Activation-Induced FoxP3 Expression Regulates Cytokine Production in Conventional T Cells Stimulated with Autologous Dendritic Cells

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A defining feature of dendritic cells (DCs) is their ability to induce the proliferation of autologous T cells in the absence of foreign antigen—a process termed the “autologous mixed leukocyte reaction” (AMLR). We report that equine monocyte-derived DCs, but not macrophages, are potent inducers of the AMLR. The response is contact dependent and major histocompatibility complex class II dependent and primarily involves CD3+ CD4+ CD8− T cells. Upon stimulation with DCs or the mitogen concanavalin A, a subset of the proliferating T cells expresses the regulatory T-cell (Treg) transcription factor FoxP3. Although many of these FoxP3+ T cells are capable of producing the effector cytokines interleukin-4 (IL-4) and gamma interferon (IFN-γ), they are more likely to produce IL-10 and less likely to produce IFN-γ than equivalent FoxP3− cells. Therefore, FoxP3 expression is an inherent component of equine T cell activation and is associated with a more immunosuppressive cytokine profile. These results confirm that FoxP3 expression in the horse, in contrast to the mouse, is regulated similarly to FOXP3 expression in humans and provide evidence that FoxP3 expression by conventional T cells may help regulate the developing immune response.

Dendritic cells (DCs) represent a heterogeneous population of innate immune cells specialized in immunosurveillance, antigen presentation, and initiation of the adaptive immune response. DCs are uniquely capable of stimulating naive T cells and can generate a variety of effector responses, anergy, or tolerance (34). Therefore, DCs are an essential component of a successful immune response, and a detailed understanding of the interactions between DCs and T cells is relevant to many areas of immunology.

DCs take up antigen from the peripheral tissues, process it, and present it to naive T cells in the draining lymph node. Depending on the local cytokine environment during DC-mediated T cell activation, the antigen-specific T cell can proliferate and differentiate into one of a variety of effector T cell phenotypes, including (i) gamma interferon (IFN-γ)-producing Th1 cells that are important for immunity to intracellular pathogens and tumors; (ii) interleukin-4 (IL-4)−, IL-5−, and IL-13−producing Th2 cells that promote antibody production, help prevent parasitic diseases, and are involved in allergic responses; and (iii) IL-17−producing Th17 cells that protect against extracellular infections (58). DCs also play a critical role in immune regulation because they are capable of both expanding thymus-derived FoxP3+ (designated FOXP3 in humans) natural regulatory T cells (nTregs) and inducing naive T cells to develop into induced regulatory T cells (iTregs) in the periphery (55, 56). In turn, Tregs use a variety of mechanisms (such as the production of the anti-inflammatory cytokines IL-10 and transforming growth factor β [TGF-β]) to keep effector responses in check and to prevent immune-mediated disease (7).

DCs also possess the unique ability to efficiently induce proliferation of autologous T cells in the absence of exogenous antigen. This process was first demonstrated with murine DCs several decades ago and was termed the “autologous mixed leukocyte reaction” (AMLR) (39). The AMLR has since been described with human cells and is thought to represent polyclonal activation of autoreactive T cells specific for self-antigens presented by DCs (2, 3, 10, 38, 44). Interestingly, the AMLR displays characteristics of a normal immune response (including specificity and memory) and is reduced in a variety of human disease states (23, 53). Furthermore, the self-reactive T cells in the AMLR demonstrate a capacity for immunosuppression and increased transcription of the Treg transcription factor FOXP3, suggesting that such DC-stimulated T cells are involved in immune regulation during the normal immune response in vivo (25, 45, 51).

A protocol for generating monocyte-derived DCs has been described in the horse, and the phenotype of these cells has been characterized by several groups (9, 12, 19). However, relatively little is known about the ability of equine DCs to induce proliferation and differentiation of autologous T cells. Our objective in the present study was to monitor the magnitude and nature of the equine DC-induced AMLR. Previously, a detailed analysis of this interaction was limited by the use of heterogeneous cell populations and [3H]thymidine proliferation assays. We developed a method for isolating and coculturing relatively pure populations of equine monocyte-derived DCs with autologous 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-stained peripheral blood T cells. We used multicolor flow cytometry to measure T cell proliferation, surface marker expression, and cytokine production.

