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Investigating the Impact of Helminth Products on Immune Responsiveness Using a TCR Transgenic Adoptive Transfer System[1]

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Helminth infections and their products have a potent immunomodulatory effect on the host immune system and can impair immune responses against unrelated Ags. In vitro studies have suggested that the immunomodulation by helminth extracts may be the result of bystander response bias toward a Th2 phenotype and/or an Ag-specific T lymphocyte proliferative hyporesponsiveness. The aim of this study was to determine the role of these potential mechanisms of immunosuppression in vivo. Therefore, using a sensitive model of CFSE-labeled OVA-specific TCR transgenic T lymphocyte adoptive transfer, we analyzed the effect of *Ascaris suum* body fluid (ABF) on the kinetics and amplitude of a primary OVA-specific T cell response as well as the Th1/Th2 profile of the response in wild-type and IL-4 knockout (KO) mice. We find that inhibition of delayed-type hypersensitivity by ABF was associated with a Th1/Th2 shift in wild-type animals, but not in IL-4 KO mice. The use of this model has allowed us to demonstrate that although the kinetics of the OVA-specific primary response was not affected by ABF, the expansion of the OVA-specific T lymphocytes was significantly inhibited in both wild-type and IL-4 KO mice. This inhibition was associated with a reduced proliferative capacity of these cells in vivo, distinct from anergy. ***The Journal of Immunology*****, 2003, 171: 447–454.**

Helminth infections have a potent systemic immunomodulatory effect on the host immune system, impairing immune responses to heterologous antigenic challenge (1, 2), allografts (3–6), and concurrent viral (7, 8) or other helminth (9, 10) infections. As many of these infections are pandemic, impaired vaccine-induced immunity as a result of concurrent parasitic infections is of huge potential health significance (11–14), and understanding the mechanisms of the immunomodulation by parasites has obvious consequences for the design of vaccines.

The Th2 response that usually accompanies helminth infections can skew the immune responses to bystander Ags from a Th1 toward a Th2 phenotype (8, 15, 16). Similarly, coimmunization with parasite extracts can modulate the Th1 response to an unrelated Ag via an IL-4- (16) and/or IL-10-dependent mechanism (17, 18). It has therefore been suggested that the immunomodulation observed in helminth infections is the result of skewing toward a Th2 phenotype. Some studies report, however, that in addition to inhibiting Th1 responses, helminth infections can inhibit Th2 responses against Ags (17, 18) and allergens (19).

The diminished immune response may also result from Ag-specific T lymphocyte hyporesponsiveness. This is reflected by the decreased ability of T cells from helminth infected mice, or T cells from uninfected mice cultured with nematode excretory/secretory products, to proliferate in vitro when restimulated with specific Ags and/or polyclonal mitogens (2, 17, 20, 21).

We have previously shown that the gastrointestinal helminth *Ascaris suum* body fluid (ABF)[3] inhibits the delayed-type hypersensitivity (DTH) response to a heterologous protein, OVA. Although we were able to demonstrate that ABF affected the induction phase of the immune response, we were unable to visualize primary Ag-specific cell responses in vivo because of the low frequency of, and the inability to distinguish, Ag-specific lymphocytes in normal mice. As such, we were unable to assess whether any changes observed were due to modifications in clonal expansion, cell death, differentiation, or localization of Ag-specific T cells. The aim of the present study was to determine whether immunomodulation by ABF was related to modification in kinetics and/or amplitude of the primary Ag-specific T cell response in vivo and to assess the potential implication of IL-4 in any effects observed. To make these determinations, we exploited a model where wild-type and IL-4 knockout (KO) BALB/c mice were transferred with CFSE-labeled transgenic (Tg) T lymphocytes expressing a TCR specific for OVA detectable by staining with an anti-clonotypic mAb, KJ1-26 (22).

The results presented in this study show that the DTH inhibition by ABF was not solely dependent upon a shift from Th1 toward Th2 phenotype of the OVA-specific response. For the first time, we demonstrated that ABF immunomodulation correlated with an IL-4-independent inhibition of Ag-specific T cell clonal expansion during the primary response. This phenomenon was related to a reduced proliferative capacity of the T cells but was not associated with anergy.

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[3] Abbreviations used in this paper: ABF, *Ascaris suum* body fluid; DTH, delayed-type hypersensitivity; HAO, heat-aggregated OVA; KO, knockout; LN, lymph nodes; PLN, peripheral LN; Tg, transgenic.

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Materials and Methods

Mice

IL-4-deficient (IL-4 KO) BALB/c mice, obtained from Dr. F. Brombacher (University of Cape Town, South Africa), and wild-type BALB/c mice were bred in the animal facility of University of Strathclyde from stock. Mice homozygous for chicken OVA peptide 323–339/I-A^d-specific DO11.10 TCR transgenes on a BALB/c background (23) were obtained from Dr. P. Garside (University of Glasgow, Glasgow, U.K.). Mice were kept under specific pathogen-free conditions and maintained on OVA-free diets in the animal facility of University of Strathclyde in accordance with local and home office regulations.

Preparation of cell suspensions for adoptive transfer and CFSE-labeling of donor cells

Cell suspensions were prepared from pooled lymph nodes (LN) and spleens from 6–10-wk-old female DO11.10 TCR-Tg mice by forcing them through a Nitex mesh (Cadisch Precision Meshes, London, U.K.) and washed in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) supplemented with 10% FCS (Harlan Sera-Lab, Crawley Down, U.K.). The cells were resuspended in sterile PBS (Life Technologies, Paisley, U.K.) at a concentration of 5×10^7 cells/ml, and the percentage of $CD4^+$/KJ1-26^+ cells was determined by flow cytometry as described below. Cells were then incubated with 5 μM CFSE (Molecular Probes, Leiden, The Netherlands) for 8 min at room temperature. Labeling was stopped by the addition of an equal amount of FCS, and cells were washed and resuspended in RPMI 1640 with 10% FCS. Before transfer, cells were centrifuged, resuspended in RPMI 1640, and passed through Nitex mesh. A total of 2.5×10^6 $CD4^+$/KJ1-26^+ cells in 200 μl of this cell suspension were injected into the tail vein of the 6–10-wk-old female recipient mice.

Flow cytometry

To determine the percentage of $CD4^+$/KJ1-26^+ cells before transfer, an aliquot of the cell suspension was incubated with Fc receptor-blocking buffer (anti-CD16/32 hybridoma supernatant, 10% FCS, and 0.1% sodium azide) for 10 min at 4°C and then incubated with PE-conjugated anti-CD4 mAb (BD PharMingen, Oxford, U.K.) and biotinylated KJ1.26 mAb (24) for 20 min at 4°C. Cells were washed in FACS buffer (PBS, 2% FCS, and 0.05% sodium azide) then incubated with FITC-conjugated streptavidin (BD PharMingen) for 20 min at 4°C.

