Cellular Immunology 257 (2009) 88–96

Contents lists available at ScienceDirect

Cellular Immunology

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

Engagement of transgenic Ly49A inhibits mouse CD4 cell activation by disrupting T cell receptor, but not CD28, signaling

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ARTICLE INFO

Article history:
Received 15 November 2008
Accepted 6 March 2009
Available online 11 April 2009

Keywords:
Signal transduction
Type 1 diabetes
CD4 cells
Inhibitory receptors
Costimulatory receptors

ABSTRACT

Islet specific CD4 cells expressing inhibitory receptors may be a useful therapeutic tool for treating type 1 diabetes (T1D). Engagement of transgenic Ly49A inhibits CD4 cell activation and delays onset of T1D in mice. However, in vitro studies suggest the inhibitory effect of Ly49A is incomplete. Here we report that following simultaneous TCR and Ly49A engagement, phosphorylation of Zap70, Erk1/2 and c-Jun were significantly diminished. Kinetic studies indicated that Ly49A did not simply delay activation but had a long-lasting effect. In contrast, when only costimulatory signals were provided through CD28, Ly49A engagement did not block p38 MapK or Akt phosphorylation. Likewise, expression of the downstream targets *Bcl-xl* and *Baff* were unaffected. Together these data suggest that engagement of Ly49A selectively inhibits signals downstream of the TCR but spares those unique to CD28. These results suggest that when considering its use as an immunotherapy, the potency of inhibitory receptors such as Ly49A may be further improved by pairing them with costimulatory blockade.

1. Introduction

Activated islet-specific T cells are central to the destructive autoimmune response observed in T1D [1,2]. Creation and use of inert T cell populations able to usurp inflammatory mediators may yield therapeutic benefits for T cell mediated autoimmunity. One way to quell T cell activation is by ectopic expression of inhibitory receptors such as Ly49A. Expression of Ly49A on islet-specific CD4 cells creates an inert population of T cells that are capable of trafficking to antigen rich sites but, unlike pathogenic islet-specific T cells, fail to elicit T1D in mice co-expressing Ly49A ligand [3]. Although Ly49 receptors are not found in humans, a subset of killer cell immunoglobulin-like receptors are present, serve a similar function and might be used in place of Ly49A in humans [4–6].

T cells and NK cells share common inhibitory and activation signaling elements making NK cell inhibitory receptors an attractive tool for converting otherwise autoreactive CD4 cells into inert cell populations [7]. As a member of the c-type lectin superfamily of type II transmembrane glycoprotein receptors, Ly49A is a well known homodimer naturally expressed on murine natural killer (NK) cells [4,8,9]. Ly49A inhibits NK cell function following recognition of its ligand, the MHC class I molecule H-2D^d [10–12]. Upon engagement, the immunoreceptor tyrosine-based inhibitory motif (ITIM) found within the cytoplasmic tail of Ly49A becomes phosphorylated, most likely by src family kinases, leading to the recruitment of SHP-1 and possibly SHP-2 phosphatases [13–16]. These phosphatases target intermediate kinases, including Zap70, that arise from parallel activation of other receptor signaling pathways. As a result, NK cell activation and target cell lysis are inhibited. Src family kinases and SHP phosphatases also function in T cells to regulate antigen recognition; therefore the key signaling components are in place to transduce Ly49A-mediated inhibitory signals in Ly49A transgenic CD4 cells.

NK cells also express a diverse array of activating receptors, including CD16 (FcγRIII), CD94/NKG2C, Ly49D and Ly49H, that share characteristics with T cell antigen receptors. Similar to T cell receptors (TCR), NK cell activating receptors often do not possess extensive cytoplasmic domains but rely on closely associated complexes that contain ITAMs to transduce signals. For example, CD16 associates with FcεRIγ (mouse and human) or CD3ζ (human) [17,18]. Likewise, Ly49D, Ly49H and NKG2C associate with DAP12 [19,20]. FcεRIγ, CD3ζ and DAP12 all possess ITAM domains and function in a manner analogous to the ITAM-laden CD3 complex associated with the TCR [21–23]. When NK cell activating receptors are engaged, the associated ITAM-containing signaling complexes become phosphorylated by src family kinases with subsequent recruitment and phosphorylation of spleen tyrosine kinases (syk), such as Zap70 [24]. Syk family kinases activate PI3K and scaffold proteins such as linker for activation of T cells (LAT) or non-T cell activation linker (NTAL) [25–27]. Subsequently, additional signaling cascades are activated, including the Map kinases

