Molecular Immunology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Molecular Immunology

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

NFATc2 (NFAT1) assists BCR-mediated anergy in anti-insulin B cells

Rachel H. Bonami[a], William T. Wolfle[a], James W. Thomas[a,b,*], Peggy L. Kendall[b,c]

[a] Division of Rheumatology and Immunology, Department of Medicine, Vanderbilt University, MCN T3113, 21st Avenue S., Nashville, TN 37232, USA
[b] Department of Pathology, Microbiology, and Immunology, Vanderbilt University, MCN T3113, 21st Avenue S., Nashville, TN 37232, USA
[c] Division of Allergy, Pulmonary, and Critical Care, Department of Medicine, Vanderbilt University, MCN T3113, 21st Avenue S., Nashville, TN 37232, USA

ARTICLE INFO

Article history:
Received 20 September 2013
Received in revised form
16 December 2013
Accepted 6 January 2014
Available online xxx

Keywords:
B cell
NFAT
Autoimmunity
Tolerance
Insulin
Signaling

ABSTRACT

NFAT transcription factors play critical roles in both the activation and repression of T and B lymphocyte responses. To understand the role of NFATc2 (NFAT1) in the maintenance of tolerance for anti-insulin B cells, functionally inactive NFATc2 (NFATc2$^{-/-}$) was introduced into C57BL/6 mice that harbor anergic anti-insulin 125Tg B cells. The production and peripheral maturation of anti-insulin B cells into follicular and marginal zone subsets was not altered by the absence of functional NFATc2. Surface B cell receptor expression levels, important for tonic signaling and altered by anergy, were not altered in any spleen B cell subset. The levels of anti-insulin antibodies were not different in 125Tg/B6/NFATc2$^{-/-}$ mice and the anti-insulin response remained silenced following T cell dependent immunization. However, studies addressing *in vitro* proliferation reveal the anergic state of 125Tg B cells is relieved in 125Tg/B6/NFATc2$^{-/-}$ B cells in response to BCR stimulation. In contrast, anergy is not released in 125Tg/B6/NFATc2$^{-/-}$ B cells following stimulation with anti-CD40. The relief of anergy to BCR stimulation in 125Tg/B6/NFATc2$^{-/-}$ B cells is associated with increased transcription of both NFATc1 and NFATc3 while expression of these NFATs does not change in anti-IgM stimulated 125Tg/B6/NFATc2$^{+/+}$ B cells. The data suggest that NFATc2 plays a subtle and selective role in maintaining anergy for BCR stimulation by repressing the transcription of other NFAT family members.

1. Introduction

Insulin is a 5800 Da protein that is synthesized, stored, and secreted by the pancreatic islets of all mammals. Administration of the hormone is life saving for individuals with type 1, or insulin dependent, diabetes mellitus (T1D), and is also used for metabolic control in many patients with type 2 diabetes. Although insulin was once considered too small to induce specific immunity, a large body of clinical and experimental data clearly demonstrates that immune responses to insulin arise in a variety of circumstances. Antibodies (Ab) to insulin are almost universal in treated patients and insulin autoantibodies are recognized as part of the natural autoantibody repertoire (Coutinho et al., 1995; van Haeften, 1989). In contrast to common insulin Ab that rarely cause symptoms, dramatic insulin resistance occurs as part of the insulin autoimmune syndrome in which high titers of insulin Ab require massive dosages of insulin to maintain metabolic control. The insulin autoimmune syndrome may accompany an underlying autoimmune disorder such as lupus erythematosus or it may occur as a primary immunological disorder (Blackshear et al., 1983; Lupsa et al., 2009). The most common autoimmune disorder associated with insulin autoantibodies is type 1 diabetes (T1D). Autoantibodies to insulin arise asymptomatically during the prodrome of T1D and are biomarkers of the autoimmune process that destroys pancreatic beta cells and causes diabetes (Palmer et al., 1983; Eisenbarth et al., 2002). Although Ab are not considered primary mediators of beta cell destruction, autoantibodies to insulin and other beta cell autoantigens are critically important tools for detection and early intervention in T1D. The clinical features of insulin immunity and autoimmunity indicate that the insulin molecule challenges the mechanisms that maintain tolerance for self-proteins.

