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SUPPORTING INFORMATION

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CTLA-4-competent conventional T cells back up regulatory T cells to restrain memory T-helper type 2 cell responses

To the Editor,

How inhibitory co-receptors such as CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) prevent undesired Th2 responses still remains incompletely understood. Understanding the underlying mechanisms could therefore facilitate the improvement of therapeutic treatment of Th2-driven diseases. CTLA-4 is differentially expressed on activated conventional T cells (Tconv) and regulatory T cells (Tregs) whereas the latter essentially maintain tolerance against harmless environmental antigens[1] and regulate Th2 differentiation especially in allergy.[2,3] However, their suppressive capacity does not solely rely on CTLA-4.[4,5] Instead, CTLA-4 expression on Tconv might be critically involved in the restriction of Th2 responses. Hence, we aimed to dissect the hierarchy and interplay of CTLA-4-dependent effects among Tregs and Tconv on Th2-effector and Th2-memory differentiation.

To investigate distinct roles of Tregs and Tconv on Th2 differentiation *in vivo*, various settings of highdose OVA/Alum adjuvanted *in vivo* immunization were applied including inducible genetic Treg depletion via diphtheria toxin (DT) and/or serological CTLA-4 blockade during priming (Figure 1A). Responding $CD40L^+$ Tconv cells revealed significantly higher frequencies of IL-4 and IL-13 producers in the absence of Tregs than in the Treg nondepleted control (Figure 1B). Intriguingly, an additional CTLA-4 blockade further increased frequencies of IL-4 and IL-13 producers compared to those of only Treg depleted. Even upon Treg recovery in the memory response (Figure S1), this paradigm remains long-lasting for IL-4 and IL-13 (Figure 1B). Interestingly, CTLA-4 blockade did not influence Treg activity and therefore the frequencies of Th2-cytokine producers. In terms of antigen-specific expansion, absolute numbers of $CD4^+CD40L^+$ cells indicated that the presence of both Tregs and CTLA-4 competent Tconvs is required for optimal control of primary Th2 cells (Figure 1C).

As the quality of Th cells is shaped by their ability to simultaneously produce multiple cytokines,[6] we analyzed T-cell multi-functionality. Intriguingly, the respective combined cytokine data of primary single, double, or triple Th2 producers showed that in addition to Treg depletion CTLA-4 blockade further increased their frequencies (Figure 1D). In the memory response, Treg depletion alone during sensitization significantly increased the fraction of single and triple producers (Figure 1D). However, additional CTLA-4 blockade of Tconv during priming significantly enhanced the fraction of double

