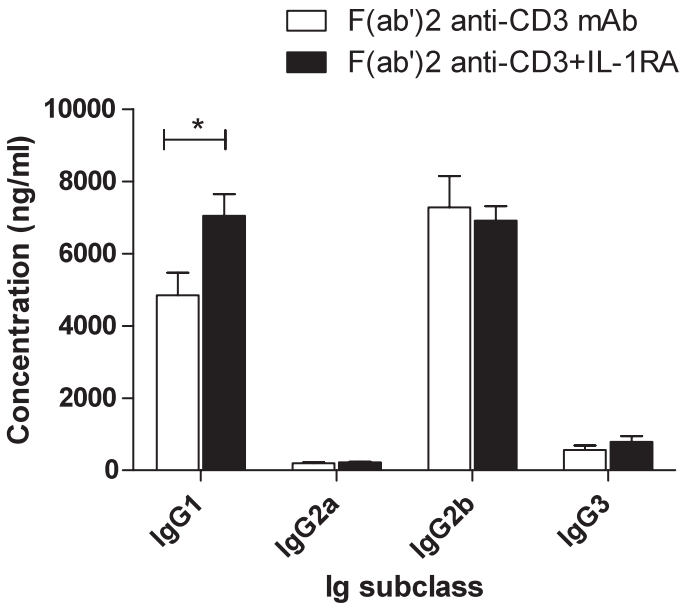

FIG. 5. Combination therapy leads to an increase in IgG1 isotype. We measured the circulating levels of IgG isotypes in the serum from day 30 mice by Luminex (n = 5–13/group). There was a significant increase in the concentration of IgG1 in mice that received the combination compared with those treated with anti-CD3 mAb alone (*P < 0.05).

DISCUSSION

We found that IL-1β modulates T-cell responses after anti-CD3 mAb in T1D. A 5-day treatment regimen with IL-1RA significantly improved the rate of reversal of T1D in hyperglycemic NOD mice treated with non-FcR binding anti-CD3 mAb compared with the anti-CD3 treatment alone, but IL-1RA itself was ineffective. Our studies with neutralizing anti–IL-1β mAb suggest that the effect of the IL-1RA is due to a specific blockade of IL-1 action. Our data suggest that the combination treatment induced long-term tolerance, since reduced inflammation was found 1 month after treatment, and the reversal did not require continuous IL-1 blockade, which may be detrimental for the immune system and even for β-cell function (43). Similar lasting effects of IL-1RA treatment on low-grade systemic inflammation and β-cell function has been reported in patients with type 2 diabetes (44).

It has been known for >20 years that IL-1β has an important role in the pathogenesis of autoimmune diabetes (45). IL-1, in synergy with other cytokines, is cytotoxic to pancreatic β-cells (5,6,10,45,46). The direct toxicity occurs