To detect the $CD4^+$/KJ1-26^+ cells in the popliteal LN and peripheral LN (PLN) at different time points following immunization, cell suspensions were prepared as previously described and stained with PerCP-conjugated anti-CD4 (L3T4) (BD PharMingen) and biotinylated KJ1.26 mAb followed by PE-labeled streptavidin (BD PharMingen).

After washing in FACS buffer, stained cells were resuspended in FACS flow (BD Biosciences, Oxford, U.K.) and analyzed with a FACScan using CellQuest software (BD Biosciences). Analysis was performed on 30,000 lymphocytes or 1000–3000 $CD4^+$/KJ1-26^+ cells.

Immunization

The day following cell transfer, groups of mice were immunized in the right footpad with 50 μl OVA (100 μg, fraction V) (Sigma-Aldrich, Poole, U.K.) emulsified in CFA (Sigma-Aldrich) with or without 500 μg of adult pseudocoelomic ABF, prepared as previously described (25).

Assessment of DTH responses in vivo

Twelve days postimmunization mice were challenged with 100 μg heat-aggregated OVA (HAO) in 50 μl saline in the left footpad. DTH responses were assessed by measuring the footpad thickness in the challenge footpad before and 24 h after challenge, using dial-gauge microcalipers (Kroeplin, Schlüchtern, Germany). The difference between the means of the two measurements gives an index of footpad swelling in millimeters, which was used for comparison between groups.

Cell culture for cytokine analysis and proliferation assay

Two days after challenge with HAO, the popliteal LN and PLN of each mouse were pooled and single-cell suspensions were prepared. Cells were incubated in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 1.25 mg/ml fungizone (complete medium, Life Technologies) alone or in the presence of 1 mg/ml OVA or 50 μg/ml ABF at 37°C in 5% CO_2. For cytokine analysis, 1.6×10^6 cells/well were cultured in 250 μl triplicate cultures for 72 h, then the culture supernatants were harvested and stored at −20°C until assayed by ELISA. For proliferation assay, 4×10^5 cells/well were incubated in 200 μl triplicate cultures for 72 h. [^{3}H]Thymidine at 0.5 μCi per well (Amersham Pharmacia Biotech, Bucks, U.K.) was added for the last 8 h of culture. The plates were then store at −20°C until the cells were harvested onto filters (Biological Instrumentation Service, Kirkham, U.K.) using a cell harvester (Skatron Instrument, Sterling, VA). Incorporated [^{3}H]thymidine was measured with a scintillation counter (Beckman Coulter, Fullerton CA).

Cytokine assays

Cytokine production was quantified by sandwich ELISAs using standards and Ab pairs specific for the corresponding cytokine according to the manufacturer's recommendations (BioSource International, Nivelles, Belgium for IFN-γ, IL-5, and IL-10; R&D Systems, Abingdon, U.K. for IL-13 assay)

Measurement of Ab responses

OVA-specific IgG1, IgG2a, and IgE in serum from mice 14 days after immunization were tested by sandwich ELISA using Immulon HBX 96-well plates (Thermo Life Sciences, Hants, U.K.) coated with OVA (20 μg/ml in 0.05 M Na_2CO_3/$NaHCO_3$, pH 9.6) and HRP-conjugated goat anti-mouse IgG2a Abs (Southern Biotechnology Associates, Birmingham, AL) at 1/10,000 dilution or 2 μg/ml biotinylated rat anti-mouse IgE Ab (BD PharMingen) plus streptavidin-HRP at dilution 1/1000 (BD PharMingen). The reactions were developed with tetramethylbenzidine (Insight Biotechnology, Middlesex, U.K.) and stopped with 0.4 M sulfuric acid. The plates were read at 405 and 650 nm on a SpectraxMax 190 ELISA reader (Molecular Devices, Sunnyvale, CA). For OVA-specific IgE test, serum was diluted by 2-fold in PBS (10% FCS). For OVA-specific IgG2a, titration curves were conducted. The results are shown as the mean OD_{650}-OD_{405} of five samples ± SEM at each dilution.

Assessment of responder frequency and proliferative capacity from CFSE profiles

Responder frequency (R) and proliferative capacity (C_p) of $CD4^+$KJ1-26^+ T cells were calculated as described by Gudmundsdottir et al. (26):

$$R = \frac{P_{sr}}{P_s} \qquad (1)$$

$$C_p = \frac{\sum_{1}^{n} E_n}{P_{sr}}$$

E_n is the number of $CD4^+$KJ1-26^+ T cells that have undergone n division cycles (as determined by CFSE fluorescence peak intensity), P_s being the precursor sample pool, and P_{sr} being the precursor sample pool that respond by dividing.

$$P_s = \sum_{0}^{n} \left(\frac{E_n}{2^n}\right) \qquad (2)$$

$$P_{sr} = \sum_{1}^{n} \left(\frac{E_n}{2^n}\right)$$

Statistical analysis

Results are represented as the mean ± SEM. The Mann-Whitney U test was used to determine the significance of differences between groups. A value of $p < 0.05$ was considered to be significant.

Results

ABF suppresses the DTH response against OVA in both wild-type and IL-4 KO transferred mice

We have previously shown that ABF-suppressed DTH responses in normal mice (25) and wished to confirm that this effect was still apparent in animals adoptively transferred with Tg T cells. Following transfer of OVA-TCR Tg lymphocytes, mice were immunized 24 h later with OVA or OVA plus ABF. The effect of ABF on the proliferation of OVA-TCR Tg lymphocytes was assessed 3, 5, and 7 days after immunization. To assess the effect of ABF on

DTH responses, mice were challenged with HAO 12 days after immunization with OVA/CFA in the presence or absence of ABF. Cytokine and proliferative responses were assessed 14 days after immunization.

HAO challenge induced a DTH reaction in both wild-type and IL-4 KO OVA-sensitized mice, which was significantly inhibited when the mice were coimmunized with ABF. However, the reduction tended to be lower, although not significantly, in IL-4 KO (60%) than in wild-type (90%) mice (Fig. 1). These results confirm our previous findings and further show that ABF can suppress DTH in animals with an increased precursor frequency of Ag-specific T cells.

ABF has no effect on the OVA-specific proliferation in vitro

To determine whether suppression of OVA-specific DTH was associated with T cell hyporesponsiveness in our model, the ability of PLN cells to proliferate following Ag restimulation in vitro was examined. Despite the profound effects on DTH, ABF did not appear to have any effect on the OVA-specific proliferative response in vitro, either in wild-type or in IL-4 KO mice (Fig. 2), similar to previous observations (25).

