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Blockade of CTLA-4 Decreases the Generation of Multifunctional Memory CD4\(^+\) T Cells In Vivo

Marion Rudolph,*†‡ Katrin Hebel,* Yoshinori Miyamura,§‖ Emanuel Maverakis,§‖ and Monika C. Brunner-Weinzierl*†‡

CTLA-4 is known as a central inhibitor of T cell responses. It terminates T cell activation and proliferation and induces resistance against activation induced cell death. However, its impact on memory formation of adaptive immune responses is still unknown. In this study, we demonstrate that although anti–CTLA-4 mAb treatment during primary immunization of mice initially enhances the number of IFN-\(\gamma\)-producing CD4\(^+\) T cells, it does not affect the size of the memory pool. Interestingly, we find that the CTLA-4 blockade modulates the quality of the memory pool: it decreases the amount of specialized “multifunctional” memory CD4\(^+\) T cells coproducing IFN-\(\gamma\), TNF-\(\alpha\), and IL-2 in response to Ag. The reduction of these cells causes an immense decrease of IFN-\(\gamma\)-producing T cells after in vivo antigenic rechallenge. Chimeric mice expressing CTLA-4–competent and –deficient cells unmask, which these CTLA-4–driven mechanisms are mediated CD4\(^+\) T cell nonautonomously. In addition, the depletion of CD25\(^+\) T cells prior to the generation of Ag-specific memory cells reveals that the constitutively CTLA-4–expressing natural regulatory T cells determine the quality of memory CD4\(^+\) T cells. Taken together, these results indicate that although the inhibitory molecule CTLA-4 dampens the primary immune response, its engagement positively regulates the formation of a high-quality memory pool equipped with multifunctional CD4\(^+\) T cells capable of mounting a robust response to Ag rechallenge. The Journal of Immunology, 2011, 186: 5580–5589.

A hallmark of the adaptive immune response is the development of memory T cells. Upon activation, CD4\(^+\) T cells undergo rapid proliferation as they differentiate into effector cells. After the Ag is cleared, most of these effector CD4\(^+\) T cells will undergo activation-induced cell death and die. Only a small percentage of CD4\(^+\) T cells survive and form the memory CD4\(^+\) T cell pool. This memory pool is composed of CD4\(^+\) T cells exhibiting diverse states of differentiation, which are able to produce a variety of cytokines and differ within their efficiency to mediate protection (1, 2). Less differentiated CD4\(^+\) T cells produce the cytokines IL-2 or TNF-\(\alpha\) and provide little protection, whereas the highly differentiated CD4\(^+\) T cells produce these cytokines simultaneously with an effector cytokine such as IFN-\(\gamma\), and they are characterized as multifunctional CD4\(^+\) T cells that mediate a particular high level of protection (1, 3). Although the heterogeneity of the memory cell population is well recognized (2), the factors that influence the selection of the various CD4\(^+\) T cells for the memory pool and determine its composition are poorly understood.

Activation and subsequent differentiation of naive CD4\(^+\) T cells require concomitant triggering of the TCR and the costimulatory molecule CD28 (4). Following activation, CTLA-4 (CD152) begins to be upregulated, with the peak of expression on day 2 (5, 6). It binds with 20-fold higher affinity to the same ligands as CD28, CD86 (B7-2), and CD80 (B7-1) present on the surface of APC (7). The successful competition of CTLA-4 with CD28 for CD86/CD80 binding and its effect on the composition of the immunological synapse and on signal transduction terminate the T cell response (8–15). The critical regulatory role of CTLA-4 becomes apparent in CTLA-4\(^{-/-}\) mice, which die by the age of 3–5 wk as a result of lymphoproliferative disorders and multiorgan inflammation (16, 17). Absence of CTLA-4 engagement during Ag priming leads to enhanced cytokine production (18–20). Moreover, CTLA-4 recently has been shown to mediate resistance to apoptosis in vitro, to enhance migration of CD4\(^+\) T cells in vivo (21–25), and to be constitutively expressed on natural regulatory T cells (nTregs) (26). Importantly, the suppressive function of nTregs is dependent on CTLA-4, which impedes activation of Ag-experienced T cells via downregulation of CD80 and CD86 on dendritic cells and induces the tryptophan catabolizing enzyme IDO (27–29).

On the basis of CTLA-4’s strong immunomodulatory effects on T cell function, we ask whether it participates in differentiation of the memory T cell pool. It has already been shown that anti–CTLA-4 mAb treatment in vivo enhances the memory CD4\(^+\) T cell response, yet inhibition has also been noted (30, 31). These contradictory results may in part be due to the investigation of bulk T cell populations instead of monitoring Ag-specific T cells at the single-cell level. Recently, it was shown that, regardless of Th1 or Th2 bias, in vivo-generated Ag-specific CD4\(^+\) T cells are individually detectable via their Ag-specific CD40L (CD154) expression after in vitro restimulation (32–34). In this study, we extended this technology for the analysis of in vivo-generated memory CD4\(^+\) T cells combined with the serological blockade with anti–CTLA-4 mAb or the genetic inhibition of CTLA-4 signaling. Our data show that the quantity of memory CD4\(^+\) T cells...
generated during an immune response is regulated independently of CTLA-4 signaling. However, the CD4+ T cell memory quality and the recall response are strongly influenced by CTLA-4: a decrease of the amount of multifunctional CD4+ T cells and the secondary immune response is found under the blockade of CTLA-4 signaling during priming. Surprisingly, this is mainly regulated CD4+ T cell nonautonomously, with nTregs playing a key role.