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doi:10.1016/j.cellimm.2009.03.003

(MapK). Similarities in NK and T cell activation signals, particularly recruitment and activation of syk family kinases to ITAM-containing activation receptor complexes, suggest that inhibitory receptors such as Ly49A may be useful as a means to artificially inhibit T cell activation.

Although Ly49A expression is best known and studied on NK cells, Ly49A also appears on a subset of other cells including CD8, NKT and γδT cells [28–30]. A number of studies support the notion that the molecular actions of Ly49 inhibitory receptors are similar for both NK and T cells by demonstrating that Ly49A engagement subdues T cell effector functions. Ly49A engagement suppresses anti-CD3 induced CD69 upregulation on Ly49A$^+$ CD8 cells [30] and IL-2 secretion and apoptosis of Ly49$^+$ T cell hybridomas [31]. Transgenic expression of Ly49A on T cells results in suppression of antigen induced proliferation, surface receptor modulation, cytokine production and cytotoxicity in vitro [3,32], as well as anti-viral [33] and anti-tumor [34] responses in vivo. Conversely, antibody blockade of Ly49A and Ly49G enhances in vitro cytotoxicity and cytokine production by sorted Ly49$^+$ T cells [28]. The influence of Ly49A extends to immature T cells, where Ly49A engagement results in developmental defects due to Ly49A mediated changes in selection thresholds [35,36]. Therefore, although Ly49A is not normally expressed on CD4 cells, ample evidence demonstrates that engagement of ectopically expressed Ly49A inhibits CD4 cell activation.

Using a murine adoptive transfer model of type 1 diabetes where islet-specific T cells express Ly49A, previous studies demonstrated that disease onset was prevented due to Ly49A-mediated inhibition of antigen-specific T cell activation [3]. Ly49A engagement inhibited proliferation, effector cytokine secretion and modulation of key surface receptors. While inhibition of T cell activation was impressive in vivo and in vitro following primary exposure to antigen, low level stimulation was observed in vitro upon secondary challenge. The ability of Ly49A T cells to respond, albeit at reduced levels, to repeated antigen challenge may limit the usefulness of this therapeutic approach for chronic autoimmune diseases. Therefore, a greater understanding of how Ly49A inhibits CD4 function may be helpful in optimizing the therapeutic use of inhibitory receptors such as Ly49A. Here we show that Ly49A blocks Zap70, extracellular signal-regulated kinase (Erk) 1/2 and c-Jun activation but leaves unique components of the CD28 signaling pathway intact. These observations will help direct future efforts at optimizing the therapeutic use of inhibitory receptors for treatment of autoimmune diseases such as type 1 diabetes.

2. Materials and methods

2.1. Mice

B10.D2 and B10.HTG mice were purchased from the Jackson Laboratory (Bar Harbor, ME). TCR-SFE single transgenic mice were kindly provided by Dr. H. von Boehmer (Harvard Medical School, Boston, MA) [37]. Ly49A/TCR-SFE double-transgenic mice were generated as described previously and maintained on a B10.HTG background [3]. All mice were housed in specific pathogen-free conditions at the Southern Illinois University School of Medicine in accordance with National Institutes of Health and institutional guidelines.

