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Differential Response of Regulatory and Conventional CD4$^+$ Lymphocytes to CD3 Engagement: Clues to a Possible Mechanism of Anti-CD3 Action?

Li Li,* Junko Nishio,* André van Maurik,† Diane Mathis,* and Christophe Benoist*

Several clinical trials have shown anti-CD3 treatment to be a promising therapy for autoimmune diabetes, but its mechanism of action remains unclear. Foxp3$^+$ regulatory T cells (Tregs) are likely to be involved, but through unknown mechanistic pathways. We profiled the transcriptional consequences in CD4$^+$ Tregs and conventional T cells (Tconvs) in the first hours and days after anti-CD3 treatment of NOD mice. Anti-CD3 treatment led to a transient transcriptional response, terminating faster than most Ag-induced responses. Most transcripts were similarly induced in Tregs and Tconvs, but several were differential, in particular, those encoding the IL-7R and transcription factors Id2/3 and Gfi1, upregulated in Tregs but repressed in Tconvs. Because IL-7R was a plausible candidate for driving the homeostatic response of Tregs to anti-CD3, we tested its relevance by supplementation of anti-CD3 treatment with IL-7/anti–IL-7 complexes. Although ineffective alone, IL-7 significantly improved the rate of remission induced by anti-CD3. Four anti-human CD3 mAbs exhibited the same differential effect on IL-7R expression in human as in mouse cells, suggesting that the mechanism also underlies therapeutic effect in human cells, and perhaps a rationale for testing a combination of anti-CD3 and IL-7 for the treatment of recent-onset human type 1 diabetes. Thus, systems-level analysis of the response to anti-CD3 in the early phase of the treatment demonstrates different responses in Tregs and Tconvs, and provides new leads to a mechanistic understanding of its mechanism of action in reverting recent-onset diabetes. ***The Journal of Immunology*****, 2013, 191: 000–000.**

Among the treatments being explored for type 1 diabetes (T1D), anti-CD3 is one of the most promising. Based on leads from the NOD mouse model (1–4), anti-CD3 proved to have some efficacy when used in a short-course treatment in patients with recently diagnosed diabetes (5–7). Stabilization of disease and maintenance of endogenous capacity for insulin production were observed in two independent clinical trials with different anti-CD3 reagents (5, 6, 8); more recent phase 3 trials did not meet their clinical end points (9, 10), although long-term preservation of C-peptide was still observed in one case (9), and failure in the other case may well be attributable to insufficient dosing (11). The anti-CD3 treatment effect tends to wane after a few years (12).

It is essential to understand the mechanisms underlying the effects that have been observed, to improve therapeutic protocols in terms of timing regimen, dose, and potential outcomes. Unfortunately, there is only a limited understanding of anti-CD3's mechanism of action. For instance, it is not clear whether the "cytokine storm" induced by anti-CD3, which is not without side effects, is actually required for therapeutic efficacy. Because mechanistic studies on human patients are of necessity limited to blood cells, which give an incomplete representation of events occurring in lymphoid organs or in the pancreas, most results have been obtained in the NOD model of T1D (or, more recently, in humanized mice) (13). The effects of anti-CD3 on autoimmune disease are typically long-lasting, in NOD mice as well as human patients, persisting long after clearance of the Ab, which implies some resetting of the balance between autoreactive effector cells and regulatory cells, lasting beyond the relatively short time frame (a few days) during which the TCR is blocked or internalized by anti-CD3 engagement. Induction of conventional T cell (Tconv) anergy, perturbation of the Th1 versus Th2 balance, or inactivation of autoreactive T cells has been invoked (14–17). Several investigators have suggested that dominant tolerance may be induced by anti-CD3 therapy (18), in particular via effects on CD4$^+$Foxp3$^+$ regulatory T cells (Tregs) (13, 14, 19–22).

Foxp3$^+$ Tregs play an important part in the control of immunologic self-tolerance, as well as of anti-infectious and antitumor responses (23). These diverse regulatory activities involve several specialized subphenotypes and molecular pathways (24, 25). Tregs clearly influence the development of T1D: their experimental depletion or genetic deficiency in their numbers or activity promote a more aggressive disease (26, 27), whereas their transfer or therapeutic enhancement is protective (26, 28, 29).

Studies on anti-CD3–treated mice have shown variable modifications of Tregs, no or quantitatively modest effects (17, 20–22, 30), or effects restricted to particular anatomic locations (14, 20). The consensus seems to be, then, that there are no large-scale

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The sequences presented in this article have been submitted to National Center for Biotechnology Information Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE48210) under accession number GSE48210.

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Abbreviations used in this article: PLN, pancreatic lymph node; T1D, type 1 diabetes; Tconv, conventional T cell; TF, transcription factor; Treg, regulatory T cell.

changes in Treg populations in anti-CD3–treated mice. However, we have recently shown that anti-CD3 has profound effects on Tregs whose expansion is constrained by homeostatic limitations, lifting these niche constraints through a striking and selective burst of amplification during the first few days after anti-CD3 administration (22). Using a humanized mouse model, Waldron-Lynch et al. (13) reported that anti-hCD3 induced only a modest depletion, but instead caused the increased differentiation of a gut-homing population of $FOXP3^+$ and IL-10–producing $CD4^+$ T cells. Thus, anti-CD3 may induce, rather than a wholesale expansion of Tregs, specific modifications in the homeostatic control of their repertoire or phenotypic redistribution.

The burst of DNA replication and proliferation in the first few days of anti-CD3 treatment was substantially more extensive in Tregs than in Tconvs (22), implying that the two populations respond differentially to what is otherwise the same activating ligand. In general, little is known about the comparative transcriptional responses of Tregs and Tconvs to TCR engagement. To address these issues, and more generally to obtain a better perspective on the immediate effects of anti-CD3 in vivo, we have dissected the responses of Tregs and Tconvs to anti-CD3 treatment. Our data argue for the potential of anti-CD3/IL-7 combination therapy.

Materials and Methods

Mice and treatments

NOD/LtJDoi, $Foxp3^{GFP}$NOD mice (31, 32) were obtained from the JDRF Center for Immunological Tolerance at Harvard Transgenic Mouse Core, and maintained in specific pathogen-free facilities at Harvard Medical School (Institutional Animal Care and Use Committee 02954). Diabetes was monitored with tests of urine glucose, confirmed by blood glucose measurements when positive and after diagnosis. Mice were considered diabetic with 2 reads >250 mg/dl on consecutive days. For anti-CD3 treatment, mice were injected i.v. with anti-CD3 mAb (clone KT3, protein G purified), typically 50 μg daily for 5 consecutive days. For IL-7 treatment, IL-7/anti–IL-7 complexes were generated by mixing 0.75 μg recombinant human IL-7 (Peprotech) with 15 μg anti–human IL-7 M25 mAb (BioXCell) in PBS at total volume 30 μl for 15 min at room temperature, before dilution in 100 μl PBS for i.p. injection three times at 3-d intervals.

Human samples

For isolation of mononuclear cells from human peripheral blood, freshly collected human peripheral blood was diluted with equal volume of PBS with 2 mM EDTA, layered over Ficoll-Hypaque solution, and centrifuged at 900 × *g*, at 25°C for 20 min. Remaining RBCs were lysed by RBC lysis buffer, and the remaining cells were washed twice with PBS/2 mM EDTA. Platelets were removed by overlaying the cells with FBS, centrifuged, and the mononuclear cells were resuspended in complete RPMI 1640 media supplemented with 10% FBS.

In vitro cultures

For in vitro activation assay, mouse spleen cell suspensions were prepared in ice-cold PBS. The spleen cell suspension was resuspended at 1×10^6 cells/ml in culture medium consisting of RPMI 1640 supplemented with 50 μM 2-ME (Sigma), 1× nonessential amino acids, 1 mM sodium pyruvate, and 100 U/ml penicillin. Spleen cells were incubated in a 24-well flat-bottom microtiter plate (1 ml/well) with addition of soluble anti-CD3 mAb using. Purified human PBMCs were cultured in the same conditions, with stimulation of anti-human CD3 mAbs (HIT3A, OKT3, UCHT1, and TRX4).