Materials and Methods

Mice
BALB/c, C57BL/6 (CD45.2), C57BL/6 (CD45.1; C57BL/6-Ly5.1), and CTLA-4<sup>−/−</sup> C57BL/6 (CD45.2) were bred under specific pathogen-free conditions in the animal facility of the Bundesinstitut für Risikobewertung Berlin or the University Hospital Magdeburg, or they were purchased from Charles River Laboratories. Mice were used at the age of 8–10 wk, except for bone marrow (BM) preparations, which were done at the age of 3 wk. CTLA-4<sup>−/−</sup> mice were genotyped by PCR as described elsewhere (23). All animal experiments were performed in accordance with institutional, state, and federal guidelines.

Cell staining and flow cytometry
Fluorescently labeled Abs specific for CD4 (RM4-5), CD25 (7D4), CD127 (SB/199), CD45.2 (104), IL-4 (11B11), TNF-α (MP6-XT22), CTLA-4 (UC10-4F10), and IgG1 were purchased from BD Pharmingen, for CD45.1 (A20), CD44 (DM7), IFN-γ (XMGl2), and IL-2 (Jes6-5H4) from eBioscience, and for CD40L (CD154) from Miltenyi Biotec. For intracellular staining, fixed cells were permeabilized with 0.5% saponin (Sigma-Aldrich). Data collection was performed on a FACSCalibur or LSRII (BD Biosciences) and was analyzed with FlowJo software (Tree Star).

Cell enrichment
Splenocytes from mice 8–10 wk after priming or 1 wk after rechallenge were stained for 30 min at 37°C with anti–CD127-PE (SB/199, BD Pharmingen) or IgG2b-PE, washed, and stained with anti–CD44-Cy7 (eBioscience) and anti–CD40L-PE (BD Pharmingen). CD4+ T cells were enriched by usage of anti-MHCII microbeads (Miltenyi Biotec) and congeneric APCs by anti-MHC II microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. Subsequently, the CD4+ T cells were sorted using FACSaria (cell sorting facility: German Rheumatism Research Center, Berlin, Germany) by indicated gates (Fig. 1D).

In vitro culture
A total of 1 × 10<sup>7</sup> CD4+ splenocytes/ml were cultured in RPMI 1640 medium (50 µM 2-ME, 2 mM L-glutamine [Invitrogen], 10% FCS [PAA], 100 U/ml benzyl penicillin, and 100 µg/ml streptomycin sulfate [Sigma-Aldrich]) supplemented with 100 µg/ml OVA, BSA, or hen egg lysozyme (HEL; Sigma-Aldrich) for 4 h before 5 µg/ml brefeldin A (Sigma-Aldrich) was added for an additional 4 h. For CD3R analysis, 5 × 10<sup>5</sup> inguinal

FIGURE 1. CD40L expression identifies Ag-specific memory CD4+ T cells generated in vivo. C57BL/6 mice were primed with OVA/CFA via a single ear pinna, and the Ag depot was removed after 1 or 2 wk. Some mice were rechallenged with OVA/IFA 8 wk later via the remaining ear pinna. Mice were sacrificed at indicated time points, and splenocytes were restimulated with OVA or BSA (8 h, plus brefeldin A after 4 h) in vitro. Cells were fixed, stained for CD4 and intracellularly for CD40L and cytokines, followed by flow cytometry analysis. Data represent one of at least two independent experiments with n = 5/group and experiment. A, Representative dot blots of intracellular stainings for CD40L of CD4+ splenocytes from indicated mice and time points. B, Absolute numbers of CD40L+CD4+ T cells producing indicated cytokines 8 and 9 wk (i.e., 1 wk after Ag rechallenge) after priming (**p = 0.0043; mean ± SEM). C, Fold increase of the CD40L MFI of CD40L+CD4+ cytokine compared with none cytokine producers from mice analyzed at week 9 (i.e., 1 wk after Ag rechallenge [*p = 0.0411, **p = 0.0022; mean ± SEM]). D, Presorted CD4+ splenocytes from primed/rechallenged mice were stained for CD127 and CD44 and FACS sorted according to the gates (left panels). Sorted cell fractions were restimulated in vitro with APC from C57BL/6 mice, OVA, and brefeldin A. CD4+ T cells were analyzed for intracellular CD40L expression by flow cytometry.
lymph node cells/ml were cultured for 48 h with HEL (10 μg/ml; E. Sercarz, Torrey Pines Institute for Molecular Studies, San Diego, CA) in serum-free HL-1 medium (BioWhittaker) supplemented with 50 μM 2-ME, 4 mM L-glutamine, 100 U/ml benzyl penicillin, and 100 μg/ml streptomycin sulfate. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂.