ABF skews the immune response to OVA from a Th1 to Th2 type in wild-type but not IL-4 KO mice

The effect of ABF on OVA-specific cytokine production by PLN cells was analyzed. Upon in vitro restimulation with OVA, PLN cells from OVA plus ABF-immunized wild-type mice produced significantly lower levels of IFN-γ than did cells from mice immunized with OVA only (Fig. 3*A*) and increased amounts of IL-5 (Fig. 3*B*), IL-13 (Fig. 3*C*), and IL-10 (Fig. 3*D*). OVA-specific IFN-γ production in IL-4 KO mice was significantly higher than in wild-type mice. The mean level of IFN-γ upon restimulation by OVA was reduced when the IL-4 KO mice had been immunized with OVA plus ABF compared with OVA alone but the difference did not reach significance (Fig. 3*E*). The levels of IL-5 (Fig. 3*F*) and IL-13 (Fig. 3*G*) in IL-4 KO mice were low and not modified by ABF. Upon in vitro restimulation with ABF, high levels of IL-5, IL-13, and IL-10 were produced by PLN cells from OVA plus ABF-immunized wild-type mice (Fig. 3, *B–D*) but not from their IL-4 KO counterparts (Fig. 3, *F* and *G*). Neither wild-type nor IL-4 KO mice secreted IFN-γ in response to ABF (Fig. 3, *A* and *E*). IL-4 was not detected in either wild-type or IL-4 KO mice following immunization with either OVA or OVA plus ABF.

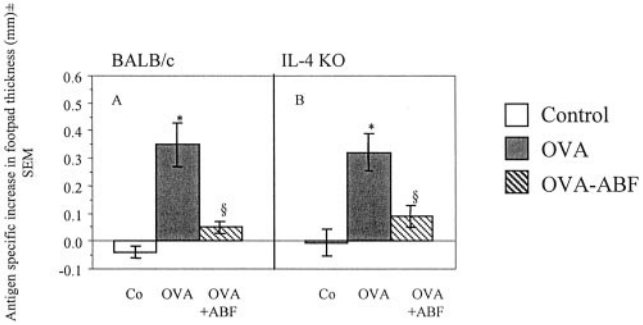

FIGURE 1. Suppression of OVA-specific DTH response by ABF in wild-type and IL-4 KO mice. Groups of OVA-TCR Tg lymphocyte transferred wild-type (*A*) and IL-4 KO (*B*) mice were immunized s.c. with 100 μg OVA or 100 μg OVA plus 500 μg ABF or left nonimmunized (Control) and challenged 12 days later in the opposite footpad with 100 μg HAO. Results represent five animals per group and are presented as the mean specific increase in footpad thickness ± SEM 24 h post-challenge. Similar results were obtained in two additional experiments. *, Significantly different ($p \leq 0.05$) from nonimmunized mice; §, significantly different ($p \leq 0.05$) from OVA-immunized mice.

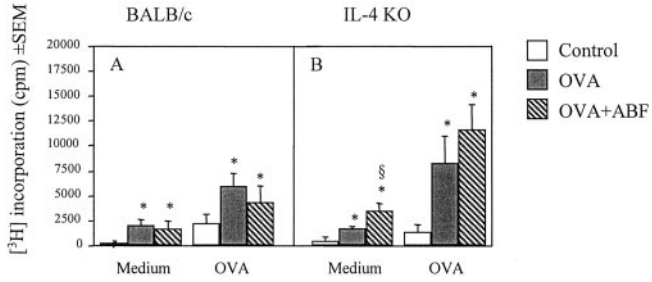

FIGURE 2. Proliferation upon in vitro restimulation. Groups of OVA-TCR Tg lymphocyte transferred wild-type (*A*) and IL-4 KO (*B*) mice were immunized with OVA or OVA+ABF or left nonimmunized and challenged with HAO 12 days later. PLN cells were harvested 2 days post-challenge and proliferation was measured by [^{3}H]thymidine incorporation after 72 h culture in complete medium (medium) ± 1 mg/ml OVA. Data shown are mean ± SEM of three to four mice assayed individually. Similar results were obtained in two additional experiments. *, Significantly different ($p \leq 0.05$) from nonimmunized mice; §, significantly different ($p \leq 0.05$) from OVA-immunized mice.

As an indicator of the effects of these cytokines in vivo, OVA-specific serum IgG1, IgG2a, and IgE titers were measured. Serum from OVA-immunized wild-type and IL-4 KO contained high levels of OVA-specific IgG2a, whereas levels of IgG1 were significantly decreased in IL-4 KO mice. ABF significantly decreased the OVA-specific IgG2a levels in IL-4 KO mice but not as drastically as in wild-type mice ($p < 0.02$). Although levels of IgG1 were significantly decreased in wild-type mice immunized with OVA plus ABF, no significant differences were observed in IL-4 KO mice (Fig. 4, *A* and *B*). OVA-specific IgE (Fig. 4*C*) was increased by ABF in wild-type but not in IL-4 KO mice, and similar results were observed for total IgE (data not shown).

These data were consistent with our previous observations (25): inhibition of DTH by ABF did not appear to be due to T cell anergy, as the ability of the draining popliteal LN cells to proliferate under OVA-restimulation in vitro was not affected by ABF. DTH suppression by ABF was associated with a shift from Th1 to Th2 cytokine profile in wild-type mice. Although we were unable to detect any IL-4, we were able to detect other OVA-specific Th2 cytokines in LN cell supernatant from OVA plus ABF immunized wild-type mice, in contrast to previous findings (25), probably as a result of the higher percentage of OVA-specific T cells due to the adoptive transfer. However, no IL-5 or IL-13 was detected in LN cell supernatants from IL-4 KO mice. Thus, in IL-4 KO mice DTH inhibition by ABF was observed in the absence of induction of a Th2 response. It is possible that IL-4 KO mice were reconstituted with IL-4-producing $CD4^+$ cells along with the transferred $CD4^+KJ1.26^+$ cells; however, similar results were obtained when mice were transferred with IL-4 KO $CD4^+KJ1.26^+$ cells (data not shown).

We therefore wished to determine whether this inhibition by ABF could be the result of the induction of T lymphocyte hyporesponsiveness in vivo. As immunomodulation by ABF has been shown to be restricted to the induction phase of the immune response (i.e., immunization) and not the effector phase (HAO challenge) (25), we monitored the effect of ABF on the primary response at a cellular level in vivo. We determined whether the kinetics and amplitude of OVA-specific T cell proliferation and accumulation were affected by coimmunization with ABF.