Flow cytometry

Intracellular staining for Foxp3 staining was performed with manufacturer's protocols (clone FJK-16s; eBioscience). For costaining of Foxp3 and EdU, mice were injected with 1 mg EdU 6 h before sacrifice. EdU staining (Click-iT EdU Flow Cytometry Assay Kits; Invitrogen) was performed after Foxp3 staining. To determine the amount of free and Ab-complexed CD3 molecules at the cell surface, we stained splenocytes with FITC-conjugated goat anti-rat or anti-human IgG, with or without preincubation with saturating amounts of the same anti-CD3 Ab as used for treatment (for mouse cells, KT3; for human cells, HIT3A, OKT3, UCHT1 [Biolegend], or TRX4 [otelixizumab]), yielding measures of total and occupied CD3 (free CD3 being the difference between the two). Results were expressed as a proportion of the mean fluorescence intensity relative to that on untreated cells.

Gene-expression profiling

Tconv ($CD3^+CD4^+GFP^-$) and Treg ($CD3^+CD4^+GFP^+$) splenocytes were double-sorted from 8-wk-old male Foxp3-iGFP NOD mice treated with anti-CD3 (KT3) for different lengths of time (each sample from a single mouse). Cells were collected directly into TRIzol. RNA was purified and was used for probe synthesis for hybridization to Affymetrix Mouse Gene M1.0 ST microarrays. Raw data were background-corrected and normalized with the RMA algorithm in the GenePattern software package. Two independent duplicate experiments were performed. Because there was slight contamination with B cells and erythrocytes in one set, hemoglobin genes and genes overexpressed in B cells (>5-fold ratio in ImmGen $CD19^+$ B versus $CD4^+$ T cell ImmGen data) were filtered out, as were genes with discordant fold-change (>2.5 and <1.3). Microarray data have been deposited at National Center for Biotechnology Information Gene Expression Omnibus under accession no. GSE48210 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE48210).

Statistical analyses

Student *t* test was used for comparisons of cell frequencies, and the incidence of diabetes was compared by Kaplan–Meier log rank test. Findings were considered significant at $p \leq 0.05$.

Results

Tregs and Tconvs respond differently to injection of anti-CD3

To study the earliest transcriptional effects of anti-CD3 treatment, we used knock-in mice in which Tregs can be distinguished by expression of a fluorescent reporter, that is, a mouse line carrying a bicistronic IRES-spaced Foxp3-GFP reporter gene (31) backcrossed onto the NOD genetic background (32) (hereafter referred to as NOD.$Foxp3^{igfp}$). Adult NOD.Foxp3iGFP mice were injected at 8 wk of age with the usual 50-μg dose of the anti-CD3 mAb, KT3, used in our published diabetes reversal studies (22). Under these conditions, KT3 induces a limited cytokine storm (33), and in previous studies, this protocol reverted the hyperglycemia in ∼50% of recently diabetic mice. This dose leads to a slight (≤2-fold) reduction in Tconvs in the spleen and pancreas in the few days after treatment (data not shown). $CD4^+GFP^+$ Tregs and $CD4^+GFP^-$ Tconvs were sorted from spleens of treated mice at 2, 8, 24, and 72 h after initiation of treatment, and genome-wide gene-expression profiling was performed.

To assess the relative engagement of the TCR in Tregs and Tconvs after such treatment, we first analyzed the response of CD69, an early response marker directly downstream of TCR signaling. CD69 induction was early and comparable in both cell types (Fig. 1A, *left panel*). In contrast, the CD44 response, whose induction is more complex, was higher in Tregs. Overall results are depicted in Fig. 1B, which compares the induction ratio (relative to the same populations from untreated mice) for Tregs and Tconvs at various times. Several conclusions can be drawn from these plots. First, anti-CD3 elicited an abortive response in both T cell populations: although a robust response was evident 2 h after administration (at an arbitrary fold-change cutoff of 2.5, 416 and 550 genes being induced in Tconvs and Tregs, respectively), it had faded out by 72 h (only 70 and 64 changes apparent). This transient activation differs from gene-expression profiles observed when T cells were activated in vivo by persistent cognate Ag or strong costimulation, where responses continued to amplify at 72 h (e.g., see Ref. 34 or T cell activation data at http://www.immgen.org).

Second, the changes induced in Tregs and Tconvs were very comparable for the majority of genes, as indicated by the diagonal disposition of the transcripts in Fig. 1B. However, the slightly off-diagonal placement indicates that the immediate response was

