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B7/CD28 in central tolerance: costimulation promotes maturation of regulatory T cell precursors and prevents their clonal deletion

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According to the "two-step model," the intrathymic generation of $CD4^+$ regulatory T (T_{reg}) cells segregates into a first, T cell receptor (TCR)-driven phase and a second, cytokine-dependent phase. The initial TCR stimulus gives rise to a $CD25^+Foxp3^-$ developmental intermediate. These precursors subsequently require cytokine signaling to establish the mature $CD25^+Foxp3^+$ T_{reg} cell phenotype. In addition, costimulation via CD28/B7 (CD80/86) axis is important for the generation of a T_{reg} cell repertoire of normal size. Recent data suggest that CD28 or B7 deficient mice lack $CD25^+Foxp3^-$ T_{reg} cell progenitors. However, these data leave open whether costimulation is also required at subsequent stages of T_{reg} differentiation. Also, the fate of "presumptive" T_{reg} cells carrying a permissive TCR specificity in the absence of costimulation remains to be established. Here, we have used a previously described TCR transgenic model of agonist-driven T_{reg} differentiation in order to address these issues. Intrathymic adoptive transfer of T_{reg} precursors indicated that costimulation is dispensable once the intermediate $CD25^+Foxp3^-$ stage has been reached. Furthermore, lack of costimulation led to the physical loss of presumptive T_{reg} cells rather than their escape from central tolerance and differentiation into the conventional $CD4^+$ T cell lineage. Our findings suggest that CD28 signaling does not primarily operate through enhancing the TCR signal strength in order to pass the threshold intensity required to initiate T_{reg} cell specification. Instead, costimulation seems to deliver unique and qualitatively distinct signals that coordinately foster the developmental progression of T_{reg} precursors and prevent their negative selection.

Keywords: regulatory T cell, thymocyte development, thymus, tolerance, costimulation, thymus epithelium, CD28, B7

INTRODUCTION

$CD4^+$ regulatory T (T_{reg}) cells expressing the transcription factor Foxp3 exert an essential function for the maintenance of self-tolerance and immune homeostasis (Sakaguchi, 2004). There is good evidence that a substantial fraction of the T_{reg} cell repertoire originates from the thymus; for instance, there is a large degree of sequence-overlap between the T cell receptor (TCR) repertoires of thymic and peripheral $Foxp3^+$ cells (Hsieh et al., 2006; Pacholczyk et al., 2006; Lio and Hsieh, 2011).

Entry into the T_{reg} cell lineage during thymocyte development is believed to depend upon instructive processes ensuing from self-antigen recognition (Wirnsberger et al., 2011). Evidence for this has been obtained in TCR/neo-self-antigen double transgenic systems (Jordan et al., 2001; Apostolou et al., 2002; Kawahata et al., 2002; Aschenbrenner et al., 2007) and also stems from observations that polyclonal thymocytes bearing superantigen-reactive TCRs are substantially enriched in $Foxp3^+$ cells (Papiernik et al., 1998; Ribot et al., 2006). The exact parameters and modalities of antigen recognition that specify whether an autoreactive MHC II-restricted thymocyte enters the T_{reg} lineage or is subject to negative selection remain to be established; however, there is some consensus that interactions of intermediate avidity may favor T_{reg} cell differentiation over clonal deletion (Feuerer et al., 2007; Atibalentja et al., 2009; Picca et al., 2009; Hinterberger et al., 2010). Furthermore, co-signals provided by common γ-chain cytokines [interleukin (IL)-2 in particular, but also IL-7 and -15; Fontenot et al., 2005a; Mayack and Berg, 2006; Yao et al., 2007; Bayer et al., 2008; Vang et al., 2008] as well as costimulation through CD28/B7 interactions are required for efficient intrathymic differentiation of T_{reg} cells.

Mice deficient in CD28 or its ligands CD80 and CD86 (B7.1 and B7.2, respectively) display a significant decrease in the number of thymic and peripheral T_{reg} cells (Salomon et al., 2000; Tang et al., 2003; Lohr et al., 2004; Tai et al., 2005). Although costimulation has been implicated in IL-2 production (Lindstein et al., 1989; Fraser et al., 1991; Jenkins et al., 1991), the failure of $Cd28^{-/-}$ or $Cd80/Cd86^{-/-}$ mice to generate a T_{reg} cell pool of normal size is not directly linked to cytokine deprivation. Thus, the inefficient entry of $Cd28^{-/-}$ thymocytes into the T_{reg} lineage is not "rescued" by the presence of bystander $Cd28^{+/+}$ cells in mixed bone marrow chimeras, indicating that the paucity of thymic T_{reg} cells in costimulation deficient mice primarily reflects a T cell-intrinsic function of the CD28 signaling axis (Tai et al., 2005).

The "two-step model" of intrathymic T_{reg} differentiation suggests a sub-division into an antigen-driven instruction phase and a cytokine-dependent (but largely antigen independent) consolidation phase. Accordingly, $CD25^+Foxp3^-$ CD4 single-positive (SP) cells represent TCR-instructed, T_{reg} lineage committed intermediates that require continual cytokine (IL-2, IL-7, or IL-15) signaling, but are largely independent of TCR stimulation, for their differentiation into "mature" $CD25^+Foxp3^+$ T_{reg} cells (Burchill et al., 2008; Lio and Hsieh, 2008). Recent data support the idea that CD28 costimulation and common γ-chain cytokine signaling operate at distinct stages of intrathymic T_{reg} differentiation. Specifically, polyclonal $CD25^+Foxp3^-$ cells, which are believed to contain T_{reg} precursors that arise through TCR-mediated instruction ("step one") are strongly diminished in the thymus of *Cd28*$^{-/-}$ mice (Lio et al., 2010; Vang et al., 2010).

The principle requirement for costimulation during intrathymic generation of the T_{reg} cell pool has been well documented in polyclonal systems. However, assessing the number of $Foxp3^+$ cells in a diverse TCR repertoire does not reveal insights into the "alternative" fate of presumptive T_{reg} cells in the absence of costimulation. Thus, it is as yet unclear whether the respective TCR specificities are physically lost from the repertoire, i.e., negatively selected, or whether these cells instead escape from central tolerance induction and enter the pool of mainstream CD4 T cells. To address this issue, we have made use of a previously described TCR transgenic model of agonist antigen-driven T_{reg} differentiation.

MATERIALS AND METHODS

MICE

T cell receptor–hemagglutinin (HA; Kirberg et al., 1994) and AIRE–HA (Aschenbrenner et al., 2007) have been described previously. $Foxp3^{gfp}$ reporter mice (Fontenot et al., 2005b) were kindly provided by A. Rudensky (Memorial Sloan Kettering Institute, New York). *Cd28*$^{-/-}$ (Shahinian et al., 1993), *CD80/86*$^{-/-}$, *CD80*$^{-/-}$, and *CD86*$^{-/-}$ mice (Borriello et al., 1997) were purchased from Jackson Laboratories. BALB/c mice were purchased from Charles River. Mice were maintained in individually ventilated cages. Animal studies were approved by local authorities (Regierung von Oberbayern, 55.2.1.54.2531-7-08).

INTRATHYMIC TRANSFER

About 5×10^5 CD4 SP thymocytes or 4×10^5 cells of sorted subpopulations from TCR–HA × AIRE–HA donors (CD45.1) were injected in 3 µl PBS into one thymic lobe of CD45.2 recipients of the indicated genotype. The analysis of injected thymi was carried out by depletion of $CD8^+$ cells, staining for the indicated surface markers and analysis of the entire thymus by flow cytometry.

