Regulatory T cells require renal antigen recognition through the TCR to protect against injury in nephritis

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Abstract: Regulatory T cells (Treg) are important for maintaining immune homeostasis. Adoptive transfer of Tregs is protective in renal disease models in both immunocompetent and immunodeficient mice. However the involvement of TCR recognition of renal antigens remains to be clarified. To address this question, we made use of Tregs from the DO11.10 mouse (a TCR transgenic (Tg) mouse), that recognise the non-murine antigen Ovalbumin (OVA) and therefore are not activated by renal antigens. DO11.10 Tregs were assessed functionally in vitro and demonstrated equivalent suppression to WT BALB/c Tregs. Adriamycin Nephropathy (AN) was induced in mice which had been transfused with CD4+CD25+ Tregs isolated from DO11.10 or BALB/c mice. To eliminate the memory/activation state as a cause of differences in activity, the protective capacity of DO11.10 Tregs pre-activated with OVA in vivo was assessed. Transfer of WT BALB/c Tregs significantly attenuated the development of AN with less glomerulosclerosis, tubular atrophy and macrophage infiltration as compared to AN mice without Treg transfer. However, mice receiving either naïve or pre-activated DO11.10 Tregs were not protected from AN. The lack of protection by DO11.10 Tregs was not due to failure to traffic to the affected kidney. These results suggest that antigen recognition in the kidney is important for Treg protection against injury.

Keywords: DO11.10 Tregs, antigen specificity, Adriamycin Nephropathy

Introduction

Tregs play an important role in maintaining immune homeostasis and defects in Treg cell number or function are associated with various autoimmune diseases, including myasthenia gravis, multiple sclerosis, and polyglandular syndrome [1-3]. Different subsets of Tregs have been identified, among which CD4+CD25+ Tregs are the best characterised [4, 5]. The suppressive functions of CD4+CD25+ Tregs are either cell contact-dependent [6] or mediated by secretion of immunosuppressive cytokines such as IL-10 and TGF-β [7, 8]. Other mechanisms involve expression of costimulatory molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) [9], and enzymes like CD39 [10, 11]. They appear to act on a wide variety of cells in both the cognate and innate immune system. Natural CD4+CD25+ Tregs are generated in the thymus via recognition of self-antigens [12] and expression of various homing receptors such as CCR4, CCR5, CCR7, CD62L and CD103 allow Tregs to traffic to the sites of inflammation and exert their suppressive effects [13, 14]. Furthermore, it has been shown that peripheral shaping of TCR repertoire plays an important role in Treg function [15-18].

Antigen-specific Tregs appear to be more potent than polyclonal Tregs in suppressing autoimmune diabetes, graft-versus-host disease (GVHD) and inducing transplantation tolerance [19-24], however the role of antigen recognition in the action of antigen-specific Tregs is not fully defined in nephritis. In vitro studies have shown that Treg activation requires T cell receptor (TCR) engagement with specific antigen, but activated Tregs function in a bystander mode [25]. We therefore compared the efficacy of
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**Materials and methods**

**Mice**

Breeding pairs of DO11.10 mice were kindly provided by Prof Stephen R Holdsworth from Monash University. 6-8 week old male BALB/c mice were purchased from the Animal Resources Centre in Perth, Australia. All animals were maintained under standard sterile conditions in the Department of Animal Care at Westmead Hospital. Experiments were carried out in accordance with protocols approved by the Animal Ethics Committee of Sydney West Area Health Service.

**Antibodies**

Antibodies used for immunohistochemistry included: rat anti-mouse F4/80 (Serotec, Oxford, UK), rat anti-mouse/rat Foxp3 (eBioscience, CA) and biotinylated goat anti-rat immunoglobulin (Zymed Laboratories, San Francisco, CA). APC-rat anti-mouse CD4 (BD Pharmingen™, CA), FITC-rat anti-mouse KJ1-26 (Biolegend, CA), PE-Cy5-rat anti-mouse/rat Foxp3 (eBioscience, CA) and PE-rat anti-mouse CD25 (BD Pharmingen™, CA) were used for flow cytometry stainings. Anti-mouse CD3 antibody used for *in vitro* suppression assay was from BD Pharmingen™, CA.

