CD25<sup>+</sup>Foxp3<sup>+</sup> Regulatory T Cells Facilitate CD4<sup>+</sup> T Cell Clonal Anergy Induction during the Recovery from Lymphopenia

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CD25<sup>+</sup>Foxp3<sup>+</sup> Regulatory T Cells Facilitate CD4<sup>+</sup> T Cell Clonal Anergy Induction during the Recovery from Lymphopenia<sup>1</sup>

Tracy L. Vanasek,* Sarada L. Nandiwada,* Marc K. Jenkins, † and Daniel L. Mueller<sup>2*</sup>

T cell clonal anergy induction in lymphopenic nu/nu mice was found to be ineffective. Exposure to a tolerizing peptide Ag regimen instead induced aggressive CD4<sup>+</sup> cell cycle progression and increased Ag responsiveness (priming). Reconstitution of T cell-deficient mice by an adoptive transfer of mature peripheral lymphocytes was accompanied by the development of a CD25<sup>+</sup>Foxp3<sup>+</sup>CTLA-4<sup>+</sup>CD4<sup>+</sup> regulatory T cell population that acted to dampen Ag-driven cell cycle progression and facilitate the induction of clonal anergy in nearby responder CD25<sup>+</sup>CD4<sup>+</sup> T cells. Thus, an early recovery of CD25<sup>+</sup> regulatory T cells following a lymphopenic event can prevent exuberant Ag-stimulated CD4<sup>+</sup> cell cycle progression and promote the development of clonal anergy. The Journal of Immunology, 2006, 176: 5880–5889.

Individuals with primary immunodeficiency and reduced lymphocyte counts are at heightened risk for the development of autoimmunity (1). Likewise, lymphopenia is not uncommon in systemic inflammatory diseases such as systemic lupus erythematosus (SLE), and those affected with rheumatoid arthritis or multiple sclerosis demonstrate a primary defect in either thymic output or peripheral T cell homeostasis (2, 3). Mouse models of systemic lupus erythematosus and diabetes demonstrate similar evidence of premature thymic atrophy and/or peripheral lymphopenia (4, 5). Finally, lymphopenia is associated with resistance to the development of transplantation tolerance (6). Thus, clinical and experimental data suggest an association between T cell lymphopenia and defects in immune self-tolerance. Despite these data, immune-depleting agents such as cyclophosphamide and antithymocyte globulin remain the standard of care for the treatment of severe necrotizing vasculitis and prevention of acute allograft rejection, respectively (7, 8). Although effective to quickly suppress dangerous immunopathology, such treatments are not designed to achieve durable Ag-specific tolerance and carry substantial risk of infection.

Under nonlymphopenic conditions, naive CD4<sup>+</sup> T cells can be induced into an unresponsive state termed clonal anergy by repeated systemic exposure to soluble Ag (9). Anergy is an inability of CD4<sup>+</sup> T cells to produce IL-2 or to proliferate upon subsequent Ag challenge, as a consequence of multiple intracellular signaling defects (10–12). This contrasts with the aggressive priming of CD4<sup>+</sup> T effector cells and generation of immunological memory that follows the recognition of Ag in the presence of adjuvant, tissue injury, and/or infection (9, 13, 14). Certain biochemical signals (e.g., activation of the mammalian target of rapamycin) that follow the recognition of Ag in the presence of adjuvant, tissue injury, and/or infection (9, 13, 14). Certain biochemical signals (e.g., activation of the mammalian target of rapamycin) that follow the recognition of Ag in the presence of adjuvant, tissue injury, and/or infection (9, 13, 14). Certain biochemical signals (e.g., activation of the mammalian target of rapamycin) that follow the recognition of Ag in the presence of adjuvant, tissue injury, and/or infection (9, 13, 14). Certain biochemical signals (e.g., activation of the mammalian target of rapamycin) that follow the recognition of Ag in the presence of adjuvant, tissue injury, and/or infection (9, 13, 14). Certain biochemical signals (e.g., activation of the mammalian target of rapamycin) that follow the recognition of Ag in the presence of adjuvant, tissue injury, and/or infection (9, 13, 14).

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Materials and Methods

Mice

DO11.10 (DO11) TCR-transgenic (TCR-Tg) mice were bred to homozygosity and maintained in our animal facility (29). CD4+ T cells in these mice are uniformly reactive to chicken OVA peptide 323–339 (OVAp)/A Rac complexes and express a clonotypic TCR detectable with the mAb KJ1-26 (30). Rag-2−/− DO11 TCR-Tg mice were purchased from Taconic Farms through the National Cancer Institute at the National Institutes of Health (Frederick, MD). All mice were housed under specific pathogen-free conditions and used in accordance with National Institutes of Health guidelines and the University of Minnesota Institutional Animal Care and Use Committee. Mice were age and sex matched for all experiments.

