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Identification of Circulating Human Antigen- Reactive CD4+ FOXP3+ Natural Regulatory T Cells

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Circulating human CD4+CD25++CD127− FOXP3+ T cells with a persistent demethylated regulatory T cell (Treg)-specific demethylated region FOXP3 gene are considered natural Tregs (nTregs). We have shown that it is possible to identify functional Ag-reactive nTregs cells for a range of different common viral and vaccination Ags. The frequency of these Ag-reactive nTregs within the nTreg population is strikingly similar to the frequency of Ag-reactive Teff cells within the CD4+ T cell population. The Ag-reactive nTregs could be recognized with great specificity by induction of CD154 expression. These CD154+ Ag-reactive nTregs showed a memory phenotype and shared all phenotypical and functional characteristics of nTregs. The isolated CD154+ nTregs could be most efficiently expanded by specific antigenic stimulation, while their Ag-reactive suppressive activity was maintained. After an in vivo booster Ag challenge, the ratio of Ag-reactive T cells to Ag-reactive Tregs increased substantially, which could be attributed to the rise in effector T cells but not Tregs. In conclusion, the nTreg population mirrors the effector T cell population in the frequency of Ag-reactive T cells. Isolation and expansion of functional Ag-reactive nTregs is possible and of potential benefit for specific therapeutic goals. The Journal of Immunology, 2012, 188: 1083–1090.

Regulatory CD4+ T cells (Tregs) are considered to be crucial for the suppression of excessive T cell responses that may cause damage to the organism. FOXP3 is a key molecule for identification of suppressive function of Tregs. Deficiency or dysfunction of FOXP3+ Tregs in humans and Foxp3 Tregs in rodents are associated with T cell-mediated autoimmune diseases, allergies, and inflammatory bowel disease. Additionally, Tregs may be important in establishing tolerance to organ transplants.

The ontogeny of Tregs may involve several differentiation pathways, with thymus-derived and induced Tregs (iTregs) being the main ones. Tregs generated within the thymus are called natural Tregs (nTregs) (2). Additionally, at least in vitro Tregs may be induced from a FOXP3+ T cell population using the appropriate cytokine milieu, including TGF-β. These iTregs are phenotypically indistinguishable from nTregs, are FOXP3+, and can be generated as Ag-reactive T cells as opposed to nTregs (4). Recently, demethylation at a highly conserved region within the FOXP3 gene (Treg-specific demethylated region [TSDR]) was found to discriminate between natural Tregs (highly demethylated TSDR) and induced Tregs (highly methylated TSDR) (5, 6).

Within the peripheral blood of healthy humans a relative stable population of Tregs can be recognized that comprise ~5–10% of the total CD4+ T cell population. These FOXP3+ cells express high levels of CD25 and low levels of CD127 (FOXP3+CD25++ CD127−CD4+ T cells) (7). Recently, it has been shown that this cell population is composed of Tregs with a naive and memory T cell phenotype based on differential expression of the CD45 isoforms CD45RO (memory phenotype) and CD45RA (naive phenotype) (8, 9). The memory Tregs behave similar to memory cells of effector CD4+ T cells (Teff), as they have short telomeres and a remarkably high turnover rate (10). Sequence-based T cell clonotype tracing of human peripheral blood and transfer of CD45RA+ Tregs into NOD/SCID/common γ-chain–deficient mice showed conversion of these CD45RA+ Tregs into CD45RA+ FOXP3+ cells (8).

An unresolved issue that remains is whether Ag-reactive Tregs are present within the human nTreg population, as such cells cannot be directly identified. Previous studies have indicated the existence of HIV and CMV Ag-reactive Tregs in the peripheral blood but failed to show their existence on the single cell level. It has been postulated that these Ag-reactive Tregs may originate from activated CD4+ Teff and are in fact iTregs (7).

Alternatively, Ag-reactive Tregs could stem from conversion of naive FOXP3+ nTregs into memory nTregs, in a similar fashion as the Ag-reactive memory CD4+ Teff develop from FOXP3+ naive CD4+ T cells (11). Identification on the single cell level of Ag-reactive nTregs would allow for tracking Ag-reactive responses. Isolation and propagation of such Ag-reactive nTregs could be highly useful for immunotherapy, as this would avoid any unwanted generalized suppression of immune responses (12). Previously, we have used membrane-bound CD154 (CD40L) expression in a live cell assay to detect and isolate human Ag-reactive CD4+ Teff with high sensitivity and specificity (13, 14). In this study we followed this strategy to show the presence and characteristics of Ag-reactive nTregs.