ANTIBODIES AND FLOW CYTOMETRY

Phycoerythrin-conjugated annexin-V, phycoerythrin-conjugated monoclonal antibodies (mAbs) to GITR (DTA-1) and PD-1 (J43), cychrome-conjugated mAb to CD8 (53-6.7), phycoerythrin-indotricarbocyanine-conjugated mAb to CD25 (PC61), allophycocyanin-conjugated mAb to CD45.1 (A20), allophycocyanin-conjugated mAb to BrdU (Cat. No. 51-23619L), and allophycocyanin indotricarbocyanine-conjugated mAb to CD4 (GK1.5) were obtained from Becton Dickinson. Phycoerythrin-conjugated mAb to Foxp3 (FJK-16s) was from eBiosciences. The mAb to the TCR–HA was purified from hybridoma (6.5) supernatants and conjugated to phycoerythrin or Alexa Fluor 647 in our lab.

BRDU LABELING

One milligram of BrdU (Becton Dickinson) in 200 µl PBS was intraperitoneally injected into recipient mice. 24 h after injection mice were sacrificed and thymocytes were stained with the indicated surface markers. Subsequently cells were fixed, permeabilized, treated with DNase I, and stained with a mAb specific to BrdU according to the manufacturers protocol (BrdU Flow Kit, Becton Dickinson).

BONE MARROW CHIMERAS

Bone marrow was depleted of T cells with biotinylated mAbs to CD8 and CD4 followed by depletion with streptavidin MACS beads (Miltenyi Biotec) according to standard procedures. BALB/c recipient mice were irradiated with two split doses of 450 rad and were reconstituted with 8×10^6 bone marrow cells.

PURIFICATION OF CD4 SP CELLS OR T_{REG} PRECURSORS

CD4 SP cells or subpopulations of CD4 SP cells (T_{reg} precursors) were purified by CD8 depletion, staining for the indicated surface markers, and sorting with a FACSAria cell sorter (Becton Dickinson).

STATISTICAL ANALYSIS

Statistical significance was assessed by the two-tailed Student's *t*-test with unequal variance.

RESULTS

LOSS OF PRESUMPTIVE T_{REG} CELLS IN THE ABSENCE OF COSTIMULATION

Studies in polyclonal systems have clearly indicated a substantial reduction in the thymic production of T_{reg} cells in the absence of costimulation (Bour-Jordan et al., 2011). However, these analyses did not reveal the fate of presumptive T_{reg} cells under these circumstances, that is, whether the respective TCR specificities are physically lost from the repertoire or instead enter the naïve pool of CD4 T cells. To address this issue, we used TCR–HA × AIRE–HA double transgenic mice. In these animals, expression and presentation of cognate antigen by medullary thymic epithelial cells (mTECs) promotes the negative selection of the majority of *influenza* HA specific CD4 SP thymocytes, while at the same time a distinct and traceable cohort of HA-specific CD4 SP cells differentiate into T_{reg} cells (Aschenbrenner et al., 2007; Hinterberger et al., 2010). T cells expressing the HA-specific TCR (TCR–HA) can conveniently be traced using the anticlonotypic antibody 6.5.

In the absence of cognate antigen, about 30% of CD4 SP cells express the HA-specific TCR–HA (**Figure 1A**). Expectedly, in TCR–HA single-transgenic mice, the fraction of TCR–HA^+ CD4 SP thymocytes was indistinguishable irrespective of whether costimulation was provided or not (data not shown). By contrast, when TCR–HA × AIRE–HA mice were bred onto a CD28 or CD80/CD86 deficient background, we observed a significantly altered thymic phenotype. Specifically, there was a substantially decreased frequency of TCR–HA^+ cells among CD4 SP

