TCR ligand density and affinity determine peripheral induction of Foxp3 in vivo

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T cell receptor (TCR) ligation is required for the extrathymic differentiation of forkhead box p3+ (Foxp3+) regulatory T cells. Several lines of evidence indicate that weak TCR stimulation favors induction of Foxp3 in the periphery; however, it remains to be determined how TCR ligand potency influences this process. We characterized the density and affinity of TCR ligand favorable for Foxp3 induction and found that a low dose of a strong agonist resulted in maximal induction of Foxp3 in vivo. Initial Foxp3 induction by weak agonist peptide could be enhanced by disruption of TCR–peptide major histocompatibility complex (pMHC) interactions or alteration of peptide dose. However, time course experiments revealed that Foxp3-positive cells induced by weak agonist stimulation are deleted, along with their Foxp3-negative counterparts, whereas Foxp3-positive cells induced by low doses of the strong agonist persist. Our results suggest that, together, pMHC ligand potency, density, and duration of TCR interactions define a cumulative quantity of TCR stimulation that determines initial peripheral Foxp3 induction. However, in the persistence of induced Foxp3+ T cells, TCR ligand potency and density are noninterchangeable factors that influence the route to peripheral tolerance.

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Abbreviations used: Foxp3, forkhead box p3; MCC, moth cytochrome c; pMHC, peptide MHC.

CD4+ T cells recognize and respond to peptide antigens in the context of MHCII. The nature of TCR–peptide MHC (pMHC) interactions determines the stimulation threshold for positive and negative selection of T cells in the thymus, and it has also been shown to influence the lineage decisions of the developing cells (Singer et al., 2008). For example, strong TCR signals have been proposed to guide double-positive thymocytes toward the CD4 fate (Itano et al., 1996). In peripheral T cells, the potency of TCR ligand can have a profound effect on the extent of activation; higher affinity TCR–pMHC interactions generally lead to increased signaling downstream of the TCR and, subsequently, more robust proliferation and cytokine production (Davis et al., 1998; Germain and Stefanová, 1999). In addition to influencing the magnitude of the T cell response, the potency and density of pMHC affinity may also instruct CD4+ helper differentiation (Constant et al., 1995; Hosken et al., 1995; Tao et al., 1997; Rogers and Croft, 1999).

Regulatory T cell (T reg cell) differentiation and function is also dependent on TCR stimulation (Josefowicz and Rudensky, 2009; Shevach, 2009). T reg cells are defined by their expression of the winged helix/forkhead transcription factor forkhead box p3 (Foxp3) and have been shown to suppress both pathological and healthy immune responses (Sakaguchi, 2004; Fontenot and Rudensky, 2005; Belkaid, 2007). Foxp3 is required for the development, maintenance, and suppressive function of these cells, as indicated by the multiorgan autoimmunity resulting from its loss of function in both mice and humans (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003; Gavin et al., 2007; Zheng and Rudensky, 2007). Foxp3+ T reg cells can be divided into two categories based on their site of origin: thymic T reg cells and induced T reg cells, which leave the thymus as naive CD4+ Foxp3-negative T cells but then acquire Foxp3 expression and suppressor function in the periphery (Curotto de Lafaille and Lafaille, 2009).

The requirement for TCR stimulation in the thymic development of T reg cell is illustrated by the failure of TCR transgenic T cells to express Foxp3 in the absence of endogenous

TCR rearrangement, unless their cognate antigen is present (Olivares-Villagómez et al., 1998; Itoh et al., 1999; Jordan et al., 2001; Apostolou et al., 2002; Kawahata et al., 2002). The selection of T reg cells upon encounter of transgenically expressed neo-autoantigens suggests that TCR specificity for self could play a role in T reg cell development, which is consistent with a study demonstrating that T reg cell TCRs are more self-reactive than their non–T reg cell counterparts (Hsieh et al., 2004). Thymic T reg cell selection may be associated with relatively strong TCR stimulation because thymocytes expressing a TCR more weakly stimulated by its antigen were not selected to be T reg cells (Jordan et al., 2001). Another study implicated superior survival of Foxp3^{+} thymocytes in contributing to the increased frequency of T reg cells observed in TCR transgenic systems where the cognate antigen was expressed (van Santen et al., 2004). Differences in the strength of TCR–pMHC interactions could determine T reg cell selection versus deletion of self-reactive thymocytes. That TCRs preferentially used by T reg cells in wild-type mice are also present in the repertoires of Foxp3-deficient mice is consistent with the notion that these TCR–self-pMHC interactions fall between the avidity ranges resulting in positive and negative selection (Hsieh et al., 2006).

TCR specificity has also been implicated in Foxp3 expression by induced T reg cells (Lathrop et al., 2008). Stimulation of adoptively transferred TCR transgenic T cells demonstrated that peripheral Foxp3 induction is associated with suboptimal activation and inversely correlates with proliferation (Kretschmer et al., 2005). Consistent with these in vivo findings, more recent in vitro studies have suggested a mechanism by which extensive TCR stimulation is detrimental for the generation of induced T reg cells; constitutive or prolonged signaling through the Akt–PI3K–mTor pathway, which is downstream of the TCR, antagonizes the induction of Foxp3 (Haxhinasto et al., 2008; Sauer et al., 2008). Another possible explanation for the negative impact of robust TCR signaling on induced T reg cell generation is cell cycle–dependent maintenance of a silenced state of the Foxp3 locus (Josefowicz et al., 2009). Collectively, these data suggest that weak TCR stimulation is favorable for the peripheral induction of Foxp3 (Kretschmer et al., 2005; Haxhinasto et al., 2008; Sauer et al., 2008; Josefowicz et al., 2009).

Although it is clear that the level of TCR stimulation influences Foxp3 induction, several factors impact the cumulative TCR stimulation a cell receives, such as TCR ligand density and the affinity and duration of TCR–pMHC interactions. In this study, we sought to define the TCR ligand characteristics ideal for in vitro and in vivo induction of Foxp3 using TCR transgenic T cells recognizing a panel of peptide ligands with varying affinities for the TCR. Given the current assumption that weaker TCR stimulation favors peripheral Foxp3 induction, we were surprised to find that a low-affinity agonist was greatly diminished in its ability to induce a persistent population of Foxp3-expressing cells in vivo. Modulating the duration of TCR–pMHC interactions and the density of pMHC resulted in efficient initial Foxp3 induction by weak agonist
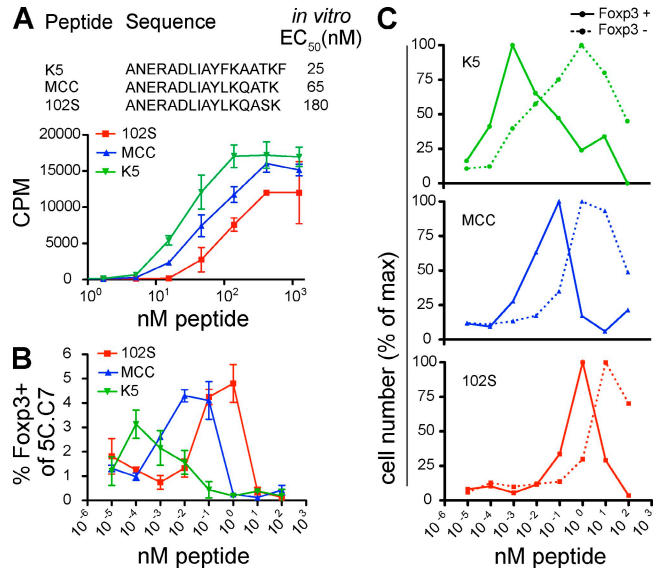