Materials and Methods

Blood samples

PBMCs were isolated from healthy donor-derived buffy coats obtained from the local blood bank (Sanquin Blood Bank, Rotterdam, The Netherlands), as described before in detail (14). The vaccination of healthy volunteers was performed with standard doses of tetanus toxoid vaccine and hepatitis B
surface Ag (HBs Ag) with weekly isolation of PBMCs thereafter, using the protocols published previously (15, 16). All individuals included gave informed consent, and the local Medical Ethical Committee approved the study. It was conducted according to the principles of the Declaration of Helsinki and in compliance with International Conference on Harmonization/Good Clinical Practice regulations.

Cell sorting experiments

PBMCs were enriched for T lymphocytes using the Pan T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Enriched T lymphocytes were prepared for sorting experiments using the following mAbs: AmCyAn-labeled CD3 (BD Pharmingen, Erembodegem, Belgium), Pacific Blue-labeled CD4 (BD Pharmingen), PE-labeled CD7 (BD Pharmingen), and PE-Cy7-labeled CD25 (epitope A; BD Pharmingen), PE-labeled CD127 (BD Pharmingen), and inclusion of a live/ dead marker ViaProBe (7-aminoactinomycin D [7-AAD]; BD Pharmingen). After staining, the cells were washed and resuspended at 20–25 × 10^6/ml and sorted (BD FACSAria II SORP; BD Biosciences) into CD3<sup>+</sup>CD4<sup>-</sup> (Teff depleted). Percentages of cytokine-producing cells were determined by stimulating freshly isolated, the Ag-reactive, and/or the polyclonal expanded Teg fractions (unseparated or CD154-enriched and CD154-depleted). Briefly, Tregs were transferred in triplicates to the wells and used at different ratios (i.e., 1:5 to 1:2500). Subsequently, 5 × 10^5 CD4<sup>+</sup> Teff (responder cells) were added in triplicate to the wells. Irradiated (40 Gy) autologous PBMCs either loaded with the different Ags—that is, CMV Ag (34 μg/ml), HBs Ag (5 μg/ml), Tet-Tox (37.5 μg/ml), and PHA (1 μg/ml; Roche Diagnostics, Mannheim, Germany)—or not were used as stimulators and used at a concentration of 5 × 10^5/ml. At day 5, [H]thymidine was added and following 16–18 h incubation, the plates were harvested, and radioactivity was counted using a liquid scintillation counter. The cpm or proliferation in presence of Tregs was related to proliferation in the absence of Tregs and depicted as percentages.

Cytokine-producing capacity

Percentages of cytokine-producing cells were determined by stimulating both Tregs and CD4<sup>+</sup> Teff either in the presence of costimulation only (anti-CD28 and anti-CD49d both at 1 μg/ml) or with the combination of PMA (50 ng/ml; Sigma-Aldrich, St. Louis, MO) and ionomycin (1 μg/ml; Sigma-Aldrich) for 24 h, of which the last 12 h were in the presence of GolgiPlug (BD Biosciences). Subsequently, the cells were harvested and the cell surface was stained using Abs directed against CD3, CD4, and the viability marker (7-AAD). Following fixation and permeabilization, intracellular staining was performed using mAbs directed against CD3, CD4, and CD25 (BD Pharmingen), IL-2, IFN-γ, or TNF-α (the last three from BD Pharmingen) (18, 19). Percentages of cytokine-producing cells within the Tregs were performed in the context of FOXP3 expression.

Statistical analyses

For comparisons between groups, the t test, Mann–Whitney U test, one-way ANOVA, or Kruskal–Wallis test was used, as appropriate. Post hoc analysis was performed using the Bonferroni test for multiple comparisons or the Mann–Whitney U test. A p value of <0.05 for two sides was considered statistically significant.