**Isolation of CD4^+CD25^+ Tregs**

Mouse spleen was harvested from BALB/c or DO11.10 mice and passed through a 40 µm cell strainer to obtain a single-cell suspension, which was followed by red blood cell lysis. CD4^+CD25^+ Tregs were isolated using a mouse CD4^+CD25^+ regulatory T cell isolation kit according to manufacturer’s manual (Miltenyi Biotec, GmbH, Germany). Briefly, non-CD4^+ T cells were depleted using biotinylated antibody cocktail and anti-biotin microbeads. CD4^+CD25^+ T cells were separated from CD4^+CD25^- T cells by positive selection using PE anti-CD25 mAb and anti-PE microbeads.

**Figure 1.** DO11.10 Tregs induced by OVA injection. Flow cytometry analysis of DO11.10 Tregs (KJ1-26^-) in the spleen was conducted after single i.v. injection of OVA. BALB/c mice were adoptively transferred with 1.5 million DO11.10 Tregs (day 0), followed by i.v. injection of 5 µg OVA (day 1). Spleens of transferred mice were harvested on day 4 and day 9 and analysed by flow cytometry.
Adoptive transfer of CD4+CD25+Tregs and induction of AN

After cell isolation, ~1.5 million CD4+CD25+Tregs were injected into the tail vein of mice. Eight days after the adoptive transfer or OVA injection, AN was induced by tail vein injection of Adriamycin (9.8 mg/kg body weight). Mice were divided into five groups: (1) BALB/c Treg-AN group (n=6); (2) naïve DO11.10 Treg-AN group (n=7); (3) activated DO11.10 Treg-AN group (mice injected with 5 µg of OVA one day after adoptive transfer and 8 days prior AN induction) (n=7); (4) normal group (n=4); (5) AN group (n=5).

Renal function

Blood and urine samples were collected every two weeks after the induction of AN. Urine protein concentration was measured using colorimetric assay (Bio-Rad, Hercules, CA) based on the method of Bradford. Urine creatinine and serum creatinine were analysed using an automated chemistry analyser VITROS (Ortho Clinical Diagnostics, Johnson & Johnson) by staff at the Institute of Clinical Pathology and Medical Research at Westmead Hospital.

Renal histology and immunohistochemistry

Coronal sections of the kidney were fixed in 10% neutral-buffered formalin and embedded in paraffin. 4 µm sections of paraffin block were stained with periodic acid-Schiff’s (PAS) reagent and counterstained with haematoxylin. Renal histopathology was graded as previously described [29]. Immunohistochemical staining was performed to determine macrophage infiltration in the kidney. Sections were incubated with primary antibody (16 h, 4°C) followed by secondary antibody incubation (30 min, RT) as previously described previously [33, 34].

Flow cytometry analysis

Single cell suspensions were directly stained with fluorochrome-conjugated rat anti-mouse antibody to cell surface antigens followed by permeabilization and intracellular staining of Foxp3. All samples were analysed on LSR II flow cytometer (Becton Dickinson, Mountain View, CA). Flowjo software was used for analysis (Tree Star, Inc. Ashland, Oregon).

In vitro proliferation assay

Cells were cultured in triplicate in round-bottomed 96-well plates in RPMI1640 (Lonza, Basel, Switzerland) supplemented with 10% FCS, 10 mM Hapes, 50 u/ml Penicillin/50 µg/ml streptomycin, 50 µM 2-ME and 2 mM L-Glutamine. BALB/c CD4+CD25+ indicator T cells were stimulated with plate-bound anti-mouse CD3 antibody (clone number: 145-2C11) (plates pre-coated with 100 µl of 1 µg/ml anti-mouse CD3 antibody, overnight, 4°C) in the presence of irradiated BALB/c antigen-presenting cells (APC) from splenocytes. DO11.10
CD4+CD25+ indicator T cells were stimulated with irradiated BALB/c APC pulsed with 10 µg/ml OVA peptide (2 h, 37°C). Coculture consisted of 5x10^4 indicator T cells, 7.5x10^4 APC and Tregs at 3 different doses (5x10^4, 2.5x10^4 and 1.25x10^4). After 3 days of culture, proliferation was determined by overnight (18 h) incorporation of [3H] thymidine (1 µCi per well). Cells were harvested onto glass fiber filters (PerkinElmer, Massachusetts) and the radioactivity incorpo-
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Real-time PCR