Figure 1. There is an inverse relationship between TCR/pMHC affinity and the optimal peptide concentration for Foxp3 induction in vitro. (A) EC_{50} values for the experiment shown were determined as the peptide concentration resulting in 50% of maximal proliferation. LN cells from 5C.C7 TCR transgenic RAG2^{−/−} mice were stimulated with irradiated splenocytes and the indicated peptide for 60 h. ^{3}H-methyl-thymidine was used to assess proliferation. (B and C) 5C.C7 RAG2^{−/−} LN cells were cultured with irradiated splenocytes in the presence of IL-2 and the indicated peptide for 4 d before flow cytometry analysis of the frequency (B) or number (C) of Foxp3^{+} cells. (C) Percentage of maximum cell number is shown, with Foxp3^{+} 5C.C7 represented as a solid line and Foxp3^{−} cells as a dashed line. In A and B, error bars show mean ± SD, with $n = 3$ or $n = 2$ wells, respectively. Data are representative of at least three independent experiments.

peptides in our system. However, Foxp3^{+} cells induced by the weak agonist peptides did not persist compared with those generated by the higher affinity ligand. Our data suggest that it is the cumulative level of TCR stimulation that determines initial induction of Foxp3 in the periphery but that TCR ligand density and potency are noninterchangeable factors in the persistence of the induced Foxp3^{+} population.

RESULTS

There is an inverse relationship between TCR/pMHC affinity and optimal peptide concentration for Foxp3 induction in vitro

To explore the impact of TCR/pMHC affinity on peripheral Foxp3 induction we used 5C.C7 T cells, which recognize a peptide from moth cytochrome *c* (MCC; 88–103) in the context of the MHCII molecule I-E[k] (Davis et al., 1998). The 5C.C7 TCR is ideal for addressing this question because, in addition to the natural ligand MCC, a wide variety of related peptide ligands have been characterized for their TCR binding properties and in vitro activating potencies (Reay et al., 1994; Rabinowitz et al., 1996; Wilson et al., 1999; Li et al., 2004; Krogsgaard et al., 2005). In addition to MCC, we assessed Foxp3 induction by the weak agonist peptide 102S (Reay et al., 1994; Rabinowitz et al., 1996) and the superagonist K5 (Krogsgaard et al., 2003, 2005; Li et al., 2004), both of which
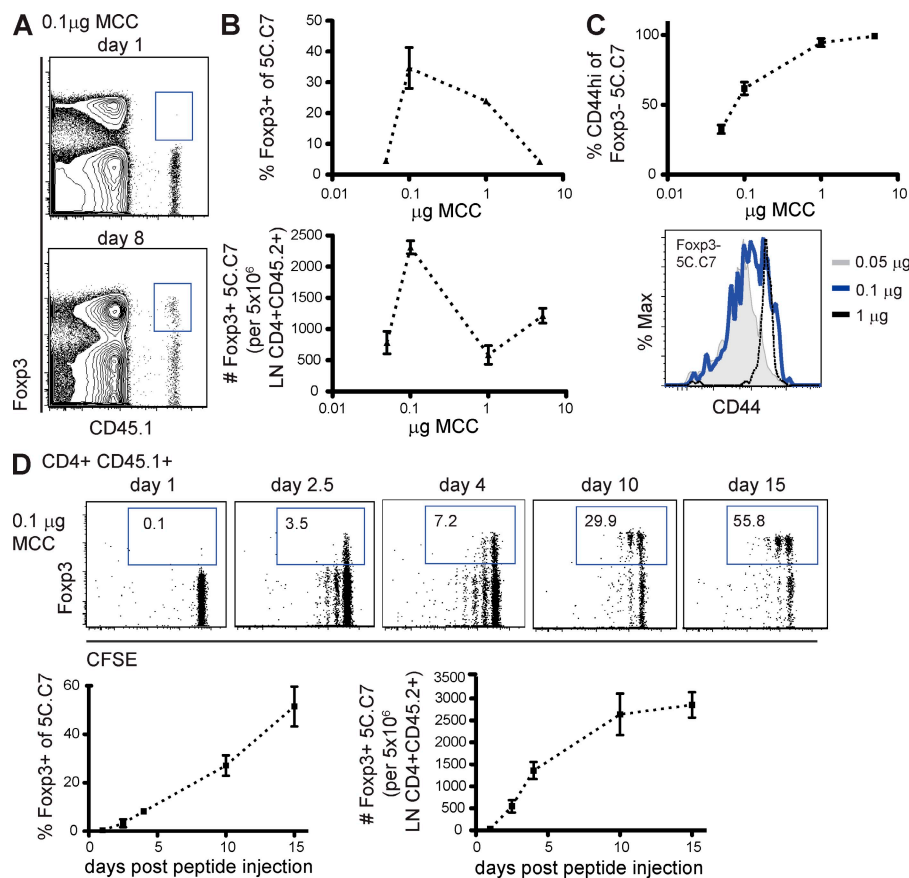
Figure 2. A single low dose of intravenous MCC peptide results in efficient peripheral induction of Foxp3. B10.A mice containing 10[6] naive adoptively transferred 5C.C7 RAG2^{−/−} CD45.1 T cells were injected intravenously with the indicated dose of MCC peptide. CD4+CD45.1+ cells were assessed for expression of Foxp3 (A and B) and CD44 (in the Foxp3− population; C). (D) A time course was performed to assess Foxp3 expression and CFSE dilution of adoptively transferred 5C.C7 at each indicated day after peptide injection. The frequency of Foxp3+ cells, as a percentage of total 5C.C7, is shown on the plots. Error bars show mean ± SD of two mice per group, and data are representative of at least three independent experiments. Dot plots in A are gated on CD4+ cells, whereas all other plots and histograms are gated on CD4+CD45.1+, unless otherwise indicated.

response to two peptides differing in their EC$_{50}$ values (Turner et al., 2009), and with in vivo data indicating that Foxp3 induction is favored by conditions suboptimal for proliferation of responding cells (Kretschmer et al., 2005).

The addition of exogenous TGF-β, a factor known to facilitate TCR-dependent Foxp3 induction (Chen et al., 2003; Selvaraj and Geiger, 2007), widened the range of TCR stimulation over which Foxp3 induction occurred in vitro (Fig. S1). Furthermore, the superagonist K5 resulted in the most Foxp3+ cells, peaking at concentrations of peptide higher than favorable for Foxp3 induction in the absence of exogenous TGF-β. Thus, in terms of Foxp3 induction, TGF-β reduces the sensitivity to strong stimulation through the TCR. This is consistent with the requirement of TGF-β and other negative regulators for Foxp3 induction in systems relying on relatively strong TCR stimulation (Chen et al., 2003; Zheng et al., 2006; Selvaraj and Geiger, 2007). Our in vitro data suggest that the combination of ligand density and affinity determine an optimal range of TCR stimulation conducive for expression of Foxp3. Sensitivity to variations in these parameters is decreased in the presence of exogenous TGF-β.