Results

Ag-reactive Tregs can be detected by CD154 expression in a live cell assay

Typically, Ag-reactive T cells are found at very low frequencies (1:10,000 or less of total circulating T cells) in the peripheral blood of healthy individuals. The exception to this rule is CMV Ag-reactive T cells that can be detected at a much higher frequency of 1:1000 to 1:100. Therefore, we started our study with CMV Ag, as this allowed for relatively easy detection and isolation of Ag-reactive T cells, using the CD154 live cell assay. PBMCs were enriched for T cells and the viable CD4<sup>+</sup>T cells were sorted in a Teff (CD4<sup>+</sup>Teff/CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>+</sup>) and Treg (CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>Bright/CD127<sup>+</sup>) population based on the differential expression of CD25 and CD127 (Fig. 1). This resulted in
CD154+ cells were observed at 6 h. However, sorting of both expression, whereas in the Tregs (Fig. 2) a phasic response (maximum at 6 and 24 h) of cell surface CD154 was following sorting, the CD4+ Teff (G) and Tregs (H) were stained intracellularly for FOXP3 (the open histogram resembles the isotype control staining in each of the fractions). Additionally, the percentage of demethylated TSDR FOXP3 was also determined for the different fractions (I). Results are a typical example from 10–15 experiments except for I, which represents mean and SEM for each fraction.

A Treg population with an average 95 ± 2.4% FOXP3-expressing cells (mean fluorescence intensity [MFI], 4475 ± 220) (Fig. 1H) as compared with CD4+ Teff that contained on average 7 ± 1% FOXP3-expressing cells with an overall MFI of 318 ± 157 (Fig. 1G). Moreover, the Tregs showed highly demethylated TSDR FOX3 as compared with CD4+ Teff that contained predominantly methylated TSDR FOX3 (Fig. 1I). In the presence of CMV Ag, CD154+ T cells were readily and consistently detected in both T cell populations in CMV-seropositive donors (Fig. 2). In contrast, CMV-seronegative donors did not have a CD154 signal above background (data not shown) (14). The background was very low in both populations (<0.01%) when freshly isolated T cells were used. Kinetic analysis of Ag-reactive CD154+ T cells showed a typical difference between CD4+ Teff and Tregs (Fig. 2A–F). The CD4+ Teff (Fig. 2A, 2B, 2E) showed a rapid but biphasic response (maximum at 6 and 24 h) of cell surface CD154 expression, whereas in the Tregs (Fig. 2C, 2D, 2F) only a few CD4+ cells were observed at 6 h. However, sorting of both populations was necessary to allow for discrimination of the CD154+CD4+ Teff and CD154+ Tregs at 24 h because the activated CD154+CD4+ Teff increased their expression of CD25 (Fig. 2G) and downregulated expression of CD127. This resulted in an average 30–50% overlap between both populations of Ag-reactive T cells at 24 h. It is known that activated CD4+ Teff may become FoxP3+ (20), but at 24 h the CD154+ Tregs still showed a significant higher FoxP3 expression (Fig. 2G). Additionally, the percentage demethylated TSDR FOX3 remained high in CD154+ Tregs as compared with CD154+CD4+ Teff (Fig. 2H), even after expansion of >80% of the TSDR FOX3 of CD154+Tregs was demethylated (data not shown).

Membrane-bound CD137 has been used to detect Ag-reactive T cells (21), and CD137+ cells could be detected after CMV Ag stimulation in the sorted Tregs (Fig. 2M). However, in contrast to the CD154 live cell assay, the expression in time of CD137+ Ag-reactive T cells was similar for CD4+ Teff and Tregs and reached the maximum at 24 h (Fig. 2N, 2O). We continued the experiments with the CD154 live assay, as CD137 background signals were in general higher and could vary considerably among donors.

CD154+ Tregs have a memory phenotype and express inflammatory cytokines after polyclonal stimulation

Within the CD4+ Teff population, cells can be classified in subsets based on the expression of CCR7 and CD45RO isoform on their cell membrane. This leads to a division into three major subsets—naïve T cells (CCR7+CD45RO-), central memory T cells (CCR7+CD45RO+), and effector memory T cells (CCR7-CD45RO+)—that correlate with increasing T cell differentiation (16). Within the Treg population a similar subset distribution was observed as for the CD4+ Teff population (Fig. 3B and 3A, respectively). The CD154+CD4+ Teff and CD154+ Tregs were predominantly found within the memory compartment (Fig. 3C).