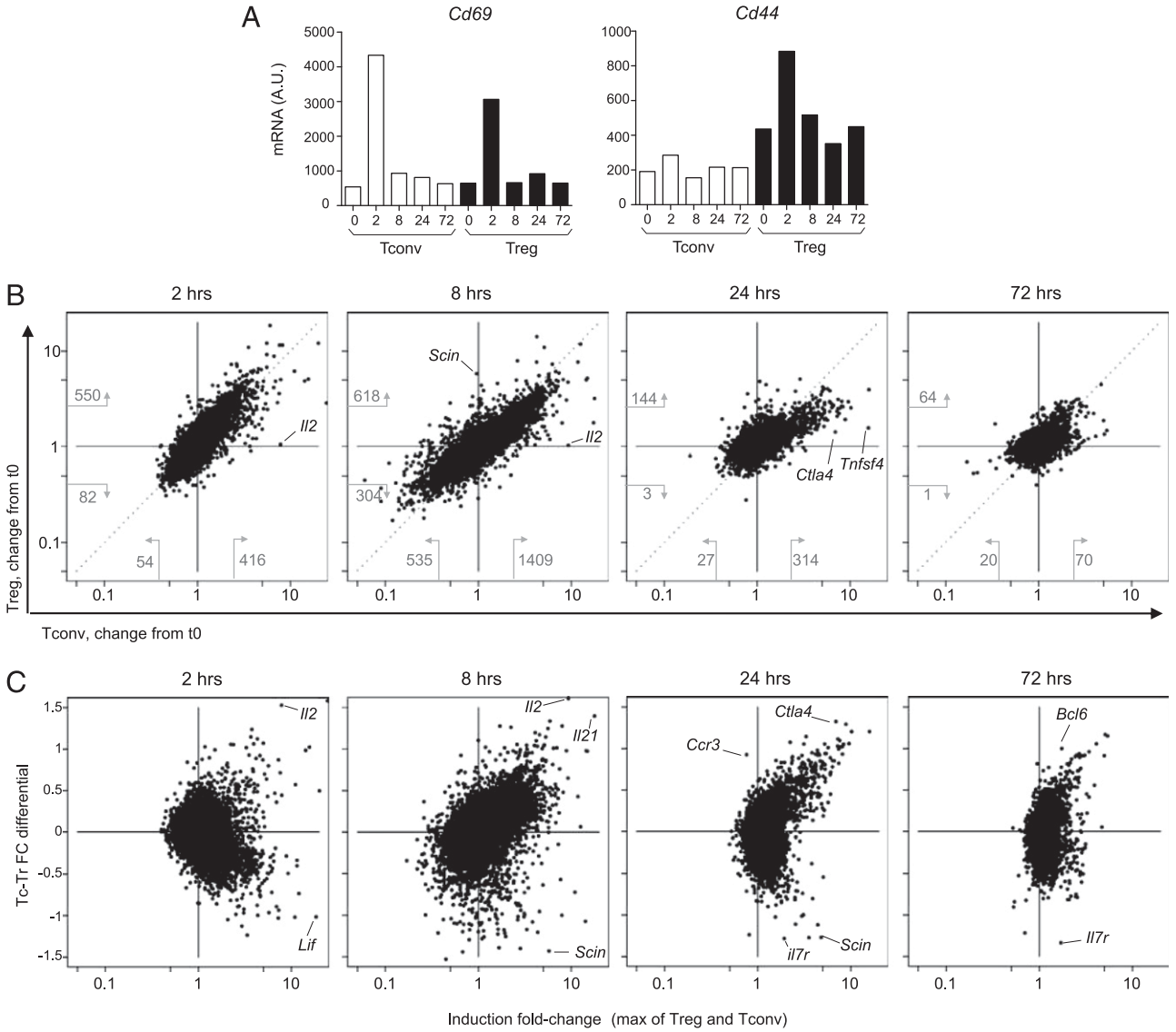

FIGURE 1. Tconvs and Tregs respond differently to anti-CD3. Transcriptional profiles were generated from Tconv and Treg CD4$^+$ splenocytes from pooled mice (n = 3) at 2–72 h after treatment with anti-CD3. (**A**) Changes in activation markers that reflect TCR signaling. (**B**) Comparison of the changes elicited in Tregs and Tconvs (visualized as the ratio of expression at indicated times relative to untreated [t0]); numbers indicate the number of transcripts that change by ≥2.5-fold. (**C**) A Tconv-Treg differential index was computed for each gene to reflect these differential changes (genes toward the top of the graph preferentially respond in Tconvs, those at the bottom in Tregs) and is plotted against the maximum change observed.

more intense in Tregs at 2 h (above the diagonal; also indicated by the number of responding transcripts), whereas the response in Tconvs dominated at later times (below the diagonal). This faster start in Tregs may be linked to their preactivated state.

Third, the behavior of a number of transcripts did differ in the two cell types, being uniquely or preferentially induced in one or the other. This observation was true at both early (e.g., *Il2*, strikingly unique to Tconvs; or *Scin*, found only in Tregs) and late times (*Tnfsf4* in Tconvs). To better visualize these differences, we computed a Tconv/Treg differential response index (by subtracting the fold-change in Treg from that in Tconv, and normalizing to the mean change), where a positive value reflects a preferential response in Tconvs and a negative one indicates a preferential induction in Tregs). In Fig. 1C, this index was plotted against the maximum change observed in Tregs and Tconvs (see Supplemental Table I for gene details). The general disposition at 2 and 8 h after anti-CD3 administration reflected the different intensity of the responses noted earlier; but at 24 and 72 h, the profiles showed clear "horns" of genes preferentially induced in one or the other of the two cell types. At 24 h, more of the induction was seen in Tconvs, but the residual induction at 72 h was more marked in Tregs (Fig. 1C).

These findings raised the question of whether the differential response to anti-CD3 in Tregs and Tconvs reflected differences in baseline expression profiles between the two cell types (i.e., the "Treg signature" (35). The Tc/Tr differential response index versus the baseline Tconv/Treg ratio in unstimulated cells is plotted in Fig. 2 (see also Supplemental Fig. 1 for 2- and 24-h time points). To a great extent, the differential transcript changes in the two cell types amounted to a "catching up," where genes underexpressed at the start in one cell type were preferentially activated there. For example, *Il2ra* (CD25), *Ctla4*, *Gpr83*, and *Tigit* were underrepresented in naive Tconvs, and their induction was stronger than in Tregs, where they were already expressed; similarly, *Pde3b*, *Vipr*, and *Igfbp4* responded preferentially in Tregs. On the other hand, some transcriptional responses were unique to Tconvs, such as the increase in *Il2*, *Il21*, and *Tnfsf4* (OX40L) transcripts mentioned earlier, suggesting that their expression was completely

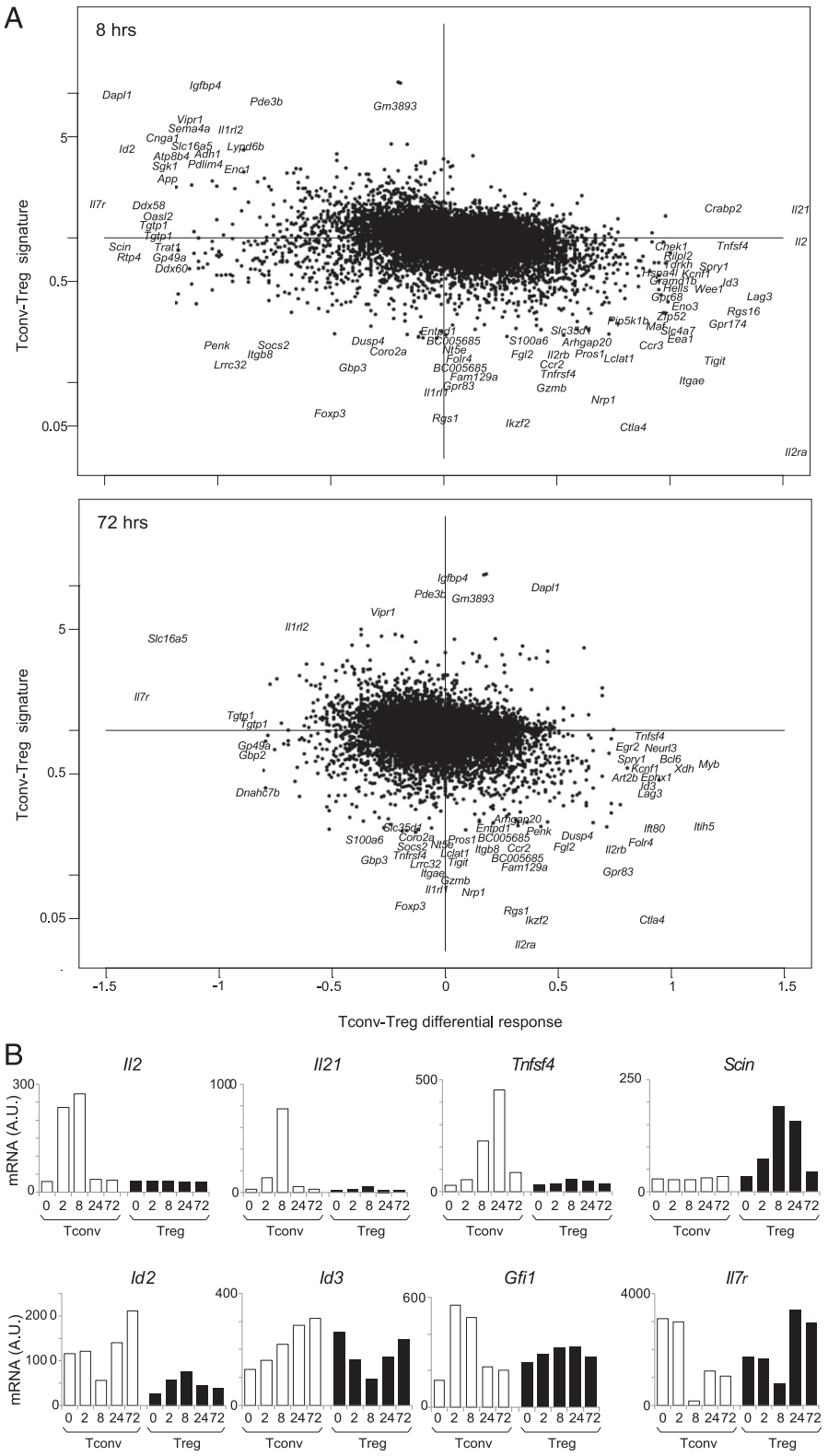

FIGURE 2. Elements of the differential response of Tconv and Treg to anti-CD3. (**A**) The Tconv-Treg differential response, computed as in Fig. 1B, at 8 (*top panel*) and 72 h (*bottom panel*) is plotted against the difference in expression between Tconvs and Tregs at baseline (*y*-axis), and the most extreme transcripts are shown by name. (**B**) Gene-expression values (arbitrary units) for selected transcripts that are distinctively induced by anti-CD3 in Tconvs and Tregs. Values shown are average from two independent experiments.

repressed in Tregs (Fig. 2B); the converse was true for *Scin* induction in Treg vis-à-vis Tconvs. Genes encoding transcription factors (TFs) followed these trends, and we noted an interesting pattern in the response of the E-protein–regulating factors Id2 and Id3, which control several aspects of T cell homeostasis: *Id2,* preferentially expressed in Tconvs, was temporarily shut down there, with an inverse pattern in Tregs; conversely, its homolog *Id3* was induced in Tconvs, but transiently downregulated in Tregs. Most intriguing was the behavior of the *Il7r* gene: in the context of the breach of homeostatic control elicited by anti-CD3, it was the most strongly differential transcript at both 8 and 72 h (Fig. 2A), being strongly repressed in Tconvs, but much less so, and actually induced at 24 and 72 h in Tregs (Fig. 2B).