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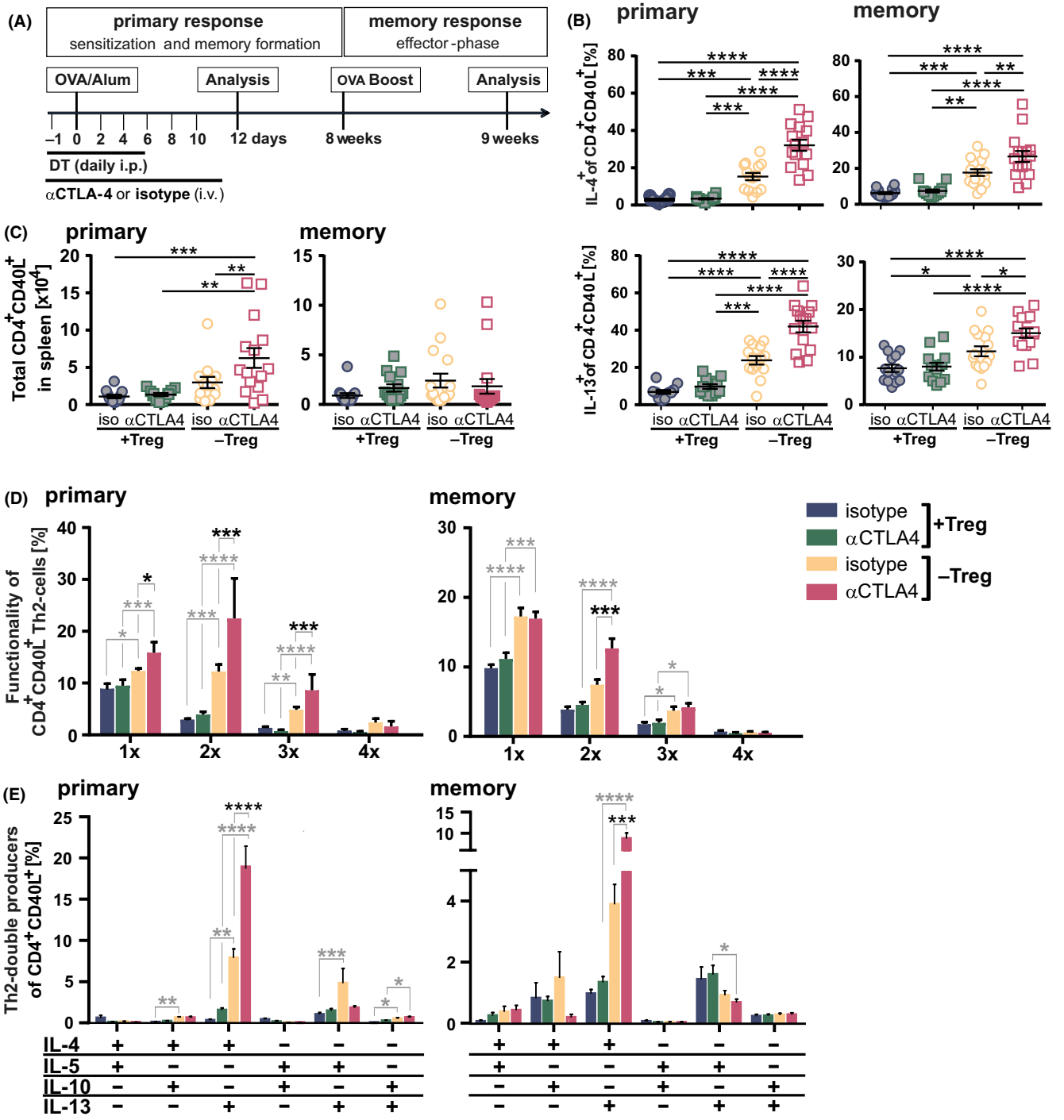

FIGURE 1 Effect of CTLA-4 blockade and Treg depletion on primary and memory Th2 responses *in vivo*. A, Experimental protocol. DEREG (–Treg) and WT (+Treg) mice were sensitized with 100 µg OVA/Alum (i.p.). Tregs were depleted by diphtheria toxin (DT, i.p.). B-E, Mice were treated i.v. with iso or αCTLA-4 antibodies in the presence or absence of Tregs, respectively. Frequencies of primary and memory IL-4$^+$ or IL13$^+$ Th2 cells of CD4$^+$CD40L$^+$ splenocytes (B) and their absolute numbers (C). Frequencies of combined functional (D) and individual Th2–double-producing Th2 cells (E) among all CD4$^+$CD40L$^+$; n = 3 with 12-15 mice per group; mean ± SEM; **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001 (one-way ANOVA, Kruskal-Wallis)

producers (Figure 1D). Dissecting the individual functional subsets, most of the Th2-double producers were downregulated by Tregs, whereas differentiation into IL-4/IL-13 co-producers was profoundly inhibited by CTLA-4 of Tconv in absence of Tregs (Figure 1E). Of note, Treg depletion plus CTLA-4 blockade during priming was long-lasting in terms of accumulated IL-4/IL-13 co-producers (Figure 1E). These results demonstrate the relevance of CTLA-4 expression on Tconv along with Tregs in controlling Th2 subpopulations. According to the model that multi-functional T cells are superior to single producers and more likely transform into memory T cells,[6] our data show that