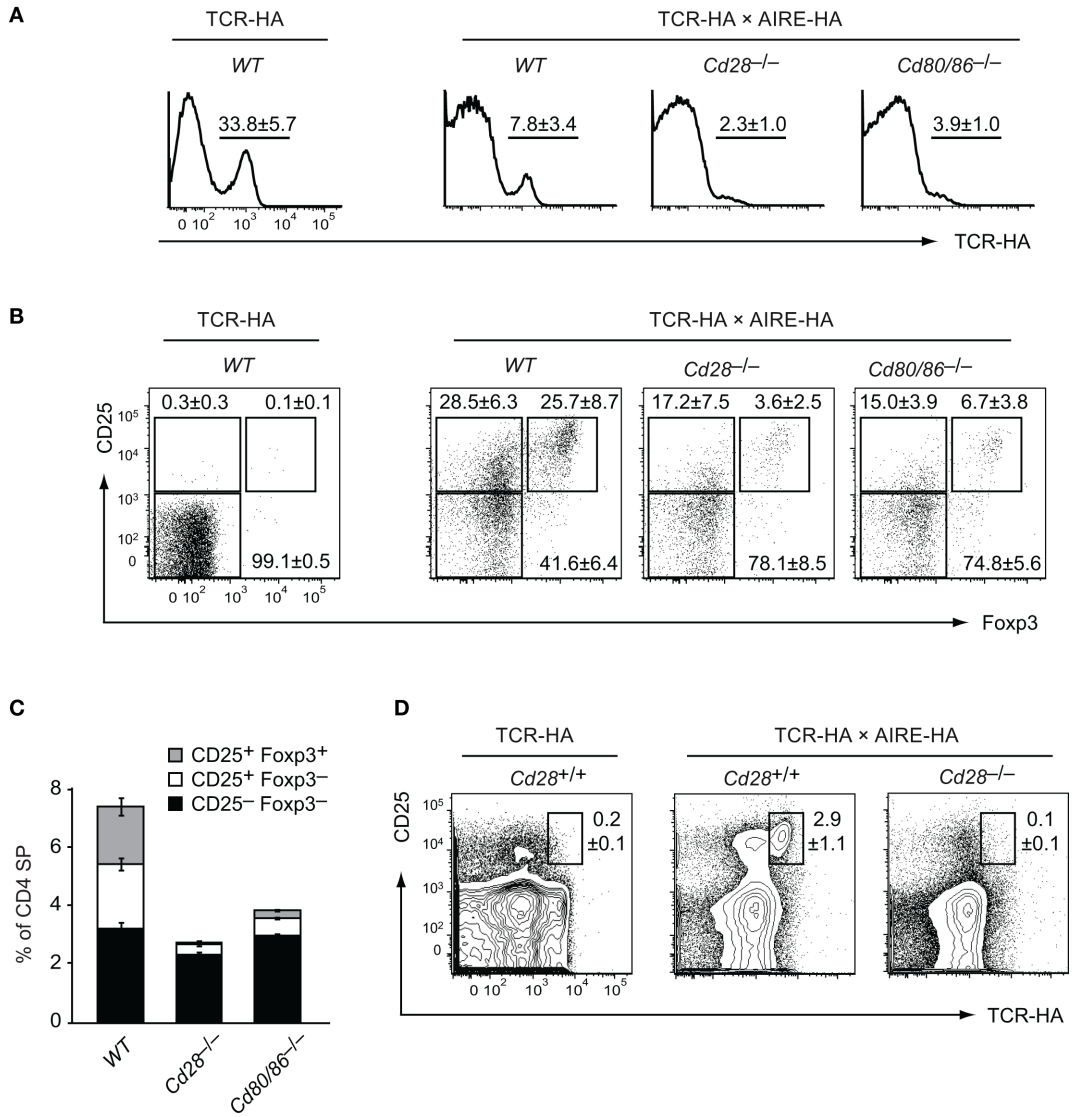

FIGURE 1 | Loss of HA-specific thymic T_{reg} precursor cells in costimulation deficient mice. Thymocytes from 6-week-old TCR–HA single-transgenic mice and TCR–HA × AIRE–HA mice on a costimulation sufficient (=*WT*), *Cd28*$^{-/-}$ or *Cd80/86*$^{-/-}$ background were stained for CD4, CD8, TCR–HA, CD25, and Foxp3 ($n = 5$ for TCR–HA, $n = 36$ for *WT* TCR–HA × AIRE–HA, $n = 14$ for *Cd28*$^{-/-}$ TCR–HA × AIRE–HA, $n = 13$ for *Cd80/86*$^{-/-}$ TCR–HA × AIRE–HA). **(A)** Frequency of TCR–HA^{+} cells (±SD) among CD4 SP cells ($P = 3 \times 10^{-11}$ for *WT* vs. *Cd28*$^{-/-}$ and $P = 2 \times 10^{-7}$ for *WT* vs. *Cd80/Cd86*$^{-/-}$). **(B)** Expression of CD25 and Foxp3 by gated TCR–HA^{+} CD4 SP thymocytes. **(C)** Relative abundance (±SD) of TCR–HA positive CD25^{-}Foxp3^{-} and CD25^{+}Foxp3^{-} T_{reg} precursor subpopulations and mature CD25^{+}Foxp3^{+} T_{reg} cells among gated CD4 SP thymocytes (CD25^{-}Foxp3^{-} subsets: $P = 0.0002$ for *WT* vs. *Cd28*$^{-/-}$ and $P = 0.3$ for *WT* vs. *Cd80/Cd86*$^{-/-}$; CD25^{+}Foxp3^{-} subsets: $P = 5 \times 10^{-12}$ for *WT* vs. *Cd28*$^{-/-}$ and $P = 1 \times 10^{-10}$ for *WT* vs. *Cd80/Cd86*$^{-/-}$; CD25^{+}Foxp3^{+} subsets: $P = 3 \times 10^{-16}$ for *WT* vs. *Cd28*$^{-/-}$; and $P = 4 \times 10^{-15}$ for *WT* vs. *Cd80/Cd86*$^{-/-}$). The relative and absolute abundance of CD4 SP thymocytes was not significantly different between the various genotypes (data not shown). **(D)** Expression of CD25 and TCR–HA by gated CD4^{+} T cells from peripheral lymph nodes of the indicated genotype.

thymocytes when compared to costimulation competent TCR–HA × AIRE–HA controls (**Figure 1A**). These somewhat surprising initial findings indicated that lack of costimulation augmented the antigen-driven loss of HA-specific CD4 SP cells.

Among TCR–HA^{+} CD4 SP thymocytes of costimulation sufficient TCR–HA × AIRE–HA mice, we found that CD25^{-}Foxp3^{-}, CD25^{+}Foxp3^{-}, and CD25^{+} Foxp3^{+} cells are represented at roughly equal proportions (**Figure 1B**, and Wirnsberger et al., 2009). Consistent with the "two-step" model of T_{reg} cell development (Lio and Hsieh, 2008), we have shown previously that these subsets represent consecutive stages of agonist induced T_{reg} cell development (CD25^{-}Foxp3^{-} → CD25^{+}Foxp3^{-} → CD25^{+}Foxp3^{+}; Wirnsberger et al., 2009). In the absence of CD28 or CD80/CD86 costimulation, the percentage of "mature" CD25^{+}Foxp3^{+} T_{reg} cells among TCR–HA^{+} CD4 SP thymocytes and their

immediate $CD25^+Foxp3^-$ precursors was considerably decreased (**Figures 1B,C**). Instead, the majority of residual TCR–HA^+ CD4 SP cells were $CD25^-Foxp3^-$, suggesting a developmental bottleneck at the transition from a $CD25^-Foxp3^-$ to a $CD25^+Foxp3^-$ phenotype, i.e., at the TCR-driven "step one" of T_{reg} cell differentiation.

The $CD25^-Foxp3^-$ surface phenotype of the majority of TCR–HA^+ CD4 SP cells in costimulation deficient mice might have indicated that these cells are naive cells that have not received a "T_{reg} instructing" TCR signal of appropriate strength. Potentially, such cells might escape from central tolerance induction and seed peripheral lymphoid organs. If this were the case, one might expect to find TCR–HA^+ non-T_{reg} $CD4^+$ T cells in the periphery of costimulation deficient TCR–HA × AIRE–HA mice. However, inspection of peripheral CD4 T cell compartments revealed the complete absence of TCR–HA^+ cells in costimulation deficient mice (**Figure 1D**). Specifically, not only was the distinct population of TCR–HA^+ $CD25^+$ T_{reg} cells that is seen in costimulation sufficient mice absent, but there was also no discernible emergence of TCR–HA^+ $CD25^-$ cells in peripheral lymphoid organs (**Figure 1D**).

In order to address in how far either CD80 or CD86 provided the essential signals for T_{reg} cell differentiation, we bred the TCR–HA × AIRE–HA system onto the respective single knockout background. This revealed a degree of redundancy of the two B7 family members in that both *Cd80*$^{-/-}$ and *Cd86*$^{-/-}$ mice only showed a relatively mild reduction of $CD25^+Foxp3^-$ precursors and their "mature" $CD25^+Foxp3^+$ progeny among TCR–HA^+ CD4 SP thymocytes (**Figures 2A,B**).

In sum, these observations are consistent with a role of costimulation in the TCR-driven development of early intermediates of thymic T_{reg} development. A similar conclusion has recently been drawn from the absence of $CD25^+GITR^+CD122^+$ cells among polyclonal CD4 SP cells of *Cd28*$^{-/-}$ mice (Lio et al., 2010; Vang et al., 2010). Importantly, our data suggest that lack of costimulation, rather than allowing these presumptive T_{reg} cells to escape from clonal deviation and to enter the naïve repertoire, leads to physical loss of the respective specificities. In other words, under conditions that are otherwise permissive for T_{reg} cell differentiation (i.e., appropriate strength of TCR stimulus), lack of costimulation results in the conversion of T_{reg} differentiation into negative selection.

THE FUNCTION OF COSTIMULATION EXTENDS BEYOND IL-2 SIGNALING AND IS CELL-INTRINSIC

CD28 costimulation has been implicated in IL-2 production (Lindstein et al., 1989; Fraser et al., 1991; Jenkins et al., 1991). Hence, its abrogation may impinge on T_{reg} cell differentiation through lack of IL-2 mediated cell extrinsic survival and/or differentiation signals that orchestrate the cytokine-dependent "second" phase of T_{reg} cell differentiation (Burchill et al., 2008; Lio and Hsieh, 2008; Wirnsberger et al., 2009). However, upon breeding onto an *Il2*$^{-/-}$ background, thymi of TCR–HA × AIRE–HA mice – in contrast to what was observed in *Cd28*$^{-/-}$ or *Cd80/86*$^{-/-}$ mice – did not show a reduction of TCR–HA^+ CD4 SP cells and of mature $CD25^+$ cells within this population (**Figure 3**). This is consistent with earlier observations that IL-2 acts on thymic T_{reg} cell differentiation in an at least partly redundant manner with other common γ-chain cytokines such IL-7 or IL-15 (D'Cruz and Klein, 2005; Fontenot et al., 2005a; Vang et al., 2008) and indicates that the apparent developmental blockade and loss of TCR–HA^+ T_{reg} cells in CD28 or CD80/86 deficient TCR–HA × AIRE–HA mice cannot be explained by an eventual requirement of CD28/B7 costimulation solely for IL-2 production.