Adaptive transfer and in vivo treatments of mice

Lymph nodes (axillary, brachial, inguinal, and mesenteric) and spleens of TCR-Tg mice were harvested into complete media containing 10% FCS (Atlas Biologicals), 2 mM l-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 0.5% M-2 media (Mediatech) prepared for adoptive transfer as described previously (9). In some experiments, cells were labeled with CFSE (Molecular Probes) before transfer, using a modification of a technique previously described (32). Briefly, lymph node and spleen cells in PBS at a concentration of 1 × 10^7 cells/ml were incubated in 2.5 μM CFSE for 5 min at 37°C. The labeling reaction was stopped by the addition of complete media. The CFSE-labeled T cells were washed twice with PBS before i.v. transfer. 2.5–5 × 10^6 cells were injected into recipient syngeneic mice. OVAp 323–339 was prepared in our microchemical facility (University of Minnesota), dissolved in PBS, and filter sterilized for use. HAp 111–119 was kindly provided by A. Khoruts (University of Minnesota). OVAp and HAp were delivered i.p. at doses of 100 or 250 μg. Rapamycin (RAPA) was obtained from S. N. Sehgal (Wyeth-Ayerst Research, Princeton, NJ). A stock solution of 1 mg/ml RAPA in 100% ethanol was prepared. RAPA was then suspended in 0.2% carboxymethylcellulose, as previously described (33), and delivered i.p. at a dose of 0.5 mg/kg/day for 4 days beginning on the day of adoptive transfer.

To explore clonal anergy induction in the setting of T cell lymphopenia, OVAp-reactive DO11 CD4+ T cells were adoptively transferred into athymic nu/nu (nude) BALB/c recipient mice and then immediately exposed to repeated (three times) i.v. injections of Ag (OVAp) in the absence of an adjuvant. In a parallel group of WT recipient animals, this regimen of prolonged TCR stimulation led to a state of unresponsiveness to Ag rechallenge by day 13 that resulted in a defect in the in vivo production of IL-2 upon Ag rechallenge (Fig. 1A). In contrast, the KJ1-26 CD4+ T cells exposed to the 3× OVAp regimen within the nude mice retained a significantly higher capacity to synthesize IL-2 (p = 0.001).

Spleen cells were washed with staining buffer (PBS containing 2% FCS and 0.05% azide), and then immediately exposed to repeated (three times) i.v. injections using a modification of a technique previously described (32). Briefly, lymph node and spleen cells in PBS at a concentration of 1 × 10^7 cells/ml were incubated in 2.5 μM CFSE for 5 min at 37°C. The labeling reaction was stopped by the addition of complete media. The CFSE-labeled T cells were washed twice with PBS before i.v. transfer. 2.5–5 × 10^6 cells were injected into recipient syngeneic mice. OVAp 323–339 was prepared in our microchemical facility (University of Minnesota), dissolved in PBS, and filter sterilized for use. HAp 111–119 was kindly provided by A. Khoruts (University of Minnesota). OVAp and HAp were delivered i.p. at doses of 100 or 250 μg. Rapamycin (RAPA) was obtained from S. N. Sehgal (Wyeth-Ayerst Research, Princeton, NJ). A stock solution of 1 mg/ml RAPA in 100% ethanol was prepared. RAPA was then suspended in 0.2% carboxymethylcellulose, as previously described (33), and delivered i.p. at a dose of 0.5 mg/kg/day for 4 days beginning on the day of adoptive transfer.

The rat anti-mouse CD25 mAb PC61 obtained from A. Khoruts (University of Minnesota) and were maintained in our animal facility. CD4+ T cells in these mice are specific for peptide 111–119 of influenza hemagglutinin (HAp) presented by class II MHC molecules and can be detected with the anti-clonotypic mAb 6.5 (31). Wild-type (WT) BALB/c and BALB/c mAb (400 g) was injected i.p. into mice every 3 days during the experiment.

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Cell purification and in vitro suppression assay

The suppressive properties of CD4+ subpopulations isolated from intact DO11 mice, or from DO11-reconstituted nude mice, were tested by their addition to CD25+ cell-depleted normal DO11 lymph node and spleen cells. In some experiments, CD25+ T cells from normal DO11 mice were positively selected using CD25 mAb and MACS magnetic streptavidin microbeads (Miltenyi Biotec), according to manufacturer’s instructions. To obtain CD25+ CD4+ and CD25- CD4+ subpopulations from DO11-reconstituted nude mice, CD4+ cells were first isolated using CD4 Dynabeads (Dynal Biotech) followed by CD4 Detachbead to remove the CD4 magnetic beads. The CD25+ CD4+ and CD25- CD4+ subpopulations were then separated using CD25 mAb and MACS-positive selection (Miltenyi Biotec). To assay for in vitro suppression, the purified CD25+ CD4+ and CD25- CD4+ subsets from DO11-reconstituted nude mice following addition to CD25-depleted DO11 lymph node and spleen cell cultures in the absence or presence of 10 μM OVAp. IL-2 secretion was measured in the 48 h supernatants by capture ELISA.