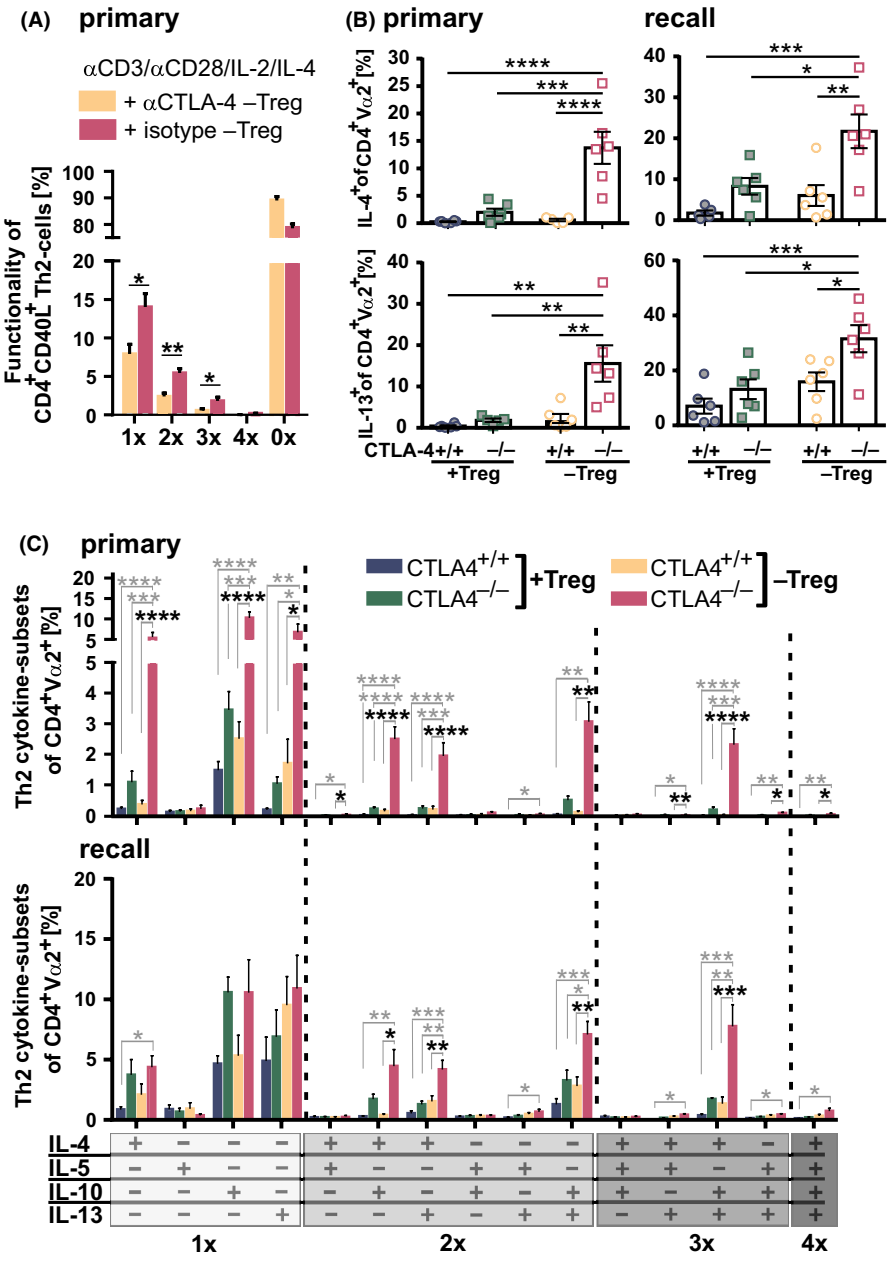

FIGURE 2 The absence of Tregs sensitizes Tconv for CTLA-4–mediated signals *in vitro*. A, Frequencies of combined functional CD40L$^+$ Th2 cells on day 6 stimulated with immobilized αCD3/αCD28/αCTLA-4 or control; n = 5; *P < .05, **P < .01 (Student unpaired two-tailed t test or nonparametric Mann-Whitney). B-C, Naïve OT-II CTLA-4$^{+/+}$ or CTLA-4$^{-/-}$ Tconv was primary stimulated for 6 d with $OVA_{323-339}$ in the presence or absence of Tregs (OT-II CTLA-4$^{+/+}$), respectively. For recall responses, primary cultures were stimulated for 3 d with congenic APCs and $OVA_{323-339}$. Frequencies of primary and recall IL-4- or IL-13-producers (B). Frequencies and multi-functionality of primary and recall Th2 subsets (C); n = 6; mean ± SEM; *P < .05, **P < .01, ***P < .001, ****P < .0001 (one-way ANOVA, Kruskal-Wallis)

mainly Tregs control long-lasting Th2 responses of high quality; however, CTLA-4-competent Tconv critically back up Treg dysfunction.