We found that equine DCs potently induced the AMLR in the absence of foreign antigen. The AMLR-responsive T cells exhibited activation-induced FoxP3 expression—confirming that FoxP3 is regulated similarly in horses and humans and is an inherent component of T cell activation and proliferation. Further-
more, the induced expression of Foxp3 by activated T cells was associated with a regulatory cytokine profile. This analysis contributes to our knowledge of the early events in the immune response, including concomitant regulatory mechanisms, with potential implications for immunotherapeutic strategies (11).

**MATERIALS AND METHODS**

**Monocyte isolation and generation of DCs.** Peripheral blood was collected into 10-mL heparinized Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) by jugular venipuncture from healthy adult horses according to an approved Institutional Animal Care and Use Committee (IACUC) protocol. Peripheral blood mononuclear cells (PBMC) were isolated via 1077 Ficoll-Paque (Amersham Biosciences, Piscataway, NJ) density centrifugation at 700 × g for 15 min. We modified previously described magnetic bead positive selection and plastic adherence protocols to perform monocyte isolation (12, 46). Briefly, 107 PBMC were labeled with anti-CD14-conjugated monoclonal antibody (MAB; hybridoma clone 105, kindly provided by Bettina Wagner, Cornell University, Ithaca, NY) (26), washed with phosphate-buffered saline (PBS), incubated with anti-mouse IgG1 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany), and positively selected over an LS column (Miltenyi Biotech) according to the manufacturer’s protocol. To reduce nonspecific binding, the cells were labeled in buffer containing 10% heat-inactivated autologous serum (AS), and the column was pretreated with 500 μL of AS as described previously (48). Fetal bovine serum (FBS) was used instead of AS where indicated to evaluate lymphocyte proliferation in the presence of bovine proteins. Three million 14C17 cells were plated onto a 35-mm petri dish (Fisher Scientific, Pittsburgh, PA) in 3 mL of AIM-V medium (Gibco-Invitrogen, Grand Island, NY) with 10% AS and allowed to adhere for 1 h at 5% CO2 and 37°C. Nonadherent cells were removed by gentle washing with Dulbecco modified Eagle medium plus F-12 medium (DMEM-F12; Invitrogen). DCs were produced by culturing the adherent monocyte-enriched population (~2.5 × 106 cells) in 3 mL of DMEM-F12 complete medium (Gibco-Invitrogen) supplemented with 10% AS, 1× antibiotics/antimycotics (Gibco-Invitrogen), 10 ng of recombinant equine IL-4 (rEqIL-4; kindly provided by David Horroh, University of Kentucky, Lexington, KY)/mL, and 50 ng of recombinant human granulocyte-macrophage colony-stimulating factor (rHuGM-CSF; R&D Systems, Minneapolis, MN)/mL for 3 days at 5% CO2 and 37°C (19). For comparison, macrophages were generated by culturing monocytes in complete medium with 10% AS, 1× antibiotics/antimycotics, and no exogenous cytokines.

**Autologous T cell isolation and CFSE staining.** On the third day of DC culture, blood was collected from the same horse and PBMC were isolated via 1077 Ficoll-paque density centrifugation. For negative selection of peripheral blood T cells, monocytes and neutrophils were depleted with anti-canine CD172a (hybridoma clone B-ly4; Becton Dickinson) (33). A total of 107 PBMC were labeled with anti-canine CD172a and anti-human CD21 (hybridoma clone B-ly4; Becton Dickinson) (33). A total of 105 PBMC were cultured with anti-canine CD172a and anti-human CD21, washed in PBS, incubated with anti-mouse IgG1 microbeads, and eluted from an LD column according to the manufacturer’s protocol (Miltenyi Biotech). The purified T cells were washed in PBS, resuspended in 0.25 μM CFSE (Sigma-Aldrich, St. Louis, MO) diluted in PBS, and incubated on a spinning rack at room temperature in the dark for 10 min (14). An equal volume of FBS (Gibco-Invitrogen) was added to inactivate the extracellular CFSE, and the cells were washed three times in PBS and then resuspended in lymphocyte proliferation medium consisting of RPMI 1640 medium (Gibco-Invitrogen) enriched with 10% AS, 25 μM 2-mercaptoethanol (Sigma-Aldrich), and 1× antibiotics/antimycotics (Gibco-Invitrogen).