To better understand how tolerance is maintained for anti-insulin B lymphocytes, our laboratory generated mice that harbor VH or VL genes from anti-insulin mAb125 as BCR (IgM) transgenes. Mab125 was obtained from the primary immune response of a non-autoimmune BALB/c mouse and binds rodent insulin with an affinity of 1×10^{-7} M. In contrast to transgenic models that employ high affinity BCRs, this model assesses the fate and function of B

Abbreviations: Ab, antibody; BCR, B cell receptor; FBS, fetal bovine serum; FO, follicular; HBSS, Hanks buffered salt solution; HEL, hen egg lysozyme; MFI, mean fluorescence intensity; MZ, marginal zone; NOD, nonobese diabetic mouse; T1, transitional 1; T1D, type 1 diabetes; TD, T cell-dependent.

* Corresponding author at: Division of Rheumatology and Immunology, Department of Medicine, Vanderbilt University, MCN T3113, 21st Avenue S., Nashville, TN 37232, USA. Tel.: +1 615 322 4746; fax: +1 615 322 6248.
E-mail address: james.w.thomas@vanderbilt.edu (J.W. Thomas).

0161-5890/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.molimm.2014.01.003

cells whose BCRs represent an early or primary immune response. Mice that carry both VH125 and Vκ125 transgenes (125Tg) have a large population of insulin-binding B cells (Rojas et al., 2001). Studies in this model demonstrate that a majority of BCRs on anti-insulin B cells in the periphery are occupied by endogenous insulin, and the initial encounter with insulin has been tracked to early B cell development in the bone marrow parenchyma (Henry-Bonami et al., 2013). In contrast to high-affinity models of B cell tolerance, insulin-binding B cells are not developmentally arrested; they enter mature follicular (FO) and marginal zone (MZ) compartments, with an increase in MZ B cells. Although anti-insulin B cells reside in mature subsets, their ability to produce Ab following T cell-dependent immunization is silenced (Rojas et al., 2001). Similarly, *in vitro* studies on freshly isolated anti-insulin B cells demonstrate impaired lymphocyte proliferation following stimulation through the BCR, TLR4 and CD40 (Acevedo-Suarez et al., 2005). This form of "split" tolerance, in which functional silencing and anergy are separated from alterations in B cell development, is similarly maintained in both non-autoimmune C57BL/6 and autoimmune nonobese diabetic (NOD) mice (Acevedo-Suarez et al., 2005).

The mechanisms that maintain tolerance for anti-insulin B cells also differ from the high-affinity models of B cell tolerance. Anti-insulin B cells do not demonstrate elevated basal levels of intracellular calcium present in the hen egg lysozyme (HEL)/anti-HEL BCR transgenic model, and they do not have impaired tyrosine kinase substrate phosphorylation following BCR stimulation with anti-IgM (Acevedo-Suarez et al., 2006). However, intracellular levels of inositol 1,4,5-triphosphate are increased, and NFATc1 levels are reduced in 125Tg/B6 B cells. In contrast to antigen-naïve B cells, anti-insulin B cells from the spleen are unable to signal calcium transients in response to insulin (Henry et al., 2009; Kendall et al., 2013). Further, bone marrow cultures that investigate the encounter of naïve immature anti-insulin B cells with insulin show the induction of anergy is associated with reduced intracellular mobilization of calcium (Henry et al., 2009). Immature B cell proliferation to anti-CD40 is blunted by insulin antigen exposure or calcineurin inhibition, also indicating a possible role for NFAT in maintaining anergy (Henry et al., 2009). Together, these data suggest that low amplitude, calcium-mediated signals play a key role in the induction and maintenance of tolerance for anti-insulin B cells.

Low amplitude calcium signaling is recognized to activate calcineurin phosphatase, which dephosphorylates NFATs and allows nuclear entry of the cytoplasmic transcription factor without perturbation of other pathways (Dolmetsch et al., 1997). NFATc2 (also known as NFAT1) is recognized to play a key role in regulating tolerance in T cells and is necessary for induction of anergy with anti-CD3 or ionomycin *in vitro* (Macian et al., 2002). Using bone marrow chimeras in the high-affinity HEL/anti-HEL model, NFATc2 was found to have a role in regulating B cell tolerance (Barrington et al., 2006). In that study, B cell anergy was relieved in NFATc2$^{-/-}$ mice as evidenced by increased amounts of circulating autoantibody, increased numbers of mature B cells and increased responses to allo-T cell help (Barrington et al., 2006). However, a role for NFATs in inducing B cell tolerance in the same model has been questioned by the observation that features of tolerance (reduced surface IgM and autoantibody production) are not changed in the absence of calcineurin B (Winslow et al., 2006).