Real-time quantitative RT-PCR

CD25+ CD4+ and CD25- CD4+ cell populations from intact DO11 mice and DO11-reconstituted nude mice were purified as described above. One million cells were lysed with TRIzol (Invitrogen Life Technologies) and RNA was extracted according to the manufacturer’s instructions. RNA was further purified using the RNA Easy Mini kit (Qiagen). Total RNA equivalent to the cell number from each sample was reverse transcribed using the Superscript II Platinum Two Step qRT-PCR kit (Invitrogen Life Technologies). PCR primers were synthesized in our microchemical facility (University of Minnesota) and real-time PCR was conducted using a Cepheid Smart Thermocycler by adding SybrGreen (Molecular Probes) to the reaction mixtures. Primers were designed to amplify the junction of exons 7 and 8 and contained the following sequences: Foxp3 (forward): 5′-AAA GGA GAA GAC GGG AGC ATT G-3′; Foxp3 (reverse): 5′-CCT GAG TAC TGG CTA CGA T-3′. Hprt mRNA was used as a positive control to normalize the Foxp3 data. The Hprt primers were designed to amplify the junction region of exons 7 and 8 of the Foxp3 mRNA. The primers contained the following sequences: Foxp3 (forward): 5′-TGA AGT ACT GTA ATG AGT CA-3′; Hprt (reverse): 5′-AGC AAG CTT GCA ACC 'TTA ACC A-3′. Data are expressed as the amount of Foxp3 mRNA present in a sample relative to Hprt.

Results

Clonal anergy induction is defective in the setting of T cell lymphopenia

To explore clonal anergy induction in the setting of T cell lymphopenia, OVAp-reactive DO11 CD4+ T cells were adoptively transferred into athymic nu/nu (nude) BALB/c recipient mice and then immediately exposed to repeated (three times) i.v. injections of Ag (OVAp) in the absence of an adjuvant. In a parallel group of WT recipient animals, this regimen of prolonged TCR stimulation led to a state of unresponsiveness to Ag rechallenge by day 13 that resulted in a defect in the in vivo production of IL-2 upon Ag rechallenge (Fig. 1A). In contrast, the KJ1-26 CD4+ T cells exposed to the 3× OVAp regimen within the nude mice retained a significantly higher capacity to synthesize IL-2 (p = 0.001).
Therefore, clonal anergy could not successfully be induced in the lymphopenic environment.

Ag-stimulated cell cycle progression is stronger during T cell lymphopenia

Further analysis of the proportion of Ag-reactive KJ1-26+ CD4+ T cells that remained within the spleens of the WT mice after the 3× OVAp infusion regimen revealed little change in their frequency as compared with 3× PBS-treated control animals, consistent with an ineffective clonal expansion response in the absence of infection or adjuvant (Fig. 1B) (9). In contrast, a significant increase in the percentage and total number of KJ1-26+ CD4+ T cells was observed within the spleens of nude recipient mice chronically exposed to Ag, as compared with nude mice exposed to PBS alone (Fig. 1B and data not shown). This enhanced clonal expansion response together with the persistent recall Ag responsiveness of the 3× OVAp-treated KJ1-26+ CD4+ T cells in lymphopenic mice thus was more reminiscent of a successful T cell priming event than of an induction of immunological tolerance.

Previously, our work had indicated that aggressive in vivo cell cycle progression during the primary response to Ag antagonizes the development of clonal anergy (15). We, therefore, postulated that the resistance to anergy induction observed in the nude mice was caused by this lymphopenia-induced enhanced drive for cell cycle progression. To test this, the intensity of cell cycle progression in lymphopenic mice was characterized based on the rate of CFSE dye dilution in KJ1-26+ CD4+ T cells immediately exposed to a single infusion of Ag (1× OVAp). KJ1-26+ CD4+ T cells in both WT and nude recipients did demonstrate a reduced CFSE fluorescence intensity indicative of multiple rounds of cell division in response to Ag (Fig. 2A). However, the CFSE fluorescence of the KJ1-26+ CD4+ T cells recovered from 1× OVAp-treated nude mice was always much lower than in the WT mice, consistent with a faster rate of cell division. A mathematical examination of the flow cytometry data confirmed that KJ1-26+ CD4+ T cells in nude mice had a significantly higher average cell division rate (5.58 ± 0.35 divisions/T cell over 5 days) than T cells stimulated within the WT recipients (2.79 ± 0.05; p = 0.016) (Fig. 2B). Such an increase in the rate of cell division predicted a generation of daughter cells in the lymphopenic mice that was nearly eight times greater than in the WT case. Therefore, an enhanced cell cycle progression may have accounted at least in part for the greater clonal expansion originally observed in the 3× OVAp-treated lymphopenic animals.