In order to test whether the requirement for costimulation was cell-intrinsic, we generated mixed bone marrow chimeras. Irradiated AIRE–HA mice or wild-type controls were reconstituted with a 1/1 mixture of TCR–HA transgenic *Cd28*$^{+/+}$ and *Cd28*$^{-/-}$

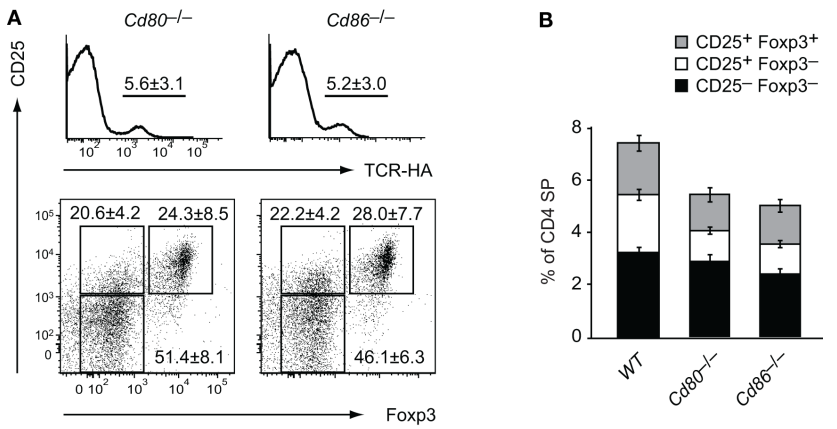

FIGURE 2 | Partially redundant role of CD80 and CD86 for intrathymic T_{reg} development. Thymocytes from 6-week-old TCR–HA × AIRE–HA mice on a *Cd80*$^{-/-}$ ($n = 14$) or *Cd86*$^{-/-}$ ($n = 20$) background were stained for CD4, CD8, TCR–HA, CD25, and Foxp3. **(A)** Frequency of TCR–HA^+ cells (±SD) among CD4 SP cells ($P = 0.09$ for *WT* vs. *Cd80*$^{-/-}$ and $P = 0.03$ for *WT* vs. *Cd86*$^{-/-}$; upper panel). The lower panel depicts the expression of CD25 and Foxp3 by gated TCR–HA^+ CD4 SP thymocytes. **(B)** Relative abundance (±SD) of TCR–HA positive $CD25^-Foxp3^-$ and $CD25^+Foxp3^-$ T_{reg} precursor subpopulations and mature $CD25^+Foxp3^+$ T_{reg} cells among gated CD4 SP thymocytes ($CD25^-Foxp3^-$ subsets: $P = 0.8$ for *WT* vs. *Cd80*$^{-/-}$ and $P = 0.4$ for *WT* vs. *Cd86*$^{-/-}$; $CD25^+Foxp3^-$ subsets: $P = 0.003$ for *WT* vs. *Cd80*$^{-/-}$ and $P = 0.0002$ for *WT* vs. *Cd86*$^{-/-}$; $CD25^+Foxp3^+$ subsets: $P = 0.08$ for *WT* vs. *Cd80*$^{-/-}$ and $P = 0.06$ for *WT* vs. *Cd80/Cd86*$^{-/-}$).

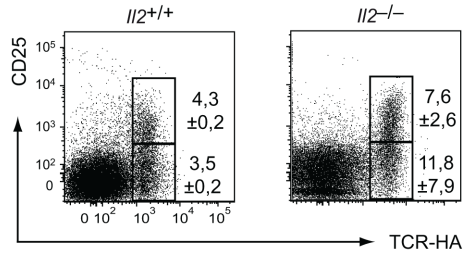

FIGURE 3 | Efficient intrathymic T_{reg} differentiation in IL-2 deficient TCR–HA × AIRE–HA mice. Thymocytes from 3-week-old TCR–HA × AIRE–HA mice on an *Il2*$^{+/+}$ ($n = 3$) or *Il2*$^{-/-}$ ($n = 3$) background were stained for CD4, CD8, TCR–HA, CD25, and Foxp3. Numbers indicate the average frequency (±SD) of cells within gates.

bone marrow cells (**Figure 3**). As expected, in the absence of cognate antigen, *Cd28*$^{+/+}$, and *Cd28*$^{-/-}$ cells equally contributed to all thymocyte subsets (not shown). In the presence of cognate antigen, TCR–HA$^+$ cells represented about 6% of *Cd28*$^{+/+}$ cells among CD4 SP thymocytes and segregated into CD25$^-$Foxp3$^-$, CD25$^+$Foxp3$^-$, and CD25$^+$Foxp3$^+$ subsets similar to what was observed in TCR–HA × AIRE–HA mice (**Figure 4B**; compare **Figures 1A,B**). By contrast, TCR–HA$^+$ cells made up for only about 3% of *Cd28*$^{-/-}$ cells among CD4 SP cells, and the majority of these cells had a CD25$^-$Foxp3$^-$ phenotype (**Figure 4B**). Overall, the contribution of *Cd28*$^{+/+}$ and *Cd28*$^{-/-}$ cells to CD25$^-$Foxp3$^-$ TCR–HA$^+$ thymocytes reflected the 1/1 input ratio, whereas *Cd28*$^{-/-}$ cells were strongly underrepresented among the subsequent CD25$^+$Foxp3$^-$ "intermediate" population and were barely detectable within the "mature" CD25$^+$Foxp3$^+$ subset (**Figure 4C**).

Together, these findings clearly indicated that costimulation sufficient bystander cells do not rescue the progression of CD28 deficient cells toward a mature T_{reg} cell phenotype, for instance through provision of IL-2 or other factors *in trans*. Instead, there is a cell-intrinsic requirement for CD28 signaling at the earliest stages of T_{reg} cell differentiation that is unrelated to the presumed role of IL-2 at a subsequent stage of this process.

CD28 DEFICIENT HA-SPECIFIC CD25$^-$FOXP3$^-$ CELLS ARE NOT NAIVE

Our results so far revealed that in the presence of cognate antigen, HA–specific CD4 SP cells with a CD25$^-$Foxp3$^-$ phenotype could be found in similar proportions irrespective of whether or not CD28/B7 costimulation was available, whereas CD25$^+$Foxp3$^-$ and CD25$^+$Foxp3$^+$ cells were strongly reduced in the absence of costimulation. This suggested a developmental blockade at the transition to a CD25$^+$Foxp3$^-$ phenotype, i.e., at "step one" of T_{reg} cell differentiation. Alternatively, it was possible that CD25$^-$Foxp3$^-$ CD4 SP cells only in a costimulation sufficient environment represented a true T_{reg} intermediate downstream of the initiating TCR stimulus, whereas in the absence of costimulation, CD25$^-$Foxp3$^-$ CD4 SP cells may instead actually be naïve cells.

In order to distinguish these two possibilities, we performed a more detailed surface marker analysis of *Cd28*$^{+/+}$ and *Cd28*$^{-/-}$ CD25$^-$Foxp3$^-$ CD4 SP thymocytes in the mixed bone marrow chimeras depicted in **Figure 4A** and compared their phenotype to *bona fide* "naïve" CD25$^-$Foxp3$^-$ CD4 SP thymocytes from TCR–HA single-transgenic mice (**Figure 4D**). Both *Cd28*$^{+/+}$ and *Cd28*$^{-/-}$ CD25$^-$Foxp3$^-$ CD4 SP thymocytes displayed a similar up-regulation of the surface molecules PD-1 and GITR, whereas truly naïve CD4 SP cells were PD-1 negative and GITR^{low}. In further support that *Cd28*$^{+/+}$ and *Cd28*$^{-/-}$ CD25$^-$Foxp3$^-$ CD4 SP thymocytes had received a similar TCR stimulus, expression of the TCR was similarly down-regulated on either population, presumably as a result of cognate antigen encounter (**Figure 4D**).

In sum, these findings provided further evidence that in the absence of costimulation, HA-specific cells do not escape as naïve T cells. Instead, our observations support the idea that irrespective of whether or not costimulation is provided, TCR–HA$^+$ progenitors receive a TCR signal that is sufficient to mediate the acquisition of an "early" T_{reg} progenitor phenotype. However, in the absence of CD28 signals, these cells only very inefficiently progress toward the subsequent CD25$^+$Foxp3$^-$ stage and the mature CD25$^+$Foxp3$^+$ T_{reg} phenotype.

COSTIMULATION DOES NOT ACT VIA PROLIFERATIVE EXPANSION OF T_{REG} CELL PRECURSORS

So far, we have considered that in the absence of costimulation, the earliest phase of T_{reg} differentiation represents a developmental dead end. An alternative explanation for the paucity of CD25$^+$Foxp3$^-$ cells and their CD25$^+$Foxp3$^+$ progeny in CD28 or CD80/86 deficient mice would be that costimulation would orchestrate the entry of T_{reg} cell precursors into cell cycling, thereby mediating the proliferative expansion of intermediate T_{reg} precursors rather than their actual developmental progression. Of note, despite a certain consensus that cycling of "mature" Foxp3$^+$ thymocytes is barely detectable, it is as yet unclear whether T_{reg} cell differentiation involves an early expansion phase prior to Foxp3 expression. This is particularly relevant for the earliest CD25$^-$Foxp3$^-$ progenitor stage, because in a polyclonal repertoire these early T_{reg} precursors are essentially impossible to distinguish from the bulk of "naïve" non-T_{reg} cell precursors.