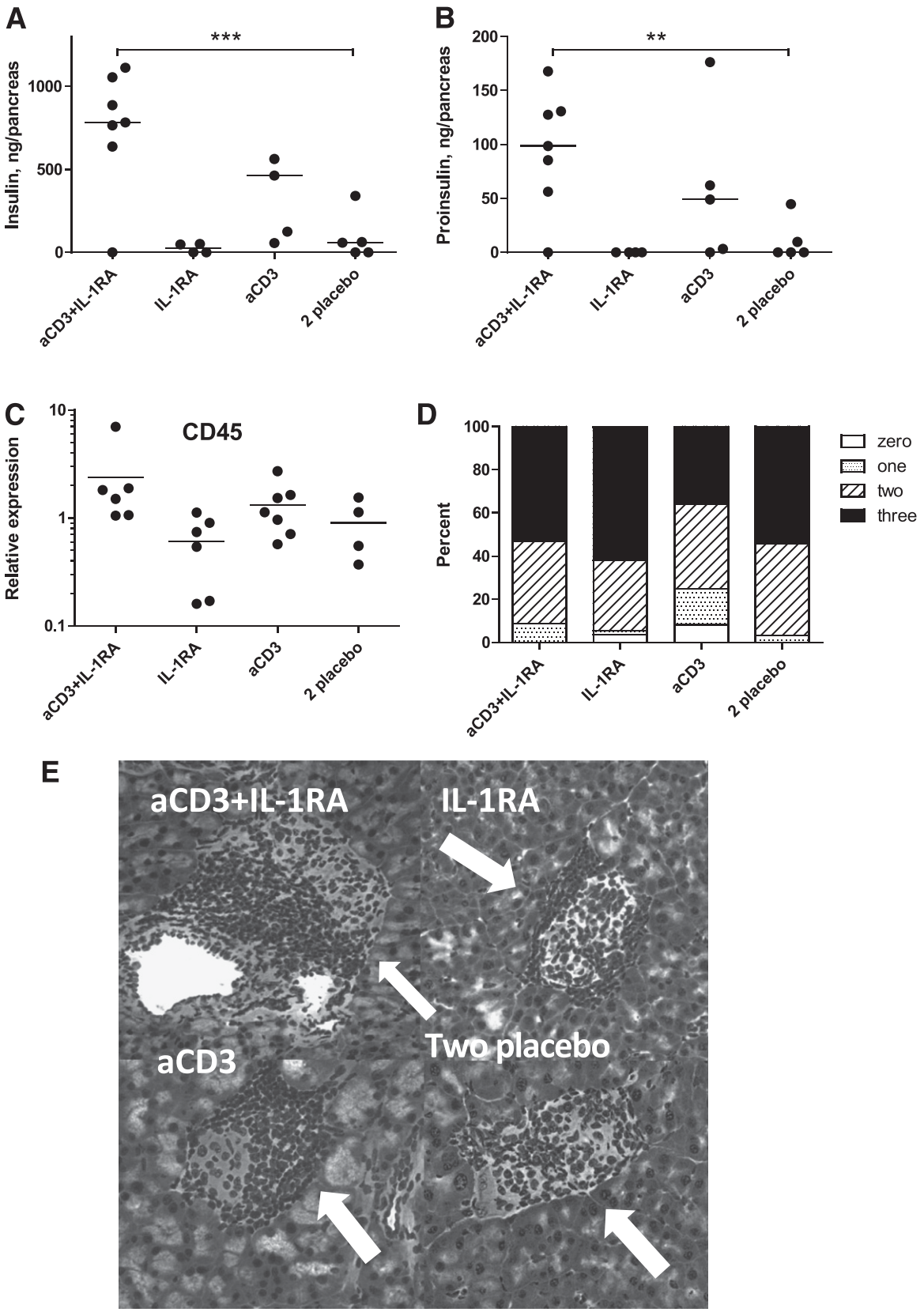

FIG. 6. Analysis of day 30 pancreata for insulin content and insulitis. We extracted and measured the concentration of insulin (*A*) and proinsulin (*B*) from the pancreata of day 30 (n = 4–7 mice/group). The total amount of insulin or proinsulin from each pancreas is shown ($P < 0.02$; ***$P < 0.01$). *C*: The expression of CD45 in the pancreata was studied by RT-PCR. *D*: Individual islets were scored for insulitis (36–59 islets/group), and the proportion of islets with each grade of insulitis is shown. *E*: Photomicrographs of representative islets (*arrows*) from mice in each treatment group.**