As expected, substantially lower numbers of Tregs secreting IL-2 (6.4 ± 2.4%), IFN-γ (1.5 ± 0.6%), or TNF-α (8.7 ± 2.8%) were found compared with the CD4+ Teff population (IL-2+, 30.3 ± 3.3%; IFN-γ, 9.9 ± 2%; and TNF-α, 22.8 ± 6.6%) after polyclonal stimulation with PMA/ionomycin (Fig. 3H–K and 3D–G, respectively). More than 98% of the cytokine-producing Tregs coexpressed FOXP3 (Fig. 3I–K).

CD154+ Tregs can be expanded in an Ag-specific manner and are potent suppressors

Next, the CMV Ag-specific CD154+ Tregs were sorted (Fig. 4A–C) and compared with CD154-depleted Tregs for their suppressive function either immediately (Fig. 4D, 4E) or following expansion (Fig. 4I, where only results of CMV Ag-expanded
CD154-expressing Tregs are displayed). The CD154+ Tregs were significantly better suppressors of CMV Ag-induced T cell proliferation compared with the CD154-depleted Tregs (Fig. 4D, 4F), although both cell populations showed a similar efficacy in suppressing polyclonal PHA-induced T cell proliferation (Fig. 4E, 4F).

FIGURE 2. CD154 and CD137 expression following CMV Ag stimulation in CD4+ Teff and Tregs. CD4+ Teff and Tregs were stimulated with CFSE-labeled autologous PBMCs, either loaded with CMV Ag or not, in the presence of costimulation (anti-CD28 and anti-CD49d mAbs) and anti-CD40 to stain for CD154 on the cell surface (A–F) or without anti-CD40 to stain for CD137 (J–O). At 6 and 24 h CMV Ag stimulation, CD154 (A–D) or CD137 (J–M) expression (black dots) was determined within CD4+ Teff and Tregs. The kinetics of CD154- and CD137-expressing cells within CD4+ Teff (E, N) and Tregs (F, O) was determined immediately following isolation and upon stimulation with or without CMV Ag for different time points. One of three representative examples is shown. MFI of FOXP3 within total and CD154-expressing CD4+ Teff and Tregs following stimulation with CMV Ag for 24 h (G) was analyzed as well as the percentage of demethylated TSDR FOXP3 (H) and MFI of CD25 (I). Results are means ± SEM of three to five independent experiments. *p < 0.05.
It was possible to expand unseparated or CD154-expressing (CMV Ag-reactive) Tregs of a CMV-seropositive donor in the presence of CMV Ag, IL-2, and IL-15. No proliferation of Tregs was observed when CMV Ag expansion was performed without IL-2 and IL-15 (data not shown) or when cells of a seronegative individual were chosen for expansion (Fig. 4G). These results could be replicated by using VZV Ag in cultures of Tregs derived from VZV-seropositive donors, but again not in VZV-seronegative donors (data not shown). The CD154-enriched Tregs could be expanded more efficiently (yielding higher cell numbers) when compared with unseparated Tregs (Fig. 4G) using CMV Ag. In contrast, CD154-depleted Treg cultures showed no significant expansion in response to CMV Ag, and expansion was comparable to cytokine-only conditions. Expanded CMVAg-specific CD154+ Tregs showed stable high FOXP3 expression (Fig. 4I); that is, >95% of these Tregs still expressed FOXP3. These CMV Ag-expanded CD154+ Tregs were potent suppressors of CMVAg-induced T cell proliferation (Fig. 4I). Additionally, they showed a modest suppression of T cell proliferation to other common Ags but only at the highest ratios of Tregs/CD3+ Teff (Fig. 4I). These results indicate that CMV Ag-reactive CD154+ Tregs, when expanded in an Ag-specific manner, remain predominantly Ag-reactive in their suppressor function.

Proliferation in cpm is given in Supplemental Fig. IA–D. Additionally, a kinetic analysis did not reveal a shift in the proliferative response of CD3+ Teff to CMV Ag in the presence of 1:5 CMV Ag-expanded CD154+ Tregs. The strong suppression observed at day 6 was therefore unlikely to be caused by exhaustion of this culture (Supplemental Fig. 1E).

