A model of suppression of the antigen-specific CD4 T cell response by regulatory CD25+CD4 T cells \textit{in vivo}

Kristen M. Thorstenson*, Laura Herzovi* and Alexander Khoruts

Center for Immunology and the Department of Medicine, University of Minnesota, 6-134 BSBE Building, 312 Church Street, SE Minneapolis, MN 55455, USA

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Abstract

Despite intense recent interest, the suppressive mechanisms of regulatory CD25+CD4 T cells remain poorly understood. One deficiency in the field is the lack of \textit{in vivo} models where the effects of regulatory CD25+CD4 T cells on antigen-specific responder T cells can be measured quantitatively. We describe one such model here. We compared responses of adoptively transferred naive wild-type antigen-specific CD4 T cells in syngeneic CD28−/− and wild-type recipient mice toward a nominal antigen. The cells exhibited a greater degree of proliferation and differentiation in CD28−/− mice and could not be rendered functionally hyporesponsive by systemic exposure to adjuvant-free antigen. The only reason we were able to find to explain this difference was the deficiency of regulatory CD25+CD4 T cells in the CD28−/− mice. Use of CD28−/− mice as adoptive transfer recipients provides a simple model that reveals the contribution of regulatory CD25+CD4 T cells in controlling antigen-driven responses \textit{in vivo}.

Introduction

Regulatory CD25+CD4 T cells are well recognized to play an essential role in the maintenance of immunological tolerance, but their suppressive mechanisms remain unclear (1–4). There is broad agreement that their ability to suppress \textit{in vitro} is dependent on direct contact with the responder T cells and independent of any soluble factors or inhibitory effects on the function of antigen-presenting cells (APCs) (4–6). However, different mechanisms may play a dominant role \textit{in vivo}. In fact, distinct mechanisms may operate in lymphopenic and non-lymphopenic hosts, as well as within secondary lymphoid tissues and peripheral tissues. Mechanistic studies of T cell responses \textit{in vivo} can be greatly enhanced by the ability to track the fate of antigen-specific responder T cells. The goal of this study was to establish a relatively simple \textit{in vivo} model system that can be used to quantify the effects of regulatory CD25+CD4 T cells on antigen-specific T cell responses in normocytic animals.

Regulatory CD25+CD4 T cells are present in greatly decreased numbers in CD28−/− mice (7, 8). Thus, we compared responses of adoptively transferred antigen-specific naive TCR transgenic (Tg) CD4 T cells in wild-type and CD28−/− recipients. We found that the TCR Tg CD4 T cell responders proliferate to a significantly greater extent in CD28−/− hosts following antigenic challenge, and re-constitution of CD28-deficient hosts with wild-type CD25+CD4 T cells inhibits proliferative responses of antigen-specific CD4 T cells in a dose-dependent manner. Furthermore, enhanced cell cycle progression of antigen-specific CD4 T cells in CD28−/− hosts correlates with their increased differentiation into IFN-γ and IL-4-producing effector cells and a defect in tolerance induction. These results compliment previously published reports of \textit{in vivo} suppression by antigen-specific CD25+CD4 T cells (9–11) and introduce some differences which may lead to eventual greater mechanistic insight.

Methods

Mice

DO11.10 TCR Tg RAG-2−/− mice extensively backcrossed onto the BALB/c background were used as donors of CD4 T cells. The mice were bred and maintained as previously described (12). In the majority of the experiments, normal female BALB/c mice purchased from the National Cancer Institute (Frederick, MD, USA) was used as recipients at 6–10 weeks of age. CD28−/− mice on the BALB/c background were originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and bred subsequently at our facility. Thy1.1 congenic BALB/c mice were a generous gift of Laurence Turka.
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(University of Pennsylvania, Philadelphia, PA, USA). HA TCR Tg RAG-2–/– mice were used as intermediate hosts to generate effector DO11.10 T cells (28). All mice were maintained in a specific pathogen-free facility in microisolator cages with filtered air according to the National Institutes of Health guidelines. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Adoptive transfer and immunizations

