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## Dexamethasone Regulates Macrophage and Cd4+Cd25+ Cell Numbers in the Chicken Spleen

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### ■ Keywords

Coccidiosis, *Eimeria*, Avian immunology, Flow cytometry, Neuroimmunomodulation.

### ABSTRACT

Dexamethasone (DEX) is a corticoid hormone that is experimentally used to mimic the effects of increased levels of endogenous corticosterone observed during the stress response. Currently, stress is considered one of the major predisposing factors for diseases in the poultry industry. The aim of this study was to analyze the effects of DEX and/or of a 20-fold coccidial vaccine dose on leukocyte phenotypes in the spleen and cecal tonsils of chickens. Twenty specific-pathogen-free (SPF) Leghorn chickens were divided into four groups: a non-treated group (NT), a DEX-treated group (Dex), a vaccinated group (V) and a DEX-treated+vaccinated group (Dex+V). On experimental day (ED) 42, each bird in the vaccinated groups received a anti-coccidial vaccine. DEX was injected in the birds of the Dex and Dex+V groups (0.9 mg/kg) on ED42 and ED45. The immunophenotyping was performed by flow cytometry analysis of splenocytes and cecal tonsils cells on ED48. DEX treatment *per se* was unable to change CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>+</sup> and CD4<sup>+</sup>CD8<sup>+</sup> populations with TCRγδ or CD28 in the spleen, or macrophages and T lymphocytes in the cecal tonsils. V group birds presented higher numbers of splenic macrophages compared with those measured in the Dex+V group. The number of CD4<sup>+</sup>CD25<sup>+</sup> cells in the spleen of birds of the V group was higher than those measured in the other experimental groups. Our data suggest that CD4<sup>+</sup>CD25<sup>+</sup> cells and macrophages might be influenced by DEX treatment in spleen, but not in the cecal tonsils of chickens inoculated with *Eimeria*.

### INTRODUCTION

Dexamethasone (DEX) is an immunosuppressant drug that is widely used to study some stressor effects in disease development and course in poultry (Huff *et al.*, 2001, 2013; Shini *et al.*, 2010). Given its similarity to endogenous corticosteroids, DEX treatment is thought to reproduce the effects of high levels of corticosterone, thus mimicking stress-associated signaling pathways (Devenport *et al.*, 1989).

DEX treatment of chickens infected with *Eimeria mivati* was reported to reduce the CD8<sup>+</sup>, TCRγδ and MHC-II<sup>+</sup> cells and to increase in the CD3<sup>+</sup> and CD4<sup>+</sup> cells in their spleen; these findings were followed by a reduction in lymphocyte activity, which was related to the reported increase in *E. mivati* infection (Isobe & Lillehoj, 1993).

Coccidial infection induces complex immune responses. Different strains of *Eimeria* are known to produce variable immune responses under natural conditions (Lillehoj & Trout, 1996). The determination of the immunomodulatory effects of drugs such as DEX, which mimics an increase in endogenous corticosteroid levels, may aid the understanding the course of coccidiosis infection and vaccination failures during stress.



Recent biotechnological developments have contributed for the characterization of novel phenotypes of avian immune cells (Kaiser, 2010). A better understanding of certain cells populations activities, such as regulatory T lymphocytes (i.e., regulatory CD4<sup>+</sup>CD25<sup>+</sup> cells) may open new avenues to ascertain poultry's immune responses to infectious agents (Shanmugasundaram & Selvaraj, 2011, 2012a, 2012b; Selvaraj, 2013). Studies in the field of neuroimmunomodulation are showing that these lymphocyte populations and mainly the resident cells present in lymphoid organs, such as those of the spleen and cecal tonsils, play relevant and definitive roles in the course of infectious diseases (Shanmugasundaram & Selvaraj, 2011, 2012a, 2012b; Selvaraj, 2013).

The aim of this study was to analyze the effects of a DEX treatment and/or a coccidial overdose vaccination on B and T lymphocytes and macrophages phenotypes in the spleen and cecal tonsils of chickens.