The weak agonist 102S yields diminished frequency and number of induced Foxp3+ 5C.C7 T cells in vivo

To dissect the effects of antigen density and affinity on Foxp3 induction in vivo, we opted for a single intravenous dose of peptide, which has previously been shown to tolerize a monoclonal population of antigen-specific T cells (Thorstenson and Khoruts, 2001). Low doses of MCC peptide were able to induce Foxp3 expression in adoptively transferred 5C.C7 T cells (Fig. 2 A). Over a 100-fold titration of peptide, we found 0.1 µg MCC to be optimal for peripheral Foxp3 induction,

contain mutations in TCR contact residues that result in a shorter or longer half-life of 5C.C7 TCR–pMHC interactions, respectively (Corse et al., 2010; Huppa et al., 2010). All of the peptides used in our study bind to MHC with comparable affinity (Krogsgaard et al., 2003), which is essential to separate the influences of TCR ligand potency versus density of pMHC ligand upon T cell responses. To confirm the rank order of the peptides in our experimental setting, we stimulated 5C.C7 T cells with irradiated splenocytes and the indicated titration of peptide (Fig. 1 A). As expected, at equivalent doses of peptide 102S induced less 5C.C7 proliferation and K5 more proliferation, relative to the natural ligand MCC. This difference in in vitro potency is apparent in the higher and lower EC$_{50}$ values for 102S and K5, respectively (Fig. 1 A).

To assess the ability of the various ligands to induce Foxp3 in vitro, 5C.C7 T cells were stimulated in the presence of exogenous IL-2 and a titration of peptide. Interestingly, to reach peak in vitro Foxp3 induction for each ligand, the peptide concentration had to be adjusted to compensate for affinity. Relative to MCC, a higher concentration of 102S and a lower concentration of K5 was required (Fig. 1 B). For all three peptides, the optimal concentration for in vitro Foxp3 induction is below the concentration of peptide required for expansion of the Foxp3-negative compartment within the same cultures (Fig. 1 C). These results are consistent with a recently published study, comparing in vitro Foxp3 induction and proliferation in
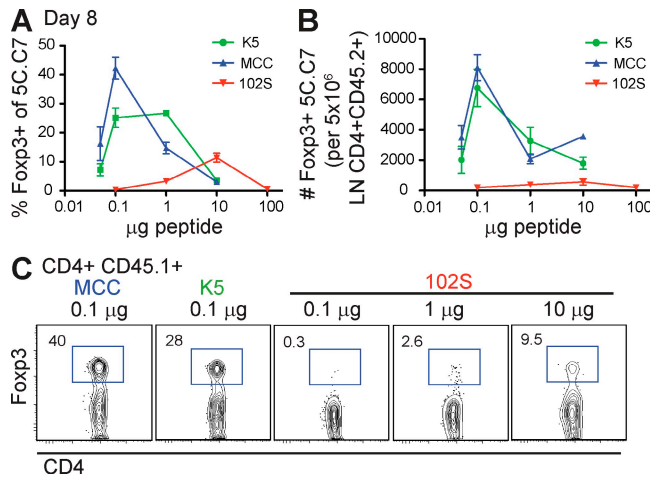
Figure 3. The weak agonist 102S results in a diminished frequency and number of induced Foxp3+ 5C.C7 T cells in vivo. B10.A recipients received 5C.C7 RAG2−/− CD45.1 T cells and were subsequently injected with the indicated dose of 102S, MCC, or K5 peptide. After 8 d, LN cells were assessed for the percentage (A) and number, as normalized to the endogenous CD4+CD45.2+ population (B), of 5C.C7 T cells expressing Foxp3. Error bars show mean ± SD of two mice per group and data are representative of at least three independent experiments. (C) Below the graphs are representative CD4+CD45.1+ gated dot plots, with the percentage of Foxp3+ cells shown on the plot.

both in terms of the percentage of 5C.C7 expressing Foxp3 and the number of 5C.C7 Foxp3+ cells (Fig. 2 B). This dose was below the threshold of activation required for complete CD44 up–regulation of the 5C.C7 Foxp3− cells (Fig. 2 C) and resulted in only partial CFSE dilution (Fig. 2 D). This suggests that in our system, induction of Foxp3 expression is coincident with a weaker TCR signal (in terms of a lower peptide dose). A time course after injection of the optimal dose showed that although Foxp3 can be detected in some 5C.C7 T cells as early as 2.5 d after immunization, peak accumulation of Foxp3+ 5C.C7 T cells occurs between 4 and 10 d after immunization (Fig. 2 D). 5C.C7 T cells induced to express Foxp3 under our experimental conditions express high levels of CD25 and CTLA-4 and display phenotypic characteristics common to T reg cells (Fig. S2). Consistent with previous studies (Kretschmer et al., 2005; Josefowicz et al., 2009), these Foxp3+ 5C.C7 T cells had undergone only a few cell divisions, with the majority remaining undivided at early time points (Fig. 2 D, days 2.5 and 4; and Fig. S2 D).

We addressed the influence of TCR/pMHC affinity on in vivo Foxp3 induction by comparing varying doses of the weak agonist 102S and the superagonist K5 with MCC 8 d after peptide injection (Fig. 3). All three peptides, including the weak agonist 102S, are able to stimulate canonical in vivo T cell responses, including proliferation, cytokine production, and memory, upon subcutaneous immunization with LPS (Corse et al., 2010). In response to intravenous peptide injection, we found K5 to induce comparable numbers of Foxp3+ 5C.C7 T cells as MCC at the same low doses of peptide (Fig. 3 B). Given results that low levels of TCR stimulation favor

Foxp3 induction (Kretschmer et al., 2005; Haxhinasto et al., 2008; Sauer et al., 2008; Josefowicz et al., 2009), we were surprised to find that the weak agonist 102S appeared diminished in its ability to induce expression of Foxp3. Although intravenous administration of 102S peptide did result in some Foxp3 expression, the percentage of 5C.C7 expressing Foxp3 was fourfold less than that observed with the optimal dose of MCC 8 d after peptide injection (Fig. 3, A and C). Furthermore, normalization to remove the effect of the Foxp3− 5C.C7 T cells from the calculation revealed a drastically smaller number Foxp3+ 5C.C7 in the 102S-treated mice, compared with both high-affinity ligands (Fig. 3 B). We detected even less Foxp3 induction upon further increase of the dose to 100 µg (Fig. 3, A and B). This indicates that optimal Foxp3 induction by the 102S ligand cannot be accomplished by further increasing the concentration of peptide. This could not be attributed to decreased in vivo stability of 102S compared with MCC, as both peptides appeared to be cleared at a comparable rate (Fig. S3).