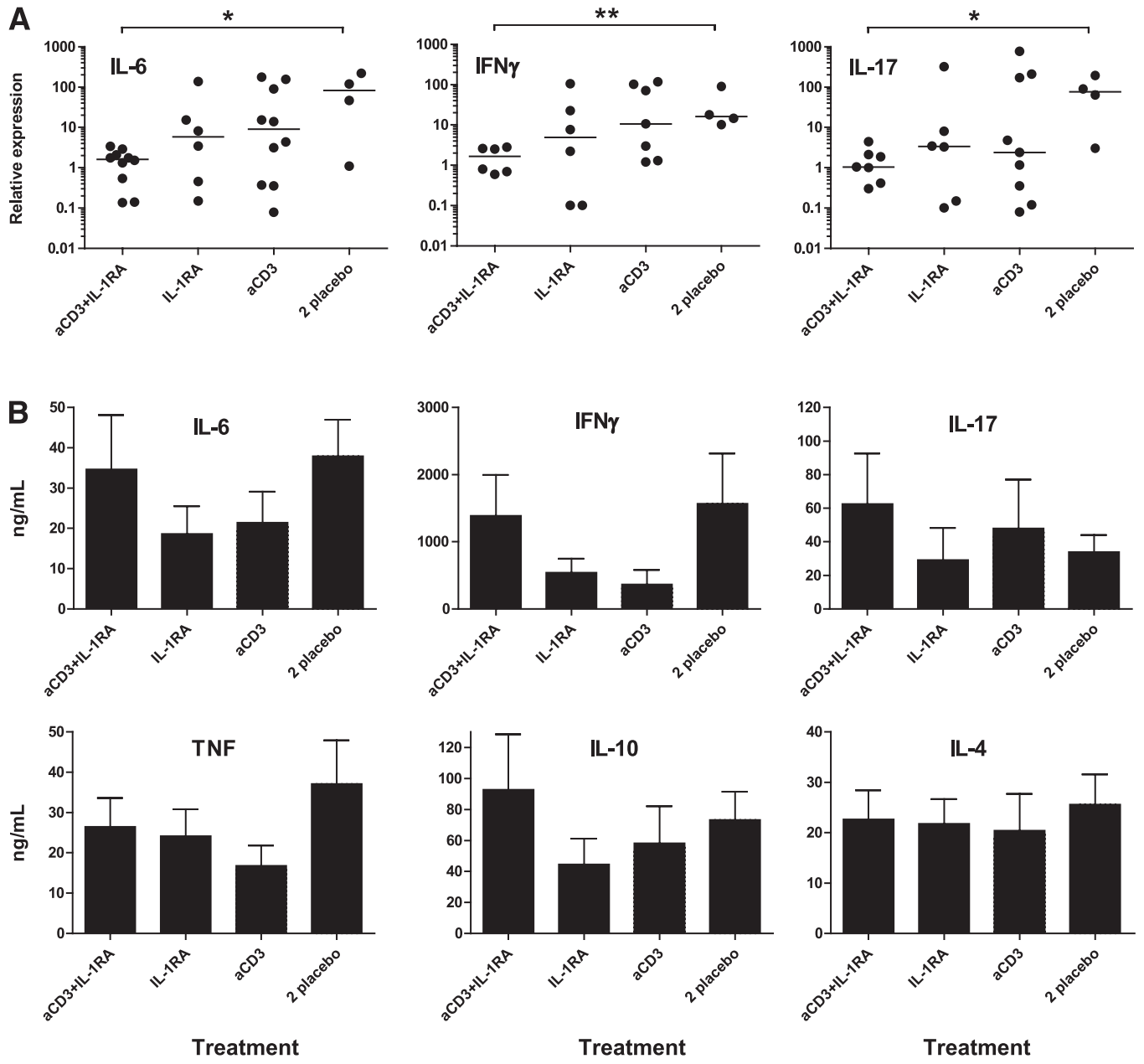

FIG. 7. Cytokine gene expression in pancreata and cytokine secretion by spleen cells in vitro on day 30. *A*: RNA was prepared from day 30 pancreata and analyzed for cytokine gene expression by qPCR (each dot represents an individual mouse pancreas). There were significant (*$P < 0.05$, **$P < 0.01$) reductions in the expression of IL-6, IFN-γ, and IL-17 in the pancreata from the mice that received combination therapy. *B*: Splenocytes were prepared from the same mice as in *A* and were cultured for 48 h with anti-CD3 mAb. The supernatants were harvested and the indicated cytokine levels were measured by Luminex. The differences between the subgroups of treated mice were not statistically significant.

by signaling through IL-1 receptors that are expressed on β-cells and activation of the nuclear factor κB and mitogen-activated protein kinase pathways, NO production, and depletion of endoplasmic reticulum calcium (14,43). IL-1β favors immune effector cell survival and expansion and reduces the regulatory compartment (29). Thus, because of the role of IL-1β as both an immune response initiator and as a direct toxin to β-cells as well as its potential role in mediating insulin resistance through the elaboration of other inflammatory mediators, it is not surprising that its blockade would be of benefit in diabetes. Indeed, Larsen et al. (47) found that hemoglobin A_{1c} levels were improved in patients with type 2 diabetes who were treated with IL-1RA, largely because of the direct β-cell protective effects. However, previous studies from NOD mice have shown modest effects of IL-1β antagonists or knockout of the IL-1 receptor in this model, similar to our findings in which IL-1RA or anti–IL-1β mAb did not reverse diabetes (24).

Our analysis suggests that a number of mechanisms may be involved in the effects of the combination when compared with anti-CD3 mAb alone. When IL-1RA was combined with anti-CD3 mAb, there was significant elimination of certain pathogenic T cells, increased insulin content in the pancreata of mice at the time that the drug treatment was completed, and increased regulatory function of splenocytes in preventing adoptive transfer of diabetes. This result is consistent with a local effect of the IL-1 antagonist on islet inflammation and IL-1–driven expansion of effector