A single i.v. infusion of Ag in the absence of adjuvant is a relatively poor stimulus for T cell clonal anergy induction in WT mice (15); nevertheless, it was apparent in these experiments that the T cells exposed to 1× OVAp in nude mice achieved an even greater level of recall Ag responsiveness as they underwent progressively more rounds of cell division (Fig. 2C). To determine

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Defective tolerance induction in the setting of lymphopenia. WT or nude mice were pretreated i.v. three times 3 days apart with 100 μg of OVAp (or PBS as a control) beginning 1 day after adoptive transfer of 4× 10^6 DO11 T cells. Six days later, recipient mice were challenged i.v. with 250 μg of OVAp for 3 h. A, Intracellular IL-2 content among Ag-specific KJ1-26+ CD4+ T cells, as a percentage of the 3× PBS-treated controls. B, Percentage of KJ1-26+ CD4+ T cells accumulating within the spleens of partially reconstituted nude mice on day 13 of the analysis. Error bars represent the SEM for duplicate mice. The p value was determined using the Student t test. This experiment was repeated with similar results.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Increased recall Ag responsiveness following Ag-stimulated cell cycle progression in lymphopenic recipients. WT or nude mice adoptively transferred with 4× 10^6 CFSE-labeled DO11 T cells were treated with a single i.v. infusion of 100 μg of OVAp or PBS as a control. Some animals were treated with simultaneous i.p. infusions of 0.5 mg/kg RAPA in carboxymethylcellulose, whereas other mice received the vehicle alone. Five days later, animals were rechallenged i.v. with 250 μg of OVAp for 3 h. A, CFSE dye dilution (log FL1) of KJ1-26+ CD4+ T cells recovered from the spleens of WT (filled histograms) or nude (open histograms) recipient mice. B, Average division calculation for the Ag-stimulated KJ1-26+ CD4+ T cell groups as shown in A. C, Plot of the relationship between cell division history and mean recall Ag-induced IL-2 production in KJ1-26+ CD4+ T cells from WT (filled symbol) or nude (open symbol) recipients pretreated i.v. on day 1 with 100 μg of OVAp in the presence of RAPA (circles) or vehicle control (squares). IL-2 production is calculated as the percentage of the T cell response observed in control animals (diamond symbol) receiving a PBS pretreatment alone. *, The mode number of cell divisions observed in the KJ1-26+ CD4+ T cell population as a result of the OVAp pretreatment. Error bars represent the SEM. Data shown are representative of two independent experiments.
whether the strength of cell cycle progression during primary Ag challenge directly regulated the level of recall Ag responsiveness in the nude mice, we examined the effects of the antiproliferative agent RAPA on both the lymphopenia-enhanced cell cycle progression and the eventual level of Ag responsiveness achieved. As previously described, treatment of WT mice with RAPA during a primary Ag challenge significantly inhibited cell cycle progression by the KJ1-26+CD4+ T cells (Fig. 2, A and B) (15). Similarly, treatment of nude mice with RAPA during the time of the primary i.v. OVAp administration slowed the cell cycle progression and reduced the average division rate of the KJ1-26+CD4+ T cells by 36 ± 0.09%. Interestingly, RAPA also inhibited the lymphopenia-induced proliferation of a fraction of the transferred KJ1-26+CD4+ T cell population in the absence of Ag. KJ1-26+CD4+ T cells in both WT and nude animals demonstrated reduced recall IL-2 production in association with their blunted primary proliferative response when RAPA was present at the time of priming (Fig. 2C). Thus, these data showed that within the lymphopenic host, a homeostatic drive toward immune reconstitution promotes an overly aggressive cell cycle progression response during Ag stimulation that prevents the development of clonal anergy.

Recovery from lymphopenia reduces the homeostatic drive for excessive Ag-induced T cell proliferation

We previously demonstrated that KJ1-26+CD4+ T cells will become anergic even in nude recipients when a 3x OVAp infusion regimen first begins at least 15 days after the T cell adoptive transfer (35). An adaptive tolerance that resembles this peptide-induced clonal anergy has also been observed to develop over extended periods of time in 5C.C7 TCR-Tg CD4+ T cells adoptively transferred into lymphopenic (CD3ε−/−) mice that express this T cell’s specific Ag (pigeon cytochrome c) as a transgene (36). Therefore, chronic TCR stimulation can induce T cell clonal anergy within immunodeficient mice, but only after a partial reconstitution of the lymphopenic immune system has taken place.