Unless otherwise specified, responder donor T cells from the secondary lymphoid tissues (axillary, brachial, cervical, inguinal, mesenteric lymph nodes, and spleen) were labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) using the technique previously described (13) and injected into the tail vein at 2.5–5 × 10^6 cells per specified recipient mouse (14). Some of the CD28–/– recipient mice were reconstituted with varying numbers of wild-type CD25+CD4 T cells, which were prepared in >95% purity using positive selection using streptavidin-labeled magnetic microbeads and columns (Miltenyi Biotec, Auburn, CA, USA) following staining with biotinylated secondary lymphoid tissues (axillary, brachial, cervical, inguinal, mesenteric lymph nodes, and spleen) were labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) using the technique previously described (13) and injected into the tail vein at 2.5–5 × 10^6 cells per specified recipient mouse (14). Some of the CD28–/– recipient mice were reconstituted with varying numbers of wild-type CD25+CD4 T cells, which were prepared in >95% purity using positive selection using streptavidin-labeled magnetic microbeads and columns (Miltenyi Biotec, Inc., Auburn, CA, USA) following staining with biotinylated anti-CD25 mAb, PC61.

Various immunization protocols were used. Some mice were injected subcutaneously with the ovalbumin protein emulsified in CFA (Sigma–Aldrich, St. Louis, MO, USA) into three sites—tail base and bilaterally in the upper dorsum, 50 μl per injection (1 mg of protein/per milliliter of emulsion). Some mice were immunized with varying amounts of the ovalbumin peptide (OVA) 323–339 (25–250 μg per mouse) intravenously alone or with soluble adjuvants, which included LPS (25 μg per mouse) and cholera toxin (0.5 μg per mouse). Some mice were challenged with varying amounts of LPS-free ovalbumin protein alone or with soluble adjuvants mentioned. In most of the experiments, we used 500 μg of the ovalbumin protein.

Chicken ovalbumin purchased from Sigma–Aldrich was found to have significant contamination with LPS. Therefore, LPS was removed from the commercial protein by phase separation using a protocol previously described (15). Every batch of the ovalbumin protein was tested for the ability to up-regulate B7-2 expression on splenic CD11cCD8α+ and CD11cCD8α− cells in vivo 9 h following intravenous injection of 5 μg of ovalbumin, time of peak B7 up-regulation by LPS (16). ‘Clean’ protein did not have this activity (2 mg per mouse injected intravenously), while it was easily seen with the unpurified commercial ovalbumin.

Secondary adoptive transfer

Effector DO11.10 T cells capable of efficient homing to secondary lymphoid tissues and producing IFN-γ and IL-4 were prepared using intermediate HA RAG–/– hosts. Naive DO11.10 RAG–/– T cells were adoptively transferred into HA RAG–/– mice that received 500 μg of the LPS-free ovalbumin protein and 250 μg of anti-CTLA-4 mAb, 4F10. Seven days later, the T cells were purified by negative selection against I-Ad, CD11b and DX5 using biotinylated antibodies and streptavidin-coated magnetic microbeads (Miltenyi Biotec, Inc.), labeled with CFSE, and transferred into BALB/c or CD28–/– recipients.

Flow cytometry

Single-cell suspensions were prepared separately from the lymph nodes (axillary, brachial and inguinal) and spleens by mechanical disruption. DO11.10 T cells were identified using fluorochrome-conjugated antibodies, KJ1-26 and CD4 (Caltag [San Francisco, CA, USA], BD Pharmingen [San Diego, CA, USA], eBioscience [San Diego, CA, USA]). DTA-1 hybridoma was generously provided by Shimon Sakaguchi (Kyoto University, Japan). Intracellular staining for cytokines was done as previously described following direct in vivo stimulation with the peptide (12, 17, 18). Briefly, mice received an intravenous injection of indicated dose of the OVA 323–339 and sacrificed 1 h later. Immediately after that, lymphoid tissues were disrupted into a single-cell suspension and fixed in 2% formaldehyde for 20 min. The cells were then permeabilized with a saponin-containing buffer and stained with PE-conjugated antibodies against IL-2, IL-4, IFN-γ or isotype controls (BD Pharmingen and eBioscience).

