Veterinary Immunology and Immunopathology 149 (2012) 20–27

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Regulatory T cell properties of thymic CD4$^+$CD25$^+$ cells in ducks

Revathi Shanmugasundaram, Ramesh K. Selvaraj*

Department of Animal Sciences, Ohio Agricultural Research and Development Center, Wooster, OH 44691, United States

ARTICLE INFO

Article history:
Received 5 October 2011
Received in revised form 12 April 2012
Accepted 22 May 2012

Keywords:
Tregs
Suppression
IL-10
Ducks

ABSTRACT

Thymic CD4$^+$CD25$^+$ cells from ducks were characterized for mammalian T regulatory cells' suppressive and cytokine production properties. The cross reactivity of anti-chicken CD25 monoclonal antibody with duck CD25 was confirmed by evaluating Concanavalin-A-stimulated CD25 upregulation in splenocytes. CD4$^+$CD25$^+$ cells were detectable in the thymus, spleen, cecal tonsil, and lung (airsacs), but not in the bursa. Duck CD4$^+$CD25$^+$ cells had approximately nine-fold higher IL-10 mRNA, 12-fold higher TGF-β, 16-fold higher CTLA-4, and nine-fold higher LAG-3 mRNA amounts than thymic CD4$^+$CD25$^-$ cells. Thymic CD4$^+$CD25$^+$ cells had no detectable levels of IL-2 mRNA. Duck CD4$^+$CD25$^+$ cells had a three-fold higher IL-10 mRNA amount than chicken CD4$^+$CD25$^+$ cells. Duck CD4$^+$CD25$^+$ cells were anergic *in vitro*. Duck CD4$^+$CD25$^+$ cells suppressed naive cell proliferation at effector: responder cell ratios above 0.5:1 in both contact-dependent and -independent pathways. It could be concluded that thymic CD4$^+$CD25$^+$ cells in ducks are most likely the counterpart of mammalian T regulatory cells.

1. Introduction

During infection, pathogens are killed by activated cells of the immune system. Activated immune cells, although essential for pathogen elimination, produce inflammatory cytokines and reactive oxygen species and cause undesirable host damage like muscle wasting and autoimmune diseases (Belkaid and Rouse, 2005). T regulatory cells (Tregs) are a subset of T cells that specialize in immune suppression. Tregs protect the host from excessive immune responses and maintain self tolerance and mucosal tolerance (Workman et al., 2009). On the other hand, hyperactive Tregs can impair T cells, B cells, and other immune cell activities and, therefore, are implicated in impaired microbial defenses, pathogen persistence (Li et al., 2008), impaired vaccine responses (Stober et al., 2005), and compromised anti-tumor responses.

Tregs have been extensively characterized and studied in mammals. Among markers that have been defined for Tregs, the closest that can be termed unique to Tregs is FoxP3 (Li et al., 2008). A FoxP3 ortholog has yet to be identified in chickens. *In silico* analysis and degenerate primer analysis (primer design based on mouse, human, cattle, and fish FoxP3 sequences) did not amplify any specific bands in chickens, ducks, or Japanese Quail. Thus, the presence of FoxP3 or Tregs is questioned in chickens (Shanmugasundaram and Selvaraj, 2010, 2011). In the absence of FoxP3, we recently isolated and characterized chicken Tregs based on the expression of CD25, a characteristic marker of Tregs in mammals (Shanmugasundaram and Selvaraj, 2011).

Chicken thymic CD4$^+$CD25$^+$ cells have 29-fold higher IL-10 and 16-fold higher TGF-β mRNA amount than CD4$^+$CD25$^-$ cells. Chicken thymic CD4$^+$CD25$^+$ cells do not produce IL-2 mRNA. CTLA-4 and LAG-3 are unique markers of Tregs in mammals. Chicken thymic CD4$^+$CD25$^+$ cells have eight-fold higher CTLA-4 and 12-fold higher LAG-3 mRNA amounts than CD4$^+$CD25$^-$ cells. Chicken thymic CD4$^+$CD25$^+$ cells suppress T cell proliferation

Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; Con A, concanavalin-A; PMA, phorbol myristate acetate; Tregs, regulatory T cells.

* Corresponding author. Tel.: +1 330 2633793; fax: +1 330 2633949.
E-mail address: selvaraj.7@osu.edu (R.K. Selvaraj).

0165-2427/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetimm.2012.05.019

in both contact-dependent and -independent pathways (Shanmugasundaram and Selvaraj, 2011).

The Treg population in ducks has yet to be characterized. Ducks are migratory waterfowl, and a duck immune system has several properties that are different from a chicken immune system (Lundqvist et al., 2006). For example, ducks infected with the avian influenza virus have a depressed T cell response (Laudert et al., 1993), whereas chickens infected with the avian influenza virus have increased T cell proliferation (Holt, 1990; Van Campen et al., 1989).

This experiment was conducted to compare duck Tregs to chicken Tregs. Because Tregs originate as a separate lineage of cells in the thymus, thymic $CD4^+CD25^+$ cells were studied. Thymic $CD4^+CD25^+$ cells from ducks were isolated and characterized for cytokine production profile, unique marker analysis, anergic properties, and suppressive properties compared to thymic $CD4^+CD25^+$ cells from chickens.