## MATERIALS AND METHODS

### Birds

Twenty specific-pathogen-free (SPF) male Leghorn chickens were housed in isolation chambers (Alesco, São Paulo, Brazil) with a maximum density of 10 animals per square meter from the first day post-hatching. The chickens received water and feed *ad libitum* with no additives, as these may intervene with the development of the inoculated *Eimeria*. The light/dark cycle was 12/12 h, with lights on at 7:00 a.m. The experimental protocol was approved by the Committee on the Care and Use of Laboratory Animal Resources, School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil (# 3071/2013).

### Experimental groups and treatments

On experimental day 1 (ED1), the 20 chickens were randomly allocated in four groups: a non-treated group (NT, n=5), a DEX-treated group (Dex, n=5), an *Eimeria* spp.-vaccinated group (V, n=5) and a DEX-treated + *Eimeria* spp.-vaccinated group (Dex+V, n=5).

### Coccidiosis overdose vaccination

ON ED42, birds were vaccinated (Coccivac-D, MSD Animal Health, NJ, USA) by gavage at a concentration twenty-fold higher than the manufacturer's recommendations (groups V and Dex+V). This dose was chosen to challenge the intestinal integrity as reported elsewhere for experimental necrotic enteritis

induction (Lee *et al.*, 2011). The vaccine used contains live oocysts of precocious strains of *Eimeria acervulina*, *E. brunetti*, *E. hagani*, *E. tenella*, *E. necatrix*, *E. mivati*, *E. maxima*, and *E. praecox*.

### DEX treatment

The birds in the Dex and Dex+V groups were injected with DEX (D4092, Sigma-Aldrich, MO, USA) via the intracoelomic route (dose = 0.9 mg/kg) on ED42 and ED45.

The birds of the NT group did not receive any treatment and remained undisturbed in their home chamber till the time of analysis.

### Antibodies

The primary monoclonal antibodies used in this study are shown in Table 1. The antibodies used to detect macrophages and antigen-presenting cells were as follows: mouse anti-chicken monocytes/macrophages, clone KUL01; mouse anti-chicken CD80, clone IAH:F864:DC; mouse anti-chicken CD86, clone IAH:F853:AG2; and mouse anti-chicken MHC class II, clone 2G11. The antibodies used to detect lymphocytes were as follows: human anti-chicken CD25, clone 13504; mouse anti-chicken CD3, clone CT-3; mouse anti-chicken TCR  $\gamma\delta$ , clone TCR-1; mouse anti-chicken CD45, clone LT40; mouse anti-chicken CD8 $\alpha$ , clone CT-8; mouse anti-chicken CD28, clone AV7; mouse anti-chicken CD4, clone CT-4; and mouse anti-chicken Bu-1, clone AV20. Mouse IgG1 isotype controls conjugated to Fluorescein (FITC), R-phycoerythrin (R-PE), R-phycoerythrin-Texas Red (Texas Red), Allophycocyanin (APC), Spectral Red (SPRD), Cyanine 5 (Cy5) or Alexa Fluor 647 (Alexa 647) were used as fluorescence controls. Streptavidin-conjugated APCs were used to label biotinylated primary antibodies (Table 1). Anti-mouse antibodies conjugated with Texas Red were used as secondary antibodies to detect unlabeled antibodies (Table 1). Anti-CD80, CD86 and CD25 cell marker antibodies were purchased from AbD Serotec (USA), and the other cell markers were purchased from Southern Biotech (AL, USA).

### Immunophenotyping of splenic and cecal tonsil leukocytes

On ED48, the spleen and cecal tonsils were collected in conical tubes containing 4 mL of RPMI 1640 (Sigma-Aldrich, MO, USA). The tissues were cut into sections of approximately 1 mm<sup>3</sup>. The cells were subsequently gently disrupted with the aid of a syringe plunger.



**Table 1** – Identification of cell markers used for immunophenotyping of spleen and/or cecal tonsil cells along with their specificity and the conjugate/labels used for detection.