**DC harvest.** On day 3 of culture, nonadherent DCs were removed by washing the wells with PBS and saved. Adherent cells were treated with 1 mL of Accutase (Millipore, Temecula, CA) at room temperature for 5 min and gently removed with a cell lifter. The adherent and nonadherent cells were pooled, washed in PBS, and resuspended in lymphocyte proliferation medium. Cell viability was assessed by 0.4% trypan blue exclusion (Gibco-Invitrogen) and was consistently >90%.

**Autologous mixed leukocyte reaction.** Purified, CFSE-stained T cells were plated in a 96-well, flat-bottom tissue culture plate (Becton Dickinson) at a concentration of 3 × 105 cells in 300 μL of lymphocyte proliferation medium. A total of 6 × 104 DCs were added to the appropriate wells (1 DC:50 T cells). Positive control wells were treated with 5 μg of concanavalin A (ConA; Sigma-Aldrich)/mL. All treatments were performed in triplicate for each horse. For the antibody-blocking experiment, 50 μL of anti-equine major histocompatibility complex (MHC) class II MAb (CZ11 hybridoma clone 130.8 E8D9, kindly provided by Douglas Antczak, Cornell University, Ithaca, NY) was added to each of three wells on days 0 and 3 of culture. Samples assayed for cytokine production were treated with 40 ng of phorbol myristate acetate (PMA; Sigma-Aldrich)/mL, 1 μg of ionomycin (Sigma-Aldrich)/mL, and 10 μg of brefeldin A (Sigma-Aldrich)/mL for the last 5 h of culture. Unless otherwise indicated, all samples were harvested on day 6 or 7 of culture and grown in medium containing 10% AS.

**Transwell assay.** DCs and CFSE-stained T cells were cultured in lymphocyte proliferation medium in a 96-well-multipoint insert system (Becton Dickinson). Because the surface area of the flat-bottom receiver plate was twice that of the 96-well tissue culture plate used for the previous DC-T cell cocultures, 6 × 105 T cells and 1.2 × 104 DCs were plated to achieve the same cell density and DC/T cell ratio. DCs were either absent, separated from the T cells by a polyethylene terephthalate (PET) membrane with 1-μm pores, or cultured together with the T cells in the receiver plate.

**Flow cytometry.** We analyzed all samples on a FACS Calibur flow cytometer (Becton Dickinson) equipped with argon-ion and red-diode lasers. DCs and macrophages were harvested after 3 days of culture as described, and surface marker expression was measured by incubating the cells with MABs against equine MHC class II (CZ11 hybridoma clone 130.8 E8D9), equine CD14 (hybridoma clone 105), human CD86 (phycoerythrin [PE]-conjugated hybridoma clone 2331; Becton Dickinson) (19), or human CD206 (PE-conjugated hybridoma clone 3.29B1.10; Beckman Coulter, Fullerton, CA) (47). The cells were washed, and the unconjugated primary MABs were labeled with fluorescein isothiocyanate-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA). T cell proliferation was quantified by measuring the percentage of CFSEdim cells in the lymphocyte-gated area. Surface marker expression was analyzed by incubating the cells with MABs against equine CD3 (hybridoma clone F6G3.3; Scott Lab, UC Davis), CD4 (hybridoma clone HB61A, VMRD), CD8 (hybridoma clone HT1A4, VMRD), MHC class II (CZ11 hybridoma clone 130.8 E8D9), or an isotype control (mouse anti-pyovirus, kindly provided by Colin Parrish, Cornell University, Ithaca, NY), followed by a PE-conjugated anti-mouse IgG secondary antibody (Jackson Immunoresearch). Intracellular protein expression was measured after the permeabilization of cells with a saponin-based reagent (Cytofix/Cytoperm kit; Becton Dickinson). Cells were labeled with PE-conjugated anti-mouse/rat Foxp3 (hybridoma clone FJK-168; eBioscience, San Diego, CA) (43) or rat IgG2a isotype control (hybridoma clone eB2a; eBioscience). Samples incubated with anti-mouse/rat Foxp3 antibody were also stained for expression of Alexa 647-conjugated anti-equine IL-4 (hybridoma clone 12H8; Wagner Lab, Cornell University), anti-equine IL-10 (hybridoma clone 492-2; Wagner Lab, Cornell University), or anti-bovine IFN-γ (hybridoma clone CC302; AbD Serotec, Oxford, United Kingdom) (6).