We directly compared Ag-stimulated cell cycle progression in nonreconstituted or partially reconstituted nude recipients by the adoptive transfer of a second, CFSE-labeled DO11 T cell population. OVAp challenge was found to elicit significantly fewer average cell divisions by the CFSE-labeled cohort of KJ1-26+CD4+ T cells in the partially reconstituted nude recipients (2.65 ± 0.13) as compared with nude mice that had not received an initial DO11 T cell adoptive transfer on day 0 (4.29 ± 0.31; p = 0.001) (Fig. 3A). In fact, OVAp-induced cell cycle progression in the partially reconstituted nude recipients closely resembled that observed in WT mice (data not shown). Once again, those KJ1-26+CD4+ T cells that had divided the most during the primary Ag exposure in the nonreconstituted nude mice also demonstrated a higher level of recall Ag responsiveness than naive T cells, consistent with priming (Fig. 3B). In contrast, KJ1-26+CD4+ T cells exposed to Ag after partial reconstitution of the nude mice showed only poor production of IL-2 in response to an OVAp rechallenge. Thus, the results confirmed that a partial reconstitution of the lymphopenic immune system can reduce the drive for aggressive cell cycle progression during primary Ag challenge and restore the ability to induce clonal anergy.

Recovery from lymphopenia is associated with the expansion of a large CD25+Foxp3+ CTLA-4+CD4+ regulatory T cell subpopulation

Knoechel et al. (28) recently reported that following an adoptive transfer of Rag−/− DO11 T cells into Rag-deficient and lymphopenic mice that constitutively expressed a soluble form of OVA as a transgene, the T cells caused an early wasting disease that resulted in the death of about half of the recipients. Nevertheless, beyond 14 days after the T cell adoptive transfer (in surviving animals) a tolerance to OVA developed and these partially reconstituted lymphopenic animals regained their health. In their studies, this late immune tolerance was associated with a self Ag (OVA)-dependent generation of a subpopulation of CD25+Foxp3+ KJ1-26+CD4+ regulatory T cells. Both Rag-sufficient TCR-Tg and polyclonal CD25+CD4+ regulatory T cells have been previously shown to undergo an MHC class II-dependent clonal expansion following their adoptive transfer into Rag-deficient lymphopenic hosts, and still retain their suppressive activity (27). Therefore, self Ag-specific CD25+CD4+ regulatory T cells might also be expected to arise over time following a partial reconstitution of lymphopenic nude mice with DO11 T cells to promote the establishment of immunological tolerance.

Based on this information, we sought evidence that our nude mice are resistant to clonal anergy induction because they lack regulatory T cells. Freshly isolated Rag-sufficient DO11 T cells were found to contain a small CD25+CD4+ subpopulation (data not shown), but these putative regulatory T cells did not become enriched in response to an OVAp primary Ag challenge performed immediately after T cell adoptive transfer (Fig. 3C). In contrast, 24 days after their adoptive transfer into nude recipients in the absence of OVAp, a sizable proportion of the reconstituting Rag-sufficient KJ1-26+CD4+ T cells appeared to have undergone multiple rounds of cell division (data not shown) and expressed a high level of CD25 (Fig. 3C).

Consistent with a regulatory T cell phenotype, the CD25+ KJ1-26+CD4+ T cells that reconstituted nude mice demonstrated a high level of intracellular CTLA-4 and reduced expression of CD45RB (Fig. 4A and data not shown). Furthermore, in response to the infusion of OVAp this CD25+ subpopulation demonstrated little capacity to accumulate intracellular IL-2. Nevertheless, a partial induction of Cd69 expression was consistently observed following stimulation, suggesting that the CD25+ KJ1-26+CD4+ T cells still retained some Ag reactivity (Fig. 4A and data not shown). Foxp3 expression has been shown to be a very good marker for the development of regulatory T cell function (37). CD25+ KJ1-26+CD4+ T cells purified from nude mice after partial immune reconstitution expressed high levels of Foxp3 mRNA, relative to CD25+CD4+ T cells found in either WT DO11 mice or the same partially reconstituted nude mice (Fig. 4B). These same CD25+ KJ1-26+CD4+ T cells also demonstrated a capacity to inhibit IL-2 production by activated CD25+ KJ1-26+CD4+ T cells in an in vitro assay system (Fig. 4C). Therefore, the CD25+ T cells that arose during a partial reconstitution of lymphopenic mice had a similar phenotype as the well-characterized natural CD25+CD4+ regulatory T cells (38–40).