In vivo applications

Mice were primed with 100 μg OVA or HEL emulsified with CFA (all Sigma-Aldrich) at the ear pinna, which was removed 1–2 wk later. For Ag rechallenge, 100 μg OVA or PBS in IFA (Sigma-Aldrich) was injected into the remaining ear pinna 8 wk after priming. For CDR3 analysis, mice were primed tail based s.c. with 100 μg HEL (provided by E. Sercarz) or PBS emulsified in CFA.

For Ab treatment of mice in vivo, anti–CTLA-4 (UC10-4F10), hamster control (560-31.1B9), and anti-CD25 (pC61.5) mAb were purified from hybridoma supernatants (German Rheumatism Research Center) and used as a tool for the identification not only of in vivo differentiated Ag-specific effector but also of memory CD4+ T cells; mice were primed with OVA/IFA in a single ear pinna, which allowed us to remove the Ag depot later by abscising the outer ear pinna; chronic Ag-provocations were thereby excluded. To determine Ag-specific CD4+ T cells early (effector) and late (memory) after immunization, mice were analyzed 2 and 8 wk after priming. The CD4+ T cell recall response was studied in a third group of mice rechallenged with OVA/IFA via the contra lateral ear pinna 8 wk after priming and sacrificed 1 wk later.

Ag-specific CD40L+CD4+ T cells were clearly detectable in all primed mice (Fig. 1A) with a 5- to 13-fold increase after rechallenge, depending on Ag depot availability during priming (1 or 2 wk, respectively). The absolute number of cells among the CD40L+CD4+ T cell subsets producing IFN-γ alone or in combination with other cytokines increased strongly after rechallenge (Fig. 1B; total number of CD40L+CD4+ T cells: 3.7 × 10⁴ ± 6.1 × 10³ [8 wk after priming] to 2.0 × 10⁵ ± 5.0 × 10⁴ [9 wk, e.g., 1 wk after rechallenge]; p = 0.0007). Notably, within the population of CD40L+CD4+ T cells detected at 8 wk following Ag priming, most of the cells produced simultaneously IFN-γ, TNF-α, and IL-2 like the recently described multifunctional memory T cells (1, 3, 36, 37). The increase of CD40L production per cell, determined on the basis of the mean fluorescence intensity (MFI; Fig. 1C) within this cell population, supported the assumption of

Statistics

Significations were determined by two-tailed Mann–Whitney U test and data are presented as mean ± SEM. All statistical analyses were performed with GraphPad Prism V4.03.

Results

In vivo-generated Ag-specific memory CD4+ T cells are detectable via CD40L

An important aim of our study was to investigate the effects of CTLA-4 signaling on the generation of memory CD4+ T cells on the basis of individual cells under substantially physiological conditions. Therefore, we used the CD40L molecule that was recently described as a marker for the identification of Ag-specific CD4+ T cells derived from the natural, endogenous CD4+ T cell pool in vivo (32–34). First, we investigated whether CD40L can be used as a tool for the identification not only of in vivo differentiated Ag-specific effector but also of memory CD4+ T cells; mice were primed with OVA/IFA in a single ear pinna, which allowed us to remove the Ag depot later by abscising the outer ear pinna; chronic Ag-provocations were thereby excluded. To determine Ag-specific CD4+ T cells early (effector) and late (memory) after immunization, mice were analyzed 2 and 8 wk after priming. The CD4+ T cell recall response was studied in a third group of mice rechallenged with OVA/IFA via the contra lateral ear pinna 8 wk after priming and sacrificed 1 wk later.

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their high protective function proposed earlier (1, 3). The Ag depot availability lasting for 1 or 2 wk did not affect the cytokine production (data not depicted). Therefore, subsequent experiments were performed using 1 wk of Ag exposure (Figs. 2–6).

To evaluate the detection of CD40L expression as a tool to identify memory CD4+ T cells, we analyzed the distribution of CD40L+CD4+ T cells within the naive (CD44+CD127+), activated (CD44+CD127−), and memory (CD44+CD127+) subpopulations 8 wk after Ag priming and again at week 9 (i.e., 1 wk after Ag rechallenge). Subpopulations were enriched ex vivo according to the coexpression of CD4, CD44, and CD127 (Fig. 1D, left panels). As expected, unimmunized controls and the naive subpopulation (CD44+CD127+) did not contain CD40L+CD4+ T cells upon activation with congenic APC plus OVA (Fig. 1D). CD40L+CD4+ T cells from mice analyzed after rechallenge with OVA/IFA were found mainly in the CD44+CD127− effector cell fraction (79%; Fig. 1D). Most importantly, the majority (84%) of CD40L+CD4+ T cells analyzed 8 wk after priming ex vivo were found within the memory subpopulation (CD44+CD127+). Thus, strikingly, not only in vivo-generated Ag-specific effector CD4+ T cells but also memory CD4+ T cells can be identified by their CD40L expression.