In order to address this question, we performed BrdU labeling experiments. 24 h after a single injection of BrdU into *Cd28*$^{+/+}$ TCR–HA × AIRE–HA mice, a substantial fraction of TCR–HA$^+$ CD25$^-$Foxp3$^-$ cells and to a lesser extent also of CD25$^+$Foxp3$^-$ "intermediate" precursors had incorporated BrdU, whereas BrdU$^+$ cells were very rare among mature Foxp3$^+$ cells (**Figure 5A**). In the absence of costimulation (in *Cd28*$^{-/-}$ TCR–HA × AIRE–HA mice), TCR–HA$^+$ CD25$^-$Foxp3$^-$ cells incorporated similar amounts of BrdU when compared to their counterparts in *Cd28*$^{+/+}$ mice, indicating that entry into the cell cycle of this early T_{reg} cell precursor-population is independent of CD28/B7-mediated costimulatory signals (**Figure 5A**). Somewhat surprisingly, the incorporation of BrdU by CD25$^+$Foxp3$^-$ cells and also by "mature" CD25$^+$Foxp3$^+$ thymocytes was even increased rather than diminished in the absence of CD28 co-signals (**Figures 5A,B**).

In order to address whether these observations similarly applied to non-transgenic polyclonal TCR specificities, we also compared the BrdU incorporation by TCR–HA$^-$ CD4 SP thymocytes of *Cd28*$^{+/+}$ and *Cd28*$^{-/-}$ TCR–HA × AIRE–HA mice. These cells express endogenously rearranged TCRs, and their eventual entry into the T_{reg} lineage reflects polyclonal T_{reg} development. Indeed, a clear tendency toward more proliferation in the absence of costimulation was also observed for CD25$^+$Foxp3$^-$ and

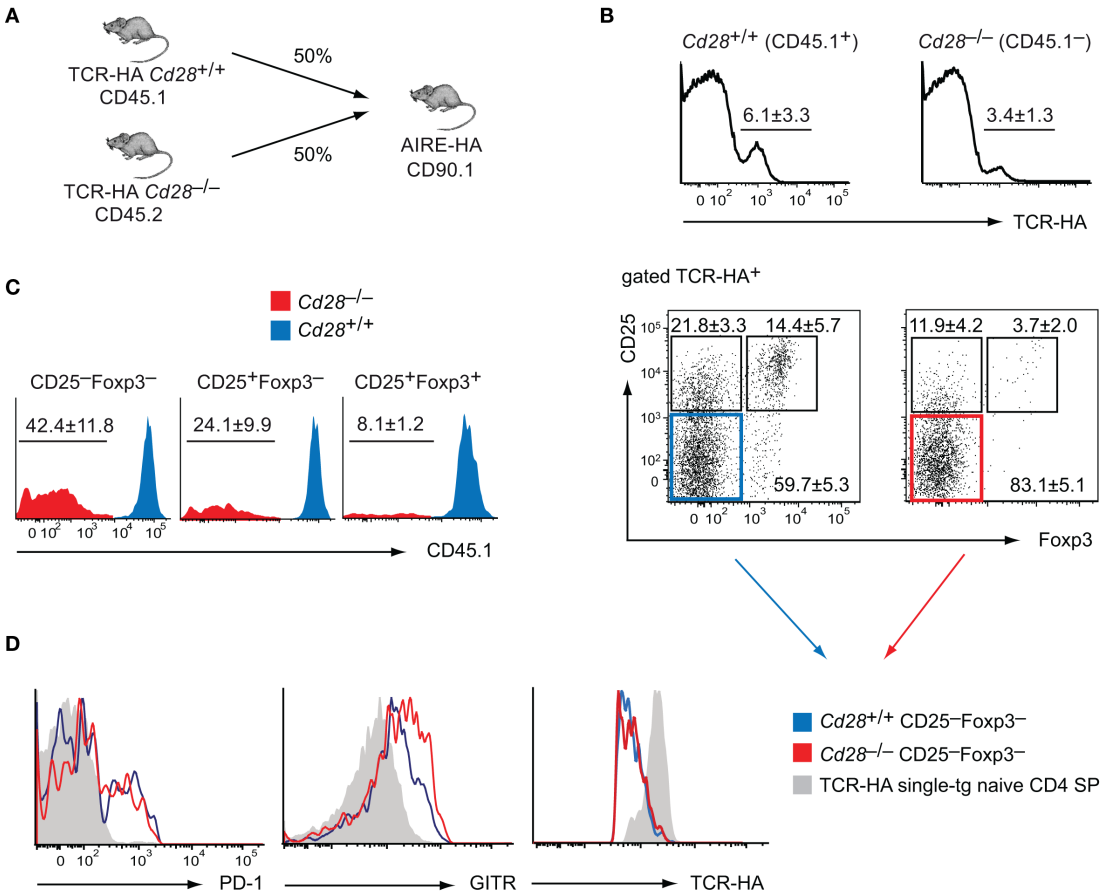

FIGURE 4 | Cell-intrinsic function of CD28 in intrathymic T_{reg} development. (A) Experimental strategy to generate mixed bone marrow (bm) chimeras. Specifically, 4×10^6 *Cd28*$^{+/+}$ TCR–HA bm cells (CD45.1) and 4×10^6 *Cd28*$^{-/-}$ TCR–HA bm cells (CD45.2) were i.v. injected into irradiated AIRE–HA recipients ($n = 9$). Six weeks after bm-reconstitution, CD8 depleted thymocytes were stained for CD4, TCR–HA, CD25, CD45.1, CD45.2, and Foxp3. **(B)** Frequency of TCR–HA$^+$ cells (±SD) among *Cd28*$^{+/+}$ (CD45.1$^+$) and *Cd28*$^{-/-}$ (CD45.1$^-$) CD4 SP cells (upper panel; $P = 0.04$). The lower panel depicts the expression of CD25 and Foxp3 by gated *Cd28*$^{+/+}$ (CD45.1$^+$) or *Cd28*$^{-/-}$ (CD45.1$^-$) TCR–HA$^+$ CD4 SP thymocytes. **(C)** Relative abundance of *Cd28*$^{+/+}$ (CD45.1$^+$; depicted in blue) vs. *Cd28*$^{-/-}$ (CD45.1$^-$; depicted in red) cells among CD25$^-$Foxp3$^-$ and CD25$^+$Foxp3$^-$ T_{reg} precursor subpopulations or mature CD25$^+$Foxp3$^+$ T_{reg} cells, gated on all TCR–HA$^+$ CD4 SP thymocytes. Numbers indicate the average frequency (±SD) of cells within gates. **(D)** Sub-fractions of cells were also stained for PD-1 or GITR. The expression of PD-1 or GITR as well as TCR–HA on gated CD25$^-$Foxp3$^-$TCR–HA$^+$ CD4 SP thymocytes of *Cd28*$^{+/+}$ (blue histogram) or *Cd28*$^{-/-}$ (red histogram) origin was assessed. The gray histogram indicates the expression of the respective markers on "naïve" CD25$^-$Foxp3$^-$TCR–HA$^+$ CD4 SP thymocytes from TCR–HA single-transgenic animals.

"mature" CD25$^+$Foxp3$^+$ cells among TCR–HA$^-$ CD4 SP thymocytes, emphasizing that our observations for TCR transgenic T_{reg} cells and their precursors faithfully recapitulated the behavior of polyclonal T cells (**Figure 5B**).

Taken together, our findings suggest that the early specification into the T_{reg} cell lineage indeed coincides with entry of "pre-Foxp3" T_{reg} precursors into cell cycling. However, our data strongly argue against a requirement for CD28/B7 costimulation for proliferative expansion of a minute "TCR-primed" precursor-population.