Cell surface antigens	Specificity	Conjugate/labels
Macrophage/Monocyte <sup>a</sup>	Chicken monocytes and macrophages	R-PE
MHC Class II <sup>b</sup>	Antigen-presenting cells, including activated B cells, activated T cells, macrophages and dendritic cells	R-PE or FITC
CD80 <sup>c</sup>	Antigen-presenting cells, including activated B cells, activated T cells, macrophages and dendritic cells	Unlabeled-Texas Red
CD86 <sup>d</sup>	Antigen-presenting cells, including activated B cells, activated T cells, macrophages and dendritic cells	Unlabeled-Texas Red
CD3 <sup>e</sup>	T cells	SPRD
CD45 <sup>f</sup>	Leukocytes	APC
CD8α <sup>g</sup>	Cytotoxic T cells	Cy5
CD4 <sup>h</sup>	T helper cells	CD4
Bu-1 <sup>i</sup>	B cells	Alexa 647
CD25 <sup>j</sup>	IL-2 receptor alpha chain in T cells	FITC
CD28 <sup>k</sup>	T cells except TCRγδ lymphocytes	Biotin-streptavidin-APC
TCRγδ <sup>l</sup>	TCRγδ lymphocytes	FITC

Notes: Alexa 647 – Alexa Fluor 647; APC – Allophycocyanin; Cy5 – Cyanine 5; FITC – Fluorescein isothiocyanate; R-PE – R-Phycoerythrin; Texas Red – R-Phycoerythrin-Texas Red; APC – Allophycocyanin; SPRD – Spectral red. <sup>a</sup>Mast et al., 1998; <sup>b</sup>Solomonsen et al., 1991; <sup>c</sup>Vervelde et al., 2013; <sup>d</sup>Wu et al., 2006; <sup>e</sup>Chen et al., 1986; <sup>f</sup>Paramithiotis et al., 1991; <sup>g,h</sup>Chan et al., 1988; <sup>i</sup>Rothwell et al., 1996; <sup>j</sup>Neulen et al., 2015; <sup>k</sup>Young et al., 1994; <sup>l</sup>Sowder et al., 1988.

The contents were transferred to a polystyrene tube coupled with a cell strainer (Becton Dickinson, NJ, USA) with a 0.7 µm-pore mesh. Leukocytes were counted in a Neubauer chamber using trypan blue for exclusion of the dead cells to determine the number of viable leukocytes.

Splenic leukocytes (1x10<sup>6</sup> cells) were submitted to red blood cell lysis via the addition of 2 mL of FACS lysis buffer (Becton Dickinson, NJ, USA) for 5 min at 37°C. This procedure was not performed on cecal tonsil cells.

Each sample analyzed comprised 1x10<sup>6</sup> cells per cytometer tube (spleen and cecal tonsil cells). Single staining was performed using the antibodies described above diluted at 1 to 3 µg in 100 µL of PBS (pH 7.4). The suspensions of cells with antibodies were kept at 4°C for 45 min in the dark. After the incubation period, the cells were washed once with 2 mL of PBS and centrifuged at 400 x g for 8 min. The cells were then resuspended in 100 µL of PBS for fluorescence measurement by flow cytometry or subjected to secondary antibody labeling. Unlabeled antibodies and biotin-conjugated antibodies were incubated with secondary anti-mouse antibodies conjugated with Texas Red and streptavidin-APC conjugates, respectively, at 4°C for 45 min in the dark. After the incubation period, the cells were washed once with 2 mL of PBS and centrifuged at 400 x g for 8 min. The cells were then resuspended

in 100 µL of PBS for fluorescence measurement by flow cytometry.

Multiple staining was performed to assess macrophages/monocytes and T cell subsets. The populations analyzed in the cecal tonsils were CD45/CD3, CD45/TCRγδ/CD8α/CD4, monocyte/macrophage/MHCII, and Bu-1. The populations analyzed in the spleen were CD45/CD3, CD45/TCRγδ/CD8α/CD4, Bu-1, CD4/CD8/CD25, CD4/CD8/CD28, monocyte/macrophage/MHCII/CD80 and CD86.

Measurements were performed using a BD FACSCalibur (BD Biosciences CA, USA) flow cytometer. This flow cytometer was equipped with a 488-nm (blue) laser allowing the detection of three colors (530/30, 575/26 and 650 LP [long pass] filters) and a 635-nm (red) laser that enabled single-color detection (670 LP [long pass] filter). A total of 10,000 measurements were performed for each sample. All antibodies were titrated prior to the experiment to determine the optimal staining concentrations, and a multicolor panel was evaluated using fluorescence-minus-one (FMO) controls and isotype controls for each fluorophore. The analyses were performed using the software FlowJo v10.0.7 (FlowJo, OR, USA).

### Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad 6 Software Inc., CA, USA). The data were analyzed using one-way analysis of



variance (ANOVA) followed by the Tukey-Kramer test for multiple comparisons. The probability of  $p < 0.05$  was taken as showing statistical significant differences among the data. Results are presented as means  $\pm$  SD.

## RESULTS

### Immunophenotyping of lymphocytes and macrophages in the cecal tonsils

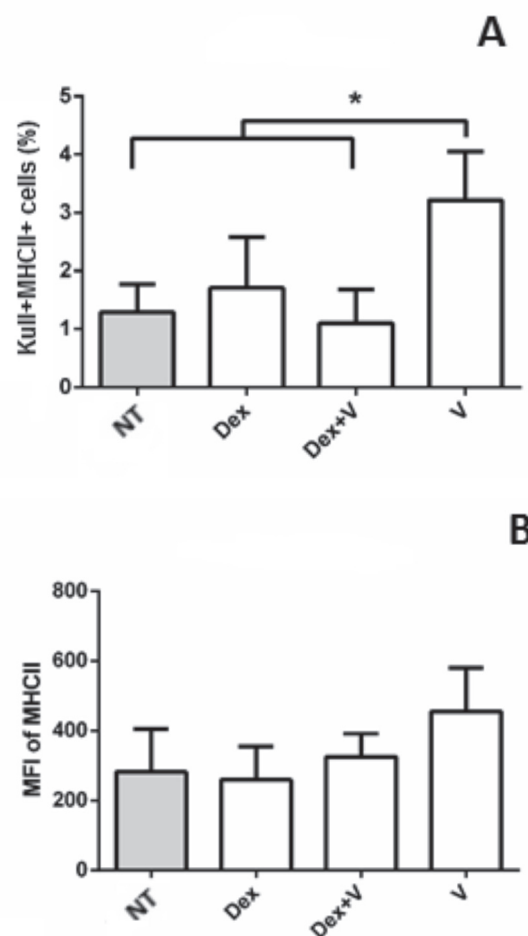
*Eimeria* vaccination (V group) and DEX injection (DEX group) *per se* were unable to change the number of CD45<sup>+</sup> cells among the different groups (mean number of positive cells measured =  $60.80 \pm 10.20\%$ , data not shown). Similarly, there were no significant differences in the numbers of CD3<sup>+</sup> cells among the groups (mean number of positive cells of  $36.80 \pm 8.22\%$ , data not shown). Similarly, significant differences were not found between birds of the Dex+V and V groups for the number of T cells (CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, or CD4<sup>-</sup>CD8<sup>+</sup>, with or without the presence of TCR $\gamma\delta$ ), B lymphocytes (Bu-1<sup>+</sup>), and for macrophages in the cecal tonsils (data not shown).

### Immunophenotyping of macrophages in the chicken spleen

The birds inoculated with precocious strains of coccidia (V group) showed a significant increase ( $p < 0.05$ ) in the number of splenic macrophages in relation to those measured in the non-treated birds (NT group); however, this increase was not significantly different from the data observed in the birds of the Dex group ( $p > 0.05$ ; Figure 1). However, the DEX treatment of the birds vaccinated with the *Eimeria* spp. strains (Dex+V group) abrogated the increase in the number of splenic macrophages found in the vaccinated animals (V group;  $p < 0.05$ ; Figure 1A).

Significant differences were also not observed in the mean fluorescence intensity (MFI) of MHC-II<sup>+</sup> expression in splenic macrophages; however, a tendency towards an increase of MHC-II<sup>+</sup> expression was observed in the coccidia-vaccinated birds (V group;  $p > 0.05$ ; Figure 1b). This trend was not observed in the DEX-treated vaccinated birds (Dex+V group; Figure 1B) compared with the birds of both NT and DEX groups.

Finally, significant differences were not found in the number of splenic CD80<sup>+</sup> or CD86<sup>+</sup> macrophages among the four groups.