Similar responses were elicited by a non–FcR-binding form of anti-CD3 (the $F(ab')_2$ fragment of the 2C11mAb; data not shown). We also asked whether these patterns could be reproduced after

anti-CD3 treatment in vitro, which would open the possibility of experimental manipulation and of analysis of responses by human cells. To most closely mimic the in vivo conditions, we cultured whole splenocytes with soluble anti-CD3 (1.25 μg/ml) for the same times as earlier. As illustrated in Supplemental Fig. 2A–D, the patterns of differential responses in Tconvs versus Tregs found in vivo were reproduced to a substantial extent in vitro (with perhaps the exception of *Il2* and *Il21*, less responsive in vitro).

Differential IL-7R protein expression in response to anti-CD3 treatment

The transcriptional response of the *Il7r* gene immediately caught our attention given the role of IL-7 as a key trophic cytokine throughout lymphocyte differentiation and memory formation (36), its differential expression in Tregs and Tconvs at baseline (37), and its role in the early resetting of Treg homeostasis after anti-CD3 administration (22). Changes in the expression of IL-2R subunits followed a different course (Supplemental Fig. 2E). The effect of anti-CD3 on the *Il7r* gene is consistent with the described downregulation of IL-7R protein during the activation of Tconvs (38–40), and with its upregulation in Tregs activated in vivo and in vitro (41). We verified that the transcriptional changes we observed were also reflected in the amount of protein. Indeed, anti-CD3 treatment induced comparable changes in surface levels of IL-7R protein, with a reciprocal shift up in Tregs and shift down in Tconvs from spleen and lymph node (72 h posttreatment in Fig. 3A). These effects were dose dependent (Fig. 3B) and were relatively transient, with a return to pretreatment levels by day 16 in both the spleen and pancreatic lymph nodes (PLNs).

We then asked how the reciprocal changes in IL-7R levels reflected the extent of anti-CD3 engagement in the TCR complex and its ensuing clearance by internalization or shedding. The levels of free or engaged CD3 were measured by indirect staining with anti-CD3 (KT3), detected with anti-rat IgG (Fig. 4A displays data at 72 h posttreatment). At the dose used in most of the experiments described earlier (50 μg i.p.), the majority of the CD3 molecules were lost from the cell surface, and the remainder were mainly occupied by the mAb; free complexes were more abundant at lower doses of injected KT3. As expected, surface levels of the CD3ε molecule reflected levels of the whole TCR complex, which was also cleared from the cell surface after similar kinetics (detected with anti–TCR-Vβ reagent; Supplemental Fig. 2F, 2G). The extent of TCR clearance was identical on Tregs and Tconvs (Fig. 4B), indicating that the differential transcriptional responses were not due to unequal TCR engagement or internalization. Importantly, the posttreatment levels of IL-7R correlated strongly with the amount of remaining TCR, both for the disappearance of IL-7R in Tconvs and its increase in Tregs (Fig. 4C). Similar responses were observed for $CD4^+$ T cells when splenocytes were cultured in vitro with graded concentrations of anti-CD3 (Fig. 4D): a dose-dependent decline in surface IL-7R on Tconvs, which correlated with levels of remaining CD3; an increase on Tregs, most apparent after 72 h (after a transient decline at early time points, but mainly at high concentrations). Thus, the transcriptional changes at the *Il7r* result in alterations in the IL-7R, and these alterations are directly related to TCR engagement by anti-CD3.

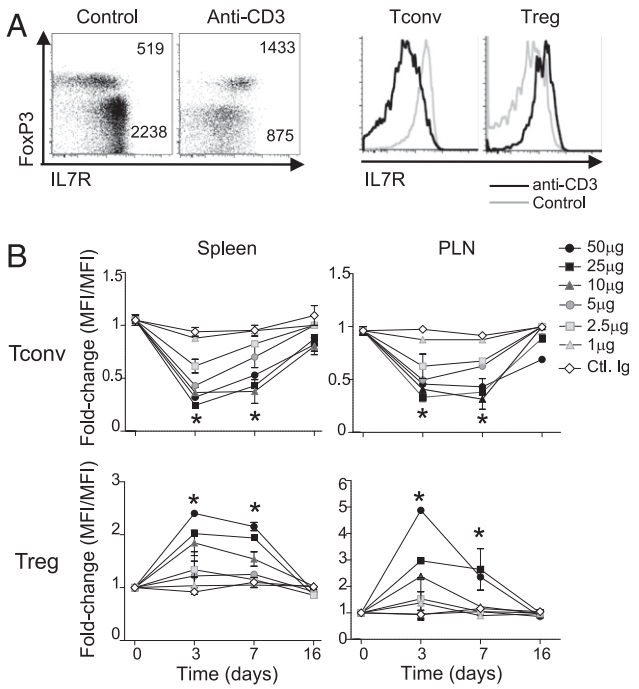

FIGURE 3. IL-7R expression on Tconvs and Tregs in response to anti-CD3. (**A**) Representative flow cytometric profile of IL-7R and Foxp3 expression on $TCR\beta^+CD4^+$ splenocytes of NOD mice, 72 h after treatment with anti-CD3 mAb (profiles representative of more than five different experiments. (**B**) Temporal analysis of IL-7R expression on Tconv and Treg $CD4^+$ splenocytes at different times after treatment with graded doses of anti-CD3 mAb i.p. $*p \leq 0.01$ (Student *t* test). Data are combined from three independent experiments, with six mice per dose.

IL-7 synergizes with anti-CD3 in vivo

The differential expression of *Il7r* mRNA and protein was an attractive explanation for the differences in population dynamics between Tregs and Tconvs after anti-CD3 treatment. Thus, we asked whether supplementation with IL-7 could enhance the effects of anti-CD3 treatment. We chose to add IL-7/anti–IL-7 complexes, in which the nonblocking Ab increases the stability and availability of the cytokine (42). The dose of 0.75 μg IL-7 with 15 μg anti–IL-7 mAb M25 (hereafter simplified as "IL-7"), administered daily at the same time as anti-CD3, resulted in only modest increases in the number of B or $CD8^+$ T lymphocytes (Supplemental Fig. 2H). Our protocol led to a slight increase in the proportion of $Foxp3^+$ cells among $CD4^+$ T cells in spleen, PLNs, and pancreas over what was achieved by anti-CD3 alone, an increase that was not statistically significant (Fig. 5A). IL-7 induced an expansion of Tregs in the spleen (Fig. 5A, *lower panel*), which was also seen for Tconvs, explaining the limited effect on Treg proportions. These increases corresponded to a boost in cell cycling, as might be expected, evidenced by incorporation of EdU (Fig. 5B).