THE TCR-DRIVEN INSTRUCTIVE BUT NOT THE CYTOKINE-DEPENDENT CONSOLIDATION PHASE OF T_{REG} DIFFERENTIATION REQUIRES COSTIMULATION

A precise assessment of where and when costimulation is required during intrathymic T_{reg} cell development is difficult to achieve when studying steady state thymocyte differentiation. For instance, it is possible that the requirement for costimulation even precedes the TCR stimulus, whereby costimulation may somehow prime cells for a subsequent instructive signal. Similarly, an early bottleneck in T_{reg} differentiation may mask a continual requirement for costimulation also at a subsequent stage of T_{reg} differentiation.

Our observations so far did not reveal whether the costimulatory interactions that support T_{reg} differentiation occur before the CD4 SP T cell stage, for instance concomitant to positive selection. We have shown previously that T_{reg} differentiation in the TCR–HA × AIRE–HA thymus can be dissociated from positive selection and CD4 lineage commitment. Specifically, injection of CD4 SP cells from TCR–HA *Rag2*$^{-/-}$ mice, i.e., truly naïve, monoclonal cells that did not contain any pre-existing Foxp3$^+$ cells, into AIRE–HA thymi resulted in a substantial fraction of cells entering

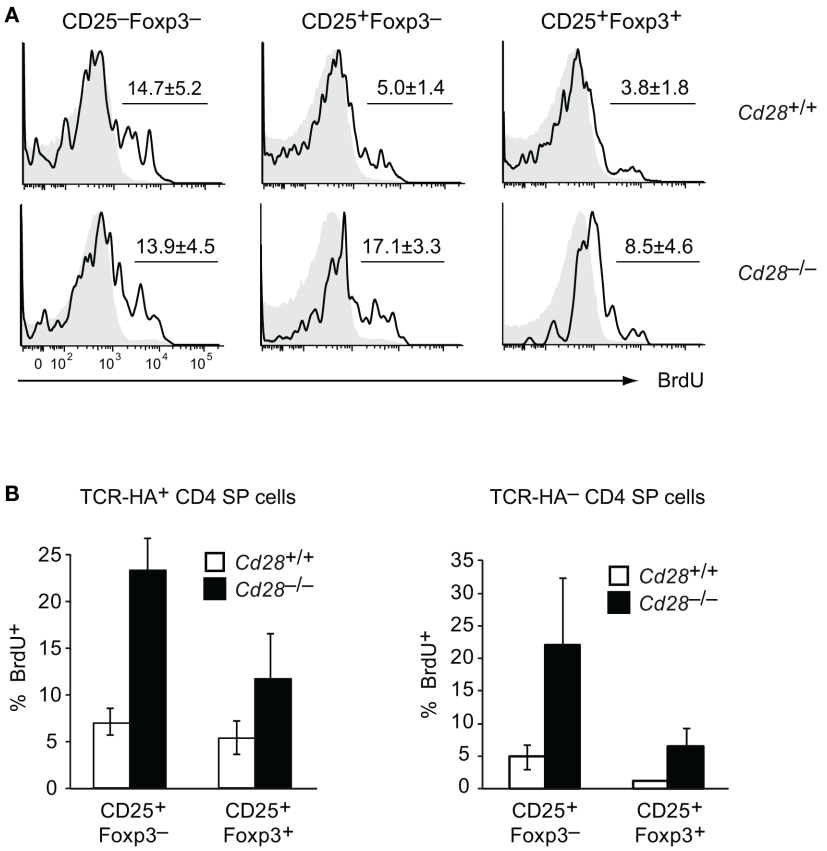

FIGURE 5 | Proliferation of TCR–HA$^+$ T_{reg} precursors is not reduced in the absence of CD28 and recapitulates the behavior of thymocytes expressing diverse TCRs. 24 h after a single injection of BrdU, thymocytes from TCR–HA × AIRE–HA mice on a *Cd28*$^{+/+}$ ($n = 4$) or *Cd28*$^{-/-}$ ($n = 7$) background were stained for CD4, CD8, TCR–HA, CD25, Foxp3, and BrdU incorporation. **(A)** Extent of BrdU incorporation (black open histogram) by gated TCR–HA$^+$ CD4 SP T_{reg} precursors (CD25$^-$Foxp3$^-$ or CD25$^+$Foxp3$^-$) or mature TCR–HA$^+$ CD4 SP T_{reg} cells (CD25$^+$Foxp3$^+$) in *Cd28*$^{+/+}$ (upper panels) or *Cd28*$^{-/-}$ (lower panels) mice. Isotype control staining of the respective samples are shown as histogram overlay (gray filled; $P = 0.8$ for CD25$^-$Foxp3$^-$ subsets; $P = 2 \times 10^{-4}$ for CD25$^+$Foxp3$^-$ subsets; $P = 0.04$ for CD25$^+$Foxp3$^+$ subsets) **(B)** Comparison of BrdU incorporation by *Cd28*$^{+/+}$ (white bars) or *Cd28*$^{-/-}$ (black bars) CD25$^+$Foxp3$^-$ or CD25$^+$Foxp3$^+$ CD4 SP thymocytes that either express the transgenic TCR–HA (left panel) or express endogenously rearranged TCRs (TCR–HA$^-$, right panel).

the CD25$^+$Foxp3$^+$ T_{reg} cell lineage (Wirnsberger et al., 2009; see also **Figure 6**). These finding indicated that self-antigen-driven intrathymic T_{reg} differentiation can be initiated in the absence of "nominal" antigen encounter prior to the CD4 SP stage.

In order to dissociate positive selection in the absence or presence of CD28/B7 costimulatory interactions from cognate antigen encounter at the CD4 SP stage in the absence or presence of costimulation, we intrathymically (i.t.) injected CD28 deficient *Rag2*$^{-/-}$ TCR–HA SP thymocytes into AIRE–HA recipients. In a "reciprocal" setting, we injected *Rag2*$^{-/-}$ TCR–HA SP cells from costimulation sufficient animals into *Cd80/86*$^{-/-}$ recipients (**Figure 6**). Both sets of experiments yielded essentially identical outcomes, namely an almost complete absence of T_{reg} differentiation, suggesting that costimulation is necessary concomitant to or immediately subsequent to the instructing TCR stimulus (**Figure 6**).

Our data so far revealed an essential requirement for costimulation simultaneous to or in close temporal proximity to the instructing TCR stimulus. When analyzing steady state T_{reg} cell development in the absence of costimulation, the early developmental arrest at the CD25$^-$Foxp3$^-$ stage precludes the analysis of an eventually continual requirement for CD28/B7 interactions at subsequent stages of T_{reg} differentiation. In order to address this issue, we isolated CD25$^-$Foxp3$^-$GITR$^+$ cells (i.e., the earliest distinct subset of TCR-triggered T_{reg} cell precursors) and cells at the subsequent CD25$^+$Foxp3$^-$ intermediate stage (i.e., cells that require common γ-chain cytokines – but not TCR stimulation – to mature into CD25$^+$Foxp3$^+$ cells) from costimulation sufficient TCR–HA × AIRE–HA mice and injected them into *Cd80/86*$^{-/-}$ recipient thymi (**Figure 7A**). This revealed that CD25$^-$Foxp3$^-$GITR$^+$ input cells were strongly dependent upon persistent costimulation to progress toward a mature T_{reg} phenotype, whereas CD25$^+$Foxp3$^-$ cells gave rise to mature T_{reg} cells irrespective of whether or not continual costimulation was provided in the host microenvironment (although T_{reg} occurred perhaps slightly less efficient in *Cd80/86*$^{-/-}$ recipients; **Figure 7B**). Taken together, these data support a model whereby B7/CD28 costimulation