**Statistical analysis.** For each analysis, a Shapiro-Wilk test was used to determine whether data were Gaussian and the appropriate parametric or nonparametric analysis was performed using Statistix 9.0 software (2008 Analytical Software, Tallahassee, FL). Paired t tests were used to compare the ability of DCs and macrophages to induce the AMLR (two-sided), to test whether proliferating T cells increased MHC class II expression (one-sided), and to compare cytokine production between FoxP3+ and FoxP3− cells (two-sided). A Spearman’s rank correlation was used separately for AS and FBS samples to measure the percentage of CFSEdim cells in the lymphocyte-gated area. For each analysis, a Shapiro-Wilk test was used to determine whether data were Gaussian and the appropriate parametric or nonparametric analysis was performed using Statistix 9.0 software (2008 Analytical Software, Tallahassee, FL). Paired t tests were used to compare the ability of DCs and macrophages to induce the AMLR (two-sided), to test whether proliferating T cells increased MHC class II expression (one-sided), and to compare cytokine production between FoxP3+ and FoxP3− cells (two-sided). A Spearman’s rank correlation was used separately for AS and FBS samples to measure...
negative cells are within the M1 region.

Within the monocyte gate, and all gated cells were CD14

Centrifugation (Fig. 1A), and purified monocytes by positive selection and plastic adherence. More than 90% of the cells were
detected within the lymphocyte gate, and more than 95% of these cells were CD3⁺ (Fig. 1C). The T cells were stained with CFSE, cocultured with autologous DCs, and monitored for proliferation.

Equine DCs induce proliferation of autologous T cells in the absence of exogenous antigen. As expected, minimal proliferation was detected in cultures of nonstimulated T cells (Fig. 2A), and a reliable proliferative response was present in T cell cultures stimulated with ConA (Fig. 2B). However, DCs induced autologous T cell proliferation in the absence of exogenous antigen (Fig. 2C). Of note, the DC-stimulated, proliferating T cells (Fig. 2C, cell population within 10⁸ and 10⁹ fluorescence) further diluted the CFSE in comparison to the ConA-stimulated, proliferating T cells (Fig. 2B, cell population within 10⁹ and 10¹⁰ fluorescence) on day 6 of culture. The degree of T cell proliferation was highly correlated with the DC concentration when the cells were grown in medium with 10% AS (r = 0.94, P < 0.0001) or 10% FBS (r = 0.98, P < 0.0001) (Fig. 3A). Furthermore, T cell proliferation was greater in cultures grown in medium supplemented with FBS compared to AS when data were grouped by serum treatment (P = 0.02). When the cells were cocultured at a ratio of 1 DC to 50 T cells, a significant increase in T cell proliferation over nonstimulated controls was detected starting on day 3 of culture (P = 0.003) and was greatest on day 6 (Fig. 3B). T cells cultured in the absence of DCs did not proliferate over baseline levels at any time point tested (P > 0.045, which is nonsignificant with the Bonferroni-corrected P value).

DCs stimulate T cells in a contact-dependent, MHC class II-dependent manner. We attempted to determine the nature of the DC stimulatory signal in the AMLR because some cells (such as memory T cells) can undergo homeostatic proliferation in the presence of DC-derived cytokines (18). We used a transwell system to prevent direct contact between DCs and T cells while permitting diffusion of cytokines between the two cell types. When direct contact with DCs was prevented by a permeable membrane, T cell proliferation was reduced to background levels (both P < 0.005) (Fig. 4A). Preventing T cell receptor (TCR) recognition of MHC class II-peptide complexes with anti-MHC class II blocking antibody decreased T cell proliferation significantly (all P ≤ 0.02) in all horses tested, but not completely to baseline levels in one of three horses (P = 0.045, which is nonsignificant with the Bonferroni-corrected P value) (Fig. 4B).

DCs are more potent inducers of the AMLR compared to macrophages. The ability to efficiently induce the AMLR is a defining characteristic of DCs (15, 39, 44). Therefore, we wanted to compare the ability of equine monocyte-derived DCs and macrophages to induce the proliferation of autologous T cells in the absence of foreign antigen. Flow cytometric phenotyping revealed that DCs were MHC class II⁺⁺⁺, CD14⁻, CD206low cells and consistently included a small population of CD86 high, which is consistent with an immature phenotype, although a subset (ca. 5%) of cells with increased expression of MHC class II. As expected from these phenotypic profiles, equine DCs were