Total RNA was extracted using RNeasy Mini kit (QIAGEN, Hilden, Germany) and reverse transcription was performed using SuperScript™ III First-Strand Synthesis system (Invitrogen, Carlsbad, CA). cDNA was subjected to quantitative PCR analysis using Taqman® Gene Expression Assays specific for the genes of interest (Applied Biosystem, Australia). PCR reaction mix was subjected to Rotor-Gene 3000 thermal cycler (Corbett Life Science) for acquisition and analysis.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) for multiple comparisons. For comparison between two groups, student t-test was performed. Results are expressed as the group mean ± SD. Differences were considered significant at P<0.05. Statistical analysis was performed using Prism 5 (GraphPad Software).

Results

In vivo OVA challenge induced the proliferation of a KJ1-26^+Foxp3^+ Treg population in the spleen

Activation of antigen-specific Tregs requires TCR engagement with specific antigen [25]. Previous studies have shown that single intravenous (i.v.) injection of OVA could induce the proliferation of peripheral OVA specific Tregs [35]. We adoptively transferred 1.5 million Tregs from DO11.10 mice into BALB/c mice, followed by i.v. injection of 5 µg of OVA the following day. Anti-KJ1-26 antibody specifically binds the TCR expressed on DO11.10 mice, which allows differentiation between the adoptively transferred DO11.10 and the endogenous Treg population. Splenic KJ1-26^+Foxp3^+ Tregs were assessed by flow cytometry at day 3 and day 8 post OVA injection. As shown in Figure 1, KJ1-26^+Foxp3^+ Tregs proliferated in the presence of OVA, whereas in the absence of OVA, a reduction of this population was observed.
WT Tregs were more potent than activated or naïve DO11 Tregs

Our previous studies have shown that CD4+CD25+ Tregs from normal BALB/c mice protect against AN [28, 29], however the involvement of TCR recognition of renal antigen in Treg’s protective effects is not known. We addressed this question with the use of DO11.10 mice, TCR transgenic mice whose T cells recognise a non-renal antigen OVA. Tregs have been shown to function in a non-antigen-specific way after the initial activation with specific antigen [25]. We compared the effects of adoptively transferred BALB/c Tregs to DO11.10 Tregs in AN. To activate adoptively transferred DO11.10 Tregs, OVA was injected one day after adoptive transfer (activated DO11.10 Treg-AN) and 9 days prior to ADR administration and for comparison purposes, a group of mice was adoptively transferred with DO11.10 Tregs without OVA injection (naïve DO11.10 Treg-AN). Urine protein/creatinine and serum creatinine were measured 4 weeks after induction of AN. WT Tregs provided greater protection that activated or naïve DO.11 Tregs (Figure 2).

Figure 5. Foxp3+ Tregs in the spleen. Immunohistochemistry staining of Foxp3+ Tregs were performed on spleen sections collected at week 4 post AN. Representative pictures of the staining were shown (magnification 400x).
WT Tregs but not activated DO11.10 Tregs ameliorated renal structural injury

Renal injury was assessed histologically by evaluating glomerulosclerosis and tubular atrophy (Figure 3A). WT BALB/c Tregs significantly reduced renal injury whereas activated and naive DO11.10 Tregs did not ameliorate either glomerulosclerosis or tubular atrophy, when compared to AN group (Figure 3B).