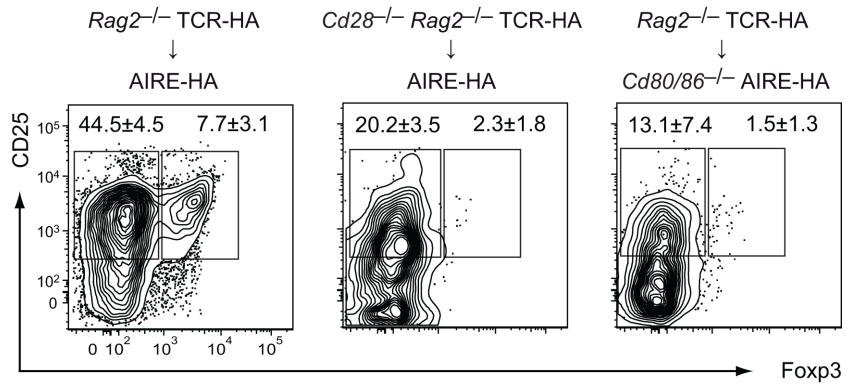

FIGURE 6 | Costimulation is required simultaneous to or in close temporal proximity to the T_{reg} lineage-instructing TCR stimulus at the CD4 SP stage. 5×10^5 CD4 SP cells from TCR–HA Foxp3^{gfp} transgenic animals on a *Rag2*$^{-/-}$ background (CD45.1) were intrathymically injected into *Cd80/86*$^{+/+}$ (left) or *Cd80/86*$^{-/-}$ AIRE–HA (right) recipients (CD45.2). In a "reciprocal" setting, we injected CD4 SP cells from *Cd28*$^{-/-}$ TCR–HA Foxp3^{gfp} *Rag2*$^{-/-}$ mice into the thymus of costimulation sufficient (*Cd80/86*$^{+/+}$) AIRE–HA recipients (middle). 6 days after transfer, injected cells were analyzed for CD25 and Foxp3 expression. Numbers indicate the average frequency (±SD) of cells within gates ($n = 4$ for all groups).

is tightly linked to the TCR-driven first phase of T_{reg} differentiation, but is dispensable at the cytokine-dependent second phase.

DISCUSSION

Our findings suggest that the critical function of B7/CD28 costimulation is to support the development and survival of the CD25$^+$Foxp3$^-$ intermediate stage of T_{reg} differentiation. Furthermore, using adoptive transfer of T_{reg} precursors, we could show that costimulation is largely dispensable once the CD25$^+$Foxp3$^-$ intermediate stage of T_{reg} differentiation has been reached. Hence, the B7 co-stimulus is mainly required simultaneous to or in close temporal proximity to the instructive TCR signal, i.e., at "step one" of T_{reg} differentiation. These findings are consistent with two recent reports indicating that there is a substantial diminution of polyclonal CD25$^+$Foxp3$^-$ T_{reg} precursor cells in CD28 deficient mice (Lio et al., 2010; Vang et al., 2010). Importantly, these analyses of polyclonal T_{reg} development did not identify the actual fate of "presumptive" T_{reg} cells in the absence of B7/CD28 costimulation. Here, the use of a TCR transgenic model of cognate antigen-driven T_{reg} differentiation allowed us to reveal that lack of costimulation leads to the physical loss of T_{reg} precursors from the T cell repertoire. As a net effect, it thus appears that CD28 signaling protects T_{reg} precursors from clonal deletion and thereby promotes the emergence of a T_{reg} repertoire of normal size.

Our findings have obvious implications for the observation that autoimmune prone NOD mice on a CD28 or B7 deficient background develop a more severe and accelerated form of diabetes (Salomon et al., 2000). Thus, it appears that the aggressive form of diabetes in this setting is caused by a deficiency in T_{reg} cells rather than by escape of otherwise "vetoed" T cells specificities from central tolerance. Consistent with this, adoptive transfer of polyclonal or islet antigen specific T_{reg} cells prevented diabetes in NOD *Cd28*$^{-/-}$ mice (Salomon et al., 2000; Tang et al., 2004).

The avidity model of T_{reg} differentiation posits that T_{reg} differentiation ensues from cognate antigen interactions whose strength lies in between the signaling intensity required for positive selection on the one hand and clonal deletion on the other hand (Feuerer et al., 2007; Atibalentja et al., 2009; Picca et al., 2009; Simons et al., 2010). We have recently obtained further evidence for this hypothesis by attenuating antigen presentation in the TCR–HA × AIRE–HA model through "designer micro-RNA" mediated knock-down of MHC class II on mTECs. This resulted in a diminished extent of negative selection and an increased emergence of T_{reg} cells, which is consistent with the notion that intermediate avidity-interactions favor T_{reg} differentiation over negative selection (Hinterberger et al., 2010). Considering the predictions of the avidity hypothesis, one may have expected TCR–HA$^+$ cells to escape from negative selection and T_{reg} induction and to eventually enter the naïve CD4 T cell pool, if B7/CD28 costimulation merely were to amplify the strength of an integrated signal downstream of the TCR and CD28. However, this is clearly not the case. Instead, lack of costimulation increases the antigen-driven net loss of TCR–HA$^+$ cells. Hence, our findings indicate that CD28 signaling does not operate primarily through amplifying the TCR signal, but through qualitatively changing the interpretation of the TCR signal and thereby initiating a distinct genetic program. Consistent with this, we found that in the presence of the AIRE–HA transgene, TCR–HA$^+$ CD25$^-$Foxp3$^-$cells displayed identical signs of early activation (up-regulation of PD-1 and GITR and down-regulation of the TCR) irrespective of whether they were *Cd28*$^{+/+}$ or *Cd28*$^{-/-}$. Parallel signals emanating from CD28/B7 costimulation may then support the progression toward the cytokine-dependent "step two" of T_{reg} differentiation. It remains possible that the early events associated with entry into the T_{reg} lineage can even be set off by a TCR signal of matching strength independent of costimulation.

Generally, CD28 co-signals are thought to stabilize mRNAs and amplify the activation of nuclear factor of activated T cells (NFAT) and nuclear factor-κB (NF-κB), thereby supporting T cell cytokine production, proliferation, survival, and differentiation (Rudd et al., 2009). Concerning a potential role of CD28 signaling in cytokine production, it is hard to see how this should