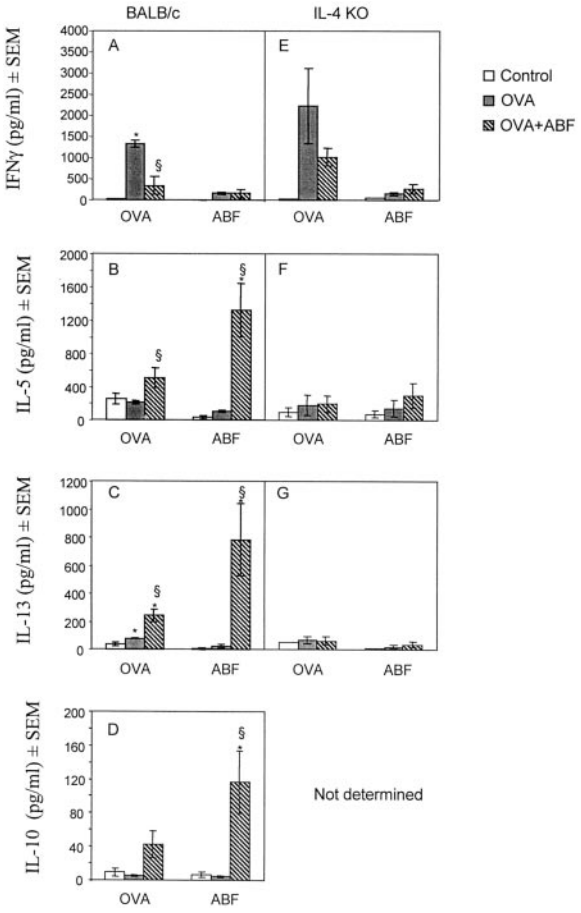

FIGURE 3. Cytokine production upon restimulation in vitro. Groups of OVA-TCR Tg lymphocyte transferred wild-type (*A–D*) and IL-4 KO (*E–G*) mice were immunized with OVA or OVA+ABF or left nonimmunized and challenged with HAO 12 days later. PLN cells were harvested 2 days postchallenge and cultured in complete medium containing 1 mg/ml OVA or 50 μg/ml ABF. IFN-γ (*A* and *E*), IL-5 (*B* and *F*), IL-13 (*C* and *G*), and IL-10 (*D*) were assayed in 72-h supernatant by ELISA. The results are displayed as the mean cytokine concentration for four to five mice per group ± SEM. PLN cells from the nonimmunized IL-4 KO mice were pooled as insufficient numbers of cells could be obtained from individual animals, and thus no statistical test was possible. Similar results were obtained in two additional experiments. ∗, Significantly different ($p \leq 0.05$) from nonimmunized mice; §, significantly different ($p \leq 0.05$) from OVA-immunized mice.

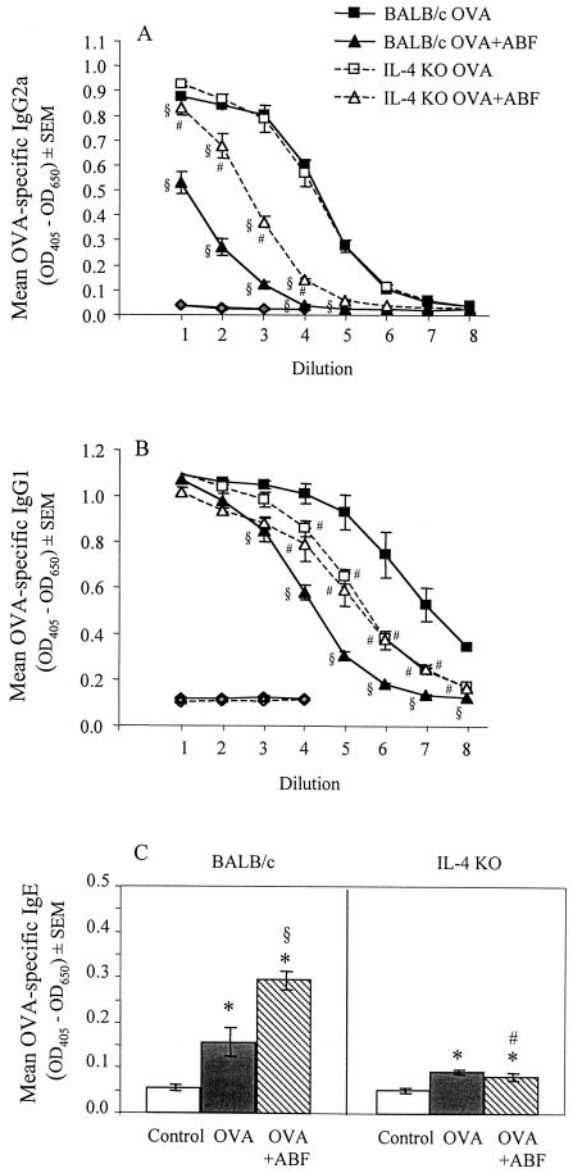

FIGURE 4. Effect of ABF on Ab responses to OVA. OVA-specific Abs were assessed by ELISA in the serum of OVA-TCR Tg lymphocyte transferred wild-type and IL-4 KO mice 14 days postimmunization with OVA or OVA+ABF. IgG2a (*A*), IgG1 (*B*), and IgE (*C*) responses in nonimmunized, OVA-immunized, and OVA+ABF immunized wild-type and IL-4 KO mice. The results represent the mean ± SEM from five mice per group. Similar results were obtained in two additional experiments. ∗, Significantly different ($p \leq 0.05$) from control group; §, significantly different ($p \leq 0.05$) from OVA-immunized group; #, significantly different ($p \leq 0.05$) from wild-type mice immunized in the same conditions.

ABF inhibits the increase in percentage of OVA-specific T cells in the draining popliteal LN following immunization in both wild-type and IL-4 KO mice

The percentage and absolute number of $CD4^+KJ1\text{-}26^-$ and $CD4^+KJ1\text{-}26^+$ cells in draining popliteal LNs and PLNs were monitored at different time points following footpad immunization with OVA in CFA with or without ABF.

As previously described (27), the percentage of popliteal lymphocytes being $CD4^+KJ1\text{-}26^+$ in both wild-type and IL-4 KO mice increased following immunization with OVA/CFA, reaching a maximum around day 5 postimmunization, and then decreased (Fig. 5*A*). Although treatment with ABF did not appear to affect the kinetics of OVA-specific T cell clonal expansion, there was significant inhibition of the increase in percentage of OVA-specific T cells in the draining popliteal LN of both wild-type and IL-4 KO mice.

The total number of $CD4^+KJ1\text{-}26^+$ cells in the popliteal LN dramatically increased following immunization, in both wild-type and IL-4 mice (Fig. 5*B*). OVA-specific lymphocyte accumulation in the draining popliteal LN was not affected by ABF in wild-type mice but was significantly reduced in IL-4 KO mice. At each time point following immunization, OVA-specific lymphocyte numbers in popliteal LN from OVA plus ABF immunized IL-4 KO mice was approximately half that observed for the other groups. Similar results were seen in PLN, although the increase was delayed in time and was of a lesser amplitude (data not shown).