Monocyte and T cell isolation can be obtained by magnetic sorting. To facilitate analysis of the interaction between equine monocyte-derived DCs and autologous T cells, we established a protocol for developing enriched cell populations using magnetic sorting. We isolated PBMC from healthy adult horses by Ficoll gradient centrifugation (Fig. 1A), and purified monocytes by positive selection and plastic adherence. More than 90% of the cells were

within the monocyte gate, and all gated cells were CD14⁺ (Fig. 1B). Monocytes were cultured in complete medium supplemented with rHuGM-CSF and rEqIL-4 to generate equine monocyte-derived DCs. On day 3 of DC culture, PBMC were again obtained from the same horse, and autologous T cells were purified by negative selection. More than 95% of the eluted cells were
detected within the lymphocyte gate, and more than 95% of these cells were CD3⁺ (Fig. 1C). The T cells were stained with CFSE, cocultured with autologous DCs, and monitored for proliferation.

RESULTS

Monocyte and T cell isolation can be obtained by magnetic sorting. Forward scatter (FSC) versus side scatter (SSC) dot plots of presorted PBMC (A), positively selected, adherent CD14⁺ monocytes (B), and negatively selected CD3⁻ T cells (C) are shown. Region 1 (R1), lymphocyte gate; region 2 (R2), monocyte gate; region 3 (R3), neutrophil gate, based on cell size and granularity and cell-lineage surface marker staining. Percentage values indicate the percentage of total cells within each gate. Histogram analyses show the fluorescence intensity for CD14⁺ cells (B, M2) and CD3⁻ cells (C, M2); negative cells are within the M1 region.

FIG 1 Flow cytometric dot plot and histogram analyses of peripheral blood cell sorting. Forward scatter (FSC) versus side scatter (SSC) dot plots of presorted PBMC (A), positively selected, adherent CD14⁺ monocytes (B), and negatively selected CD3⁻ T cells (C) are shown. Region 1 (R1), lymphocyte gate; region 2 (R2), monocyte gate; region 3 (R3), neutrophil gate, based on cell size and granularity and cell-lineage surface marker staining. Percentage values indicate the percentage of total cells within each gate. Histogram analyses show the fluorescence intensity for CD14⁺ cells (B, M2) and CD3⁻ cells (C, M2); negative cells are within the M1 region.
more potent inducers of T cell proliferation in the AMLR com-
pared to macrophages \( (P < 0.002) \) (Fig. 5B).

**Proliferating T cells in the AMLR are primarily CD3^+ CD4^+ CD8^+ T cells.** CFSE-stained, DC-stimulated T cells were labeled with surface markers to characterize the proliferating T cell population after 6 days of culture. As expected, virtually all cells in the lymphocyte gate were CD3^+ T cells (Fig. 6A). The proliferating CFSE^dim^ cells were primarily CD4^+ CD8^− T cells. The T cells were positive for MHC class II, a feature demonstrated previously for adult equine T cells, and MHC class II expression was increased in the proliferating cells \( (P < 0.05) \), a finding consistent with an activated phenotype \( (4,30) \).

**A subset of DC-stimulated T cells expresses FoxP3.** Murine DCs induce expansion of nTregs and differentiation of naive T cells to become FoxP3^+ iTregs by presenting self-antigens in the periphery \( (37) \). Because the T cells in the equine AMLR appeared to be responding to DCs in the absence of exogenous antigen, we measured their expression of the Treg transcription factor FoxP3. A subset of the proliferating T cells (5 to 15%) expressed FoxP3 after 6 days of culture with autologous DCs (Fig. 6B).

Proliferating FoxP3^+ cells can produce effector cytokines but more of these cells produce IL-10 and fewer produce IFN-γ. Although initial experiments in mice lead researchers to believe that FoxP3 is a Treg-specific transcription factor, accumulating evidence suggests that FOXP3 is also expressed transiently in activated human conventional T cells \( (50) \). In addition, mitogenic stimulation increases FoxP3 expression in equine peripheral blood T cells \( (43) \). Therefore, acquisition of FoxP3 expression in DC-stimulated equine T cells might simply be a transient marker of cellular activation and might not be associated with a regulatory phenotype. To investigate this question further, we treated DC-stimulated, CFSE-stained T cells with PMA plus ionomycin and compared cytokine production by CFSE^dim^ FoxP3^+^ cells with that of CFSE^dim^ FoxP3^−^ cells (Fig. 7A.). We found that approximately equivalent subpopulations of FoxP3^+^ T cells were capable of producing the effector cytokines IFN-γ and IL-4, as well as the regulatory cytokine IL-10 (Fig. 7B). However, compared to the FoxP3^−^ cells, more of the FoxP3^+^ cells produced IL-10 \( (P = 0.015) \) and fewer produced IFN-γ \( (P = 0.013) \) (Fig. 7C). Both cell populations contained statistically equivalent numbers of IL-4-compotent cells \( (P = 0.095) \). Approximately 60% of the FoxP3^+^ and FoxP3^−^ cells did not express detectable levels of IFN-γ, IL-4, or IL-10.