CTLA-4 blockade modulates the differentiation of memory CD4+ T cells

To study the impact of CTLA-4 on the generation of endogenous CD4+ T cell memory responses, CTLA-4 engagement was blocked serologically by applying anti–CTLA-4 mAb to OVA/CFA-primed mice (Fig. 2A). Comparing Ag-specific CD40L-expressing CD4+ T cells from mice primed in the presence of anti–CTLA-4 mAb to controls, we found a higher frequency of Ag-specific CD4+ T cells in mice treated with anti–CTLA-4 mAb, pointing to a greater expansion as a result of the blockade of CTLA-4 (Fig. 2B, upper panel). Moreover, the frequencies of IFN-γ–producing endogenous Ag-specific CD4+ T cells were clearly enhanced in the anti–CTLA-4 mAb-treated mice (Fig. 3A, upper panel). Anti-CTLA-4 mAb-treated mice had compared with control mice a significantly increased total number of Ag-specific CD4+ T cells (9.7 × 10^4 ± 2.4 × 10^4 [anti–CTLA-4] to 2.8 × 10^4 ± 3.6 × 10^3 [control] CD40L+CD4+ T cells; p = 0.0089) at nearly every differentiation state (Fig. 3B). The possibility that bystander activation in response to CTLA-4 blockade was responsible for the increased frequencies of CD40L+CD4+ T cells was excluded by applying the blocking mAb to PBS/CFA-primed control mice.
(data not depicted). At 8 wk, the frequencies of Ag-specific memory CD40L+CD4+ T cells were comparable irrespective of the treatment (Fig. 2B, middle panel; total number of CD40L+CD4+ T cells: 4.8 × 10^5 ± 1.9 × 10^5 [anti-CTLA-4] to 4.0 × 10^5 ± 4.8 × 10^5 [control]; *p* = 0.2844). However, there was a major difference between the two treatment groups with regards to the cytokines produced by the CD40L+CD4+ T cells (Fig. 3A, middle panel, 3C, left panel, 3D): the control group showed a high frequency of multifunctional CD4+ T cells producing IFN-γ, TNF-α, and IL-2 (i.e., triple producers), whereas the CTLA-4 blockade resulted mainly in IL-2/TNF-α single and double or no cytokine producers.

Taken together, our results implicate that although the blockade of the CTLA-4 signal enhances the primary immune response, it does not change the quantity of generated memory CD4+ T cells. However, it profoundly affects the quality of the memory pool by decreasing the survival of multifunctional memory CD4+ T cells.

**The CTLA-4 signal in the primary response determines the secondary immune response**

Next, we asked whether the impact of CTLA-4 on the quality of memory CD4+ T cells affects the secondary immune response after rechallenge with OVA/CFA. Only within the control group a strong increase of Ag-specific CD40L+CD4+ T cells was detected (Fig. 2B, lower panel; total number of CD40L+CD4+ T cells: 1.8 × 10^5 ± 3.0 × 10^5 [anti-CTLA-4] to 4.4 × 10^5 ± 4.0 × 10^5 [control]; *p* = 0.0003). Mice treated with anti–CTLA-4 mAb during the primary response showed a significantly reduced secondary response, irrespective of the fact that CTLA-4 signaling was present during Ag rechallenge. The frequency of IFN-γ producers was clearly higher in the control mice, whereas the anti–CTLA-4 mAb-treated animals again showed a higher frequency of IL-2/non–IFN-γ producers (Fig. 3A, lower panel).

Compared with anti–CTLA-4 mAb-treated mice, control mice had an impressive 6-fold higher number of IFN-γ–producing CD4+ T cells after antigenic rechallenge (Fig. 3C, right panel). Varying duration of application (2, 6, or 14 d) of anti–CTLA-4 mAb had little effect (Fig. 3E).

CTLA-4 blockade can lead to a Th2-skewed immune response (38). In this respect, we detected a low but significantly higher number of Ag-specific IL-4 producers in the anti–CTLA-4 mAb-treated animals 2 wk after Ag priming (Fig. 3F). However, the number of IFN-γ producers from the same anti–CTLA-4 mAb-treated mice was 4.6-fold higher than that of IL-4 producers, thus showing a Th1-prone immune response. Moreover, the increase in IL-4 production seen after Ag rechallenge of anti–CTLA-4 mAb-treated mice was rather modest; anti–CTLA-4 mAb-treated animals had 3% of Ag-specific IL-4 producers compared with 1% in control mice. Hence, the establishment of a Th2-like response cannot explain our results.

Collectively, these data indicate that blocking CTLA-4 signaling exclusively during the primary immune response has profound effects on formation of memory CD4+ T cells and thereby on the subsequent CTLA-4–competent secondary immune response.

**CTLA-4 engagement does not shape the TCR repertoire of responding CD4+ T cells**

Our investigations on CTLA-4 are based on the analysis of endogenous Ag-specific CD4+ T cells, ensuring availability of the whole TCR repertoire for responder cells. To investigate any bias of the CD4+ T cell repertoire in the absence of CTLA-4 signaling, we performed CDR3-length repertoire analysis. For this purpose, anti–CTLA-4– or control mAb-treated BALB/c mice were primed with HEL/CFA and then sacrificed 1 wk later. Subsequently, Ag-specific CD4+ T cells were expanded for 2 d in vitro. All Vβ17 combinations were analyzed for Vβs 4, 6, 8.2, 8.3, and 13 of anti–CTLA-4 or control mAb-treated HEL/CFA-primed BALB/c mice. Within the majority of the Vβ17 spectra analyzed, we could detect a representative Gaussian distribution (e.g., shown for the Vβ8.2J1.1 spectra), reflecting no HEL-specific CD4+ T cell expansions (Fig. 4, top panel). Consistent HEL–specific CD4+ T cell expansions were detected within the Vβ8.2J1.4 and Vβ8.2J1.5 spectra, with comparable expansions in Vβ8.2J1.4 (Fig. 4, middle panel). Vβ8.2J1.5, the most frequently selected TCR rearrangement of BALB/c in response to HEL, showed a consistently larger expansion within the anti–CTLA-4 mAb–treated group (Fig. 4, lower panel) (39, 40). Of note, the total number of clonal expansions detected within the anti–CTLA-4 mAb–treated animals was virtually the same as for the control mAb–treated animals.