The total number of bystander $CD4^+KJ1\text{-}26^-$ T cells in the popliteal LN also increased following immunization, but to a lesser extent than the OVA-specific T cell population (Fig. 5*C*). This increase was not significantly modified by the ABF in either wild-type or IL-4 KO mice. Although ABF had no effect on the total

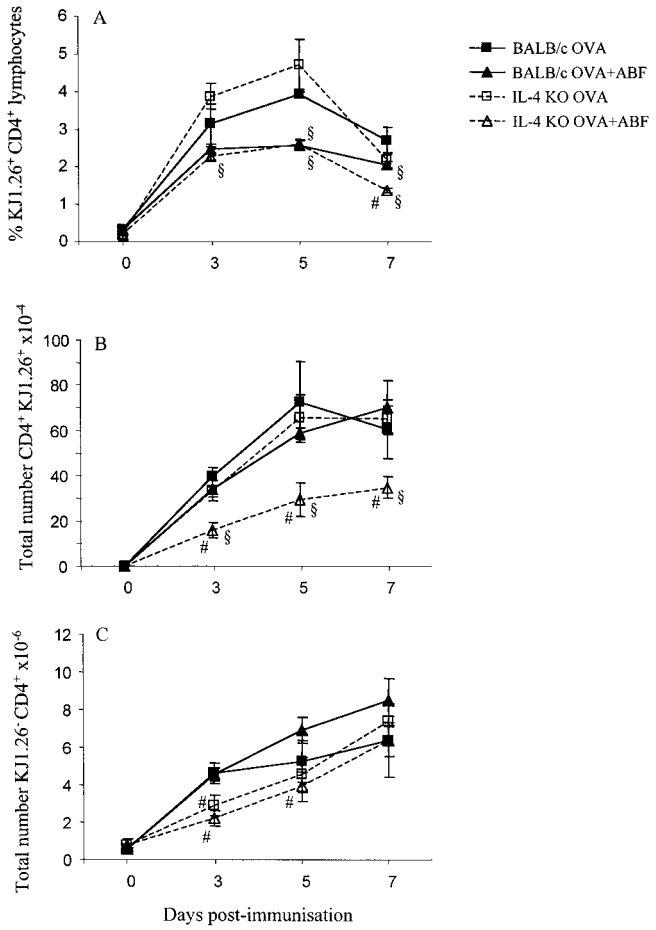

FIGURE 5. Clonal expansion of Tg T cells in the presence or absence of ABF. Wild-type and IL-4 KO BALB/c mice received an i.v. injection of OVA-specific TCR-Tg T cells (2.5×10^6/mouse) and were immunized in the footpad the following day with OVA or OVA+ABF. Percentage (*A*) and number (*B*) of Tg OVA-specific T cells ($CD4^+KJ1.26^+$) as well as total number of $CD4^+KJ1.26^-$ cells (*C*) in the draining popliteal LN were assessed by flow cytometry at various time points following immunization. Each data point represents the mean ± SEM of three individual wild-type or IL-4 KO mice immunized with OVA or OVA+ABF. Similar results were obtained in two additional experiments. §, Significantly different ($p \leq 0.05$) from OVA-immunized group; #, significantly different ($p \leq 0.05$) from wild-type mice.

number of popliteal $CD4^+KJ1\text{-}26^-$ cells in IL-4 KO mice, the total number of $CD4^+KJ1\text{-}26^-$ cells in IL-4 KO mice following immunization with either OVA or OVA plus ABF were about half that observed in wild-type mice and this, combined with the reduced proportion of $CD4^+KJ1\text{-}26^+$ following treatment with ABF probably accounts for the decreased numbers of $CD4^+KJ1\text{-}26^+$ cells in IL-4 KO mice.

ABF does not affect the responder frequency but reduces the individual OVA-specific T cell proliferative capacity in both wild-type and IL-4 KO mice

To determine whether the decreased percentage of OVA-specific T lymphocytes in the presence of ABF was related to a difference in the proliferative capacity of these cells, the DO.11.10 cells were CFSE-labeled before transfer and the number of cell divisions was assessed by flow cytometry at different time points after immunization.

At day 3 after immunization, most $CD4^+KJ1\text{-}26^+$ lymphocytes present in the draining LN from OVA-immunized mice had divided at least twice. There was a marked asynchrony in the kinetics of cell division exhibited by the $CD4^+KJ1\text{-}26^+$ population, with some cells having divided as many as nine times. There was no difference in the cell division profile between wild-type and IL-4 KO mice. Although the maximum number of cell divisions was not affected by ABF, the mean number of divisions per OVA-specific T cell was decreased by ABF in both wild-type and IL-4 KO mice. The percentage of $CD4^+KJ1\text{-}26^+$ cells that had divided more than six times was significantly reduced by ABF, in both wild-type and IL-4 KO mice, at each time point postimmunization, although the difference decreased slightly with time (Fig. 6).

The cell division profile at a specific time point after immunization is the result of two factors: the proportion of T cells that respond to activation by dividing (responder frequency) and the proliferative capacity of each responder (the number of daughter cells generated by each responding OVA-specific T cell). We determined these two factors using the formula suggested by Gudmundsdottir et al. (26). About 70% of the $CD4^+KJ1\text{-}26^+$ cells in the draining popliteal LN from both the wild-type and IL-4 KO participated in clonal expansion 4 days after OVA immunization (Fig. 7, *A* and *C*). This responder frequency was unaffected by ABF in all but one of three experiments in wild-type mice. In contrast, the proliferative capacity was significantly reduced by ABF in both wild-type and IL-4 KO mice (Fig. 7, *B* and *D*). Therefore, ABF does not affect the ability of OVA TCR Tg T cells to respond to Ag but does reduce their proliferative capacity in an IL-4-independent manner.

Discussion

The precise mechanisms by which infection with gastrointestinal nematode parasites affects concomitant immune responses remain to be determined. In the present study, we investigated the mechanisms of DTH inhibition by ABF. Using a sensitive adoptive transfer model, not only did we analyze the effect of ABF on the Th1/Th2 profile of the immune response, but also on the kinetics and amplitude of the OVA-specific primary T cell response induced in vivo. We show that immunosuppression by ABF was associated with a Th2 bias of the Ag-specific response and a reduced proliferative capacity of the Ag-specific responder T cells during the primary response. Although the Th2 skewing mediated by ABF was dependent on IL-4, inhibition of the DTH by ABF was not IL-4-dependent.