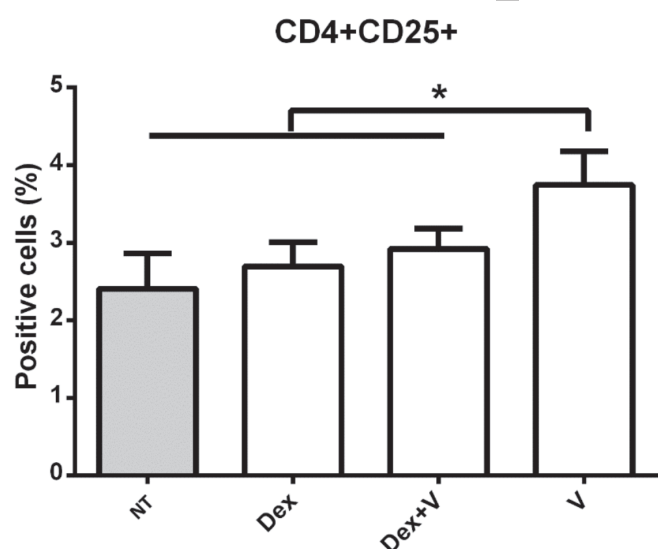
**Figure 1.** Splenic immunophenotyping of macrophages (Kull+MHCII+ cells (A) and of MHC class II fluorescence intensity per Kull+ cells (B). An asterisk on top of columns means a statistically significant differences at  $p < 0.05$  (one-way ANOVA and Tukey test for multiple comparisons). Data are mean  $\pm$  SD of 5 birds per group. NT – Non-treated group; Dex – DEX-treated group; Dex+V – DEX-treated + *Eimeria* spp.-infected group; V – *Eimeria* spp.-infected group; MFI – mean fluorescence intensity.

### Immunophenotyping of lymphocytes in the chicken spleen

Lysis of erythrocytes from the spleen cell suspension produced an average of  $95.71 \pm 3.48\%$  CD45<sup>+</sup> cells. Approximately  $63.07 \pm 10.22\%$  of these cells were CD3<sup>+</sup> lymphocytes. Significant differences were not observed in the populations of T lymphocytes (CD3<sup>+</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, or CD4<sup>-</sup>CD8<sup>+</sup>, with or without the presence of TCR $\gamma\delta$  or CD28) or B lymphocytes (Bu-1<sup>+</sup>) (data not shown).

The *Eimeria* spp. vaccinated birds (V group) presented an increased number of CD4<sup>+</sup>CD25<sup>+</sup> cells in relation to those determined in the vaccinated birds that received the DEX treatment (Dex+V group;  $p < 0.05$ ; Figure 2) and also to the uninfected birds (NT and DEX groups;  $p < 0.05$ ; Figure 2). Significant differences were not found among the numbers of CD4<sup>+</sup>CD25<sup>+</sup> cells in birds of the NT, Dex and Dex+V groups (Figure 2).





**Figure 2.** Number of splenic CD4+CD25+ lymphocytes. An asterisk on top of columns means a statistically significant differences at  $p < 0.05$  (one-way ANOVA and Tukey test for multiple comparisons). Data are mean  $\pm$  SD of 5 birds per group. NT – Non-treated group; Dex – DEX-treated group; Dex+V – DEX-treated +*Eimeria* spp.-infected group; V – *Eimeria* spp.-infected group.

## DISCUSSION

DEX has been used to induce immunosuppression in birds, thus mimicking a stress response (Shini *et al.*, 2010). In literature, DEX has been employed as an experimental model of stress effects on various diseases, such as clostridial dermatitis (Huff *et al.*, 2013). These studies have inferred that the immunomodulatory effects of DEX somehow mimic those commonly induced by stressors, i.e., they may similarly trigger the development or modify the course of poultry diseases (Huff *et al.*, 2013; Shini *et al.*, 2010). Although DEX has been used to analyze the development of avian diseases (Puvadolpirod and Thaxton, 2000; Huff *et al.*, 2005; Li *et al.*, 2009), little is known on its immunomodulatory effects during host-pathogen interactions, such as those that occur in animals with coccidiosis.