Disruption of TCR–pMHC interactions in vivo can inhibit proliferation while enhancing Foxp3 induction

To better understand the diminished ability of 102S to induce Foxp3 in vivo, we examined proliferation of Foxp3+ and Foxp3− 5C.C7 T cells in response to the various peptides and doses. In contrast to stimulation that favored Foxp3 induction (0.1 µg MCC; Fig. 2D), 10 µg 102S resulted in proliferation by the majority of 5C.C7 T cells (Fig. 4 A, left). We hypothesized that decreasing the level of cumulative TCR stimulation in conditions where the majority of 102S-stimulated cells were dividing would favor Foxp3 induction. We sought to do this by manipulating the duration of TCR stimulation. It has previously been demonstrated using two-photon imaging that T cell–APC interactions can be disrupted in vivo by injection of an MHCII antibody after peptide injection (Celli et al., 2007). Using this approach, we interfered with TCR–pMHC interactions at various times after peptide administration by injecting a monoclonal antibody to the MHCII molecule I-E^k (Fig. 4).

Strikingly, injection of anti–MHCII between 6 to 16 h after peptide administration resulted in enhanced Foxp3 induction by the weak agonist 102S (Fig. 4, A and B), which was assayed 6 d after peptide injection. This was true both in terms of the frequency and number of Foxp3+ 5C.C7 T cells (Fig. 4, B and C). Disrupting TCR–pMHC interactions during the hours in which formation and persistence of stable T cell–APC contacts dictate subsequent effector function (Hugues et al., 2004; Miller et al., 2004; Celli et al., 2007; but not 36 h after peptide injection; Fig. 4, A and B), lowered the level of cumulative stimulation, as assessed by CFSE dilution, and changed the response of the 5C.C7 T cells. This resulted in an increased frequency of Foxp3-expressing cells, whereas proliferation of the entire 5C.C7 population was reduced (Fig. 4, D and E). In contrast, administration of anti-MHCII subsequent to stimulation with the optimal dose of MCC results in fewer Foxp3+ 5C.C7 T cells (Fig. S4), suggesting that the reduced level of TCR stimulation
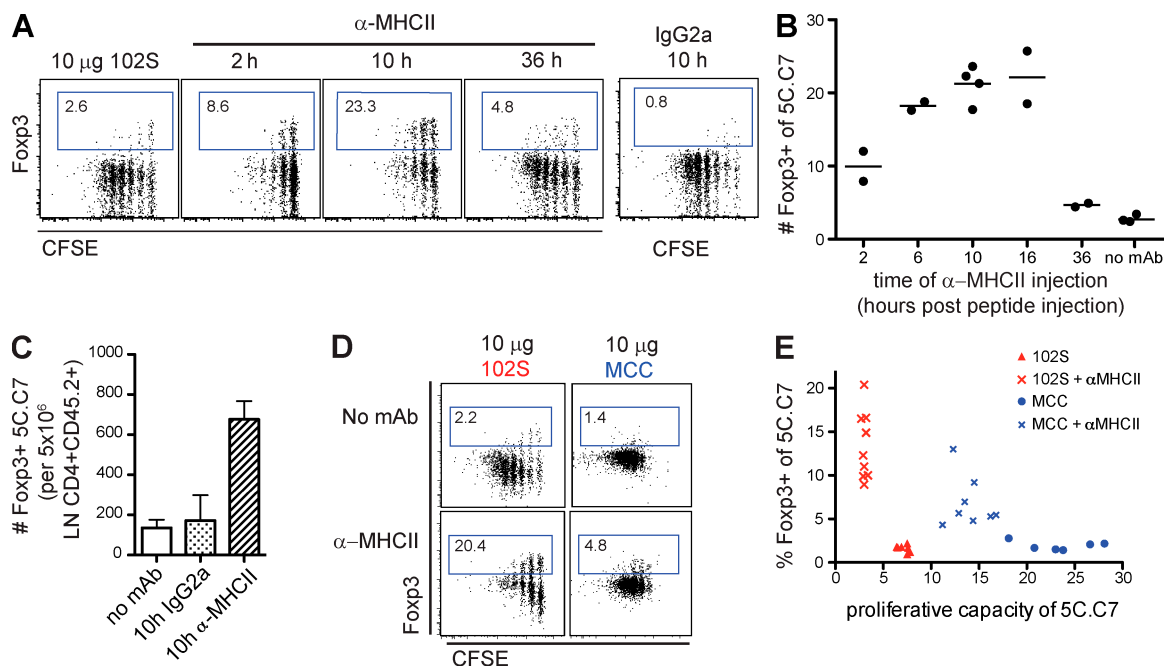
Figure 4.   Disruption of TCR–pMHC interactions in vivo can inhibit proliferation while enhancing Foxp3 induction. B10.A recipients were adoptively transferred with naive CFSE-labeled CD45.1+ 5C.C7 T cells and subsequently injected with the indicated peptide followed by intravenous anti-MHCII at the indicated times. CD45.1+ LN cells were analyzed 6 d after peptide injection. (A and B) Antibody to MHCII was injected at varying time points after injection with 10 µg 102S, and the frequency of Foxp3+ 5C.C7 T cells was assessed. Representative dot plots (A) are shown beside data pooled from two independent experiments. Each point represents one mouse and horizontal bars indicate the mean (B). (C) The influence of anti-MHCII injection on the number of Foxp3+ 5C.C7 T cells was addressed by normalizing to the endogenous CD4+CD45.2+ population. n = 2 with SD. (D and E) Anti-MHCII was injected between 8 and 10 h after injection of either MCC or 102S, as indicated. 5C.C7 CFSE dilution and Foxp3 expression was assessed (D). Percentage of 5C.C7 expressing Foxp3 versus the proliferative capacity of the total 5C.C7 population are analyzed in a scatter plot. Data are pooled from at least three independent experiments, each data point representing one mouse (E). Dot plots are gated on CD4+CD45.1+ cells, and the percentages that are positive for Foxp3 are shown. All data are representative of at least three independent experiments.

under these conditions falls below the range conducive for Foxp3 induction.

Slightly reduced proliferation and increased Foxp3 expression upon blockade was seen in mice injected with 10 µg MCC, with the effect being less drastic than for 102S (Fig. 4, D and E). This may be explained by the observation that 102S-stimulated cells express the proliferation antigen ki67 with a temporal delay, compared with MCC at the same dose of peptide, indicating that the cells responding to stimulation with the lower affinity ligand may require more time to enter the cell cycle (Fig. S5 A). At 10 µg, the majority of MCC-stimulated cells are ki67+ by 24 h, whereas ki67 expression is not detected in most 102S-stimulated cells until after 48 h (Fig. S5 A). Thus, disruption of TCR–pMHC interactions after 102S injection resulted in an increased portion of 5C.C7 T cells below the threshold of stimulation required for ki67 expression, whereas cells stimulated with 10 µg MCC are all ki67+ by 48 h, regardless of antibody treatment (Fig. S5 B). This is consistent with the modest effect of MHC blockade on the proliferative capacity and Foxp3 expression of 5C.C7 T cells responding to MCC (Fig. 4, D and E) but does not exclude the possibility that there are doses of MCC at which Foxp3 induction could be more efficiently enhanced by MHC blockade.