Results

Regulatory CD25+CD4 T cells inhibit proliferation and clonal expansion of naive CD4 T cells

The DO11.10 adoptive transfer system where responses of a limiting number of TCR Tg T cells can be physically tracked is a well-established method to measure antigen-specific T cell responses in vivo (14, 19). We used this system to compare the response of naive antigen-specific CD4 T cells in wild-type and CD28–/– recipients. Both thymic development and peripheral homeostasis of regulatory CD25+CD4 T cells are greatly dependent on B7/CD28 signals (8); thus, CD28-deficient and B7-deficient mice have a significant reduction in the numbers of these cells (7, 10, Fig. 1). Indeed, we found that clonal expansion of DO11.10 T cells is significantly greater in CD28-deficient recipients following intravenous injection of the OVA and LPS (Fig. 2A). Pre-transfer of wild-type CD25+CD4 T cells into the CD28-deficient recipients inhibited this increase in a dose-dependent manner (Fig. 2B). Augmented expansion of DO11.10 T cells was also seen following stimulation with the OVA or protein alone or in combination with adjuvants such as LPS, CFA and anti-CTLA-4 mAb (4F10) (data not shown). Pre-transfer of CD25+CD4 T cells, but not CD28–CD4 T cells, from wild-type mice was inhibitory under all conditions (data not shown). In most of the experiments described below, we used 5–10 × 10^6 CD25+CD4 T cells to achieve partial re-constitution of the CD28–/– mice. This transfer achieves a level of ~2% among the splenic CD4 T cells (Fig. 2B), compared with 5–10% CD25+ T cells within the CD4 T cell population in the wild-type mice. The relatively poor ‘parking efficiency’ of transferred CD25+CD4 T cells compared with CD25–CD4 T cells has been noted previously and proposed to be at least in part due to limited responsiveness of these cells to chemokine-induced migration into the secondary lymphoid tissues (20). Alternatively, it may merely be a consequence of the host partially clearing cells coated with anti-CD25 antibody at the time of the transfer.

Clonal expansion measured in the secondary lymphoid tissues is a function of clonal proliferation, death and migration into tissues. In order to gain better understanding of the
enhanced clonal expansion in the CD28-deficient hosts, we calculated how many times an average DO11.10 T cell divided in the course of the primary immune response by measuring the CFSE content of the DO11.10 population. Clearly, responder T cells divided more times in the CD28−/− recipients (Fig. 2A). As expected, DO11.10 T cells that divided the most were preferentially lost from the secondary lymphoid tissues of the BALB/c recipients after reaching peak expansion on day 3 after antigen exposure. This loss is in part attributable to cell death but may also be partially due to preferential migration of high dividers into non-lymphoid tissues (21–23). Interestingly, the disappearance of highly divided CD4 responders from secondary lymphoid tissues was blunted in the CD28−/− recipients. Therefore, the presence of CD25+CD4 T cells affects both the expansion and contraction phases of the primary immune response.

**CD25+CD4 T cells inhibit differentiation of naive antigen-specific CD4 T cells into Th1 and Th2 phenotypes**

The next question we wished to address is how CD25+CD4 T cells affect T cell differentiation in the course of the primary immune response. We measured cytokine production by DO11.10 T cells directly in vivo following a bolus intravenous injection of the OVA as a re-challenge. Significant proportion of DO11.10 T cells in the lymph nodes of CD28−/− animals primed with ovalbumin/CFA produced IL-4, but little IFN-γ, while no IL-4 or IFN-γ production by DO11.10 T cells was noted in the lymph nodes of wild-type BALB/c recipient mice (Fig. 4). In contrast, IFN-γ was produced by a fraction of DO11.10 T cells in the spleens of animals immunized with OVA and LPS. This fraction is increased ~2-fold in CD28-deficient recipients. However, production of both IL-4 and IFN-γ was decreased in CD28-deficient animals that were partially reconstituted with wild-type CD25+CD4 T cells (Fig. 4).