Endogenous TCR gene recombination is necessary for optimal CD25+CD4+ regulatory T cell generation during partial reconstitution of lymphopenic mice to fully dampen Ag-induced cell cycle progression

An examination of TCR transgene and CD25 expression following partial immune reconstitution of nude mice with Rag-sufficient DO11 T cells did reveal a dimming of the clonotypic TCR staining within the large CD25+ KJ1-26+CD4+ regulatory T cell population, perhaps consistent with endogenous Tcra gene rearrangement and the expression of a second TCR having self Ag specificity (Fig. 5A). Such recognition of particular self peptide/MHC specificities appears in general to be important, because Rag−/− TCR-Tg mice lacking in TCR diversity are often deficient in CD25+CD4+ regulatory T cells (41, 42). Thus, we reasoned that
A partial reconstitution of nude mice with Rag-deficient DO11 donor T cells having limited TCR diversity would fail to give rise to the CD25+KJ1-26+CD4+ subset and would test whether the control Ag-induced cell cycle progression depended on the presence of these regulatory T cells. The generation of CD25+KJ1-26+CD4+ regulatory T cells (in the absence of OVAp) was observed to be reduced following reconstitution of the nude mice with Rag−/− DO11 donor T cells (Fig. 5A). Furthermore, reconstitution with Rag−/− DO11 T cells had a decreased capacity to suppress the proliferation of CFSE-labeled OVAp-stimulated KJ1-26+CD4+ T cells as compared with Rag-sufficient donor cells (Fig. 5B). Thus, it appeared that a TCR-diverse CD25+CD4+ regulatory T cell subset that developed early on during immune reconstitution acted to reduce the intensity of Ag-induced cell cycle progression.
Suppression of OVAp-dependent cell cycle progression in lymphopenic mice can occur following reconstitution with CD4+ T cells having no reactivity to OVAp

These findings indicated that a diverse TCR repertoire and broad self peptide/MHC specificity within the population of reconstituting CD4+ T cells was more important than a high level of clonotypic TCR expression to the counterregulation of OVAp-induced proliferation within nude mice. This predicted that suppression of the proliferation of CFSE-labeled KJ1-26+CD4+ T cells by the original DO11-reconstituting T cell population was not the result of intraclonal competition for OVAp/I-A<sup>d</sup> complexes (43, 44). Consistent with this, a partial reconstitution of the nude mice with Rag<sup>+/-</sup> HA TCR-Tg CD4+ T cells proved equally effective in dampening the OVAp-induced KJ1-26+CD4+ T cell proliferation, regardless of whether these T cells were stimulated with HA (Fig. 6). Note that suppression was only found to occur when the DO11- or HA-reconstituting T cells expressed Rag proteins and were capable of endogenous Tcra gene rearrangements (Fig. 5 and data not shown).
CD25⁺CD4⁺ T cells facilitate clonal anergy induction

Taken together, the data suggested that the development of a CD25⁺Foxp3⁺CTLA-4⁺CD4⁺ regulatory T cell population early during the course of immune reconstitution of lymphopenic mice was necessary to inhibit Ag-induced cell cycle progression in the absence of adjuvant or infection, and this then led to an induction of clonal anergy. To firmly establish that CD25⁺CD4⁺ regulatory T cells were responsible for suppressing the Ag-induced cell cycle progression of CD25⁻CD4⁺ responder T cells during recovery from lymphopenia, the proliferation of CFSE-labeled KJ1-26⁺CD4⁺ T cells was examined following adoptive transfer into nude mice reconstituted in the presence of an anti-CD25 mAb capable of inhibiting the development of this CD25⁺ population. Treatment of nude mice with anti-CD25 mAb PC61 throughout the period of DO11 T cell immune reconstitution reduced the percentage of KJ1-26⁺CD4⁺ T cells expressing CD25 (as detected using the 7D4 anti-CD25 mAb) from 31 ± 2% to 12 ± 4% (Fig. 7A). Although this Ab treatment never resulted in a complete elimination of the CD25⁺CD4⁺ subpopulation, OVAp-stimulated cell division by marker KJ1-26⁺CD4⁺ T cells was nevertheless significantly enhanced (p = 0.008; Fig. 7, B and C). Remarkably, this increased cell cycle progression was also associated with a resistance to clonal anergy induction and increased recall Ag responsiveness, despite the continued development of a large CD25⁻CD4⁺ DO11-reconstituting T cell population (Fig. 7D). Thus, the rapid expansion of a CD25⁺Foxp3⁺CD4⁺ regulatory T cell population during partial immune reconstitution facilitated clonal anergy induction in nearby CD25⁺CD4⁺ T cells that recognized the presence of Ag.