Foxp3+ 5C.C7 induced by injection of weak agonist peptide do not persist

Considering that disruption of TCR–pMHC interactions in vivo leads to reduced proliferation coincident with Foxp3 induction, we examined whether there was a dose of 102S that would stimulate cell division, which is comparable to low doses of MCC. In this more refined in vivo titration, we also included the peptide 102N, another weak agonist that induces comparable or slightly less in vitro IL-2 production and conjugate formation than 102S (Egen and Allison, 2002). A titration of MCC, 102S, and 102N in vivo showed that dose could compensate for pMHC potency in stimulating comparable amounts of proliferation. There was a dose of each peptide that resulted in a similar intermediate level of CFSE dilution 5 d after peptide injection (0.3 µg MCC, 3 µg 102S, and 10 µg 102N; Fig. 5, A and B [boxed data points]; and Fig. S6 A, full titration). This readout suggests that all three peptides are capable of achieving a similar level of TCR stimulation. Consistent with this, 2 d after stimulation with these doses of MCC and 102S we detected equivalent levels of ki67 expression and Akt phosphorylation, which represents the degree of activity of Akt kinase and mTORC2 complex (Fig. 5 E). This comparable amount of Akt phosphorylation is of
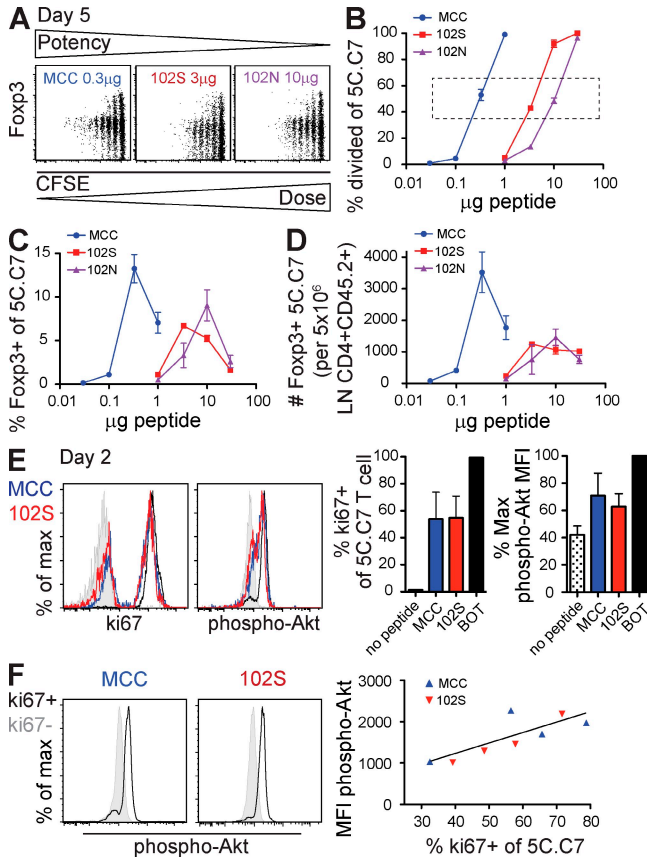
Figure 5. In vivo, peptide dose compensates for potency to reach comparable levels of proliferation and Akt phosphorylation, but not numbers, of Foxp3–expressing 5C.C7 T cells. Naive 5C.C7 RAG2^{−/−} CD45.1 T cells were adoptively transferred and stimulated in vivo by intravenous injection of the indicated peptide and dose. All dot plots and histograms are gated on CD4+CD45.1+ LN cells. (A) Day-5 representative dot plots for each peptide at the doses yielding comparable frequencies of divided cells and peak of Foxp3 induction: 0.3 µg MCC, 3 µg 102S, and 10 µg 102N. The percentage of divided (B), percentage of Foxp3+ (C), and number of Foxp3+ 5C.C7 T cells (D) 5 d after injection of the indicated peptide and dose are shown with SD. $n = 2$. The dashed rectangle in B indicates the optimal peptide doses. (E and F) 2 d after injecting doses of MCC and 102S that yield comparable CFSE dilution (0.3 µg MCC and 3 µg 102S), ki67 expression and Akt phosphorylation in total 5C.C7 T cells were measured by flow cytometry. Bar graphs in E show data pooled from three independent experiments with SD. As a control, Akt phosphorylation was compared with the maximum resulting from immunization at the base of tail with MCC and LPS, which results in a substantial portion of pAkt+ 5C.C7 T cells. Histograms in F separate CD4+CD45.1+ cells by ki67 expression, adjacent to linear regression analysis of the percentage of 5C.C7 expressing ki67 versus the phospho-Akt MFI for the total 5C.C7 population, for individual samples (best fit line shown: $R^2 = 0.647$; $P = 0.016$). All data are representative of at least three independent experiments.

particular interest considering recent studies reporting that the Akt–mTor pathway, which is essential for T cell activation and proliferation, may antagonize peripheral Foxp3 induction (Haxhinasto et al., 2008; Sauer et al., 2008). The level of phospho-Akt correlated with ki67 expression, regardless of stimulating peptide (Fig. 5 F).

As expected, the dose for each peptide that triggered partial division of the responding 5C.C7 population was also the peak of Foxp3 induction, based on the percentage of Foxp3+ 5C.C7 T cells (0.3 µg MCC, 3 µg 102S, and 10 µg 102N; Fig. 5, B and C). Although the peak of Foxp3 induction by 102S peptide in this more refined titration (3 µg) produced slightly more Foxp3 induction than 10 µg, both doses still resulted in diminished numbers of Foxp3+ 5C.C7 T cells compared with MCC. The numbers of 102S–induced Foxp3+ 5C.C7 T cells were lower at day 8 (Fig. 3) than at day 5 (Fig. 5 D). Thus, to gain more insight into the fate of cells stimulated by these strong and weak agonists, we quantitated the number of Foxp3+ and Foxp3− 5C.C7 T cells over time (Fig. 6 and Fig. S7).

We found that intravenous injection with either MCC or 102S resulted in a dose-dependent expansion and sharp contraction, just after the peak of the response (Fig. S7, A and B). As a control, we immunized with the same dose of peptide subcutaneously, at the base of tail with LPS, which generates a more typical effector response and does not result in such drastic cell loss (Fig. S7 A). The contraction of 5C.C7 T cells responding to intravenous peptide injection is consistent with studies reporting deletion of T cells stimulated by intravenous peptide as a mechanism of tolerance (Kearney et al., 1994; Liblau et al., 1996). When comparing the doses of MCC, 102S, and 102N that result in a comparable level of proliferation, we observed a similar decline in number of 5C.C7 T cells in both the LNs and the spleen (Fig. S7, C and D). This apparent deletion was coincident with the presence of a fraction of 5C.C7 T cells positive for active caspases, which was not present in recipients that did not receive peptide injection and is consistent with cell death in response to tolerizing antigen administration (Fig. S7 E).