**Ratio of Ag-reactive CD154+ Tregs to CD154+CD4+ Teff**

Not only CMV Ag, but a number of other common Ags such as VZV Ag, HBsAg, and Tet-Tox showed a CD154+ response in the Treg population (Fig. 5A). Independent of the frequency of Ag-reactive T cells, a strikingly similar ratio for the percentage of CD154+CD4+ Teff/percentage of CD154+ Tregs was found for the different Ags tested (Fig. 5B). This ratio was on average 10:1, and similar to the amount of Tregs in the circulation, when tested in the unstimulated healthy individual.

After challenging the memory T cells with a booster vaccination of HBs Ag or Tet-Tox (Fig. 5C), a time-dependent increase of Ag-reactive (CD154+) T cells was observed for CD4+ Teff. The frequency of circulating Ag-reactive CD154+ Tregs did not significantly change, leading to a temporarily increase of the CD154+ CD4+ Teff/CD154+ Treg ratio with a maximum at 2 wk (Fig. 5D).

**Discussion**

In this study we show the presence of human Ag-reactive nTregs in the peripheral blood. Reactivity for CMV Ag was initially studied, as the frequency of CMV Ag-reactive T cells is remarkably high, thereby facilitating the identification and isolation of Ag-reactive Tregs. Activated CD4+ Teff or iTregs with (temporarily) increased FOXP3 expression and increased CD25 and decreased CD127 expression may mimick nTregs (8, 20). However, there are a number of arguments in favor of the conclusion that Ag-reactive CD154+ cells within the sorted CD25++CD127−CD4+ T cell population are true nTregs.

First, the expression in time of CD154 on Tregs was different from CD4+ Teff, as the rapid biphase expression pattern (14) was not observed. Instead, similar to Ag-specific induction of CD137 on the cell surface, the expression was detectable after 6 h and reached the maximal level after 24 h stimulation. This implies that after 6 h Ag-specific stimulation all CD154+ T cells are found within the CD4+ Teff, which potentially offers the possibility to discriminate Ag-
reactive CD4+ Teff from Tregs. Unfortunately, at 24 h the differentiation between CD154+ Tregs and CD154+ Teff could not be made without previous sorting, as both CD127 and CD25 expression of CD154+CD4+ Teff substantially overlapped with Tregs at that point.

Second, the CD154+ Tregs were found in a homogeneous population of Tregs characterized by the simultaneous expression of CD127 at low levels, as well as CD25 and FOXP3 at high levels. Additionally, the FOXP3 expression in CD154+ Tregs was still significantly higher at 24 h compared with the unchanged FOXP3 expression in CD154+CD4+ Teff.

Third, the unchanged highly demethylated TSDR FOXP3 within CD154+ Tregs was in favor of the development of CMV Ag-reactive Tregs from natural occurring Tregs instead of a development from CD4+ Teff.

Fourth, the Treg population we isolated expressed low levels of inflammatory cytokines after polyclonal activation with PMA/ionomycin, as has been reported before (8, 9).

The final and most compelling argument is the observation that freshly isolated Ag-reactive CD154+ Tregs showed Ag-reactive T cell suppression and could be efficiently expanded in vitro by Ag-specific stimulation combined with exogenous IL-2 and IL-15. These Ag-expanded CD154+ Tregs were highly suppressive in an Ag-specific fashion while still displaying a nonspecific, but much less effective suppression when tested in an assay of polyclonally stimulated T cells or other recall Ags.

Based on these findings we conclude that Ag-reactive CD154+ cells within the sorted CD4+CD25+CD127- FOXP3+ T cell population are indeed nTregs and not activated CD4+ Teff or iTregs. This finding was not specific for CMV Ag, as we could...
subsequently show similar results using different Ags, including both Ags involved in natural infections and vaccination Ags.