2. Materials and methods

All birds were fed standard feed and maintained under standard animal husbandry conditions. All animal protocols were approved by the Ohio Agricultural Research and Development Center animal care and use committee.

2.1. Antibodies

Production of anti-chicken CD25 monoclonal antibody in the mouse and conjugation of the anti-chicken CD25 antibody to PE using the R-PE conjugation kit (#PJ31K, Prozyme, Hayward, CA) are described earlier (Shanmugasundaram and Selvaraj, 2011). Mouse anti-duck CD4 monoclonal antibody (AbD Serotec, Raleigh, NC) was conjugated with FITC using the FITC antibody labeling kit (#53027, Thermoscientific, Rockford, IL) or activated APC (#PJ25C, Prozyme, Hayward, CA) following manufacturer's instructions.

2.2. Upregulation of CD25 in Con A treated splenic lymphocytes

Tissues for this experiment were collected from Mammoth White Pekin ducks (Eagle Nest Poultry, Oceola, OH). Single cell suspensions from three-week-old duck spleen were enriched for lymphocytes by density centrifugation over Histopaque (1.077 g/ml, Sigma Aldrich). Splenocytes (1×10^6 cells/well) were cultured in RPMI-1640 supplemented with 5% fetal bovine serum, 1% penicillin plus streptomycin, and 10 μg/ml Concanavalin-A (Con A) (Sigma Aldrich, St. Louis, MO) for 0 or 48 h in three replications ($n = 3$). Cells were incubated with 10 μg/ml of PE-conjugated mouse anti-chicken CD25 and 1:200 dilution of unlabelled mouse IgG (AbCam, Cambridge, MA) for 45 min. The unbound antibodies were removed by centrifugation. The percentage of $CD25^+$ cells was analyzed in a flow cytometer (Guava Eascyte, Millipore).

2.3. Western blot analysis

Cellular protein was extracted from Con A treated splenic lymphocytes from Section 2.2 and analyzed for cross reactivity with anti-chicken CD25 as described previously (Shin et al., 2012) with minor modifications. Briefly, 5 or 10 μg of protein was mixed with 200 μl of lysis buffer [50 mM Tris–HCl, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, and protease inhibitor cocktail), heated for 5 min at 100 °C, and resolved by SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to PVDF membrane (Millipore, Billercia, MA) and the membrane was blocked with 5% non-fat milk powder in buffer, incubated overnight at 4 °C with anti-chicken CD25 primary antibody. The bound antibody was detected using alkaline phosphatase linked rabbit anti-mouse IgG secondary antibody (1: 5000) (Santa Cruz Biotechnology, Santa Cruz, CA) and chemiluminescent alkaline phosphatase substrate (Millipore).

2.4. $CD4^+CD25^+$ cell percentage in different organs of ducks

Tissues for this experiment were collected from Mammoth White Pekin ducks (Eagle Nest Poultry, Oceola, OH). Single cell suspensions from thymus, spleen, cecal tonsil, lung (airsacs), and bursa of three-week-old ducks ($n = 5$) were enriched for lymphocytes by density centrifugation over Histopaque (1.077 g/ml, Sigma Aldrich, St. Louis, MO). Cells (1×10^6) were incubated with 10 μg/ml of primary PE-conjugated mouse anti-chicken CD25, 10 μg/ml FITC-conjugated mouse anti-duck CD4, and 1:200 dilution of unlabelled mouse IgG for 45 min. The unbound primary antibodies were removed by centrifugation. The percentage of $CD4^+CD25^+$ cells in different organs was analyzed in a flow cytometer (Guava Eascyte, Millipore) and expressed as percentage of $CD4^+$ cells.

2.5. Thymic $CD4^+CD25^+$ cell isolation from ducks

Tissues for this experiment were collected from White Pekin ducks (Maple Leaf Farms, Milford, IN). $CD4^+CD25^+$ cells were sorted using the anti-PE multisort kit (#130-090-757, Miltenyi Biotech, Auburn, CA) following manufacturer's instructions. Single cell suspensions from the thymus (1×10^7 cells) of three-week-old ducks were enriched for lymphocytes by density centrifugation and incubated with 20 μg of PE-conjugated anti-chicken CD25 for 45 min and 1:200 dilution of unlabelled mouse IgG in 1 ml of PBS buffer supplemented with 0.5% BSA and 2 mM EDTA (buffer). The unbound antibodies were removed by centrifugation and incubated with 10 μl of anti-PE multisort PE beads in 100 μl of buffer for 15 min. The unbound beads were removed by centrifugation, and $CD25^+$ cells were collected by positive selection in an MS column (Miltenyi Biotech, Auburn, CA) following manufacturer's instructions. The multisort beads were removed using the multisort release reagent (Miltenyi Biotech, Auburn, CA) following manufacturer's instructions. The $CD25^+$ cells were incubated with 10 μg of APC-conjugated anti-duck CD4 for 10 min in 100 μl buffer. The unbound antibodies were removed by centrifugation and incubated with 10 μl of anti-APC conjugated