The two weak agonist peptides assessed in our system are able to induce a substantial frequency of Foxp3+ 5C.C7 T cells if the right dose of peptide is administered. At early time points, all three peptides induce a comparable percentage of Foxp3+ 5C.C7 (Fig. 6, days 2.5 and 5). However, Foxp3+ 5C.C7 induced by the low-affinity ligands disappear quickly after the peak of the response, within the same time frame as the Foxp3− compartment, whereas a low dose of MCC results in increased frequencies and numbers of Foxp3+ 5C.C7 throughout the course of the experiment (Fig. 6). Considering the loss of the Foxp3− 5C.C7 T cells in all of these conditions (Fig. 6 A), it is the persistence of the Foxp3+ 5C.C7 population after low-dose MCC stimulation that is specific, rather than the deletion of either 5C.C7 compartment after stimulation with weak ligand. We do not attribute the difference in Foxp3+ 5C.C7 persistence to an inability of 5C.C7 T cells responding to 102S peptide to produce IL-2 (Fig. S8).

Our measurements of the degree of proliferation and initial Foxp3 induction suggest that increasing the dose of 102S results in the same quantity of stimulation as received by MCC-stimulated cells. However, time course experiments revealed that a low dose of the strong agonist peptide, but not a high dose of weak agonist, was able to induce a persistent population
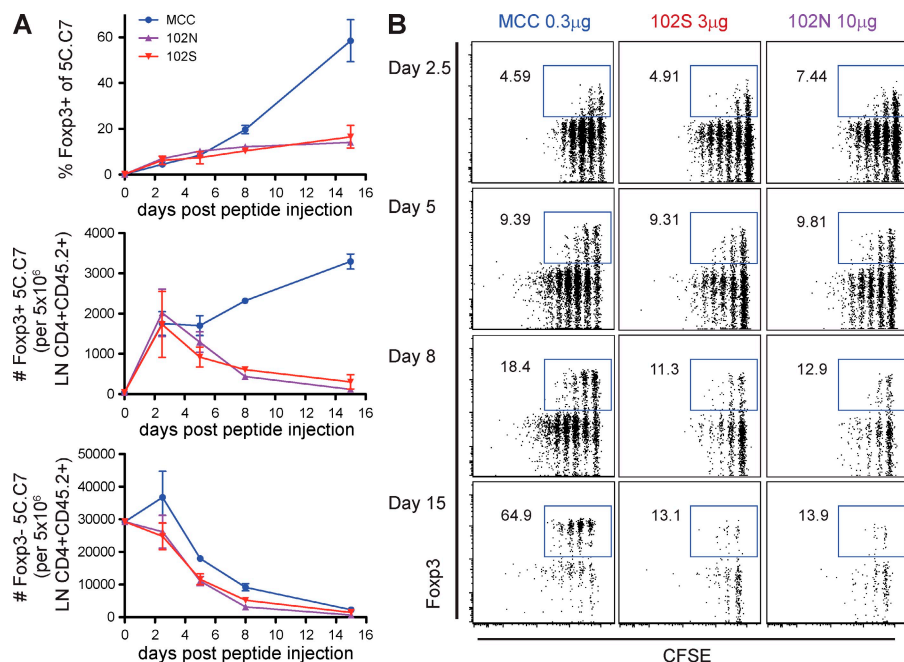
Figure 6. Foxp3+ 5C.C7 induced by injection of weak agonist peptide do not persist, compared with the high-affinity ligand MCC. After adoptive transfer of 5C.C7 RAG2$^{-/-}$ CD45.1 T cells, recipient mice were injected with varying doses of the indicated peptides, which have been previously determined to give equivalent CFSE dilution and peak Foxp3 induction (0.3 μg MCC, 3 μg 102S, and 10 μg 102N), and then sacrificed at the indicated time points after peptide injection. (A) The percentage of Foxp3$^+$ and numbers of Foxp3$^+$ and Foxp3$^-$ 5C.C7 in LN were assessed at each time point. Error bars show SD of two mice per group. Representative dot plots gated on CD4$^+$CD45.1$^+$ cells are shown in B, with the percentage of Foxp3-positive 5C.C7 shown on the plot. Data are representative of at least three independent experiments.

of Foxp3$^+$ T cells. Thus, there are two phases that must be considered. In the first phase, antigen dose can compensate for potency to achieve the optimal quantity of TCR stimulation for initial Foxp3 induction, whereas in the second phase, high doses of peptide are detrimental to the persistence of the stimulated 5C.C7 T cells, including the induced Foxp3$^+$ T cells in the case of the weak agonist peptide. This highlights limitations of comparing in vivo and in vitro systems, the latter of which do not address the issue of persistence.

DISCUSSION

Foxp3$^+$ T reg cell suppression of self-reactive effector T cells is critical for the prevention of autoimmunity (Sakaguchi, 2004). Peripheral induction of Foxp3 in mature CD4$^+$ T cells may be an important mechanism of maintaining this self-tolerance. The partial overlap of TCR repertoires of induced T reg cells and thymic T reg cells indicates that peripheral Foxp3 induction could potentially offer a second chance for self-reactive cells that escape thymic selection to enter the T reg cell lineage (Lathrop et al., 2008). Alternatively, cells could convert into T reg cells in response to non–self-antigens presented in tolerizing conditions (Curotto de Lafaille and Lafaille, 2009). Understanding the conditions optimal for peripheral Foxp3 induction in vivo could provide insight into how this process may be manipulated in a therapeutic setting.

Several studies altering antigen dose and degree of Akt and/or mTor activity have shown that efficient Foxp3 induction occurs in conditions suboptimal for activation (Kretschmer et al., 2005; Haxhinasto et al., 2008; Sauer et al., 2008), suggesting that a weaker TCR stimulation favors peripheral conversion. It was less clear how defined TCR/pMHC binding parameters, such as affinity and off rate, influence this process. In this study, we address the cumulative influence of antigen

density and the strength of TCR–pMHC interactions on the peripheral induction of Foxp3 in vivo.

Upon comparing ligands of varying affinities across an in vitro peptide titration, we found that there was a limited range of TCR stimulation over which Foxp3 induction occurred. Relative to the natural ligand MCC, peak Foxp3 induction by the superagonist K5 occurred at lower concentrations of peptide, whereas the peak induction by the weak agonist 102S occurred at higher concentrations. All three ligands were able to induce a comparable magnitude of in vitro conversion of 5C.C7 T cells, so long as peptide concentration was altered to compensate for a higher or lower affinity ligand. For each peptide, the TCR stimulation range favoring conversion was below the concentration of peptide required for expansion of the nonconverted cells. The addition of exogenous TGF-β widened the range of TCR stimulation over which conversion occurred, which allowed for Foxp3 induction at concentrations of peptide that were otherwise too high. This is consistent with previously published reports demonstrating the requirement for TGF-β and other negative regulators of T cell activation in scenarios where T cells are receiving high levels of TCR stimulation (Chen et al., 2003; Zheng et al., 2006; Selvaraj and Geiger, 2007).