We then asked whether the changes in population dynamics observed after supplementation of anti-CD3 with IL-7 might also translate to an improved therapeutic outcome. NOD mice with recent-onset diabetes were treated within 1 wk of diagnosis with the usual daily dose of anti-CD3 (50 μg), alone or supplemented with IL-7/anti–IL-7 complexes during the 5-d treatment period. As illustrated in Fig. 6, the addition of IL-7 significantly improved the outcome (Kaplan–Meier, $p = 0.02$), with 13 of 16 mice showing long-term diabetes-free survival, compared with four or nine with anti-CD3 alone. IL-7 alone had no protective effect.

It would be of interest for the practical application of anti-CD3 therapy to reduce the dose of Ab to limit its undesirable effects (i.e., the cytokine storm or the EBV reactivation observed in some patients). Therefore, we tested the effect of IL-7 in recent-onset diabetic NOD mice treated under the same regimen, but with reduced dosing of anti-CD3. At 25 μg anti-CD3, IL-7 was still effective (although with only 50% long-term remission), but little protection was seen with the less effective dose of 5 μg anti-CD3

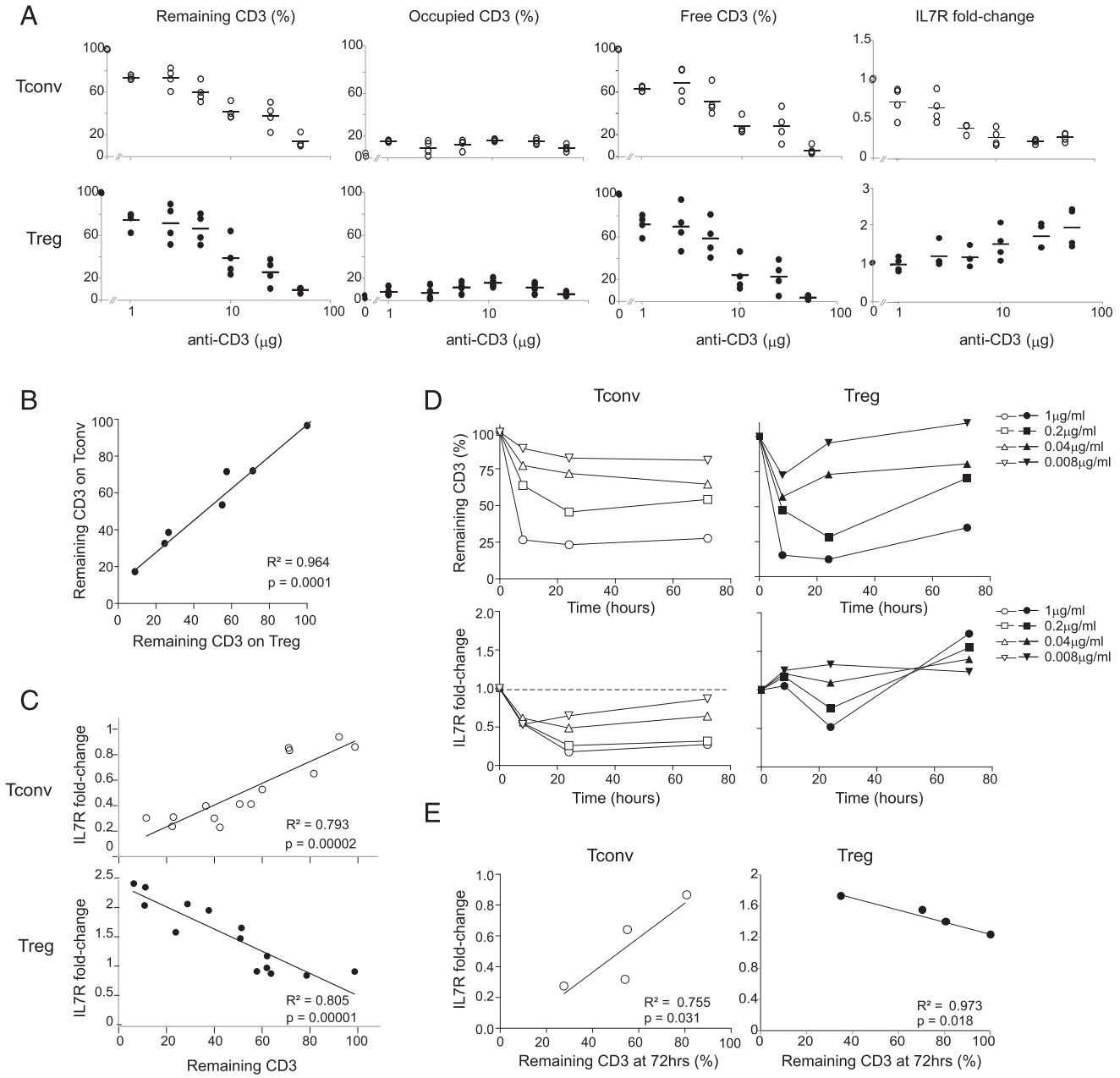

FIGURE 4. Inverse relationship between IL-7R expression and remaining surface CD3 after anti-CD3 mAb treatment. (**A**) Occupancy and clearance of CD3 molecules, and changes in IL-7R expression, over a range of doses of anti-CD3. The amount of total, Ab-bound, and free CD3 molecules, and of IL-7R (as a fold-change from untreated controls) on the surface of $CD4^+$ Tregs or Tconvs was determined by flow cytometry 72 h after treatment with a range of doses of i.p. anti-CD3. Each dot is an individual mouse. (**B**) CD3 molecules are similarly cleared in Tregs and Tconvs. Percent of remaining surface CD3 on Tconvs and Tregs from the same mouse, over a range of anti-CD3 doses (same experiments as A). (**C**) IL-7R surface expression correlates with CD3 clearance in vivo. Changes in IL-7R expression (as a fold-change from untreated controls) plotted versus the percent of remaining surface CD3 on Tconvs and Tregs 72 h after treatment with a range of doses (same experiments as A). (**D**) In vitro effects of anti-CD3. Whole splenocytes were cultured with soluble anti-CD3 mAb at the concentrations shown, and remaining CD3 and IL-7R expression on Tconvs and Tregs were determined at different times of culture. (**E**) IL-7R surface expression correlates with CD3 clearance in culture. Change of IL-7R expression plotted against the remaining CD3 expression on Tconv and Treg surface analyzed at 72 h of culture.

(Fig. 6B). Thus, supplementation with IL-7 does not seem to allow a substantial reduction in anti-CD3 dosage.

We also investigated the residual autoimmune lesion after treatment with anti-CD3 + IL-7. We and others have previously reported that anti-CD3 does not clear insulitis, which persists but in a milder and histological more organized form (similar to the "respectful insulitis" observed in the BDC2.5 TCR transgenic model on non-progressor genetic backgrounds) (43). The same result was obtained with mice protected by anti-CD3 plus IL-7 (Fig. 6C). Thus, although IL-7 reproducibly improves the efficacy of anti-CD3, it does not appear to qualitatively alter the outcome.

