Clinical Immunology (2013) 148, 162–176

available at www.sciencedirect.com

Clinical Immunology

www.elsevier.com/locate/yclim

ATRA alters humoral responses associated with amelioration of EAMG symptoms by balancing Tfh/Tfr helper cell profiles

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Received 12 December 2012; accepted with revision 15 May 2013
Available online 24 May 2013

KEYWORDS
All-trans retinoic acid;
Experimental autoimmune myasthenia gravis;
Germinal centers;
Follicular helper T cells;
Follicular regulatory T cells

Abstract All-trans retinoic acid (ATRA) is a vitamin A metabolite with diverse immunomodulatory actions used therapeutically in the treatment of some autoimmune diseases. However, the effects that ATRA may have on diminishing myasthenia gravis (MG) symptoms remain undefined. This study investigated the effect of ATRA on experimental autoimmune myasthenia gravis (EAMG) *in vivo* and *in vitro*. Data presented in this study demonstrated that intraperitoneal injection of ATRA ameliorated EAMG pathology in rats associated with reduced total anti-acetylcholine receptor (AChR) serum IgG levels. We observed that EAMG development was accompanied by an increase in follicular helper T cells (Tfh, defined as $CD4^+CXCR5^+ICOS^{high}$) and a decrease of follicular regulatory T cells (Tfr, defined as $CD4^+Foxp3^+CXCR5^+ICOS^{median}$) and that the Tfh:Tfr ratio was altered following ATRA administration. In addition, ATRA treatment restored the Th1/Th2/Th17/Treg balance. *In vitro*, ATRA inhibited AChR-specific cell proliferation and eliciting apoptosis in these cells without affecting the cell cycle. ATRA also altered the Th distribution in animals presenting with EAMG resulting in a reduction in Th1/Th17/Tfh cells and increasing the number of Th2/Treg/Tfr cell types. These results suggested that ATRA reduced EAMG severity by regulating Th cell profiles thereby providing new insights into the development of novel MG (or related) therapies.

Abbreviations: ATRA, all-trans retinoic acid; RA, retinoic acid; MG, myasthenia gravis; EAMG, experimental autoimmune myasthenia gravis; AChR, acetylcholine receptor; $R\text{-}AChR_{97-116}$, rat AChR α97–116 peptide; GCs, germinal centers; Tfh, follicular helper T cells; Tfr, follicular regulatory T cells; NMJ, neuromuscular junction.

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http://dx.doi.org/10.1016/j.clim.2013.05.009

1. Introduction

Experimental autoimmune myasthenia gravis (EAMG), an animal model for human myasthenia gravis (MG), is a B-cell mediated, T-cell-dependent autoimmune disease [1]. AChR-specific Th cells are important to the developing autoimmune response in both MG and EAMG [2].

Germinal centers (GC) represent specialized B cell microdomains located in the follicles of secondary lymphoid tissues where B cells undergo somatic hypermutation, high affinity maturation, and develop into long lived memory or plasma cells [3]. GCs consist primarily of B cells, follicular dendritic cells (DCs), macrophages, and T cells. Follicular helper cells (Tfh), a newly defined $CD4^+$ T cell population resides in the B cell follicle and are regarded as the major helper T cell population that provides B cell help, particularly during the GC reaction [4,5]. Tfh expresses CXCR5, ICOS, CD40L, PD-1, IL-21, and the transcriptional factor Bcl-6 that are critical effector molecules needed for Tfh development and function [6]. Since Tfh are potent inducers of IgG production [7,8] we screened for the presence Tfh cells (defined as CD4 + CXCR5 + $ICOS^{high}$) [9]. Another pivotal population of GC T cells is the follicular regulatory T cells (Tfr), a recently described regulatory $CD4^+$ T cell subset localized to the GCs, recent studies found that Tfr inhibited Tfh function and limited GC reactions, and the absence of $CXCR5^+$ Tregs resulted in larger GC formation and greater serum titers of Igs [10–12].