One possible explanation for this result is the direct suppression of IL-4 and IFN-γ production by CD25+CD4 T cells during the in vivo re-stimulation assay. We considered this possibility unlikely since CD25+CD4 T cells could not suppress production of IL-2 by naive CD4 T cells. Nevertheless, the possibility was tested. We measured cytokine production of primed DO11.10 T cells in vitro with phorbol myristate acetate and ionomycin following depletion of all CD25+CD4 T cells. Purified primed DO11.10 T cells from CD28-deficient recipients produced more IL-4 and IFN-γ than primed DO11.10 T cells from BALB/c mice and CD28-deficient mice reconstituted with CD25+CD4 T cells (data not shown). In addition, we tested the ability of effector DO11.10 T cells generated in HA RAG−/− mice and re-transferred into BALB/c and CD28−/− secondary recipients to produce IL-2, IFN-γ and IL-4 following a re-challenge with the OVA. No differences were found (Fig. 5A). However, similar to what we have noted for naive T cells, the effector DO11.10 T cells proliferated to a lesser extent in wild-type as compared with CD28−/− hosts following secondary antigenic challenge (Fig. 5B). Thus, in our system, CD25+CD4 T cells could not inhibit cytokine production by naive or effector CD4 T cells, but did inhibit CD4
T cell differentiation, which correlated with inhibition of the proliferative response to antigenic stimulation.

Peripheral tolerance induction following systemic antigen administration fails in CD28−/− hosts

Greater differentiation and functional responsiveness of CD4 T cells in the course of primary immune response have been associated with greater number of cell divisions (29). Since CD28−/− hosts supported greater proliferation and differentiation by adoptively transferred antigen-specific CD4 T cells, we tested whether the two variables correlate. CFSE-labeled DO11.10 RAG−/− T cells were adoptively transferred into wild-type and CD28−/− mice, which were then administered 500 μg of the LPS-free ovalbumin protein intravenously. The protocol induces a state of hyporesponsiveness in...
antigen-specific CD4 T cells, which can be prevented by the addition of adjuvant to the antigen during priming (12). Eight days after initial antigen administration, we measured the numbers of DO11.10 T cells, their CFSE content and recall cytokine production in spleens and lymph nodes of the recipients following a 1-h re-challenge with the OVA. Indeed, as expected, we saw greater IL-2 production by T cells with greater cell division history and DO11.10 T cells proliferated to a greater extent in CD28−/− hosts and made more IL-2 (Fig. 6). Interestingly, the most highly divided DO11.10 T cells also made a significant amount of IL-4 in CD28−/− animals (Fig. 6). The enhanced proliferation and differentiation of DO11.10 T cells in CD28−/− hosts in response to ovalbumin protein administration was inhibited by adoptive transfer of regulatory wild-type CD25+CD4 T cells (data not shown). The result is consistent with the idea that naturally occurring CD25+CD4 T cells normally promote tolerance induction of CD4 T cells responding to cognate antigens.

**Discussion**

Although there are several well-established *in vitro* assays of suppressive functions of regulatory CD25+CD4 T cells (4–6, 30), it is unlikely that they fully capture the suppressive mechanisms these cells employ *in vivo*. In fact, the ability of regulatory CD25+CD4 T cells to suppress proliferation of responder CD4 T cells *in vitro* is critically dependent on the suppression of IL-2 production (30), and greatest suppression is seen when IL-2 is limiting. In contrast, IL-2 is not essential for proliferation of CD4 T cells *in vivo* (18, 31), and regulatory CD25+CD4 T cells can suppress IL-2-deficient responder CD4 T cells *in vivo* (32). Suppression by CD25+CD4 T cells *in vitro* is dependent on direct contact with the responder CD4 T cells and is independent of any effects on APCs or production of soluble suppressive cytokines (4, 6, 30). In contrast, regulatory CD25+CD4 T cells have been shown to suppress elements of the innate immune system *in vivo*, and soluble cytokines, e.g. IL-10, have been shown to play a critical role in their suppressive activity (33, 34). In fact, it is possible that regulatory CD25+CD4 T cells employ multiple suppressive mechanisms *in vivo* that differ depending on the specific type of the responder cell, tissue location (e.g. secondary lymphoid tissue versus periphery) and presence or absence of lymphopenia. Thus, there is a need for robust *in vivo* models that can measure suppressive effects of regulatory CD4 T cells.