Previous studies have suggested that the ongoing Th2 response to parasite Ags creates a cytokine environment that induces a Th1/Th2 shift of the immune response to unrelated Ags, thus impairing Th1 responses (8, 15, 16, 25). In the present study, DTH inhibition was associated with a shift from Th1 to Th2 phenotype in wild-type animals but not in IL-4 KO mice. In contrast, Ferreira et al. (17) reported that coimmunization with *A. suum* extract suppressed both Th1- and Th2-related cytokines and Ab responses in wild-type DBA/2 mice. This disparity in results may be due to differences in the models employed, as both the mouse strains and the *A. suum* preparations employed were different. DTH inhibition by ABF was lower in IL-4 KO mice than in wild-type mice, as previously described, and in a model of anti-IL-4 Ab-treated mice (18, 25). This suggests that the Th2 adjuvant effect of ABF was dependent on IL-4 secretion by Ag-presenting cells and/or ABF-specific T cells and could amplify the OVA-specific Th1 response inhibition. IL-4, however, was not an indispensable component for this inhibition.

Our results indicate that the inhibition of the OVA-specific DTH response by ABF is not solely the consequence of a Th2 bias. Although DTH is an inflammatory response involving memory T cells, ABF inhibits OVA-specific DTH only if present during the

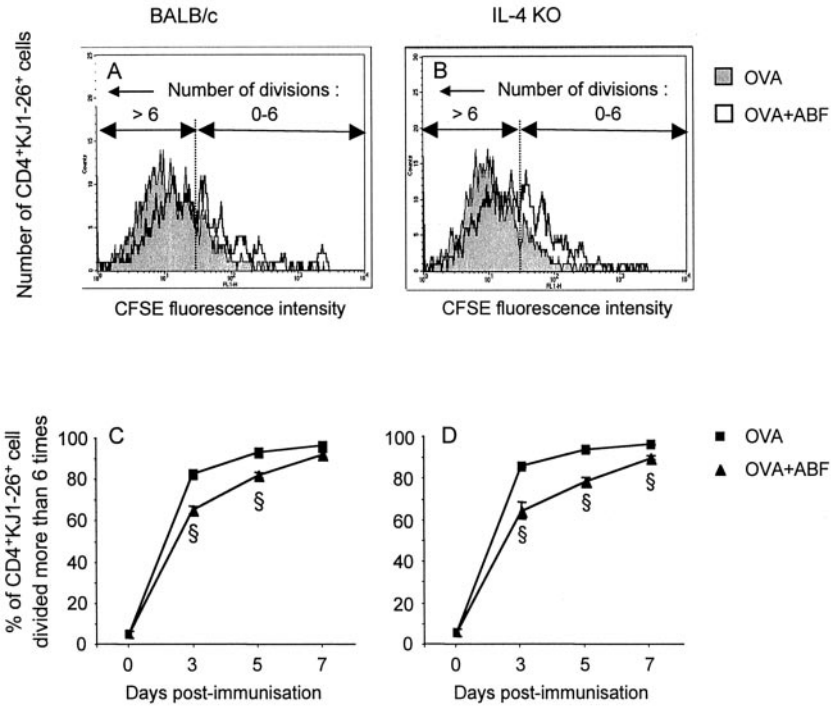

FIGURE 6. Cell division in vivo in the presence or absence of ABF. Draining popliteal LN cells from wild-type and IL-4 KO BALB/c mice transferred with CFSE-stained DO11.10 cells and immunized the following day with OVA or OVA+ABF were harvested at various time points postimmunization, and cellular CFSE profiles of $CD4^+KJ1.26^+$ cells were determined by flow cytometry. Representative CFSE profiles of $CD4^+KJ1.26^+$ cells from wild-type (*A*) and IL-4 KO (*B*) BALB/c mice immunized with OVA or OVA+ABF 4 days postimmunization with percentage of popliteal $CD4^+KJ1.26^+$ cells from OVA or OVA+ABF wild-type (*C*) and IL-4 KO (*D*) immunized mice, having divided more than six times are shown. Results are shown as the mean ± SEM of three individual mice per group. Similar results were obtained in two additional experiments. §, Significantly different ($p \leq 0.05$) from OVA-immunized group.

induction phase of the immune response (25). It has, moreover, been shown that the size of the initial clonal burst in the primary response determines the extent of $CD4^+$ memory cells that develop and are maintained (28). We thus assessed whether ABF inhibited the development of the Th1 response by modulating the amplitude of the expansion of OVA-specific clones in vivo. Tracking OVA-specific cells in an adoptive transfer model showed that ABF inhibited the increase in the percentage of OVA-specific T cells in the popliteal LNs following immunization, in both wild-type and IL-4 KO mice. In contrast, previous studies have shown that *Heligmosomoides polygyrus* infection enhanced OVA-specific T cell percentage (29), suggesting that different nematode products may have different effects on bystander Ag-specific T cells. However, this former study did not analyze the impact on the absolute number of Ag-specific T cells. The number of Ag-specific cells is the result of the percentage of these cells and cellularity of the LN. Interestingly, OVA-specific T cell number was significantly reduced by ABF in IL-4 KO mice but not in wild-type mice. As there was no concurrent reduction in the number of bystander $CD4^+KJ1.26^-$ cells in these mice, it is unlikely that ABF was inhibiting the lymphocyte entry into the LN. The number of bystander $CD4^+$ T cells in OVA plus ABF-immunized IL-4 KO mice was, on the other hand, significantly lower than in OVA plus ABF-immunized wild-type mice, manifesting that IL-4 KO mice did not develop a strong Th2 response against ABF. The diminished number of OVA-specific T lymphocytes in OVA plus ABF-immunized IL-4 KO mice may similarly reflect the fact that in wild-type animals, the inhibition of the Th1 response was compensated by a Th2 response, whereas it was not in IL-4 KO mice. Although it is possible that IL-4 KO mice were reconstituted with IL-4-producing $CD4^+$ cells along with the transferred $CD4^+KJ1.26^+$ cells, the absence of detectable IgE, IL-5, IL-13 as well as IL-4 argues against the transferred cells providing a source of IL-4 in the IL-4 KO animals. Moreover, we have now performed similar studies with IL-4-deficient $CD4^+KJ1.26^+$ cells and obtained essentially identical effects on clonal expansion and cytokine production (data not shown).