Discussion

CD25⁺Foxp3⁺CTLA-4⁺CD4⁺ regulatory T cells were shown for the first time to facilitate the induction of clonal anergy in vivo in CD25⁺CD4⁺ T cells that recognize Ag in the absence of an infection or adjuvant. CD25⁺CD4⁺ T cells accumulated in lymphopenic mice early during the course of immune reconstitution and functioned to counterregulate the homeostatic drive for excessive cell cycle progression during Ag encounter. Their inhibition of Ag-stimulated cell cycle progression enabled them to promote clonal anergy induction. Without such a partial T cell reconstitution of the nude mice, and in the absence of CD25⁺CD4⁺ regulatory T cells, Ag-stimulated cell cycle progression was exuberant and was invariably associated with the maintenance of a high level of Ag responsiveness.

It is plausible that a resistance to T cell clonal anergy induction in the absence of CD25⁺CD4⁺ regulatory T cells also accounts for the failure of transplantation tolerance-inducing regimens during lymphopenia (6). Whether CD25⁺Foxp3⁺CD4⁺ regulatory T cells play a similar role in facilitating clonal anergy induction in nonlymphopenic hosts remains uncertain. Ag-specific CD25⁺Foxp3⁺CD4⁺ regulatory T cells generated in vivo in the presence of low doses of Ag can be shown to interfere with the priming of a second cohort of naïve Ag-reactive CD25⁻CD4⁺ T cells and lead to their reduced capacity to produce IL-2 upon in vitro rechallenge (45). Nonlymphopenic CD25⁻ mice also demonstrate reduced numbers of CD25⁺CD4⁺ regulatory T cells, and anergy induction by infusion of soluble peptide Ag can be ineffective in these mice (46).

The CD25⁺CD4⁺ regulatory T cell population observed here during the recovery from lymphopenia is phenotypically and
functionally similar to naturally occurring CD25+CD4+ regulatory T cells. CD25+CD4+ T cells have previously been shown to develop from purified CD25+CD4+ T cells that have undergone extensive homeostatic proliferation upon transfer into lymphopenic recipients (27). Purified CD25+ T cells can also give rise, through extensive lymphopenia-induced proliferation, to even extensive homeostatic proliferation upon transfer into lymphopenic recipients (27). Purified CD25+ T cells can also give rise, through extensive lymphopenia-induced proliferation, to even

**FIGURE 6.** Inhibition of proliferation is not Ag specific and does not require acute activation. CFSE-labeled marker DO11 T cells were transferred into nude mice that were reconstituted for 40 days with Rag-2−/− DO11 or HA TCR-Tg cells, as indicated. One day after transfer of the DO11 marker cells (day 41), recipient mice were infused i.v. with 100 μg of OVAp alone and/or 100 μg of HAp. Three days following the peptide infusion (day 44), splenic KJ1-26+CD4+ T cell were recovered and examined for CFSE dye dilution as in Fig. 3. Plots shown are representative of duplicate mice. This experiment was repeated with similar results.

**FIGURE 7.** Depletion of CD25+CD4+ T cells during immune reconstitution leads to enhanced Ag-induced proliferation and resistance to clonal anergy induction. Nude mice were partially reconstituted with Rag-2−/− DO11 T cells either in the absence or presence of the anti-CD25 mAb PC61. Infusions of mAb (400 μg) were initiated on day 0 (the day of T cell transfer) and repeated every 3 days during the course of the experiment. One group of mice was analyzed on day 8 for the presence of CD25+KJ1-26+CD4+ T cells within the spleens following reconstitution in the absence or presence of anti-CD25 mAb, as indicated (A). Other recipient mice were transferred with a CFSE-labeled marker DO11 T cell population on the same day (day 8) and 1 day later were infused with OVAp i.v. (100 μg). Three days into the OVAp response (day 12), recipient mice received a second i.v. infusion of OVAp (250 μg) and spleens were harvested 3 h later. OVAp-induced CFSE dye dilution (B), average division (C), and IL-2 production (D) were measured within the marker KJ1-26+CD4+ T cell population recovered from recipients reconstituted in the absence or presence of anti-CD25 mAb, as indicated. Dotted and hatched lines in B are as indicated in Fig. 3. The percentage of IL-2+ KJ1-26+CD4+ T cells during recall Ag challenge was determined based on staining with an isotype control Ab (D). Data plotted in C and D are the mean ± SEM of duplicate mice. The p value was determined using the Student t test. Results shown are representative of two independent experiments.
greater numbers of CD25⁺CD4⁺ T cells that retain a capacity to suppress in vitro proliferation (27, 47). Therefore, it cannot be determined whether the CD25⁺CD4⁺ T cells generated during the course of immune reconstitution in these experiments arose from pre-existing natural regulatory T cells, or developed from naïve T cells responding to self Ag in the lymphopenic environment. We did observe that the formation of this CD25⁺CD4⁺ subset was significantly reduced when Rag⁻⁻/DO11 cells were used as the reconstituting population, suggesting that endogenous TCR α-chain-dependent recognition of self peptide/MHC regulates their development during the immune reconstitution. Perhaps a broadened TCR diversity allowed for a large expansion of the CD25⁺CD4⁺ subpopulation without too many cells competing with each other for a single self peptide/MHC niche (43, 44). Nevertheless, the capacity of HA-reconstituting CD4⁺ T cells to facilitate the induction of anergy in DO11 T cells does not suggest that competition for a single self peptide/MHC niche is their mechanism of immunoregulation (43, 44).