Here, we focused on developing a relatively simple model of suppression mediated by regulatory CD4 T cells on antigen-specific naive responder CD4 T cells in the secondary lymphoid tissues of a non-lymphopenic animal. The total numbers of T cells are comparable in wild-type and CD28−/− animals (35). However, the numbers of regulatory CD25+CD4 T cells are greatly reduced in CD28−/− and B7−/− animals because of critical importance of B7; CD28 signals for homeostasis of these cells (7, 8, 10). We found that adoptively transferred
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Fig. 5. Regulatory CD25+CD4 T cells do not diminish the cytokine production potential of differentiated T_{h1} or T_{h2} effectors. Effector DO11.10 T cells were purified from intermediate HA RAG-2−/− recipients. The spleens were taken, fixed and stained for cytokines 1 h after the peptide challenge. The bar graphs show the mean percentage of cytokine-positive DO11.10 T cells from individual mice ± variance in this experiment, which is representative of three independent experiments. (A.) The secondary recipients received an intravenous pulse of the ovalbumin protein. The spleens were taken, fixed and stained for cytokines 1 h after the peptide challenge. The bar graphs show the mean percentage of cytokine-positive DO11.10 T cells from individual mice ± variance in this experiment, which is representative of three independent experiments. (B.) The effector DO11.10 T cells were labeled with CFSE prior to secondary transfer. The secondary BALB/c and CD28−/− recipients received 500 μg of the ovalbumin protein. Bold-line histograms show CFSE content of DO11.10 T cells in spleens of CD28−/− and wild-type recipients 5 days after antigen injection. Control histograms show the CFSE content of DO11.10 T cells in animals that were not injected with antigen. Identical results were seen in the lymph nodes. The experiment is representative of three independent experiments.

responder CD4 T cells exhibited increased clonal expansion in CD28−/− recipients due to greater proliferative response and lesser degree of contraction following peak expansion. Greater proliferative response was also accompanied by greater differentiation of the antigen-specific CD4 T cells into IFN-γ and IL-4 producers, while the direction and extent of differentiation was determined by the specific adjuvant used. In addition, we found lack of tolerance induction by systemic administration of antigen without adjuvant. Although we considered and tested alternative potential explanations for these results, e.g. baseline activation of the innate immune system in immunocompromised CD28−/− hosts, the only explanation we found support for was deficiency in regulatory CD25+CD4 T cells in CD28−/− hosts.

Interestingly, we found no effect of CD25+CD4 T cells on naive or effector responder T cell function on cytokine production, which takes place within hours of antigen stimulation. In addition, the differences in proliferative responses of antigen-specific CD4 T cells in the presence or absence of CD25+CD4 T cells were not discernable until day 3 and became progressively more prominent at later time points. This result is consistent with observations made by Klein and colleagues, where antigen-specific CD25+C4 T cells did not affect the initial expansion of naive T cells following stimulation with antigen/incomplete Freund’s adjuvant but suppressed their eventual accumulation (9). These results suggest that CD25+CD4 T cells can mediate their effects after the initial T cell activation events are completed.

It should be noted that despite considerable effort, we could not demonstrate that CD28-deficient hosts were equivalent to wild-type hosts depleted with anti-CD25 antibodies, despite a trend toward greater differentiation of DO11.10 CD4 T cells in CD25-depleted animals (data not shown). Some of the reasons for the difference are probably technical. Thus, the residual antibody depleted significant numbers of responder DO11.10 cells, which expressed CD25 following antigen activation (data not shown). Although our protocol depleted CD25+ cells with >95% efficiency, we cannot fully rule out the possibility that some residual CD25+CD4 T cells still continued to provide suppression, even though our titration studies with re-constitution of CD28−取得了 hosts would argue that relatively small residual numbers of regulatory CD25+CD4 would be ineffective. It is possible that there are CD25-regulatory T cells that are also dependent on CD28 signals for their generation and/or maintenance. Suppressive CD25−GITR+CD4 T cells have been reported in wild-type mice (36). Finally, it cannot be assumed that anti-CD25 depletion exclusively targets regulatory CD25+CD4 T cells, since other cell types that may be important in the immune response toward a systemic antigen, e.g. recently described accessory cells (37), express CD25.