By labeling the TCR-Tg donor cells with CFSE, we showed that OVA-specific T cells from mice immunized in the presence of ABF had on average undergone fewer cell divisions than mice

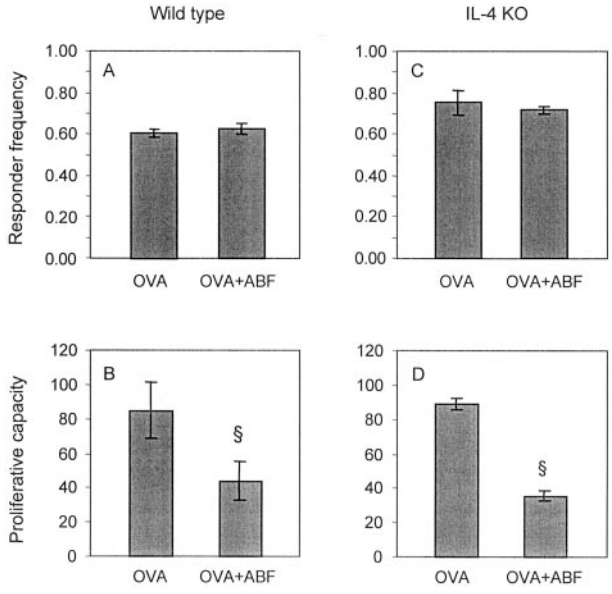

FIGURE 7. Responder frequency and proliferative capacity of the OVA-specific T cells. Wild-type (*A* and *B*) and IL-4 KO (*C* and *D*) BALB/c mice were transferred with CFSE-stained DO11.10 cells and immunized the following day with OVA or OVA+ABF. Draining popliteal LN cells were harvested 4 days postimmunization and their CFSE fluorescence intensity determined by FACS. The proportion of OVA-specific Tg T precursor cells that responded to OVA-immunization by dividing responder frequency, R, (*A* and *C*) and proliferative capacity, Cp, which is the number of daughter cells generated by the average responder cell (*B* and *D*), were deduced from the cellular CFSE fluorescent intensity profile using the formulas shown in *Materials and Methods*. Results are shown as the mean ± SEM of three individual mice per group. Similar results were obtained in two additional experiments. §, Significantly different ($p \leq 0.05$) from OVA-immunized group.

immunized with OVA/CFA alone, possibly explaining the reduced clonal expansion. As this difference did not increase with time, it is unlikely that ABF slowed the cell cycle rate (i.e., increased the doubling time of the cells). The responder frequency of the OVA-specific population was not affected by ABF either. One explanation could be that in presence of ABF, cell entry into the first round of division was delayed. Migration of Ag-loaded dendritic cells from sites of infection into draining lymphoid tissues is fundamental to the priming of T cell immune responses. It has been shown that factors from *Schistosoma mansoni* and *Leishmania major* impair departure of Langerhans cells from the epidermis (30, 31). It is therefore conceivable that ABF may likewise inhibit dendritic cell migration and as a consequence Ag presentation and T cell activation would occur later.

Our data demonstrate, for the first time to our knowledge, that ABF reduces the proliferative capacity of OVA-specific cells during primary responses in vivo. Our results furthermore suggest that the cells were not rendered anergic because the proliferative capacity of these cells upon restimulation in vitro was not affected. Interestingly, a recent in vitro study showed that pigeon cytochrome *c*-specific TCR Tg T cell proliferation was inhibited by adherent peritoneal exudate cells recruited in response to infection with the filarial nematode *Brugia malayi* but were not rendered anergic (32). Different species of nematodes have been reported to generate peritoneal macrophages (33) or splenic Ag-presenting cells (34) that suppress T cell proliferation and ABF might similarly induce suppressor APC in the draining LNs. However, whereas IL-4 is critical for the induction of suppressor peritoneal macrophages (33), the reduction in cellular proliferative capacity by ABF in our study seemed to be IL-4 independent. The reduction in proliferative capacity might be an indication that the OVA-specific cells stopped dividing after a certain number of divisions. However, because the maximum number of cell divisions was not modified, it suggests that only a fraction of the OVA-specific T cell population was undergoing limited divisions. Although the change in the absolute T cell number may not be mediating the impairment of the DTH by ABF, it is possible that a proportion of OVA-specific T cells present is required to reach a threshold before a DTH response can be induced. Furthermore, it has been reported that regulatory T cells undergo fewer divisions than do primed T cells (35). This finding, added to the fact that the DTH inhibition by ABF was independent of an OVA-specific Th2 response and was associated with OVA-specific IL-10 secretion, reinforces the suggestion that helminth immunosuppressive activity might be mediated by regulatory T cells (20, 25, 36). Therefore, we are currently investigating the role of IL-10 in our model.

The only other study to date of the effect of helminth infections or products on unrelated Ag-specific T cell expansion and proliferative capacity suggests that *H. polygyrus* infection can act as a Th2 adjuvant for the response to dietary Ags and abrogate the oral tolerance that they normally induce (29). Thus, modulating the proliferative capacity of Ag-specific T responder cells seems to be a powerful mechanism by which helminths can enhance Th2 and reduce Th1 responses.

In conclusion, using mice adoptively transferred with CFSE-stained OVA-TCR Tg T cells, we demonstrated for the first time that modulation of the immune response to bystander Ags by ABF was the results of two distinct nonexclusive mechanisms in vivo: firstly, an IL-4-dependent Th1 to Th2 shift of the Ag-specific immune response and secondly, an IL-4-independent inhibition of the Ag-specific T lymphocyte proliferative capacity during the primary response.