Effect of anti-CD3 treatment of human cells

Are the earlier results relevant for anti-CD3 therapy in humans? The similarities between the transcriptional changes induced by anti-CD3 in mouse cells in vivo and in vitro encouraged us to search for an equivalent response in human cells challenged with anti-CD3 in culture. PBMCs were obtained from healthy volunteers. To best

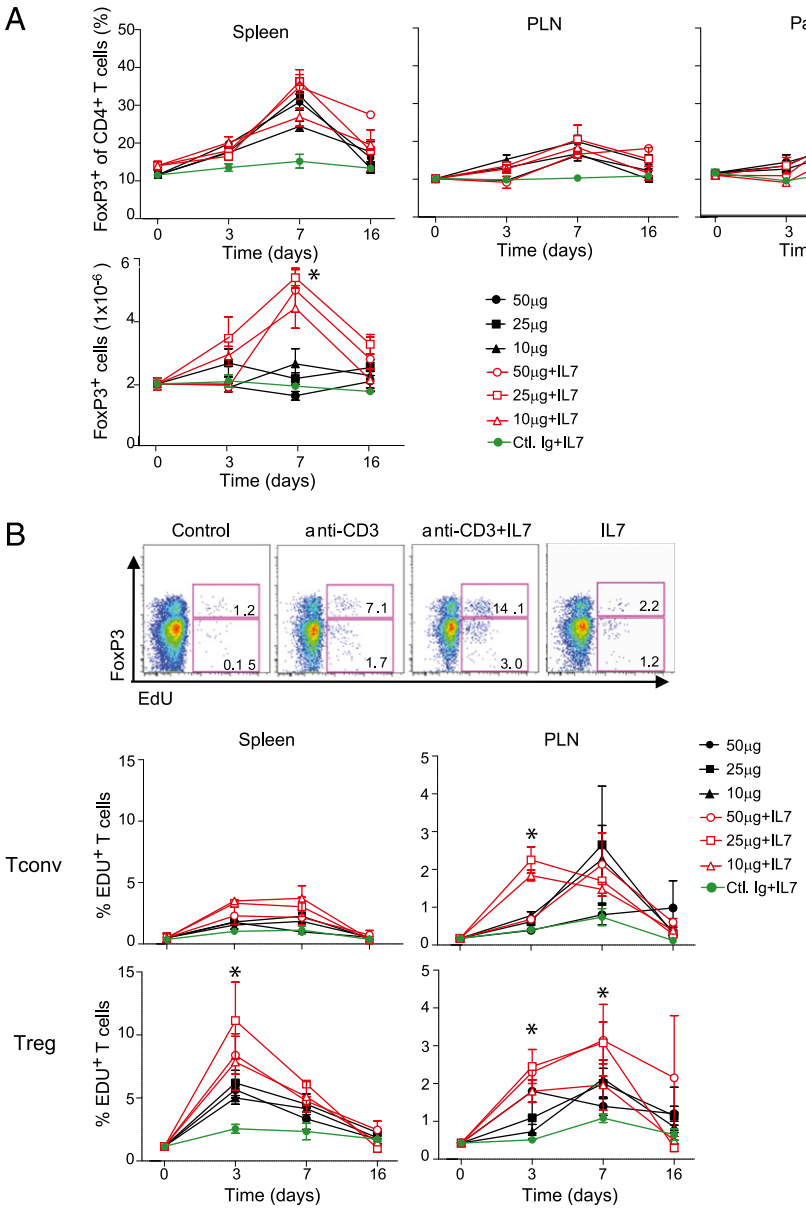

FIGURE 5. IL-7 synergizes with anti-CD3 to induce expansion of Tregs in vivo. (**A**) Percentage and absolute numbers (*bottom panel*) of Foxp3$^+$CD4$^+$ cells in the spleen, PLN, and pancreas of anti-CD3 treated (solid symbols, doses as shown) or anti-CD3 plus IL-7/anti–IL-7 (0.75 μg/15 μg) complexes treated (open symbols). Solid symbols represent IL-7/anti–IL-7 complexes alone. *$p \leq 0.0001$. Data are combined from two independent experiments with three mice for each dose. (**B**) Proliferative response. CD4$^+$ splenocytes from mice treated as indicated were analyzed by flow cytometry, 6 h after administration of with EdU. (*Top*) profiles representative of three independent experiments. (*Bottom*) Time-course analysis in response to graded doses of anti-CD3 alone or in combination with IL-7/anti–IL-7 complexes. *$p \leq 0.01$. Data are combined with two independent experiments with three mice for each dose.

approximate in vivo conditions of mixed-cell populations, we used whole PBMCs rather than purified CD4$^+$ T cells. PBMCs were cultured in the presence of anti-huCD3 mAb for 72 h and were analyzed by flow cytometry for expression of IL-7R on Tregs and Tconvs. To evaluate possible variation between mAbs of different affinity and ability to bind FcRs, we used several anti-CD3 mAb preparations, two of which have been used in clinical trials: three mouse anti-huCD3 (HIT3A, OKT3 [teplizumab], and UCHT1) and an aglycosyl form of chimeric anti-huCD3 mAb (TRX4 [otelixizumab]). The same reciprocal effects of anti-CD3 treatment on IL-7R expression by Tregs versus Tconvs were observed with human cells, although not as clearly as with mouse Tregs in vivo: a clear shift down for Tconvs and a slight upregulation of IL-7R in Tregs, particularly marked on cells expressing high levels of Foxp3 (Fig. 7A, top; titration in Supplemental Fig. 3). The effect was essentially identical with the four different mAbs examined.

Finally, we asked whether the changes in IL-7R expression might be variable between unrelated individuals. There has been variability in responses to anti-CD3 treatment in clinical trials, which could reflect differences in their diabetes status or conditions of treatment, but also perhaps pharmacogenetic variability in their response to the drug. Blood PBMCs were obtained from seven healthy donors of European ancestry (four female and three male donors; age, 21–44 y) and were treated as described earlier for each of the four anti-CD3 mAbs. Cells from all donors showed largely similar IL-7R responses at 72 h, with the usual increase in Tregs and decline in Tconvs (Fig. 7B). There was one exception, in that one donor failed to respond to UCHT1, but this observation is most likely explained by the known polymorphism in FcγRII, which conditions the ability to bind mouse IgG1 (which UCHT1 contains) and which had actually been discovered on the basis of differential responses to UCHT1 (44).

Thus, these results show that anti-CD3 elicits the same differential response by human Tregs versus Tconvs as it does in the mouse counterparts, suggesting that the underlying differential signaling pathways are the same in both species.

Discussion

The re-establishment of self-tolerance induced by anti-CD3 treatment of autoimmune diabetes likely involves the resetting of active modes of peripheral tolerance. In this article, we have analyzed potential mechanisms of action of anti-CD3, following leads that