In conclusion, neither CTLA-4 signaling nor in vivo CTLA-4 blockade during the primary immune response alters the common repertoire of responding CD4+ T cells.

**CTLA-4 controls memory CD4+ T cell differentiation by a nonautonomous mechanism**

To investigate whether the effects of CTLA-4 on the generation of the memory CD4+ T cells were mediated cell autonomously, we generated C57BL/6 chimeras reconstituted with a 1:1 mixture of BM derived from CTLA-4–knockout (KO; CTLA-4−/−, CD45.2) and CTLA-4–wild-type (WT; CTLA-4+/+, CD45.1) C57BL/6 congenic mice. Control BM chimeras were generated similarly using BM cells from CD45.1- and CD45.2-expressing CTLA-4+/− mice. This allowed us to analyze, via CD40L, Ag-specific endogenous CTLA-4–deficient and CTLA-4–competent T cells differentiated within one and the same animal (Fig. 5). The mice were primed and rechallenged as described in Fig. 2A, followed by the quantification of the number of CD40L expressing T cells

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** The Ag-specific T cell repertoire is not shaped by anti-CTLA-4 mAb treatment. Anti–CTLA-4 or control mAb (100 μg/mouse on days −1, 0, 2, 4, 6) treated BALB/c mice were primed s.c. tail based with HEL/CFA (day 0) or as a control with PBS/CFA. One week later, inguinal lymph node cells were cultured for 2 d with/without HEL (10 μg/ml) and CDR3 length repertoire analysis for all Vβ17 combinations of Vβs 4, 6, 8.2, 8.3, and 13 was conducted. In most spectra, no CD4+ T cell expansion could be detected like within the Vβ8.2J1.1 spectra (top panel). An expansion (marked by arrowhead) could be detected in the Vβ8.2J1.4 (middle panel) and Vβ8.2J1.5 (lower panel) spectra. Shown are representative spectra out of at least 3 of 4 mice per group primed in at least two independent experiments.
derived from CTLA-4+/+ and CTLA-4−/− CD4+ T cell compartments after 2 and 8 wk. With this experimental setup, we obtained results similar to those of the serological blockade experiment: At 2 wk, we detected a higher frequency of Ag-specific CD4+ T cells within the CTLA-4–deficient compared with the CTLA-4–competent CD45.2+ T cell compartment, the latter being obtained from the control chimeras (Fig. 5, right lower panel). At 8 wk, there was no difference between these two groups with regard to the number of Ag-specific CD4+ T cells detected. Furthermore, these data confirmed that the lack of CTLA-4 engagement leads to higher absolute numbers of CD40L+CD4+ T cells producing no cytokines or only the “early” ones such as IL-2 and/or TNF-α, whereas availability of CTLA-4 leads to accumulation of multifunctional CD40L+CD4+ T cells (Fig. 6A, upper panel).

However, in one respect, the results from CTLA-4 chimeric mice and the CTLA-4 blockade experiment differ: the frequency of CD40L-expressing cells derived from CTLA-4–deficient CD4+ T cells was more or less comparable to that of CTLA-4–competent CD4+ T cells after rechallenge (Fig. 5, lower panel). This is most likely explained by the fact that the blockade by anti–CTLA-4 mAb application was only transiently and restricted to the primary immune response, whereas the CD4+ T cells from the BM chimeras lacked CTLA-4 engagement in both the primary and secondary immune responses. Nevertheless, according to the results from the serological blockade, in terms of cytokine production, we detect again a higher frequency of IFN-γ producers in response to Ag rechallenge at 9 wk within the Ag-specific CD4+ T cells from CTLA-4–competent mice (data not depicted).

Interestingly, CTLA-4–competent CD45.1+CD4+ T cells, which differentiated in the presence of CTLA-4−/−/CD45.2+CD4+ T cells, were more similar to the CTLA-4–deficient T cells than they were to their counterpart CTLA-4+/+CD45.1+CD4+ T cells isolated from control chimeras (Figs. 5, 6). This is supported by an enhanced frequency of CD40L+CD4+ T cells after priming (Fig. 5) and a decrease in absolute numbers of multifunctional TNF-α/IL-2/IFN-γ triple producers detected 8 wk after priming (Fig. 6A, lower panel). Moreover, in the presence of CTLA-deficient
T cells, even the frequency of IL-4 producers among the CTLA-4–competent Ag-specific CD4+ T cells was increased, with 3% found in the control chimeras and up to 10% detected in the partially CTLA-4–deficient chimeric mice. This frequency corresponded to our results for CTLA-4–deficient CD40L−/−CD45.2+ CD4+ T cells 2 wk after Ag priming (data not depicted).