Renal macrophage infiltration correlated with disease severity

Immune cell infiltration was assessed by immunohistochemical staining of kidney sections with antibodies specific for macrophages at week 4 post AN induction. As shown in Figure 4, the BALB/c Treg-AN group had less macrophage cell infiltration than the AN group, whereas naïve or activated DO11.10 Treg-AN groups were equivalent to the AN group.

Foxp3 positive cells were detected in the spleen and kidneys of both naïve and activated DO11.10 Treg-AN mice

In order to understand why WT Tregs but not naïve or activated DO11.10 Tregs ameliorated AN, Foxp3+ T cells in the spleen and kidney sections were assessed by immunohistochemical staining. Foxp3+ cells were found in spleen sections of all the groups and no significant difference was observed among the groups (Figure 5). KJ126 staining for DO11 T cells was unsuccessful in spleen and so it was not possible to distinguish transferred Tregs from native Tregs.

We further examined expression of Foxp3 in kidney at week 4 post AN by semiquantitative real-time PCR. Foxp3 mRNA level was up-regulated in all diseased groups (Figure 6).

In vitro assessment of WT and DO.11 Treg suppressor function

Our in vivo data showed that neither activated or naïve DO11.10 Tregs protected mice against renal injury in AN, which suggested that recognition of organ-specific antigens was required
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for in vivo function of Tregs. Functional assessment of the WT and DO.11 Tregs was assessed in vitro using a standard suppression assay. Naïve DO11.10 Tregs suppressed the proliferation of BALB/c responders (Figure 7A), suggesting differing mechanisms to those with in vivo suppression. With prior activation, DO11.10 Tregs suppressed proliferation of responders to a greater degree than WT BALB/c Tregs, consistent with previous observations [25] (Figure 7B).

Discussion

Tregs are a subset of T cells generated in the thymus providing suppression of autoimmune activity in the periphery [4, 12]. They have been shown to go to specific organs including bowel and kidney and to demonstrate antigen specific expansion in resident tissues [30, 36]. However the role of antigen specificity has not been addressed in Tregs established protection against renal disease.

Treg function in renal and other immune mediated disease has been shown to be affected by depletion of Tregs leading to worsening of disease, while therapeutic delivery of natural or transduced Tregs can protect against renal injury [29]. Tregs can protect against renal injury in the absence of other cognate immune cells in renal disease in SCID mice suggesting the mechanisms of protection involve direct effects of Tregs on the innate immune system and the injured organ itself [28]. In addition to cytokines and costimulatory mechanisms, Tregs utilise a number of other strategies including the removal of dangerous extracellular purines [10, 11].

Both antigen specificity and activation status have previously been identified as key factors determining the capacity of non-renal Tregs to protect against disease [15, 17]. Tregs have been shown to develop particular effector phenotypes following activation. These have included increased capacity to traffic to inflamed tissues involving a variety of chemokine and chemokine receptors [37]. Recently antigens involved in autoimmune renal disease have been defined including PLA2R and CD10 suggesting that specific antigens may drive autoimmune renal disease and that potentially Treg protection against autoimmune renal disease may involve antigen recognition [38-40]. The D011 TCR transgenic on the same BALB/c background required for AN and recognising an exogenous antigen allows study of the requirement for renal antigen recognition in the protection against renal disease [26].

Our studies show that while DO.11 Tregs in vitro, particularly after activation, have strong suppressor activity, they do not protect against renal injury induced by Adriamycin to the same extent as WT Tregs. This suggests that renal antigen recognition is required for Treg protection in AN. This supports the use of antigen specific Tregs as cell therapy in the protection against renal disease.

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Disclosure of conflict of interest

None.

Abbreviations

AN, Adriamycin Nephropathy; APC, Antigen-Presenting Cells; CTLA-4, cytotoxic T-Lymphocyte Antigen 4; CKD, Chronic Kidney Disease; CPM, Counts Per Minute; ESRD, End-Stage Renal Disease; GVHD, Graft-Versus-Host Disease; OVA, Ovalbumin; PAS, Periodic Acid-Schiff’s; Treg, Regulatory T cells; TCR, T Cell Receptor.

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