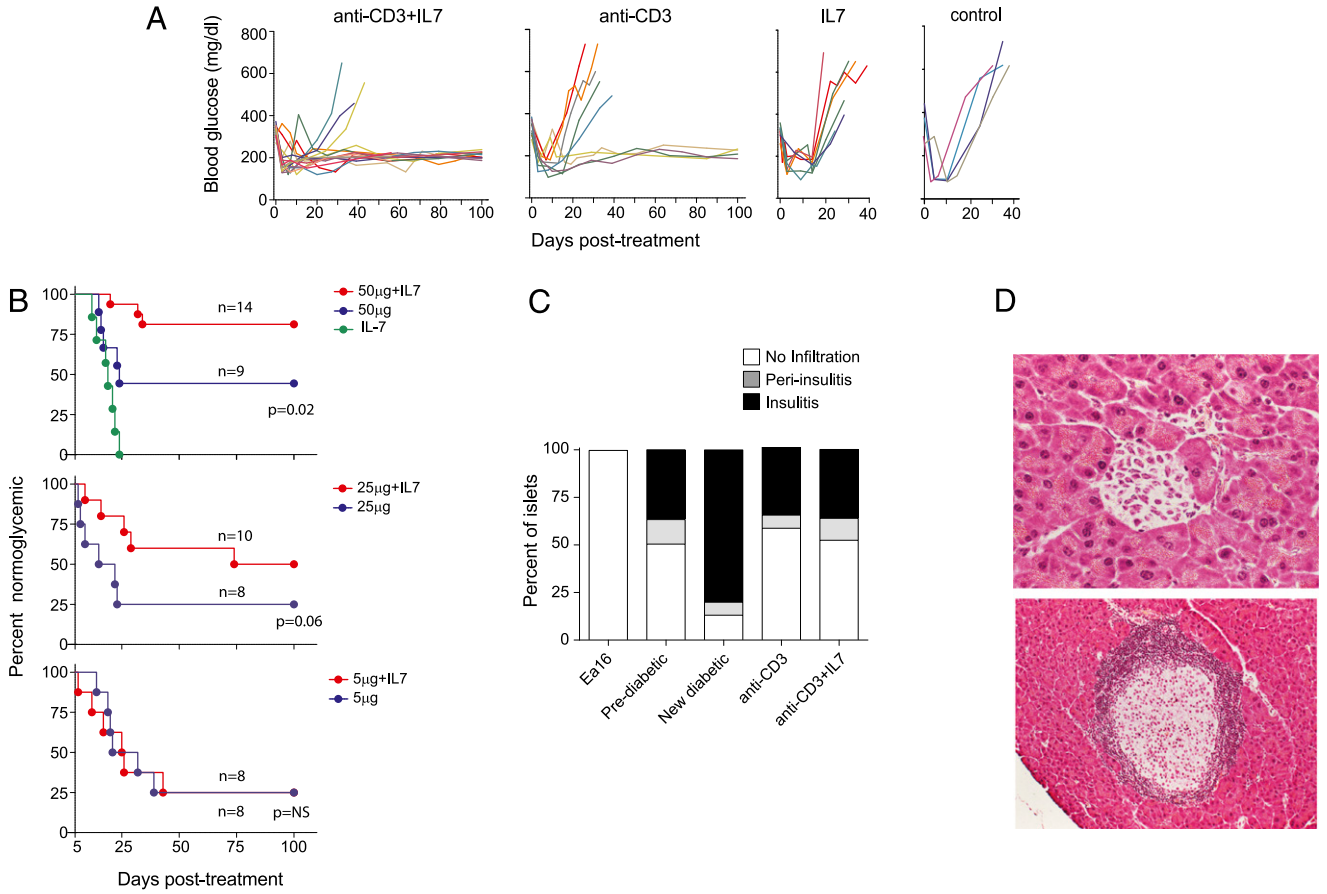

FIGURE 6. Synergistic effect of combined anti-CD3 and IL-7 treatment in newly diabetic NOD mice. (**A**) Monitoring of blood glucose in recent-onset diabetic NOD female mice treated with anti-CD3 alone (50 μg daily, $n = 9$), anti-CD3 together with IL-7/anti–IL-7 complexes ($n = 14$), IL-7 complexes alone, or none for 5 consecutive days; all mice received an insulin pellet to maintain glycemic control for 15–20 d. (**B**) Compilation of disease incidence in experiments as in (A) for several doses of anti-CD3 (IL-7/anti–IL-7 complexes at a constant dose of 0.75 μg/15 μg). (**C**) Insulitis in the pancreata of previously diabetic NOD female mice treated as in (A) (anti-CD3, $n = 5$; anti-CD3 + IL-7 complexes, $n = 5$), evaluated histologically after 100 d. Noninsulitic Ea16/NOD mice were included as a negative control. A minimum of 30 islets was examined for each mouse. (**D**) Representative islets stained with H&E from mice in long-term remission after treatment with anti-CD3 + IL-7 complexes (H&E staining, 100× and 25× objectives [*top* and *bottom*, respectively]).

pointed to differential effects on the homeostasis of $CD4^+$ Tconv and Tregs. We found that administration of anti-CD3 induced transient and clearly different responses in Tregs and Tconvs. In both human and mouse cells, one of the most distinctive divergences concerned IL-7R, a meaningful finding given the pleiotropic role of IL-7 in lymphocyte homeostasis and, most relevant, in the proliferation of Tregs unleashed by anti-CD3 (22). This result encouraged us to hypothesize, and ultimately find, a synergy between anti-CD3 and IL-7 treatments in vivo in the reversion of recent-onset diabetes in NOD mice.

The results highlight a clear difference in the transcriptional response of $CD4^+$ Tregs and Tconvs to engagement by anti-CD3. Although most alterations in gene expression were similar in the two cell types, including the activation of cell-cycle and metabolic genes, several loci showed clearly differential changes. Importantly, many of the differences observed in vivo were reproduced on anti-CD3 challenge in vitro. Some of these divergences were observed several days after treatment, reflecting indirect consequences, but others were visible at the 2-h time point, and thus reflected more immediate consequences of different signaling pathways in Tregs and Tconvs. Several reports have indicated that such differences exist: lower activation of Akt and S6 kinase in Tregs (45); a reduced recruitment of PKCΘ at the TCR synapses of Tregs, with lower Carma-1 relative to Dlgh1 activation, which might lead to relatively higher NFATc but lower NF-κB and Akt activation (46, 47); and a kinase-independent signaling pathway seeming to operate, uniquely in Tregs, between ZAP70 and the GTPase Rap1 (48). Alone or in combination, these could easily result in the quantitative differences in transcriptional activation observed in this study.

Most intriguing were the differential alterations in IL-7R expression. IL-7 is a key trophic cytokine across the immune system, affecting all lymphoid lineages. IL-7 is of demonstrated importance in the homeostasis of Tregs and in the maintenance of Foxp3 expression, particularly when IL-2 is limiting or absent (49–52), although thymic stromal lymphopoietin can partially compensate for IL-7 deficiency in the maintenance of Tregs (53). The results of Di Caro et al. (54), showing that IL-7 helps mature Tregs and enhance Foxp3 expression, are also consistent with a scenario in which Tregs gain a relative advantage over Tconvs from the these reciprocal changes in the IL-7R express. As might be expected, IL-7R is tightly regulated by many extracellular stimuli, such as TCR activation signals or IL-7 and other prosurvival cytokines (36). Low expression of IL-7R is a hallmark of Tregs (37), or at least some of them (41), perhaps via direct downregulation by FOXP3 (37). Several TFs regulate expression of *Il7r*. Ets family TFs, Ets-1 and GABPα, bind the *Il7r* promoter region at the same location as PU.1 in B cells, and promote its expression in immature thymocytes and activated $CD8^+$ T cells (55, 56). Foxo-1, a member of the forkhead family of TFs, binds an intronic *Il7r*