The contributions of NFATc2 to tolerance may differ depending on the status of signals that maintain tolerance for different autoreactive B cells. To determine how NFATc2 contributes to tolerance maintained by low amplitude calcium signaling, anti-insulin BCR transgenic mice (125Tg) were intercrossed with mice in which the Rel homology (DNA binding) domain of NFATc2 is inactivated (termed NFATc2$^{-/-}$ mice) (Hodge et al., 1996; Barrington et al., 2006; Xanthoudakis et al., 1996). Studies in these mice demonstrate that NFATc2 does not regulate the developmental fate of anti-insulin B cells that enter the mature repertoire and its presence is not required to maintain anergy to anti-CD40 stimulation *in vitro*, or for functional silencing of T cell-dependent responses in 125Tg/B6 mice. However, NFATc2 contributes to the maintenance of anergy via signaling through anti-insulin BCRs. Anti-insulin 125Tg/B6 B cells lacking functional NFATc2 generate near normal proliferative responses to BCR engagement with anti-IgM. This relieved anergy in 125Tg/B6/NFATc2$^{-/-}$ B cells is associated with heightened transcription of NFATc1 and NFATc3 following anti-IgM stimulation. These studies support a role for NFATc2 in maintaining tolerance for BCR signaling pathways by its regulation of expression of other NFATs.

2. Materials and methods

2.1. Animals

All mice were backcrossed at least 20 generations onto the C57BL/6 (B6) background. 125Tg mice harbor non-targeted anti-insulin heavy and light chain transgenes that produce an anti-insulin BCR (Rojas et al., 2001). NFATc2$^{-/-}$ mice were a kind gift from Anjana Rao (Harvard University, Boston, MA) (Xanthoudakis et al., 1996). 125Tg/B6 were intercrossed with NFATc2$^{-/-}$ to generate 125Tg/B6/NFATc2$^{-/-}$ mice. 7–28 week old male and female mice were used in all experiments. All studies were approved by the AAALAC-certified Institutional Animal Care and Use Committee of Vanderbilt University and mice were housed in specific pathogen free conditions.

2.2. Immunization and antibody detection

125Tg/B6 and B6 NFATc2$^{+/+}$ or NFATc2$^{-/-}$ mice were immunized with 50 μg beef insulin in CFA subcutaneously at the base of the tail. Sera were obtained pre-immunization and 2 weeks post-immunization from each mouse. ELISA was used to detect insulin-specific antibodies, as has been previously described (Kendall et al., 2004). Briefly, the O.D. 405 nm of wells incubated with >100-fold excess insulin in solution was subtracted from the O.D. 405 nm of uninhibited parallel wells to calculate insulin-specific binding. Goat anti-mouse IgM-alkaline phosphatase or goat anti-mouse IgG-alkaline phosphatase (Southern Biotech) was used to detect serum antibody bound to the insulin-coated ELISA plate.

2.3. Cell isolation and flow cytometry

Splenocytes were macerated in Hanks balanced salt solution (HBSS) + 10% fetal bovine serum (FBS) (HyClone) and red blood cells were lysed using Tris-buffered NH_4Cl. Lavage with HBSS was used to isolate peritoneal cavity cells. Ab reagents reactive with B220 (6B2), IgM^a (DS-1), IgM^b (AF6-78), CD5 (53–7.3), CD21 (7G6), CD23 (B3B4), or 7-aminoactinomycin D (BD Biosciences) were used for flow cytometry. Human insulin (Sigma–Aldrich) was biotinylated at pH 8 in bicine buffer using biotin N-hydroxysuccinimide ester (Sigma–Aldrich) and detected with fluorochrome-labeled streptavidin (BD Biosciences).

2.4. B lymphocyte proliferation

Splenocytes were isolated as above and magnetic activated cell sorting (MACS) was used to deplete CD43-expressing cells using LS columns (Miltenyi). Sorted B cells were plated at 2×10^{-5} cells/well in complete RPMI 1640 media (Invitrogen Life Technologies) containing 10% FBS, glutamine, gentamicin, and 2×10^{-5} M 2-ME (Invitrogen Life Technologies) in 96-well, flat-bottom plates (Corning). Stimuli include anti-IgM F(ab')$_2$ (Jackson ImmunoResearch

Please cite this article in press as: Bonami, R.H., et al., NFATc2 (NFAT1) assists BCR-mediated anergy in anti-insulin B cells. Mol. Immunol. (2014), http://dx.doi.org/10.1016/j.molimm.2014.01.003