Isobe and Lillehoj (1993) demonstrated that treatment of chickens with DEX suppressed the T-cell response in the spleen and increased the birds' susceptibility to *E. mivati* infection. In fact, that study showed that the treatment with DEX increased the subpopulations of CD4<sup>+</sup> splenic lymphocytes, simultaneously decreasing the production of IFN- $\gamma$  and IL-2 cytokines (Isobe & Lillehoj, 1993). Therefore, it is possible that reduced cytokine production, such as IFN- $\gamma$ , may be related to the decreased level of splenic macrophage activation observed in the present study (Figure 1A) (Bashyam, 2007). In turn, this decrease may have induced the trend of a reduction of the expression

of MHC class II (Figure 1B), which may be causally related to the observed reduction in macrophages' antigen recognition capability.

The observed decrease in MHC class II expression among macrophages seem to be related to an increase in regulatory T-cell activity, specifically of CD4<sup>+</sup>CD25<sup>+</sup> cells (Tiemessen *et al.*, 2007). The suppression of this lymphocyte may have induced a reduction in the level of cytokine expression (i.e., IFN- $\gamma$ ) in the birds of the present study, resulting in the effects here reported. In our study, the population of lymphocytes (CD4<sup>+</sup>CD25<sup>+</sup>) was lower in *Eimeria*-inoculated birds treated with DEX compared with those vaccinated (i.e., between Dex+V x V groups). The reduction in number of these cells may have triggered the release of small amounts of IL-10, a cytokine that is known to inhibit macrophage responses (Shanmugasundaram & Selvaraj, 2011). In this context, an abrogation of regulatory T lymphocytes is consistent with data reported elsewhere for lymphocyte proliferation (Shanmugasundaram & Selvaraj, 2011).

Birds that were vaccinated with coccidia only (V group) presented high numbers of macrophages and CD4<sup>+</sup>CD25<sup>+</sup> cells in the spleen, i.e., *Eimeria* inoculation *per se* may have induced the proliferation and/or migration of these cell populations to the spleen. As coccidial infection *per se* did not reduce the numbers of immune cells, it is possible to infer that the CD4<sup>+</sup>CD25<sup>+</sup> cell population is, in fact, a subpopulation of cells composed mainly by activated CD4<sup>+</sup> T lymphocytes, i.e., the response to *Eimeria* inoculation *per se* and not a subpopulation of cells that induces the suppression of T lymphocyte proliferation. It should be noted, however, that simple immunophenotypic characterization of CD4<sup>+</sup>CD25<sup>+</sup> cells is not enough to determine whether these cells belong to the immunosuppressive lymphocyte population. Thus, it seems reasonable to suggest that the population of CD4<sup>+</sup>CD25<sup>+</sup> cells found in our study may include a range of lymphocyte subsets with distinct activities, especially because CD25 is part of the IL-2 receptor (Teng *et al.*, 2006). Moreover, splenic memory T cells constitutively express the CD25 marker (Reinhardt *et al.*, 2012). Thus, in spite of the small number of birds per group used in the current experiment, our data strongly point towards a corticoid effect on memory T cells formation in the spleen, thus suppressing the immune response triggered by the infectious challenge. Future experiments might ascertain this hypothesis.

An important aspect of the use of DEX in our experimental model is that the drug reduced the



numbers of macrophages and CD4<sup>+</sup>CD25<sup>+</sup> cells and did not substantially affect other cell subsets (CD3<sup>+</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, and CD4<sup>-</sup>CD8<sup>+</sup> combined with TCR $\gamma\delta$  or CD28). Furthermore, it is noteworthy from our study that the effects of DEX treatment on macrophages was mainly observed in the splenic population; indeed, significant changes were not detected in the birds' cecal tonsils. The anti-inflammatory effects exerted by exogenous corticoids such as DEX on the intestinal mucosa, a fact that is known to reduce macrophage activation and migration, could tentatively explain these results.

In summary, our work showed that CD4<sup>+</sup>CD25<sup>+</sup> cells and splenic macrophages are strongly influenced by DEX treatment. However, DEX treatment was unable to modify the CD3<sup>+</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, and CD4<sup>-</sup>CD8<sup>+</sup> populations with TCR $\gamma\delta$  or CD28 within the spleen or the macrophage and CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, and CD4<sup>-</sup>CD8<sup>+</sup> populations with TCR $\gamma\delta$  within the cecal tonsils.

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