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References

1. Shimp, R. G., R. B. Crandall, and C. A. Crandall. 1975. *Heligmosomoides polygyrus* (*Nematospiroides dubius*): suppression of antibody response to orally administered sheep erythrocytes in infected mice. *Exp. Parasitol. 38:257.*
2. Stewart, G. R., M. Boussinesq, T. Coulson, L. Elson, T. Nutman, and J. E. Bradley. 1999. Onchocerciasis modulates the immune response to mycobacterial antigens. *Clin. Exp. Immunol. 117:517.*
3. Alkarmi, T., M. K. Ijaz, F. K. Dar, S. Abdou, S. Alharbi, P. Frossard, and M. Naiem. 1995. Suppression of transplant immunity in experimental trichinellosis. *Comp. Immunol. Microbiol. Infect. Dis. 18:171.*
4. Araujo, F. G., P. M. Coelho, L. H. Pereira, and J. Pellegrino. 1977. *Schistosoma mansoni*: impairment of the cell-mediated immune response in mice. *Clin. Exp. Immunol. 28:289.*
5. Ledingham, D. L., V. C. McAlister, H. N. Ehigiator, C. Giacomantonio, M. Theal, and T. D. Lee. 1996. Prolongation of rat kidney allograft survival by nematodes. *Transplantation 61:184.*
6. Liwski, R., J. Zhou, V. McAlister, and T. D. Lee. 2000. Prolongation of allograft survival by *Nippostrongylus brasiliensis* is associated with decreased allospecific cytotoxic T lymphocyte activity and development of T cytotoxic cell type 2 cells. *Transplantation 69:1912.*
7. Chowaniec, W., R. B. Wescott, and L. L. Congdon. 1972. Interaction of *Nematospiroides dubius* and influenza virus in mice. *Exp. Parasitol. 32:33.*
8. Actor, J. K., M. Shirai, M. C. Kullberg, R. M. Buller, A. Sher, and J. A. Berzofsky. 1993. Helminth infection results in decreased virus-specific $CD8^+$ cytotoxic T-cell and Th1 cytokine responses as well as delayed virus clearance. *Proc. Natl. Acad. Sci. USA 90:948.*
9. Behnke, J. M., D. Wakelin, and M. M. Wilson. 1978. *Trichinella spiralis*: delayed rejection in mice concurrently infected with *Nematospiroides dubius*. *Exp. Parasitol. 46:121.*
10. Jenkins, D. C. 1975. The influence of *Nematospiroides dubius* on subsequent *Nippostrongylus brasiliensis* infections in mice. *Parasitology 71:349.*
11. Whitelaw, D. D., J. M. Scott, H. W. Reid, P. H. Holmes, F. W. Jennings, and G. M. Urquhart. 1979. Immunosuppression in bovine trypanosomiasis: studies with louping-ill vaccine. *Res. Vet. Sci. 26:102.*
12. Ilemobade, A. A., D. S. Adegboye, O. Onoviran, and J. C. Chima. 1982. Immunodepressive effects of trypanosomal infection in cattle immunized against contagious bovine pleuropneumonia. *Parasite Immunol. 4:273.*
13. Cooper, P. J., I. Espinel, M. Wieseman, W. Paredes, M. Espinel, R. H. Guderian, and T. B. Nutman. 1999. Human onchocerciasis and tetanus vaccination: impact on the postvaccination antitetanus antibody response. *Infect. Immun. 67:5951.*
14. Onah, D. N., and D. Wakelin. 2000. Murine model study of the practical implication of trypanosome-induced immunosuppression in vaccine-based disease control programmes. *Vet. Immunol. Immunopathol. 74:271.*
15. Kullberg, M. C., E. J. Pearce, S. E. Hieny, A. Sher, and J. A. Berzofsky. 1992. Infection with *Schistosoma mansoni* alters Th1/Th2 cytokine responses to a non-parasite antigen. *J. Immunol. 148:3264.*
16. Pearlman, E., J. W. Kazura, F. E. Hazlett, Jr., and W. H. Boom. 1993. Modulation of murine cytokine responses to mycobacterial antigens by helminth-induced T helper 2 cell responses. *J. Immunol. 151:4857.*
17. Ferreira, A. P., E. S. Faquim-Mauro, I. A. Abrahamsohn, and M. S. Macedo. 1995. Immunization with *Ascaris suum* extract impairs T cell functions in mice. *Cell. Immunol. 162:202.*
18. Macedo, M. S., E. S. Faquim-Mauro, A. P. Ferreira, and I. A. Abrahamsohn. 1998. Immunomodulation induced by *Ascaris suum* extract in mice: effect of anti-interleukin-4 and anti-interleukin-10 antibodies. *Scand. J. Immunol. 47:10.*
19. Bashir, M. E., P. Andersen, I. J. Fuss, H. N. Shi, and C. Nagler-Anderson. 2002. An enteric helminth infection protects against an allergic response to dietary antigen. *J. Immunol. 169:3284.*
20. Doetze, A., J. Satoguina, G. Burchard, T. Rau, C. Loliger, B. Fleischer, and A. Hoerauf. 2000. Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T(h)3/T(r)1-type cytokines IL-10 and transforming growth factor-β but not by a T(h)1 to T(h)2 shift. *Int. Immunol. 12:623.*
21. Harnett, W., and M. M. Harnett. 2001. Modulation of the host immune system by phosphorylcholine-containing glycoproteins secreted by parasitic filarial nematodes. *Biochim. Biophys. Acta 1539:7.*
22. Kearney, E. R., K. A. Pape, D. Y. Loh, and M. K. Jenkins. 1994. Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. *Immunity 1:327.*
23. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+TCR^{lo}$ thymocytes in vivo. *Science 250:1720.*
24. Marrack, P., R. Shimonkevitz, C. Hannum, K. Haskins, and J. Kappler. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. IV. An antiidiotypic antibody predicts both antigen and I-specificity. *J. Exp. Med. 158:1635.*

25. Paterson, J. C., P. Garside, M. W. Kennedy, and C. E. Lawrence. 2002. Modulation of a heterologous immune response by the products of *Ascaris suum*. *Infect. Immun. 70:6058*.
26. Gudmundsdottir, H., A. D. Wells, and L. A. Turka. 1999. Dynamics and requirements of T cell clonal expansion in vivo at the single-cell level: effector function is linked to proliferative capacity. *J. Immunol. 162:5212*.
27. Pape, K. A., E. R. Kearney, A. Khoruts, A. Mondino, R. Merica, Z. M. Chen, E. Ingulli, J. White, J. G. Johnson, and M. K. Jenkins. 1997. Use of adoptive transfer of T-cell-antigen-receptor-transgenic T cell for the study of T-cell activation in vivo. *Immunol. Rev. 156:67*.
28. Linton, P. J., J. Harbertson, and L. M. Bradley. 2000. A critical role for B cells in the development of memory CD4 cells. *J. Immunol. 165:5558*.
29. Shi, H. N., H. Y. Liu, and C. Nagler-Anderson. 2000. Enteric infection acts as an adjuvant for the response to a model food antigen. *J. Immunol. 165:6174*.
30. Angeli, V., C. Faveeuw, O. Roye, J. Fontaine, E. Teissier, A. Capron, I. Wolowczuk, M. Capron, and F. Trottein. 2001. Role of the parasite-derived prostaglandin D_2 in the inhibition of epidermal Langerhans cell migration during schistosomiasis infection. *J. Exp. Med. 193:1135*.
31. Ponte-Sucre, A., D. Heise, and H. Moll. 2001. *Leishmania major* lipophosphoglycan modulates the phenotype and inhibits migration of murine Langerhans cells. *Immunology 104:462*.
32. Loke, P., A. S. MacDonald, and J. E. Allen. 2000. Antigen-presenting cells recruited by *Brugia malayi* induce Th2 differentiation of naive $CD4^+$ T cells. *Eur. J. Immunol. 30:1127*.
33. Allen, J. E., and A. S. MacDonald. 1998. Profound suppression of cellular proliferation mediated by the secretions of nematodes. *Parasite Immunol. 20:241*.
34. Osborne, J., and E. Devaney. 1999. Interleukin-10 and antigen-presenting cells actively suppress Th1 cells in BALB/c mice infected with the filarial parasite *Brugia pahangi*. *Infect. Immun. 67:1599*.
35. Thorstenson, K. M., and A. Khoruts. 2001. Generation of anergic and potentially immunoregulatory $CD25^+CD4$ T cells in vivo after induction of peripheral tolerance with intravenous or oral antigen. *J. Immunol. 167:188*.
36. Satoguina, J., M. Mempel, J. Larbi, M. Badusche, C. Loliger, O. Adjei, G. Gachelin, B. Fleischer, and A. Hoerauf. 2002. Antigen-specific T regulatory-1 cells are associated with immunosuppression in a chronic helminth infection (onchocerciasis). *Microbes Infect. 4:1291*.