Therefore, CTLA-4–competent CD45.1+CD4+ T cells isolated from CTLA-4−/−/CTLA-4−/− chimeric mice do not behave like CTLA-4–competent CD45.1+CD4+ T cells isolated from control CTLA-4−/−/CTLA-4−/− chimeric mice. Rather, they are similar to the wildtype KO CD45.2+CD4+ T cells of the same mouse (Fig. 6B). Hence, the data strongly suggest that CTLA-4–driven non-autonomous effects are central for shaping the memory compartment of CD4+ T cells.

nTregs control the quality of memory CD4+ T cells

nTregs constitutively express functional CTLA-4, which has been suggested to dampen together with cell autonomous CTLA-4 signals CD4+ T cell activation (21, 26, 27). To get an indication about the involvement of nTregs on the observed CTLA-4–mediated effects, we used the frequently applied temporary depletion of nTregs in vivo by application of anti-CD25 Ab 5 d before priming of the mice (41–44). The mice were divided into groups and received either anti–CTLA-4 or control mAb until day 6 as described (Fig. 2A).

Fig. 7A reveals that 8 wk after priming with OVA/CFA, mice pretreated with or without anti-CD25 had similar absolute numbers of CD40L−/−CD4+ T cells, regardless of a subsequent treatment with either anti–CTLA-4 or control mAb. In contrast, following antigenic rechallenge, there was an expansion of CD40L−/−CD4+ T cells within the non–anti-CTLA-4 mAb–treated control mice, irrespective of a pretreatment with anti-CD25 mAb. Thus, the absence of CD25+ regulatory T cells (Tregs) during the primary immune response did not alter the CTLA-4–mediated impairment of the CD4+CD40L+ T cell expansion.

However, analysis of their cytokine production demonstrates that pretreatment with anti-CD25 mAb enhanced the frequency of IL-2/TNF-α single and double producers among CD40L−/−CD4+ T cells detected at week 8 (Fig. 7B, left panel). This result is similar to those obtained with anti–CTLA-4 mAb treatment alone. Moreover, this effect was not amplified when CD25-depleted mice were additively treated with anti–CTLA-4 mAb during the priming period. In line with these data, we found that the depletion of nTregs led to a reduced frequency of IFN-γ producers (Fig. 7B, right panel) and of multifunctional (IL-2+TNF-α+IFN-γ+) CD40L−/−CD4+ T cells (Fig. 7C) to the same extent as seen for solely anti–CTLA-4 mAb–treated animals. Both effects were not enhanced by the combination of CD25 depletion and CTLA-4 mAb treatment. Hence, blockade of CTLA-4 and depletion of nTregs during priming have an equivalent impact on the cytokine profile of memory CD4+ T cells and consequently on the frequencies of Ag-specific IFN-γ producers after the CTLA-4–competent rechallenge of the mice (Fig. 7D).

Notably, in all these experiments, we detected with increasing functionality of cytokine producers a progressive increase of the MFI for the cytokines IL-2, TNF-α, and IFN-γ, as has been de-

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**FIGURE 7.** Depletion of CD25+CD4+ T cells changes the cytokine-profile but not the expansion of Ag-specific CD4+ T cells. C57BL/6 mice were treated with anti-CD25 (pc61.5) 5 d before priming (day 0) followed by anti–CTLA-4 or control mAb applications (until day 6) and priming/rechallenging according to Fig. 2A. A–D. Shown are data from two independent, combined experiments for anti–CD25–treated animals (n = 12) and one of two experiment for the group of non–anti-CTLA-4–treated mice (n ≥ 5). Data for anti-CD25–non/anti–CD25–treated mice were normalized via the results for the anti–CTLA-4 control or a control group receiving additionally the anti-CD25 control mAb. A. Absolute numbers of CD40L−/−CD4+ splenocytes from indicated mice and time points (lower bars, cell counts CD40L−/−CD4+ T cells in vitro BSA; ***p ≤ 0.0043; ***p < 0.0001; n.s., p = 0.795). B. Frequencies of IL-2, TNF-α, and IL-2/TNF-α (left panel) or IFN-γ in combination with without IL-2 and/or TNF-α (right panel) producers of CD40L−/−CD4+ T cells 8 wk after priming. C. Frequencies of IL-2, TNF-α, and IFN-γ triple producers of CD40L−/−CD4+ T cells 8 wk after priming. D. Frequencies of every combination of IFN-γ producers of CD40L−/−CD4+ T cells at 9 wk (i.e., 8 wk after priming). E. MFI of IFN-γ of CD40L−/−CD4+ splenocytes 8 wk after priming; Ab treatment and cytokine production as indicated (n = 6; *p = 0.0026, **p ≤ 0.0043; statistical analyses are shown for the comparison of anti–CTLA-4 control mAb–treated mice with or without anti-CD25 application).
scribed previously (1, 37). Differences between the groups were found only for IFN-γ producers, where both anti–CTLA-4 mAb treatment and anti-CD25 mAb application reduced the MFI (Fig. 7E). Hence, temporary CD25 depletion and CTLA-4 blockade affect the cytokine profile and the cytokine production of individual cells in the same manner.