T cells. The selective effects of the IL-1RA on effector cells in the pancreas may reflect the increased number of these cells at that location at the time of disease onset (39,40). A reduction of tetramer$^+$ CD8$^+$ T cells could be partially due to downregulation of TCR caused by anti-CD3 binding; however, it was not seen in the spleen and was not observed in the mice treated with anti-CD3 alone. The reduced effector cells alone cannot account for the effect of the combination therapy, since they were reduced in the mice that received single therapies, albeit not of statistical significance. In addition to effects on effectors, there were increased regulatory mechanisms including increased IL-10 production by activated splenocytes, increased M2 splenocytes, and induction of regulatory activity. The combination therapy led to increased serum levels of IL-4 and IL-5 on day 2 and increased IL-10 production by activated cells, which may have had an early effect on antigen presentation. Our finding of increased levels of IgG1 is consistent with diversion to a Th2 phenotype when the combination is administered. As a result of the anti-CD3 mAb treatment, there was an increase in the total number of splenocytes, a decrease in the CD4:CD8 T-cell ratio, and an increase in the number of Foxp3$^+$ splenic Tregs and ICOS$^+$CD4$^+$ T cells. ICOS$^+$Foxp3$^+$CD4$^+$ T cells have been found to be IL-10–producing Tregs (48). Thirty days after treatment, pancreatic infiltrates were not reduced, but the expression of proinflammatory genes that distinguish the cells as pathogenic effectors was decreased. This appeared to be a site-specific effect, since stimulatory responses of splenocytes were normal in these same mice. Thus, our studies have identified selective effects of anti-CD3 mAb and IL-1RA on effector and regulatory mechanisms that are involved in T1D. Individually, these effects have either no or modest effects in reducing and inducing tolerance to autoimmune diabetes. In combination, however, they are very potent and have lasting effects.

The basis for the immune regulatory changes may be the effects of IL-1RA on macrophages and dendritic cells. Whereas the proportion of CD11b$^+$ cells in the spleen was similar between the groups, these cells strongly expressed the anti-inflammatory marker *Arg1* in IL-1RA–treated mice, suggesting an increased proportion of M2 cells. Similar increase was found in the CD11c$^+$ population. Importantly, the *Arg1* gene encodes for arginase 1, an enzyme that uses L-arginine to make urea, and thus competes with iNOS for the substrate. IL-1 is a powerful inducer of iNOS, and NO is highly toxic to β-cells (49). An early increase of splenic *Arg1*, and particularly of the *Arg1* to *NOS2* ratio together with elimination of effector T cells (also a consequence of IL-1 blockade), may be one of the important mechanisms behind a rapid restoration of euglycemia and preservation of pancreatic insulin content in the combination-treated mice. Arginase 1 has also been reported as an enzyme involved in tissue repair and recruitment of other cell types. It is also possible that in addition to macrophages and dendritic cells, other cells that express these markers (e.g., NK cells) may have been affected and contribute to the effects of the combination treatment.

In agreement with this, we found a delay in transfer of diabetes when splenocytes from mice treated with the combination (but not anti-CD3 mAb alone) were mixed with pathogenic spleen cells compared with mixtures of splenocytes from placebo-treated mice. These studies would suggest that the combination therapy induces either a greater proportion or potency of cells with regulatory function. We are not clear on which cells are responsible for the enhanced activity—both IL-10–producing T cells and modified dendritic cells or macrophages may be involved. A remarkable feature of the transfer studies was that the regulatory activity was identified in the spleen soon after treatment, since, in previous studies by Belghith et al., as well as in our previous studies (27,42), the adaptive Tregs induced by anti-CD3 mAb were generally found at a later time after anti-CD3 mAb therapy.

A challenge of confronting development of immune modulatory therapies of T1D is the loss of efficacy several months after they are given, even with continued administration (30–34,50,51). In addition to the more rapid correction of diabetes, this combination therapy had lasting effects, seen 1 month after treatment. At that time, the infiltrates were not reduced in the islets, but we showed reduced expression of genes associated with pathologic phenotype(s). These findings are consistent with reports that show that IL-1β plays an important role in the differentiation of Th17$^+$ cells as well as in the expansion of CD25$^+$ effectors (8,29,52). Interestingly, blockade of cytokine gene expression was not mirrored by reduced cytokine production by activated splenocytes, suggesting that the nonresponsiveness in the pancreas may be maintained by exposure to pancreatic antigen. Further studies that can directly assess the function of the infiltrating cells and the biologic significance of the lack of cytokine gene expression that we identified in the pancreata will be needed to clarify this point.

In summary, we have shown that combining IL-1 antagonists with FcR nonbinding anti-CD3 mAb is synergistic in reversal of T1D in NOD mice. The combination affects both innate and adaptive immune pathways, resulting in lasting effects. This combinatorial approach may be useful in patients in enhancing remission of disease that has been difficult to achieve with single agents.

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V.A. planned the experiments, performed the studies in experimental animals and the analysis of specimens and materials from the mice, reviewed data, and wrote the manuscript. O.H., J.B.H., L.O.-A., and P.P.-H. performed the studies in experimental animals and the analysis of specimens and materials from the mice. P.S. and T.M.-P. reviewed data and wrote the manuscript. K.C.H. planned the experiments, reviewed data, and wrote the manuscript. K.C.H. is the guarantor and takes full responsibility for the article and its originality.

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