2.2. In vitro T cell stimulation assays

CD4 cells were purified from spleens and/or lymph nodes obtained from Ly49A/TCR-SFE or TCR-SFE mice as previously described [3]. APCs were purified from spleens isolated from B10.D2 (D^d APC) or B10.HTG (D^b APC) mice and irradiated (2500 rad) as described previously [3]. All cells were cultured at 37 °C with 5% CO_2 in RPMI 1640 (Irvine Scientific) plus 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 25 mM HEPES and 50 μM 2-ME (complete medium). CD4 cells were used immediately or stimulated with 5–10 μg/ml of plate-bound anti-CD3 (145-2C11, PharMingen) plus 1–5 μg/ml anti-CD28 (37.51, PharMingen) in the presence or absence of 10 μg/ml anti-Ly49A (A1, PharMingen) for the indicated time. Anti-CD3 was omitted from some cultures. In some cases, cells were pre-treated with 10 μM U0126 (#9903, Cell Signaling) or 10 μM SB203580 (S8307, Sigma) for 15 min before stimulation. When APCs were used as stimulators, irradiated APCs were pulsed with 5–10 mg/ml SFE peptide (influenza hemagglutinin peptide 110–119; SFERFEIFPK) for 2 h at 37 °C. CD4 cells were then incubated with APCs at a 1:3 ratio for the indicated time. In some cases following primary stimulation, CD4 cells were rested for 5 days in 20 U/ml human rIL-2 (rIL-2, Peprotech). Viable cells were collected via density gradient centrifugation (Lympholyte-M; Cedarlane Laboratories) and restimulated with D^d APC plus SFE peptide for 24 or 48 h.

2.3. Flow cytometry

Cells were incubated with PE-conjugated anti-CD4 and biotinylated anti-Vβ8 plus anti-CD69-FITC (PharMingen). Vβ8-positive cells were visualized with streptavidin–allophycocyanin (BD Biosciences). Multi-color immunofluorescence was analyzed using a FACSCalibur and CellQuest software (BD Biosciences).

2.4. Proliferation assays

CD4 cells (2×10^5 cells/well) were incubated with D^d APC (5×10^5 cells/well) plus 10 μg/ml SFE peptide in complete medium for 48 h. All samples were plated in triplicate. Plates were pulsed with 1 μCi/well [^{3}H]-thymidine (Amersham) during the last 24 h. Samples were harvested and counted on a Beckman LS600SC scintillation counter.

2.5. Immunoprecipitation and Western blot

For immunoprecipitation of Zap70, freshly isolated or activated CD4 cells were washed twice with ice-cold PBS containing phosphatase inhibitors [50 mM sodium fluoride (NaF) and 2 mM sodium orthovanadate (Na_3VO_4)]. Cells were collected in lysis buffer [1× PBS (pH 7.2) plus 1% NP-40, 50 mM NaF, 2 mM Na_3VO_4 and protease inhibitors (10 μg/ml leupeptin, 30 μg/ml aprotinin, 200 μg/ml phenyl methyl sulphonyl fluoride (PMSF) and 10 μg/ml pepstatin A)]. Cell lysates (500 μg) were pre-cleared with 50 μl protein G-sepharose (PGS, Amersham) by constant rocking for 1 h at 4 °C. Pre-cleared lysates were incubated with anti-Zap70 (2 μg) overnight at 4 °C. Antibody–protein complexes were captured with 50 μl pre-washed PGS for 1 h at 4 °C. The beads were then collected and washed four times with 1× PBS/0.5%NP-40 plus phosphatase inhibitors. Immunoprecipitates or cell lysates (for Western blots: without prior immunoprecipitation) were subjected to 10% SDS–PAGE and Western transfer as previously described [38]. Nitrocellulose membranes were blocked with TBST (150 mM NaCl, 20 mM Tris, pH 7.6, 0.05% Tween 20) containing 5% BSA for 1 h at room temperature, and then probed with the following antibodies as indicated: phospho-Tyr (#9411), phospho-Erk1/2 (#9106), phopho-p38 (#9211), phospho-AKT (#4058) or phospho-c-Jun (#9261) (Cell Signaling Technology); ZAP70 (sc-32760), Erk2 (sc-154), p38 (sc-728g) or c-Jun (sc-45) (Santa Cruz Biotechnology); anti-AKT1 (Upstate Biotechnology) or anti-β-actin (A5441) (Sigma). All of antibodies were used at 1:1000, except β-actin was used at 1:10,000. Membranes were incubated with primary antibody for 2 h at room temperature or overnight at 4 °C.