When MCC, K5, and 102S were titrated in vivo, we did not observe the same simple relationship between ligand affinity and optimal Foxp3 induction dose seen in vitro. The superagonist peptide K5, which peaked at concentrations of peptide 100-fold lower than MCC in vitro, was comparable to MCC in vivo, resulting in efficient conversion of 5C.C7 T cells at the same low dose. Strikingly, stimulation with the weak agonist 102S resulted in drastically reduced numbers of Foxp3$^+$ 5C.C7 T cells 8 d after peptide injection, compared with either of the strong ligands. This was surprising given the studies reporting

that weaker TCR stimulation favors Foxp3 induction (Kretschmer et al., 2005; Haxhinasto et al., 2008; Sauer et al., 2008). Thus, we went on to modulate multiple parameters influencing the level of TCR stimulation received by 102S-stimulated cells to optimize Foxp3 induction, and subsequently assessed the frequencies and numbers of Foxp3+ 5C.C7 T cells induced by the strong and weak agonists over time.

Upon assessing proliferation of cells responding to 10 µg 102S, the dose where we had observed a detectable frequency of Foxp3+ 5C.C7, it appeared that induction of Foxp3 by 5C.C7 T cells responding to 10 µg 102S was accompanied by proliferation more robust than that in conditions that are optimal for Foxp3 induction (i.e., 0.1 µg MCC peptide). The idea that such a strong stimulation would be detrimental to Foxp3 induction is consistent with studies demonstrating that Akt activity can antagonize expression of Foxp3 (Haxhinasto et al., 2008; Sauer et al., 2008). In addition, proliferation may oppose Foxp3 induction by cell cycle–dependent recruitment of maintenance DNA methyltransferase, which results in a silenced state of the Foxp3 locus (Josefowicz et al., 2009).

Considering these findings, we sought to decrease the cumulative TCR stimulation in response to this high density of 102S pMHC complexes by injecting antibodies to MHCII 6–16 h after injection of peptide. During this time period, T cell–APC contacts form and persist, and antibodies to MHCII have been shown to result in disruption of T cell–APC interactions (Hugues et al., 2004; Miller et al., 2004; Celli et al., 2007). By blocking TCR–pMHC interactions, we were able to both decrease the frequency of 102S-stimulated cells entering cell cycle and greatly lower their overall proliferative capacity, which correlated with increased initial induction of Foxp3. These results highlight the influence of pMHC potency, density, and duration of interactions with TCR on the overall quantity of TCR stimulation that a cell receives and, thus, its decision to divide or express Foxp3.

Together, our data are in agreement with the idea that weak TCR stimulation that is suboptimal for proliferation favors initial Foxp3 induction. Indeed, a refined titration of peptides in vivo revealed that both MCC and the two weak ligands 102S and 102N could produce this favorable quantity of TCR stimulation if the dose was adjusted to compensate for ligand strength. At these doses, which span nearly two orders of magnitude between MCC and 102N, stimulation by all three peptides resulted in equivalent Foxp3 induction, based on the frequency of Foxp3+ 5C.C7 at early time points. In conditions favoring optimal Foxp3 induction, there was no difference in the ability of strong and weak ligands to trigger Akt phosphorylation, which simply correlated with ki67 expression, regardless of stimulating peptide.

Considering only the initial induction of Foxp3, our data varying ligand dose, potency, and duration of TCR–pMHC interactions in vivo would suggest that perhaps it is simply the quantity of TCR stimulation that is important. This would be consistent with a model in which all of these factors contribute to a cumulative signal, which determines T cell responses (Rosette et al., 2001; Rachmilewitz and Lanzavecchia, 2002).

Our system suggests that the range of TCR stimulation favoring this process is very narrow, as the dose response for all three peptides is very steep. Based on our in vitro data, where the addition of TGF-β allowed for Foxp3 induction in response to higher concentrations of peptide, we would expect that a particular cytokine environment could also widen the range of TCR stimulation over which Foxp3 induction could occur in vivo. In addition, our results suggest that it may be conceivable to expand the range of antigenic conditions over which Foxp3 induction can occur in a therapeutic setting through manipulation of the duration of TCR–pMHC interactions. Disrupting T cell–APC interactions allowed us to decrease the cumulative TCR stimulation, resulting in Foxp3 induction when ligand density would otherwise be unfavorable. In doing so, it may be possible to alter the frequency of antigen-specific T reg cell, which could skew a potentially pathogenic T cell response toward tolerance.

Despite finding doses of 102S and 102N that yielded similar proliferation and initial Foxp3 induction compared with optimal doses of MCC, these weak agonists still generated diminished numbers of Foxp3+ 5C.C7 T cells at longer times after peptide injection. Time course experiments revealed that at the doses of each peptide yielding optimal initial Foxp3 induction, injection of weak agonist peptides resulted in similar deletion of both the Foxp3 negative and positive populations, whereas stimulation with MCC resulted in specific persistence of the induced Foxp3+ population of 5C.C7 T cells. Thus, there are two phases influencing the numbers of Foxp3+ 5C.C7 observed after in vivo stimulation. During initial induction of Foxp3, antigen dose could compensate for potency to achieve the optimal quantity of TCR stimulation, whereas in the second phase, maintenance versus deletion of this population appeared to be determined by unique stimulation criteria. This highlights the importance of studying in vivo systems, as persistence is not addressed in short term in vitro assays. Low-potency ligands are efficient at achieving the weak TCR signals favoring Foxp3 induction both in vitro and in vivo. However, our data demonstrate that these interactions do not necessarily induce a persistent population of Foxp3+ T cells in vivo.

Our findings demonstrate that ligand potency and density are noninterchangeable factors. Increasing the dose of low-potency ligand appeared to achieve a similar level of cumulative TCR stimulation (comparable proliferation, Akt phosphorylation, and Foxp3 induction at early time points) but failed to generate persistent Foxp3+ 5C.C7, compared with the strong agonist MCC. Therefore it is not simply a matter of getting the same quantity of TCR stimulation; there are qualitative differences that must be considered. This difference in cell fate suggests that there may be mechanisms allowing T cells to discriminate between signals comings from a few strong TCR–pMHC interactions, versus many complexes with weaker interactions. One can imagine the triggering of survival signals specifically downstream of a strong TCR–pMHC interaction, as opposed to proapoptotic signals which are induced by the triggering of many separate TCRs. There is

precedent for cell-intrinsic mechanisms of ligand discrimination via negative signaling feedback loops, which is proposed to be important in the discrimination of self- and foreign ligands (Stefanová et al., 2003). In addition, it has been suggested that the density of antigen may influence TCR signal inhibition, based on an in vivo model comparing tolerance in response to high and low levels of self-antigen (Singh and Schwartz, 2003).

Although is probable that cell-intrinsic mechanisms could be in place to aid in discrimination between high-potency versus high-density ligands, it is also possible that cell-extrinsic factors play a role in this process. This seems especially important considering that the maturation state of APCs is known to play an important role in T cell fate under tolerizing conditions. Low levels of costimulation favor Foxp3 induction (Benson et al., 2007), anergy, and deletion (Hawiger et al., 2001). Both dose and potency of peptide ligand have been reported to influence the kinetics and type of effector T cell–APC contact formation (Bousso and Robey, 2003; Skokos et al., 2007; Henrickson et al., 2008). Thus, it is possible that low doses of MCC and high doses of 102S achieve comparable cumulative TCR stimulation, but in the context of different types of T cell–APC interactions. For example, very low concentrations of peptide may only result in short interactions, which, given a potent ligand, may be sufficient to provide the minimal stimulation required for Foxp3 induction. In this scenario, more stable T cell–APC interactions resulting from higher concentrations of peptide could play a role in the subsequent deletion of these cells. Transient TCR–pMHC interactions upon low-dose injection of peptide could explain the similarity in levels of Foxp3 induction by MCC and K5 we observed, as the increased potency of the high-affinity superagonist may be minimized in the absence of more stable conjugate formation.