Our system is similar to the one described by Lohr and colleagues (10), where responder DO11.10 T cells were stimulated in vivo by ovalbumin-pulsed DCs in wild-type and B7-deficient hosts. However, there are some significant differences in the results. While our system showed clear correlation of greater functional T cell differentiation with greater cell cycle progression, no difference in cell cycling activity was seen in their experiments between wild-type and B7-deficient hosts (10). One major difference is in antigen presentation. In contrast to T cell stimulation by antigen-pulsed DCs, we administered systemic antigen, which is likely taken up by a variety of APCs including DCs, recently described accessory cells (37) and B cells, all of which may participate in driving clonal expansion. It is also formally possible that there is a difference in the amount of B7 signaling following immunization with DCs in B7-sufficient and B7-deficient hosts if antigen-non-bearing APCs provide additional B7 co-stimulation. We think this formal possibility is not critical because we also saw little difference in responder CD4 T cell proliferation in wild-type and CD28−/− hosts if we used antigen-pulsed DCs instead of systemic antigen (data not shown). Finally, it should be noted...
that endogenous T cell competitors are relatively defective in CD28−/− hosts. However, we do not believe this to be an essential difference either since the endogenous ovalbumin-specific fraction is relatively minor compared with the adoptively transferred population of DO11.10 T cells. Thus, we think it is most likely that the difference in the effects of regulatory CD25+CD4 T cells on clonal expansion between the two systems is due to a difference in the number and types of participating APCs.

We found it interesting to see IL-4 production by DO11.10 T cells in CD28−/− hosts following immunization with ovalbumin protein alone and even with CFA. We have not seen differentiation by DO11.10 T cells into Th2 cells in the adoptive transfer system using wild-type recipients in the past despite multiple efforts to do so using a variety of adjuvants, including extracts of parasitic worms and testing a range of protein and peptide antigen doses (data not shown). Interestingly, we saw differentiation of DO11.10 into IL-4-producing cells in CD28−/− hosts only with protein, but not peptide antigen, even among the most highly divided cells. The reason for the difference is not clear at this time, although obviously the half-life of the peptide antigen and its distribution among various APCs is different from the protein. It has been hypothesized that Th2 differentiation is the preferred pathway in the absence of any stimulation of pathogen-associated molecular pattern receptors (38), although clearly antigen stimulation alone is not sufficient. Our results suggest that regulatory CD25+CD4 T cells are especially potent in suppressing Th2 differentiation in vivo. However, CD28−/− hosts were also able to support Th1 differentiation of DO11.10 cells when antigen was mixed with adjuvants, such as LPS and cholera toxin (data not shown), and co-transfer of CD25+CD4 T cells was able to suppress Th1 differentiation. In fact, the ability of CD25+CD4 T cells to suppress CD4 T cell responses in the presence of LPS is somewhat surprising since it was shown that soluble factors, which include IL-6, produced by DCs following LPS stimulation, abrogate suppressive abilities of CD25+CD4 T cells in vitro (39). Clearly, a full mechanistic understanding of how CD25+CD4 T cells work can only result from a combination of in vitro and in vivo studies.

![Fig. 6. Failure of tolerance induction and differentiation into IL-4-producing effectors by DO11.10 T cells in CD28−/− hosts. CFSE-labeled DO11.10 RAG−/− T cells were adoptively transferred into wild-type BALB/c and CD28−/− recipients, which were subsequently intravenously injected with 500 μg of ovalbumin protein. Eight days later, the mice were intravenously pulsed with 250 μg of the OVA, and 1 h later the spleens were taken and splenic DO11.10 T cells were stained for intracellular cytokines. The bar graphs show the total number of DO11.10 T cells in the spleens and mean percentage of cytokine-positive DO11.10 T cells during recall response in individual mice ± variance. This experiment is representative of four independent experiments.](image-url)
Acknowledgements

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Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>CFSE</td>
<td>5,6-carboxyfluorescein diacetate succinimidyl ester</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>GITR</td>
<td>glucocorticoid-induced tumor necrosis factor receptor</td>
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