To assess the effects of CTLA-4 and Tregs on the Th2-driving transcription factors GATA3 and cMaf, we analyzed their nuclear expression. During primary response, Treg depletion fostered the enrichment of cMAf$^+$ or GATA3$^+$ Tconv and additional CTLA-4 blockade further increased these frequencies compared to those of only Treg depleted (Figure S2). However, in the memory response cMaf was enhanced only in T cells with previous CTLA-4 blockade in the presence of Tregs (Figure S2) showing that Treg- and CTLA-4–mediated control of transcription factors was not similarly recalled upon a boosting.

Th2 differentiation could be restrained by Th1 responses; however, the analyzed Th1-cytokine production was not consistent with this relation. The frequencies of primary IFNγ producers were higher in the absence of Tregs and with additional blockade of CTLA-4 than in control groups, whereas IL-2 producers behaved oppositely (Figure S3). During memory response, only minor differences in Th1-cytokine expressing cells were detectable (Figure S3). Therefore, downregulation of Th2 response by Tregs and/or CTLA-4 is unlikely caused by increased frequencies of Th1 cells.

Providing agonistic CTLA-4 signals in a Th2 environment *in vitro* by cross-linking antibodies coupled to microspheres showed that most of the Th2 subsets were significantly decreased in CTLA-4–crosslinked Tconv (Figure 2A). Therefore, in a Treg-free environment, CTLA-4–mediated signals on Tconv are capable to prevent a strong Th2 response. Of note, the strongest inhibitory effect of CTLA-4 was seen for IL-4 and IL-13 and its co-expressing subpopulations (Figure S4). To separately assess impact of Tregs or CTLA-4 on Th2-cell differentiation, we used an *in vitro* model system of CTLA-4$^{-/-}$ and CTLA-4$^{+/+}$ OVA-specific Tconv in the absence or presence of CTLA-4$^{+/+}$ OVA-specific

Tregs. Despite similar proliferation and activation, simultaneous absence of Tregs and CTLA-4 signals on Tconv during priming and recall responses were obligatory for enhanced Th2-related cytokine expression and secretion (Figure 2B, Figure S5 and S6). Furthermore, almost all primary Th2-cytokine subsets were significantly enhanced in Treg-free CTLA-4$^{-/-}$ Th2 cells, whereas in recall responses Th2 subsets predominately co-expressed IL-4 and/or IL-13 (Figure 2C). In the absence of Tregs, cMaf and GATA3 were strongly upregulated in primary CTLA-4$^{-/-}$ Tconvs; however, in recall responses this effect could only be detected for cMaf (Figure S7). These data strongly support the concept that Tregs and CTLA-4 on Tconv cooperatively restrain Th2 responses in a strict hierarchical order.

The development of allergic diseases is mainly caused by a dysregulated Treg function. In this regard, studies using genetically predisposed mice have shown that CTLA-4 blockade in the presence of Tregs enhanced allergic sensitization.[7,8] However, our data acquired in an unbiased background imply that CTLA-4 is not a requisite for Tregs to keep Th2 cells under control. Moreover, in the absence of Tregs the compartment of Tconv is rendered highly susceptible to CTLA-4 blockade. This consequently involves settings with low-Treg numbers,[9] impaired Treg function or recognition of allergens ([10]) where CTLA-4 expression on Tconv would critically control Th2-driven immune responses. Hence, deciphering CTLA-4 functioning in both Tregs and Tconvs might be highly beneficial for the development of novel therapeutic interventions in Th2-driven diseases.

CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

AUTHOR CONTRIBUTIONS

MCBW designed the study, participated in the interpretation of the findings, read, and critically revised the manuscript. MP contributed to the design of the work, performed experiments, interpreted the findings, and wrote the manuscript. AA, HL, and KV have all been involved in laboratory and mice work and processing of biological data samples.

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