Cytokine production by FoxP3^+^ versus FoxP3^−^ cells is similar after stimulation with DCs or ConA. DCs in humans and mice can adopt a tolerogenic phenotype and promote the differ-
differentiation and expansion of Treg populations (29, 57). Therefore, we wondered whether the regulatory cytokine profile detected in DC-stimulated FoxP3⁺ T cells was triggered by DC-derived signals or whether it was an inherent component of T cell activation and proliferation in the horse. We compared cytokine production by CFSEdim FoxP3⁺ cells to that by CFSEdim FoxP3⁺ cells in DC- or ConA-stimulated T cell cultures. Cells were cultured for 6 days and stimulated with PMA and ionomycin prior to analysis. Similar to the DC-stimulated samples, more of the ConA-stimulated FoxP3⁺ cells produced IL-10, and fewer produced IFN-γ (Fig. 8).

**DISCUSSION**

In this study, we showed that equine monocyte-derived DCs, but not macrophages, were capable of stimulating the AMLR in the absence of foreign antigen. To our knowledge, this is the first description of the AMLR in the horse. As in humans and mice, the equine AMLR was contact and MHC class II dependent and primarily involved CD3⁺ CD4⁺ CD8⁺ T cells (24, 27). Equine DCs express high levels of MHC class II, contain a subpopulation of cells with a mature flow cytometric phenotype, and more potently induce the AMLR compared to macrophages (9). This confirms the superior antigen-presenting and costimulatory capacity of equine DCs, because the AMLR requires DC expression of CD86 and MHC class II (44). The low levels of T cell proliferation measured in the macrophage cocultures might be due to the presence of small numbers of contaminating DCs consistently detected as MHC class IIhigh cells by flow cytometric phenotyping. These could be unsorted DCs from peripheral blood or spontaneously differentiated, monocyte-derived DCs (21).

The demonstration that T cell proliferation in the AMLR was contact and MHC class II dependent confirms that these cells were not exclusively responding to DC-derived cytokines. Instead, the T cells responded through the TCR to antigens presented by DCs. T cell proliferation was highly correlated with DC dose and, at a DC/T cell ratio of 1:50, was detectable by day 3 of culture. Maximum proliferation of T cells was detected around day 6, a finding suggestive of a primary T cell stimulation. Other researchers have shown that the murine AMLR peak response occurred around day 7 in primary cultures but, upon restimulation of T cells with fresh DCs, T cell proliferation peaked on day 3 (53).

T cell proliferation was greater in FBS than in AS (8). There-
fore, a subset of the T cells proliferating in FBS might be specific for foreign serum-derived antigens presented by DCs. In contrast, T cells cultured with DCs in AS are likely responding to autoantigens from self-proteins (38). This is supported by the kinetics of CFSE dilution, which shows that a small percentage of DC-stimulated T cells are CFSEdim at early time points and, by day 6 of culture, have markedly diluted the CFSE. In contrast, ConA-stimulated samples have a similar percentage of CFSEdim T cells by day 6, but these cells are 1 log brighter in their CFSE fluorescence. These data suggest that DCs stimulate a distinct, potentially autoreactive T cell population that undergoes multiple rounds of proliferation, whereas ConA stimulates fewer replication cycles in a larger, nonspecific population of cells. The presence of autoreactive T cells in the peripheral blood can be explained by the known imperfections in thymic negative selection and the suspected cross-reactivity of the TCR (13,32). Also, DCs may activate autoreactive T cells by presenting cryptic epitopes resulting from caspase modification of self-proteins from nearby apoptotic cells (2,3, 10).