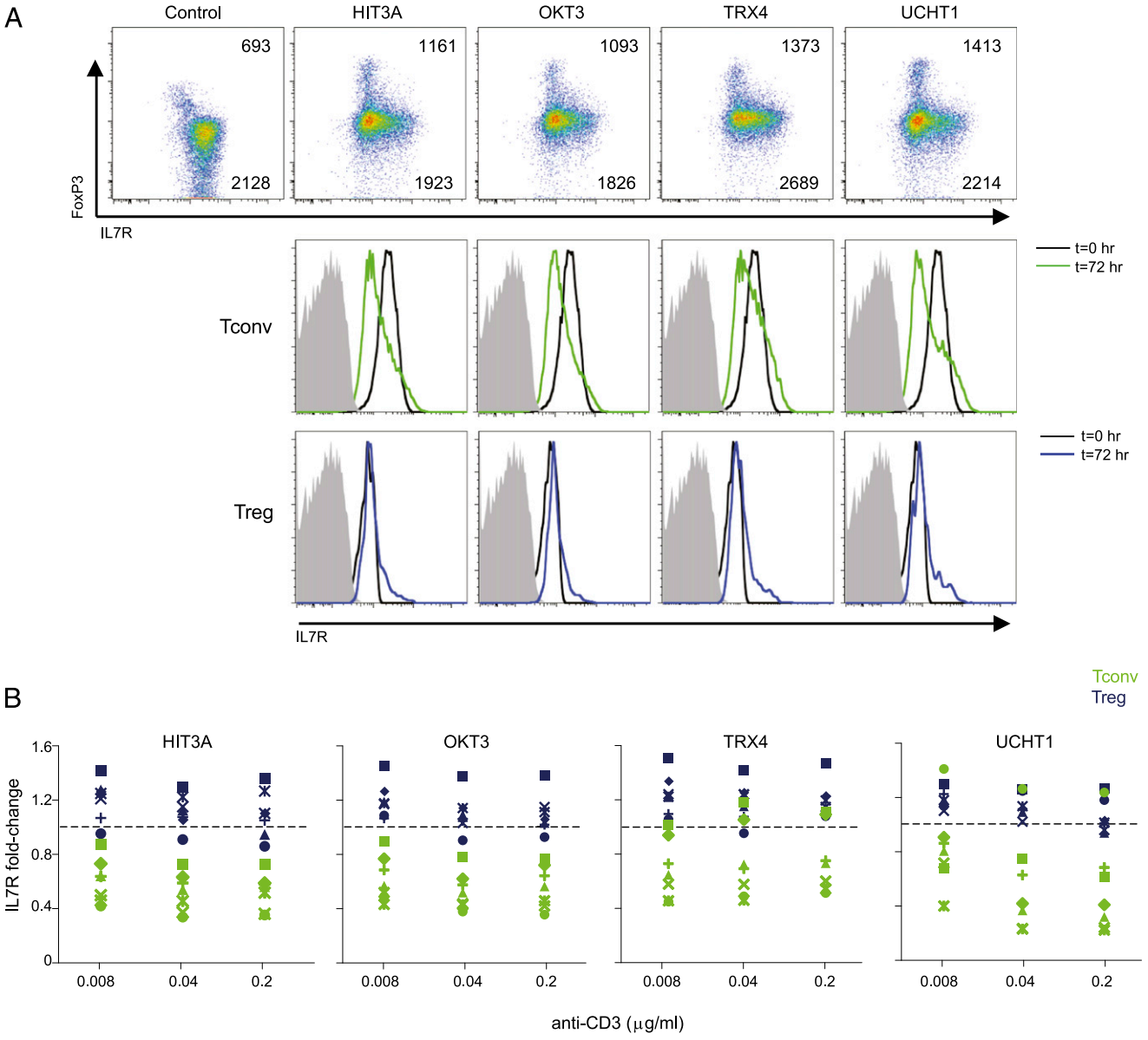

FIGURE 7. Differential IL-7R expression on Tconvs and Tregs from human blood in response to anti-CD3. PBMCs from healthy volunteers were treated in culture with soluble anti-CD3 (four different mAbs), and IL-7R expression assessed in Tregs and Tconvs after 72 h by flow cytometry. (**A**) Scatter plots of FOXP3 and IL-7R staining (*top*). Values are mean fluorescence intensity in FOXP3^{hi} and FOXP3^{-} cells. Corresponding overlaid histograms (*bottom*). (**B**) Similar responses in Tregs and Tconvs from several independent donors. Changes in IL-7R expression at 72 h (as fold-change relative to t = 0 control) induced by the different anti-CD3 mAbs on blood Tconvs (green) and Tregs (blue) from seven white donors.

enhancer and is required for the expression of *Il7r* in naive T cells (57). In contrast, Gfi-1 and FoxP1 suppress *Il7r* expression in T cells, the latter by antagonizing Foxo1 (56, 58). Finally, the E-protein inhibitor Id3 also influences the differential expression of IL-7R in effector versus memory precursor cells during CD8^{+} T cell responses (59). Thus, there appears to be a convoluted interplay of TFs responsible for *Il7r* regulation, and one can assume that different TFs affect the sequence of events that unfolds (a precipitous decline by 8 h, followed by a stagnant recovery). Interestingly, we found that anti-CD3 elicited an early decrease in Foxo1 expression, together with an induction of *Gfi1* that predominated in Tconvs, which might account for *Il7r* transcription, as well as reciprocal changes in *Id2* and *Id3* (Fig. 2B, Supplemental Table II). One or the combination of these rapid changes in TF representation might be responsible for the unique behavior of the *Il7r* locus after anti-CD3 treatment, which may be grounds for future interesting exploration.

The differential transcriptional effects elicited by anti-CD3 injection were largely reproduced by treatment of mouse splenocytes in culture, and this similarity also extended to cultured human PBMCs, for which very similar effects on IL-7R expression were detected in response to four different anti-CD3 preparations, two of which are in clinical development. Because the differential shift in IL-7R expression occurs rapidly, the likeliest explanation is that changes in mRNA/protein content are indeed taking place in each cell, but we cannot rule out that preferential survival of IL-7R cells contributes to the result. It was not possible to generate gene-expression profiles from human Tregs and Tconvs after treatment, as the activation obscures the CD25 marker normally used for sorting. Attempts were made to perform restricted signature profiling after sorting of permeabilized and anti-FOXP3–stained human cells: although too variable for robust exploitation, the results did point to differential changes in Tregs versus Tconvs for some of the genes that responded differentially in mouse cells (e.g., *Il7r*, *Il2*,

TRAT1, and *Il12rb2*; L. Li, unpublished observations). However, because the effects were generally less obvious in vitro than in vivo, it would be desirable to analyze human subjects at early times after anti-CD3 treatment. Analysis of a small group of individual donors showed no evidence of interindividual variability of the response, except for one outlier likely due to the FcγRII polymorphism that affects binding of mouse IgG1 (44), because the low response was seen only with UCHT1 and not the IgG2a mAbs (Fig. 7C).

These differential effects led us to hypothesize that a combined anti-CD3/IL-7 therapy might have enhanced efficacy in restoring glycemic control to recent-onset diabetic NOD mice. This proved to be the case, validating our interpretation, but also opening the door to potential clinical application of this new combination therapy to human T1D patients. Unfortunately, although IL-7 coadministration proved effective at increasing the penetrance of optimal doses of anti-CD3, it did not allow a reduction in dosing of anti-CD3, which would be a therapeutically desirable goal. As a side note, although our results mirrored those of Mehta et al. (60) in terms of dose-dependent TCR occupancy and clearing (∼≥90% reduction in TCR at the 50- or 25-μg therapeutic doses, only ∼50% reduction at the 5-μg dose; Fig. 4A), we did not reproduce the observation of good clinical efficacy in the 5-μg dose range. There was a clear linear relationship between the persistence of TCR and magnitude of changes affecting IL-7R, whether in mouse or human cells. Optimal effects were seen with the highest level of TCR downregulation, those at which we also fund best clinical efficacy. If our interpretation is correct, this relationship may suggest that a rapidly maximal treatment would be most effective, rather than a gradual ramp up from repeated low doses.

Whether anti-CD3 induces an alteration in dominant tolerance or an attenuation of pathogenic cells has long been an open question. Evidence has been provided for the latter (14–17), most recently with the suggestion that anti-CD3 redirects IL-17–producing pathogenic cells to the small intestine (13, 61). Although our results provide a plausible mechanism for a resetting of Treg homeostasis, they certainly do not rule out complementary effects on pathogenic T cells themselves. Indeed, the transient response observed in Tconvs, which mimics the differential response elicited with or without costimulatory signals (E. Wakamatsu, D. Mathis, and C. Benoist, unpublished observations), might underlie the anergic phenotypes reported previously (14–16).

IL-7 therapy is being attempted in several settings where it might be desirable to restore lymphoid homeostasis, such as primary or acquired immunodeficiencies or after cancer chemotherapy (62). It might seem paradoxical, then, to propose IL-7 supplementation in a context where autoreactive Tconvs are the root of the problem. IL-7 blockade by anti–IL-7 mAbs has been used as a therapy in preclinical models of autoimmune disease. It can reduce pathogenic Th17 cells in experimental allergic encephalomyelitis (63), and recent reports show it is effective in reversing recent-onset NOD diabetes (64, 65). As an adjunct to anti-CD3 therapy, however, the concept is that IL-7 serves to transiently enhance homeostatic changes that favor Treg over autoreactive effector cells.

In conclusion, we have demonstrated divergent responses of $CD4^+$ Tregs and Tconvs to anti-CD3 engagement, suggesting a molecular underpinning for the drug's mechanism of action and prompting us to propose a combination approach to resetting Treg homeostasis for therapy of autoimmune diabetes.

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Disclosures

A.v.M. is an employee of GlaxoSmithKline, which provided one of the reagents used and has an ongoing interest in anti-CD3 therapy. The other authors have no conflicts of interest.

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