The observation that a reduction in homeostatic drive for intense Ag-induced proliferation required reconstitution with Rag-sufficient TCR-Tg T cells was perhaps surprising, because one might have expected that an expanded population of DO11 Rag⁻⁻/CD25⁺CD4⁺ T cells would be fully competent to compete with newly transferred DO11 CD4⁺ responder T cells for peptide/MHC complexes and cause an inhibition of their proliferation (43). In fact, the sharing of Ag specificity between the reconstituting population and the responder CD4⁺ T cells was not required to inhibit Ag-stimulated cell cycle progression (Fig. 6). On the surface, this result appears to be at odds with that of Moses et al. (43) who showed that only TCR-Tg Rag⁻⁻/CD4⁺ T cells that compete for the same self peptide/MHC complex are capable of inhibiting the spontaneous proliferation of a particular TCR-Tg CD4⁺ T cell. It is important to note that in our experiments, the proliferative response to administered exogenous Ag given at high dose was examined rather than self peptide-dependent lymphopenia-induced proliferation.

In vitro data have also indicated that suppression by CD25⁺CD4⁺ T cells can be Ag nonspecific (48, 49). Nevertheless, it has been reported that immune regulation in vivo can appear Ag specific (50). In those experiments, HA-specific regulatory T cells were not capable of inhibiting the proliferative response of pigeon cytochrome c-specific CD4⁺ T cells responding to peptide-loaded dendritic cells (pulsed with both peptides), whereas the proliferative response of HA-specific responder T cells to the same limited stimulus was significantly reduced when HA-specific T regulatory cells were cotransferred. Perhaps in our system, the recognition of numerous self peptide/MHC complexes by the reconstituting Rag-sufficient CD25⁺CD4⁺ regulatory T cell population under lymphopenic conditions leads to a durable activation of this subset. This could then allow them to directly suppress either APCs or the effector CD4⁺ T cells themselves in an Ag-nonspecific manner, thus leading to an abortive cell cycle progression and the induction of clonal anergy in response to an Ag-recognition event.

The molecular mechanism of in vivo suppression of this Ag-induced cell cycle progression by these CD25⁺Foxp3 CD4⁺ T regulatory cells in the lymphopenic mice remains unknown. Both in vitro and in vivo investigations have indicated a capacity of CD25⁺CD4⁺ regulatory T cells to inhibit the production of IL-2 in nearby CD25⁺CD4⁺ T cells (Fig. 4C) (49, 51). In our study, the CD25⁺ regulatory cells themselves did appear anergic at the level of the IL2 gene, but they did not suppress the production of IL-2 by nearby Ag-stimulated CD25⁺ T cells in vivo (Fig. 4A). Similarly, a coexistence of anergic CD25⁺CD4⁺ regulatory T cells and IL-2-producing CD25⁺CD4⁺ effector T cells has been demonstrated in the lymph nodes of OVA-expressing lymphopenic mice that had been reconstituted 30 days earlier by an adoptive transfer of Rag⁻⁻/DO11 T cells (28). Finally, it is unclear whether IL-2 plays any role in the cell division response observed in the setting of lymphopenia (34). In our hands, the anti-CD25 mAb has demonstrated no direct inhibitory effect on OVAp-induced cell cycle progression in the nude mice (Fig. 7B and data not shown). Therefore, the mechanism of inhibition of cell cycle progression by these CD25⁺CD4⁺ regulatory T cells is likely independent of any effects on IL2 gene expression. Regardless of the molecular mechanisms involved in this suppression, during the recovery from lymphopenia CD25⁺CD4⁺ regulatory T cells act to dampen Ag-stimulated cell cycle progression and facilitate instead an induction of clonal anergy.

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Disclosures

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References