These results implicate that a CTLA-4–mediated nTreg-independent mechanism sets the expansion potential of memory CD4+ T cells. At the same time, nTregs crucially regulate the CTLA-4–mediated differentiation of multifunctional memory CD4+ T cells.

Discussion
Understanding the development of CD4+ T cell memory has been the subject of many studies, but the technical challenges of the analysis under physiological conditions have not been solved satisfactorily until now (45). In this paper, we demonstrate that the CD40L expression is a valuable tool to analyze not only effector CD4+ T cells but also memory CD4+ T cells generated from the endogenous CD4+ T cell pool. This is supported by the fact that Ag-specific CD40L-expressing CD4+ T cells detected 8 wk after Ag priming in vivo exhibit phenotypic attributes of memory CD4+ T cells: they belong to the CD127+CD44+CD4+ T cell pool, display a multifunctional (IFN-γ/TNF-α/IL-2) cytokine secretion profile, and conduct the typical fast recall response (1, 3, 36, 37). For the impact of CTLA-4 signaling on memory formation, we show in this paper that our approach, together with the treatment of mice with blocking or depleting Abs, is a beneficial framework for the analysis of the influence of various factors on the in vivo differentiation of CD4+ T cells.

In this work, we found that CTLA-4 is much more than just an inhibitory molecule; CTLA-4 has a major impact on the differentiation of memory CD4+ T cells. CTLA-4 controls the quality of the memory CD4+ T cell pool but, surprisingly, without affecting its size. Lack of CTLA-4 signaling, because of the application of blocking anti–CTLA-4 mAb or because of genetically inactivation, profoundly reduces the effector-type multifunctional memory CD4+ Th1 cells. At first sight, this could be explained by a shift of the Th1/Th2 balance in favor of a Th2 response. Indeed, we found, in agreement with the literature, slightly higher amounts of IL-4–producing CD4+ T cells under CTLA-4 blockade (38, 46). Nevertheless, the expansion of Th2 cells during priming and more importantly the formation of IL-4–producing memory CD4+ T cells as well as the Th2 recall response was compared with the Th1 memory cell formation and recall response negligibly small (Fig. 3F). Accordingly, the reduction of multifunctional CD4+ Th1 cells in anti–CTLA-4–treated mice cannot be simply explained by the fact that more Th2 memory CD4+ T cells were formed and thereby a stronger Th2 response was established. In addition, results from CDR3 spectratyping oppose the concept that our findings are the result of a shift within the repertoire of responder CD4+ T cells induced by the CTLA-4 blockade. Rather, CTLA-4 blockade influences the cytokine profile leading to a memory CD4+ T cell subset with a less differentiated phenotype in which only the “early” cytokines IL-2 and/or TNF-α are produced.

Our results showing that the CTLA-4 blockade increased the total number of Ag-specific CD4+ T cells at almost every stage of differentiation (except IL-2/TNF-α double producers) during priming (Fig. 3B), not affecting the size of the memory population, raises the question about the fate of Ag-specific CD4+ T cell populations after priming. Most likely, terminal differentiated CD4+ T cells producing only IFN-γ and “unfit” CD4+ T cells producing no cytokines at all die (2, 3). However, in addition to these cells, we detected large amounts of CD4+ T cells producing IFN-γ in combination with IL-2 and/or TNF-α in anti–CTLA-4–treated mice. A possible scenario is that, because of the lack of inhibitory as well as survival promoting CTLA-4–signals, these cells will continue their differentiation to become terminal effector cells and eventually die (3, 22, 23, 25). Less differentiated CD4+ T cells, such as IL-2 and TNF-α producers, might be less dependent on inhibition by CTLA-4 and thus could enter into the memory CD4+ T cell population. But even under this assumption, one would expect the formation of a large pool of memory CD4+ T cell in the absence of CTLA-4 because the number of IL-2 and/or TNF-α producers after priming was far greater when compared to controls. Yet, the size of memory CD4+ T cell populations could be determined not only by the cell differentiation state but also by the available niche size and/or cytokines in the environment (47–49). Thereby, the size of the memory cell population, as indicated by our results, would be set independently of the original activated number of cells, their differentiation stage (except from “unfit” and terminally differentiated), and thus independent of CTLA-4.

The phenotype of the less differentiated memory CD4+ T cells generated in anti–CTLA-4–treated mice resembles that of central memory CD4+ T cells (2). Thus, our data represent the missing link between prior contradictory results regarding the impact of CTLA-4 on memory formation (30, 31): the blockade of CTLA-4 enhances the formation of central memory and, at the same time, reduces Th1 recall responses mediated through a decrease in multifunctional memory CD4+ T cells. In contrast, in the presence of CTLA-4 signaling, the generated memory pool contains mainly multifunctional CD4+ T cells, capable of producing high amounts of IL-2 in addition to TNF-α and IFN-γ at the single-cell level, enabling them to respond robustly to Ag rechallenge.