A remarkable stable and similar ratio of Ag-reactive CD154+ CD4+ Teff and CD154+ Tregs was found for the Ags tested. The formation of Tregs in a fixed ratio, irrespective of the Ag, indicates that these cells may actually be the brake on every Teff response. Such an intimate relation between Teff and Tregs was not observed within the peripheral blood after booster vaccination, as the frequency of CD154+ Tregs did not significantly change. However, this led to a highly increased CD154+CD4+ Teff/CD154+ Treg ratio that eventually returned to baseline, but only after 6–8 wk. The difference in kinetics between CD154+ Tregs and CD154+CD4+ Teff also argues against the induction of Tregs from the CD154+CD4+ Teff.

Our data obtained in human individuals are in accordance with the data from in vivo tracking of color-coded Teff and Treg in the allograft response against pancreatic islets transplants in the mouse (22). Using this elegant technique, it was shown that natural Tregs and Teff accumulate at the same pace within the transplant. This result is very similar to the observations made on the local T cell immune response after cutaneous inoculation with tuberculin-purified protein derivative in humans (11). However, similar to our vaccination data, numbers of Tregs within the peripheral blood hardly change after islet transplantation and do not show the steep increase as observed for Teff (22). Therefore, after vaccination it may be a possibility that Ag-reactive Tregs, having a memory lymphoid homing profile (CD45RO+CCR7+), are retained and active within the lymphoid organs in the weeks following antigenic exposure. Further research in different clinical settings such as infection and autoimmunity is required to understand the relationship between circulating Ag-reactive Tregs and disease activity. The present data indicate that findings for Tregs within the peripheral blood may not accurately reflect the actual Treg response at the site of immune reaction and should be interpreted with caution.

A major conclusion of our study is that by inference of the results with several unrelated Ags, the CD4+CD25++CD127lowFOXP3+ Treg compartment is filled with Ag-specific T cells as a mirror image of the memory CD4+ Teff population. This in fact supports the hypothesis, based on the recent work by Mayari et al. (8) that, similar to Teff, the natural Treg compartment consists of thymus-derived naive T cells that will differentiate into memory T cells after appropriate antigenic stimulation. Our findings are also in line with the results from TCR analysis of Teff and Tregs, which did not show any evidence for affinity for autoreactive Ag (23), and Vβ T cell repertoire analysis, which showed that human CD4+ CD25+ Tregs share equally complex and comparable repertoires with CD4+CD25− counterparts (24). In the latter study it was also a remarkable finding that CMV seropositivity gave rise to a similar shift in Vβ repertoire in Teff and Tregs.

The possibility of direct recognition of Ag-reactive Tregs will facilitate further research in this direction, aiming a more complete understanding of the significance and interaction of Tregs in human disease and immune responses. Additionally, isolation and propagation of Ag-reactive T cells identified on the single cell level is of potential benefit for future immunotherapy with Tregs.

Disclosures
The authors have no financial conflicts of interest.
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Supplement 1 (to Figure 4) **Suppressive function of Treg fractions**

Functional analysis was performed using a proliferation assay with [3H]-thymidine incorporation as a read-out and proliferation in the presence of Tregs was compared to that without Tregs (noTregs). For this purpose, CD3+Teff were stimulated with irradiated autologous PBMC only or combined with CMVAg (A) or PHA-Ag (B) or other antigens aside from CMVAg (C) and PHA-Ag (D), i.e. VzVAg, HBsAg or TetTox (C). Different numbers of CD154-expressing (CMVAg-specific) or CD154-depleted Tregs were added to these stimulated CD3+Teff either directly upon isolation (A, B) or upon CMVAg-expansion (CD154-expressing Tregs, C and D). After 5 days of incubation, [3H]-thymidine was added and following a 16-hour incubation, the plates were harvested and proliferation was counted using a liquid scintillation counter (i.e. counts per minute, cpm are indicative of the amount of [3H]-thymidine incorporated and a measure for proliferation). A kinetic analysis was performed for the proliferation (in triplicate) of CD3+Teff (CD3+T cells that are depleted for Tregs) to CMVAg in absence and presence of 1:5 CMVAg-expanded CD154-expressing Tregs. For this purpose, [3H]-thymidine incorporation was measured at day 2, 4 and 6. Median results of three independent experiments with proliferation (in cpm) on the Y-axis and different time-points (in days) on the X-axis are given (E). The straight line depicts the proliferation of CD3+Teff to CMVAg whereas the dotted line that in presence of 1:5 CMVAg-expanded CD154+Tregs.