We propose that a low density of a high-affinity TCR ligand is optimal to achieve the quantity and quality of stimulation required to induce a persistent population of Foxp3+ T cells in vivo. In fact, considering that the Foxp3− 5C.C7 T cells are deleted in these same conditions, and that injection of high doses of either MCC or 102S results in comparable loss of 5C.C7 T cells, specificity is most apparent in the survival of the Foxp3+ 5C.C7 in response to small amounts of strong ligand. Deletion and Foxp3 induction are two proposed mechanisms of peripheral tolerance. However, how the strength and density of TCR ligand steers a self-reactive T cell between these fates is understudied. TCR repertoire analysis has suggested that T reg cells may recognize self-antigens with low avidity (Hsieh et al., 2004). We suggest that low concentrations of specific self-pMHC complexes could contribute to peripheral Foxp3 induction in response to self-antigens, even if the TCR–pMHC interaction is of a high affinity. In the case of more abundantly expressed self-pMHC complexes, our data suggest that deletion of the cognate T cells is a more likely mechanism of tolerance, regardless of the strength of the ligand and extent of Foxp3 induction. It is interesting to speculate that there could be detrimental effects of maintaining T reg cells that recognize ubiquitous self-ligands, such as bystander suppression (Masteller et al., 2005; Tang and Bluestone, 2008).

In summary, initial in vivo Foxp3 induction can be achieved by optimizing the quantity of cumulative TCR stimulation, which can be thought of as a sum of the factors of ligand potency, density, and duration of TCR–pMHC interactions. However, TCR ligand potency and density are clearly noninterchangeable in determining the persistence of the cells induced to express Foxp3. These findings provide important insight into how affinity and prevalence of antigen may determine the route to peripheral tolerance.

MATERIALS AND METHODS

Mice. 5C.C7 TCR transgenic RAG2$^{-/-}$ mice (Taconic) were bred to B10.A CD45.1 (provided by W. Paul via the National Institute of Allergy and Infectious Diseases contract facility at Taconic) to generate 5C.C7 RAG2$^{-/-}$ CD45.1 mice, which were used as donor mice in adoptive transfer experiments. Male B10.A mice (Taconic) were used as adoptive transfer recipients at between 6 and 9 wk of age. All mice were maintained in microisolator cages, and treated in accordance with the regulations of the National Institutes of Health and the American Association of Laboratory Animal Care. Experiments in this study were approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee.

In vitro T cell activation and Foxp3 induction. Cells were cultured in a 37°C humidified chamber with 5% CO$_2$ in complete RPMI1640 (supplemented with 10% FCS, 2 μM glutamine, 100 U/ml penicillin and streptomycin, and 2 μM 2-mercaptoethanol). Single cell suspensions were prepared from LNs harvested from 5C.C7 RAG2$^{-/-}$ mice (routinely >90% Vα11+Vβ3+ by flow cytometry) and stimulated in duplicate or triplicate, as indicated, in 96-well round bottom plates. In T cell proliferation assays, 3×10^4 5C.C7 cells were stimulated with 2×10^5 irradiated splenocytes and the indicated peptides for 60 h. 1 μCi/well ^{3}H-methyl-thymidine was used to monitor T cell proliferation. Cells were harvested onto glass-fiber filters using a Tomtec harvester, and filters were counted using a MicroBeta scintillation counter (PerkinElmer). For in vitro T reg cell conversion assays, 10^5 5C.C7 T cells were cultured with 4×10^5 irradiated splenocytes that had been depleted of T cells using CD90.2 microbeads (Miltenyi Biotec), the indicated peptide, and 100 U/ml of recombinant human IL-2 (PeproTech), with or without human TGF-β1 (PeproTech). Cells were cultured for 90–96 h before cells were stained for flow cytometry analysis. In some cultures the absolute number of cells was determined using an automated cell counter (Guava Technologies Inc.).

Peptides. Peptides were synthesized and HPLC purified (≥95%) by Biosynthesis, Inc.

Flow cytometry and antibodies. Flow cytometry was done on an LSRII (BD). Antibodies to surface markers were obtained from BD, eBioscience, or BioLegend. Foxp3 was detected using antibodies (eBioscience) and fixation/permeabilization reagents. For detection of phosph-Akt (Ser473), we used an Alexa Fluor 647–conjugated antibody (Cell Signaling Technology; DE9), after immediate fixation with 2% paraformaldehyde and subsequent permeabilization with cold methanol. CTLA-4 and ki67 antibodies (BD) were detected after fixation and permeabilization as required for Foxp3 or phospho-Akt analysis, depending on the experiment.

In vivo Foxp3 induction. Between 5×10^5 and 1×10^6 5C.C7 RAG2$^{-/-}$ CD45.1 T cells were transferred into B10.A recipients by tail vein injection. Mice were subsequently immunized intravenously the same or next day with 0.03–100 μg of peptide diluted in PBS. When disruption of T cell–APC interactions was desired, 500 μg of monoclonal antibody to the mouse MHC

class II molecule I-E^k (clone 14–4-4S; American Type Culture Collection; produced by Memorial Sloan Kettering Cancer Center [MSKCC] Monoclonal Antibody Core Facility [MACF]) or isotype control antibody (clone 20–8-4s, recognizing H-2Kb, also produced by the MSKCC MACF) was injected into the tail vein at the indicated time point after peptide injection. In some experiments cells were CFSE labeled before transfer. To enrich for 5C.C7 T cells before flow cytometry analysis, CD4$^+$ T cells were purified from LN suspensions by Dynal negative selection (Invitrogen).

Analysis. Flow data were analyzed using FlowJo (version 8.8.6; Tree Star, Inc.) and further processed using either Prism 5.0 (GraphPad Software, Inc.) or Excel (Microsoft). Proliferative capacity was calculated from CFSE profiles as previously described (Gudmundsdottir et al., 1999).

Online supplemental material. Fig. S1 shows the influence of exogenous TGF-β on in vitro Foxp3 induction by peptides of varying potency. Fig. S2 shows characteristics of Foxp3$^+$ 5C.C7 T cells that are common to T reg cells. Fig. S3 demonstrates comparable stability of 102S and MCC peptides in vivo. Fig. S4 shows the impact of MHCII blockade on Foxp3 induction by an optimal low dose of MCC. Fig. S5 contains analysis of ki67 expression after in vivo stimulation with varying doses of MCC and 102S, with and without disruption of TCR–pMHC interactions. Fig. S6 includes additional peptide doses from titration and timecourse experiments shown in Figs. 5 and 6. Fig. S7 characterizes loss of 5C.C7 T cells after injection of intravenous peptide, including dose dependence and expression of active caspases. Fig. S8 shows ex vivo IL-2 production by 5C.C7 T cells, harvested 6 d after in vivo peptide stimulation. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091999/DC1.

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