The vitamin A metabolite, retinoic acid (RA), influences multiple immune cell lineages and has a wide range of immunological functions through binding to retinoic acid receptors (RAR) and retinoic X receptors (RXR) [13,14]. All-trans retinoic acids (ATRA), which belong to a class of RA, are major RAR ligands that also play important roles in lymphocyte differentiation and function. ATRA can affect the nature of developing Th cell responses since it negatively regulates Th1 inflammation while enhancing Th2 mediated immune responses [15]. ATRA also modulates Th17 and Treg differentiation by favoring the development of Tregs over Th17 cells [16]. In addition, antigen (Ag)-specific IgG1 responses were impaired in vitamin A-deficient mice [17] suggesting that B cell isotype switching and antibody production were also affected by ATRA [18]. Antigen presenting cell (APC) function was also shown to be affected by ATRA in the context of cytokines released following exposure to LPS [19] and ATRA also affected DC maturation and antigen-presenting capacity [20]. Due to the numerous immunoregulatory properties attributed to ATRA a few studies have explored the therapeutic effects of ATRA in the treatment of autoimmune diseases including systemic lupus erythematosus [21], multiple sclerosis [22], and rheumatoid arthritis [23] in mouse models of disease.

This study further investigated ATRA effects on T cell-dependent autoimmune humoral immunity in EAMG as a means of assessing the therapeutic potential of ATRA for use as a novel treatment of MG.

2. Materials and methods

2.1. Animals

Female Lewis rats weighing 160–180 g were purchased from the Vital River Laboratory Animal Co. Ltd. (Beijing, People's Republic of China) and housed at the Harbin Medical University under specific-pathogen-free conditions at 21 ± 2 °C and 45 ± 5% humidity. Animal handling and experimental procedures were performed in accordance with the guidelines of the Care and Use of Laboratory Animals published by the China National Institute of Health. Rats were lightly anesthetized prior to immunization and ATRA treatments, and sacrificed after deeply anesthetizing.

2.2. Reagents

The synthetic peptide corresponding to the α97–116 region (DGDFAIVKFTKVLLDYTGHI) of the rat AChRα subunit ($R\text{-}AChR_{97–116}$) was synthesized by AC Scientific, Inc. (Xian, China) as described previously [24] and HPLC analysis demonstrated that it was 95% pure. Incomplete Freund's adjuvant (IFA), ATRA, DMSO rabbit-anti-rat neurofilament 200 (NF-200) and anti-rabbit FITC were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Mycobacterium tuberculosis* strain H37RA was purchased from Difco (Detroit, MI, USA). FITC-conjugated anti-rat-CD4, PE-conjugated anti-rat-Foxp3, PE-cy7-conjugated anti-mouse/rat-ICOS, APC-conjugated anti-rat-IL-17, and APC-conjugated anti-rat-Foxp3 were purchased from eBioscience (San Diego, USA). Percp-conjugated anti-rat-CD25, PE-conjugated anti-rat-IFN-γ, and PE-conjugated anti-rat-IL-4 were purchased from BD Biosciences (San Jose, CA, USA), goat-anti rat CXCR5 was purchased from Santa Cruz Biologicals (Santa Cruz, CA, USA), anti-goat-PE was purchased from Abcam (Cambridge, MA, USA), peanut agglutinin (PNA)-biotin was purchased from Vector Laboratories (Burlingame, CA, USA), streptavidin-HRP was purchased from Beyotime Institute of Biotechnology (China), and tetramethylrhodamine-labeled α-bungarotoxin (α-BTX) was purchased from Invitrogen (Carlsbad, CA, USA). ELISA kits were purchased from eBioscience and the Cell Counting Kit-8 kit (CCK-8) was purchased from Dojindo Molecular Technologies (Shanghai, China).

2.3. Elicitation of EAMG

Rats were randomly divided into 3 groups of 6 rats/group. Rats in EAMG and ATRA-treated groups were immunized subcutaneously at the base of tail with the $R\text{-}AChR_{97–116}$ peptide (50 μg/rat) emulsified in IFA combined with 1 mg of *M. tuberculosis* in a total volume of 200 μl (day 0) followed by a boost on day 30 with the same dose of $R\text{-}AChR_{97–116}$ in IFA. The adjuvant control group was injected with a complete Freund's adjuvant (CFA) containing IFA, *M. tuberculosis* and phosphate-buffered saline (PBS) instead of $R\text{-}AChR_{97–116}$.

On day one post immunization rats in the ATRA treatment group were injected intraperitoneally (i.p.) with a total of 0.5 mg ATRA (diluted in 0.2 ml corn oil) 3 times every other day. Rats in the adjuvant and EAMG groups were injected with the same volume of corn oil. All rats were weighed and monitored for clinical scores every other day until sacrificed.

For adoptive transfer induction, lymph node cells from EAMG rats 37 days post primary immunization were cultured at a density of 2×10^6/ml in the presence of 10 μg/ml of $R\text{-}AChR_{97–116}$. Cells were split and groups treated with ATRA (25 μM) or the same volume of DMSO. After 5–7 days