We became interested in the presence of Tregs in our equine DC-T cell cocultures because Tregs are often specific for self-antigens and because of the aforementioned suppressive capacity of AMLR-stimulated human T cells. We found that a subset of the DC-stimulated, proliferating T cells (5 to 15%) expressed the Treg transcription factor FoxP3. It was unclear whether these cells were truly Tregs because in humans, unlike in mice, conventional T cells transiently upregulate FOXP3 upon cellular activation in the absence of exogenous TGF-β (16). Similarly, increased FoxP3 levels were reported in mitogen-stimulated canine and equine PBMC, suggesting that FoxP3 gene expression is regulated similarly to humans in these species (36,43). To further investigate the role of FoxP3 in the DC-stimulated samples, we measured cytokine production and found that approximately equivalent numbers of the proliferating FoxP3+ cells were capable of producing IFN-γ, IL-4, and IL-10. This finding confirms that suppressive and effector phenotypes exist concurrently within the FoxP3+ population (43). However, compared to the proliferating FoxP3+ cells, more of the proliferating FoxP3+ cells produced IL-10 and fewer produced IFN-γ. If FoxP3 were simply a marker of activation with no functional significance, the cytokine profile of both groups of cells would be identical. Instead, the FoxP3+ cells produced a more immunosuppressive cytokine profile, demonstrating that FoxP3 expression was associated with a regulatory transcriptional program in at least some of the proliferating cells. This is likely mediated by the inhibitory effect of Foxp3 on IFN-γ expression (5). Interestingly, a similar regulatory cytokine profile was also enriched in proliferating FoxP3+ cells after polyclonal stimulation with ConA. Therefore, the expansion of FoxP3+ cells and the associated alterations in cytokine production are not induced by DC-specific signals but are, instead, an inherent characteristic of equine T cell activation and proliferation. Measuring TGF-β production by these cells would also be interesting since...
the production of this cytokine is a specific marker of regulatory activity, but this was not performed due to a lack of appropriate reagents.

The FoxP3+ cells following ConA or DC stimulation either represent the expansion of preexisting nTregs, the induction of FoxP3 in activated conventional T cells, or a combination of the two. Although ConA stimulation induces some expansion of preexisting nTreg populations in the dog, the vast majority of the FoxP3+ cells following polyclonal mitogenic stimulation are activated conventional T cells (41). In addition, because nTregs do not proliferate readily, the CFSEdim FoxP3+ cells analyzed in our experiments likely represent activated conventional T cells (42). Future analysis of expression of Helios (a recently described nTreg-specific transcription factor) might verify this distinction (49). Taken together, these results indicate that FoxP3 expression in activated equine conventional T cells is associated with a regulatory cytokine profile. The demonstration that FoxP3 is upregulated following DC stimulation proves that this effect is not mitogen exclusive and supports the concept that FoxP3 might be induced during conventional T cell stimulation in vivo.

Conflicting results have been reported in experiments measuring the suppressive capabilities of human FOXP3+ conventional T cells (28, 31, 35, 40, 52). The functional immunosuppressive significance of transient, activation-induced FOXP3 expression is therefore suspect. However, the possibility that FOXP3+ effector T cells help to fine-tune the developing immune response is an attractive hypothesis that cannot be discounted, particularly because transduction of FOXP3 in naive human T cells imparts a regulatory phenotype (31, 54). This is supported by the recent finding that stimulation of conventional equine T cells induced a population of CD4+ CD25+ T cells that were functionally suppressive and did not proliferate (20). However, these cells were activated in the presence of TGF-β, so they may represent true Tregs rather than activated conventional T cells with transient Treg-like characteristics (40). In other studies, activation-induced FOXP3 expression was insufficient to inhibit IFN-γ production by human effector T cells (IL-4 and IL-10 were not measured) (1, 17). This finding likely contrasts with our data because our analysis focused exclusively on the proliferating cells rather than both proliferating and nonproliferating populations. This allowed us to measure FoxP3 and cytokine expression only in the relevant, DC- or ConA-responsive cells.

We report herein that equine monocyte-derived DCs are capable of inducing the AMLR. This response should be considered
when measuring the ability of DCs to induce an antigen-specific T cell response in vitro. We also found that equine DCs stimulate the proliferation of autologous, potentially autoreactive helper T cells and that a subset of these cells exhibits activation-induced FoxP3 expression, which is associated with an immunoregulatory cytokine profile. Such activation-induced FoxP3 expression is an inherent component of proliferating equine T cells and might help regulate the developing immune response in vivo. These findings
are relevant to the human immune system because FoxP3 expression is regulated similarly in humans and horses.

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