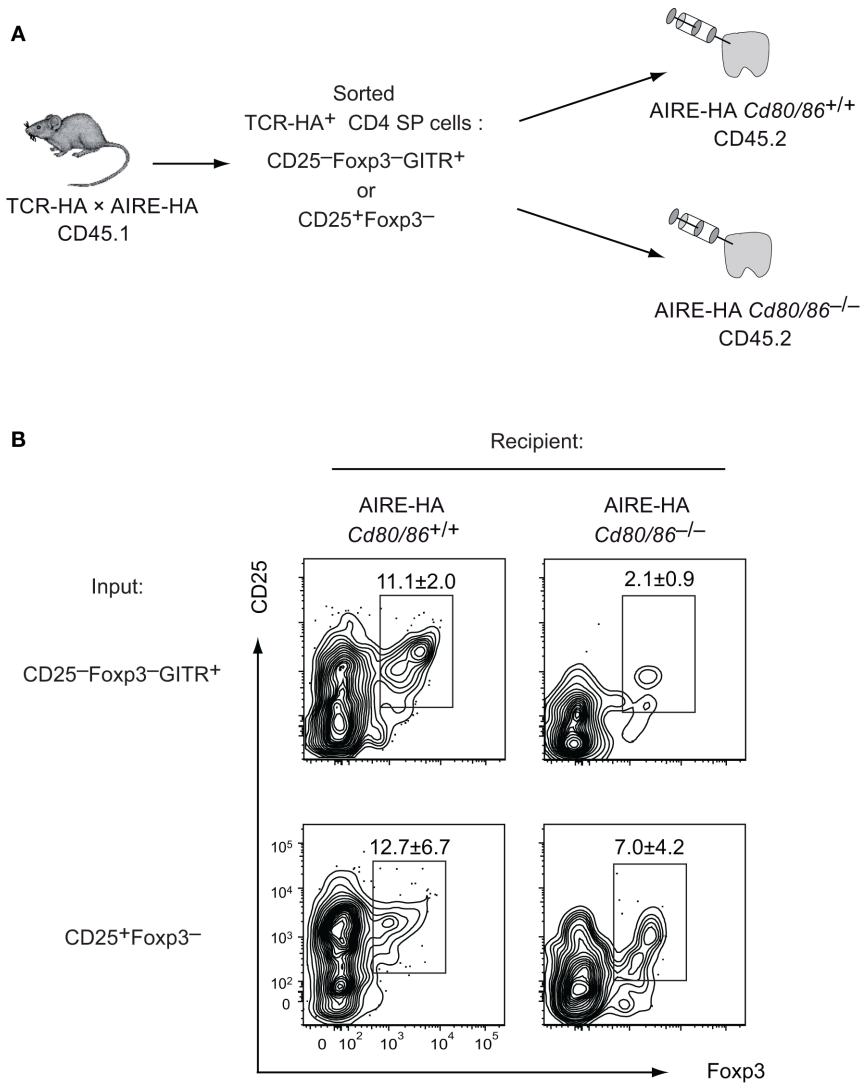

FIGURE 7 | The TCR-driven instructive but not the cytokine-dependent consolidation phase of T_{reg} differentiation requires costimulation. **(A)** Experimental design: TCR–HA$^+$ T_{reg} precursor subsets (CD25$^-$Foxp3$^-$GITR$^+$ and CD25$^+$Foxp3$^-$) were isolated from CD4 SP thymocytes of TCR–HA × AIRE–HA animals (CD45.1). 4×10^5 cells were i.t. injected into *Cd80/86*$^{+/+}$ or *Cd80/*$^{-/-}$ AIRE–HA recipients (CD45.2). **(B)** Four days after injection transferred cells were analyzed for CD25 and Foxp3 expression. Numbers indicate the average frequency (±SD) of cells within gates ($n = 3$ for transfer of CD25$^-$Foxp3$^-$GITR$^+$ cells and $n = 5$ for transfer of CD25$^+$Foxp3$^-$ cells; $P = 0.0065$ for CD25$^-$Foxp3$^-$GITR$^+$ input cells and $P = 0.15$ for CD25$^+$Foxp3$^-$ input cells).

account for the block of thymic T_{reg} development at "step one," which is believed to be TCR-driven but cytokine independent. Along these lines, we and others found that the bottleneck in T_{reg} development caused by CD28 deficiency affects a stage of T_{reg} differentiation considerably upstream of the perturbations that are caused by IL-2 deficiency (Bayer et al., 2005; D'Cruz and Klein, 2005; Fontenot et al., 2005a; Setoguchi et al., 2005; Vang et al., 2008). As already discussed above, it also appears highly unlikely that CD28 functions to merely amplify the TCR signal. Sequence analyzes of polyclonal T_{reg} cells generated in the absence or presence of costimulation also argue against this scenario (Lio et al., 2010). Thus, it was found that the residual T_{reg} cell repertoire generated in the absence of CD28 was not dramatically altered at the level of TCR specificities. Instead, the relative abundance of individual TCR specificities within the contracted T_{reg} pool of *Cd28*$^{-/-}$ mice resembled that of the WT T_{reg} repertoire, at least with regard to abundant specificities (Lio et al., 2010). On this basis, it was suggested that CD28 signaling provides signals (parallel to TCR stimulation) that facilitate T_{reg} development, but by themselves are not truly essential (Lio et al., 2010).

An alternative explanation why the polyclonal T_{reg} compartment is reduced by about 80% in *Cd28*$^{-/-}$ mice would be that some, but not other TCRs depend upon CD28 co-signals to segregate into the T_{reg} compartment. However, our observations in a TCR transgenic system are more consistent with the "facilitator" scenario, as the differentiation of quasi-monoclonal TCR–HA$^+$

T_{reg} cells is diminished by a factor of about five-fold rather than being fully abolished (or not being affected at all).

In order to explain why the defect in CD28 or B7 deficient mice is quantitative rather than qualitative, we considered the hypothesis that costimulation might foster T_{reg} generation through promoting the proliferative expansion of T_{reg} precursors rather than actually instructing their differentiation *per se*. However, we could not find any evidence that this was the case. In fact, the proliferation of T_{reg} precursors was even increased in the absence of costimulation, perhaps suggesting a compensatory mechanism. On the basis of this finding, the most plausible scenario is that CD28 signaling serves a dual, partly instructive (as *bona fide* differentiation factor) and partly permissive (as survival factor) function during T_{reg} differentiation. Of note, neither function appears to be truly essential, so that the role of costimulation is indeed perhaps better described as that of a "catalyst."

The full spectrum of molecular events downstream of CD28 signaling during T_{reg} differentiation remains to be established. However, recent work has shed light on how costimulation may support the differentiation of T_{reg} precursors through qualitatively modulating signaling events downstream of the TCR. CD28 communicates with several downstream signaling cascades through distinct motifs in its cytoplasmic tail that mediate interactions with Lck and the PI3K pathway, respectively. Several groups have reported that efficient T_{reg} cell generation does not require CD28's PI3K-binding motif, whereas the Lck-interacting P_{187}YAPP motif seems to be crucial for T_{reg} differentiation (Tai et al., 2005; Lio et al., 2010; Vang et al., 2010). Mutations in the CD28 P_{187}YAPP motif strongly diminish TCR/CD28 mediated NF-κB activation (Sanchez-Lockhart et al., 2008), and the ablation of genes involved in NF-κB activation (PKC-θ, CARMA-1, Bcl-10, IKK-2) impairs thymic T_{reg} differentiation (Schmidt-Supprian et al., 2004; Barnes et al., 2009; Medoff et al., 2009). The recent identification of c-Rel as essential NF-κB family transcription factor in T_{reg} differentiation may provide important clues as to how integrated TCR/CD28 signaling activates the transcriptional program that controls T_{reg} differentiation (Isomura et al., 2009; Long et al., 2009; Ruan et al., 2009; Deenick et al., 2010; Visekruna et al., 2010). One aspect of c-Rel's function seems to be direct control of the Foxp3 gene through binding to a DNA motif resembling the CD28-response element in the IL-2 gene (Zheng et al., 2010). It has been suggested that through opening and remodeling of the Foxp3 locus, c-Rel activation downstream of TCR/CD28 signaling may serve a *bona fide* lineage instructing function (Josefowicz and Rudensky, 2009). However, considering that T_{reg} differentiation can proceed surprisingly well in the absence of a functional Foxp3 gene (Gavin et al., 2007; Hill et al., 2007; Lin et al., 2007; Lahl et al., 2009), it appears reasonable to assume that NF-κB-signaling or other signaling pathways downstream of CD28 also initiate further – as yet unknown – instructive molecular events not related to Foxp3-induction. At the same time, it is likely that CD28 signaling in parallel elicits a transcriptional program that is of rather permissive nature. It may thereby set the stage for "step two" of intrathymic T_{reg} differentiation, for instance by up-regulating components of the IL-2 receptor (Lio et al., 2010; Vang et al., 2010). Unraveling these functions will be challenging, since the presumed lineage instructing function of IL-2 signaling in T_{reg} cells is, on the one hand, not absolute and, on the other hand, inextricably intertwined with its pro-survival function (Malek et al., 2002; D'Cruz and Klein, 2005; Fontenot et al., 2005a). Furthermore, it also remains to be established in how far CD28 costimulation may directly influence the survival of T_{reg} precursors through controlling pro-survival genes such as Bcl-x_L, akin to its function in mature, "conventional" T cells (Boise et al., 1995; Shi et al., 1995; Noel et al., 1996; Radvanyi et al., 1996). However, given the evidence that the PI3-kinase pathway is important for induction of Bcl-x_L by CD28 (Burr et al., 2001; Okkenhaug et al., 2001), yet that the PI3K interacting motif in CD28 is dispensable for efficient T_{reg} induction (Tai et al., 2005; Lio et al., 2010; Vang et al., 2010), this scenario appears less likely.

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