FIGURE 8. Model illustrating the impact of the CTLA-4 signal on the generation of multifunctional CD4+ T cells. A. The lack of the CTLA-4–mediated inhibition during the primary response provokes extensive proliferation and differentiation of effector CD4+ T cells. Large amounts of terminal differentiated IFN-γ-producing effector CD4+ T cells result, which ultimately undergo activation-induced cell death (3). Hence, the memory compartment includes mainly less differentiated CD4+ T cells (i.e., none cytokine, IL-2 or TNF-α producers). These cells produce low amounts of IL-2 per single cell, which results in conjunction with other regulatory elements in a low expansion after antigenic rechallenge (1, 37). In line with the low differentiation state, which the CD4+ T cells have adopted during first immunization, they were unable to produce large amounts of IFN-γ. B. nTregs mediate via CTLA-4 the downregulation of CD80/CD86 and induction of the tryptophan catabolizing enzyme IDO of DC (27–29). This, in addition to the CTLA-4 signal to the effector CD4+ T cell, gives the CD4+ T cells a pause. Extensive proliferation and differentiation to terminal effector CD4+ T cells is avoided so that multifunctional CD4+ T cells (i.e., IFN-γ/TNF-α/IL-2 producer) further differentiate to memory cells. After antigenic rechallenge, their expansion is enhanced by their increased IL-2 production. Their previously acquired differentiation state allows these CD4+ T cells to produce large amounts of IFN-γ.
Moreover, by depletion of nTregs prior to priming, we detected a similar impact on the memory phenotype (i.e., reduction of multifunctional CD4+ T cells) (26). An effect that was not amplifiable by additional CTLA-4 blockade. This points toward a CTLA-4-mediated role of nTregs on the development of memory CD4+ T cells. At first sight, this assumption seems to contradict our results and those of others, because the frequency of Tregs was increased by anti-CTLA-4 application (data not depicted (50)). However, differences in frequencies of Tregs under CTLA-4 blockade are most likely irrelevant, because Tregs no longer fulfill their suppressive function without available CTLA-4 (21, 26, 27, 51). Therefore, a role of Tregs and their CTLA-4 expression playing a major role on the formation of the quality of the memory pool is very well consistent with our hypothesis. However, this issue may be clarified unequivocally by using mice with a specific deficiency of CTLA-4 in Tregs (27). Nevertheless, the fact that only the anti–CTLA-4 mAb treatment and not the nTreg depletion or the combination of both resulted in a reduced secondary response implicates that additional CTLA-4–mediated nTreg-independent control elements must exist (Fig. 7A). Candidates for this could be the Ag-specific adaptive Tregs. They emerge from effector CD4+ T cells, produce IL-10, and are detectable in increased amounts after blockade of CTLA-4 in vivo (31). Taking into account that IL-10–producing cells diminish the formation of multifunctional CD4+ T cells, our results might be partially explained by the generation of adaptive IL-10–producing Tregs during CTLA-4 blockade (52). Whether adaptive Tregs can become memory cells has to be investigated; however, if that is the case, the memory pool of adaptive IL-10–producing Tregs could be more pronounced in anti–CTLA-4–treated mice. These cells could contribute to the reduction of the recall response in the anti–CTLA-4–treated animals—an aspect currently under investigation. Our data implicate that the establishment of a high-quality memory compartment and the strong secondary response upon Ag rechallenge are not only controlled autonomously by CD4+ T cells. It has been suggested recently that CTLA-4 of effector T cells and Tregs acts synergistically in the rejection of tumors and to maintain tolerance (53–55). Despite the differences of systems, our data implicate also a dual function of the CTLA-4 from effector and nTregs to control the cellular composition of the memory pool and its recall response. However, it remains to be elucidated whether the principle of the far-reaching physiological effects of CTLA-4-mediated immune regulation generally involves both cell types.

From the data presented in this paper and the results of earlier studies, we propose a model of how the inhibitory molecule CTLA-4 and Tregs imprint a signal on the T cells that determine the subsequent cellular composition of the memory pool (Fig. 8) (27–31). But why does the immune system use inhibitory mechanisms for setting up long-term immunity? In face of a threat for the organism it is important that the T cells quickly become efficient effector cells to rapidly eliminate the pathogen. Paradoxically, for the generation of memory less is more, if they are not inhibited because CTLA-4 is recruited within the immunological synapse. Therefore, inhibitory mechanisms via CTLA-4 are very useful: The inhibitory signals arrest and slow down activated T cells giving them time for differentiation into memory cells of high quality. In addition, because CTLA-4 is recruited within the immunological synapses in dependency of TCR strength, it will select mainly T cells with highly affine TCRs (56). Thereby, the CTLA-4 engagement ensures the selection of the most suitable CD4+ T cells for the development of long-lived Ag-specific multifunctional CD4+ T cells.

Given that the frequency of multifunctional CD4+ T cells directly correlates with protection against infections such as Leishmania major (1) and HIV-1 (57) our findings implicate that CTLA-4 signaling during memory formation will provide the host with the most powerful protection. Thus, our results have direct relevance for the design of CTLA-4–based immunomodulatory therapies. Antagonistic approaches of CTLA-4 signaling during infection or provocation with tumors have been suggested to rapidly clear the pathogen or tumor load, which might well work out for primary acute immune responses, but agonistic approaches to strengthen CTLA-4 signaling might be more effective at gaining long-term immunity.

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