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Solute Carrier 11A1 Is Expressed by Innate Lymphocytes and Augments Their Activation

Jodi F. Hedges, Emily Kimmel, Deann T. Snyder, Maria Jerome, and Mark A. Jutila

Solute carrier 11A1 (SLC11A1) is a divalent ion transporter formerly known as the natural resistance–associated macrophage protein (NRAMP1) and the Bcg/Lsh/Ity locus. SLC11A1 was thought to be exclusively expressed in monocyte/macrophages and to have roles in phagosome maturation and cell activation. We characterized the expression of SLC11A1 in the majority of human and bovine γδ T cells and NK cells and in human CD3⁺CD45RO⁺ T cells. Consistent with a role for iron-dependent inhibition of protein tyrosine phosphatases, SLC11A1⁺ lymphocytes were more prone to activation and retained tyrosine phosphorylation. Transfection of SLC11A1 into a human γδ T cell–like line rendered the cells more prone to activation. Nonadherent splenocytes from wild-type mice expressed significantly greater IFN-γ compared with cells from Sv/129 (SLC11A1⁻/⁻) mice. Our data suggest that SLC11A1 has a heretofore unknown role in activation of a large subset of innate lymphocytes that are critical sources of IFN-γ. SLC11A1⁺ animals have enhanced innate IFN-γ expression in response to Salmonella infection compared with SLC11A1⁻ mice, which include commonly used inbred laboratory mice. Expression of SLC11A1 in innate lymphocytes and its role in augmenting their activation may account for inconsistencies in studies of innate lymphocytes in different animal models. The Journal of Immunology, 2013, 190: 4263–4273.

Solute carrier 11A1 (SLC11A1) (also denoted natural resistance–associated macrophage protein 1 [NRAMP1] and formerly Lsh/Ity/Bcg locus) has a critical role early in bacterial infection (1). SLC11A1 is a highly conserved divalent cation transporter, and the primary substrate is iron (2) and was thought to be expressed exclusively in cells of myeloid lineage (3, 4). SLC11A1 localizes to late endosomes and lysosomes, which fuse with phagosomes only upon activation by IFN-γ (5–7). Some evidence suggests SLC11A1 protects from bacterial infection by exporting iron from phagosomes, thus restricting their availability to internalized bacteria (6) and promoting phagosome maturation (8). More recently, SLC11A1 has been shown to have a critical function in macrophage activation by promoting and sustaining MEK/ERK, JAK/STAT, and NF-κB signaling through iron-dependent inhibition of multiple protein tyrosine phosphatases (PTPs (9)). This activity is also greatly enhanced by IFN-γ (9). Regulation of PTPs is also important in lymphocyte activation (10).

The importance of SLC11A1 function is underscored by a single point mutation (D169, susceptible) that results in a misfolded protein and renders mice susceptible to intracellular bacterial pathogens, such as Mycobacterium spp., Leishmania, and Salmonella. The commonly used inbred laboratory mouse strains C57BL/6 and BALB/c possess the D169 mutation; thus, most studies done in these mice disregard a functional SLC11A1 protein (11). Susceptibilities to Mycobacterium tuberculosis and leprosy in humans (12) and Mycobacterium paratuberculosis in cattle are associated with SLC11A1 polymorphisms (13), suggesting a conserved function. There is strong evidence for increased IFN-γ expression in SLC11A1 wild-type mice in several different disease states (14–16) and during Salmonella infection in particular (17–19). Although the primary early source of this cytokine is innate lymphocytes (20), the precise mechanism of expression of IFN-γ in SLC11A1⁺ mice has not been identified. Increased IFN-γ expression has been attributed to enhanced activation of SLC11A1⁺ monocytes, which produce more IL-12 and other cytokines that in turn stimulate IFN-γ expression in vivo. However, IFN-γ is critical for maximal activation and IL-12 expression by SLC11A1⁺ macrophages (7, 21–23). This raises a question as to the mechanism of early IFN-γ expression in SLC11A1 wild-type mice.

We originally noted expression of multiple transcripts, thought to be restricted to myeloid cells, in bovine γδ T cell subsets (24, 25). A unique myeloid-like response was assigned to human and bovine γδ T cells, because they responded directly to pathogen-associated molecular patterns (26). We sought to characterize the expression and function of SLC11A1 in γδ T cells. Preferential expression of SLC11A1 transcripts in γδ T cells was detected in bovine, human, and mouse γδ T cells. SLC11A1 protein expression was identified in γδ T cells and other cellular sources of IFN-γ: NK and NK-like cells, and memory phenotype (CD3⁺, CD45RO⁺) T cells. We hypothesized that, similar to its critical role in monocytes (9), SLC11A1 enhances activation signaling in γδ T cells. SLC11A1 expression was strongly associated with enhanced activation of innate lymphocytes. Human and bovine lymphocytes lacking SLC11A1 did not express IFN-γ. SLC11A1 promotes monocyte activation by iron-dependent inhibition of PTPs (9), and a similar mechanism for SLC11A1 function in lymphocytes was explored. Chemical blockade of PTPs was used to simulate SLC11A1 function. Similar to chemical blockade of
PTPs, lymphocytes expressing SLC11A1 were both more readily activated and retained tyrosine phosphorylation. Augmenting the iron content in media induced activation of human lymphocytes, consistent with a role for iron in lymphocyte activation. Expression of SLC11A1 in a γδ TCR+ human cell line (MOLT14) rendered cells more prone to activation and IFN-γ expression. Similar to earlier findings (19), nonadherent splenocytes from Sv/129 mice expressed significantly greater IFN-γ compared with those from Sv/129 (SLC11A1+/−) mice in response to supernatant fluids from Salmonella-infected adherent cells from either strain. The expression of SLC11A1 in innate lymphocytes, their enhanced activation, and in particular expression of IFN-γ may provide a novel mechanism for enhanced immunity in SLC11A1+/− mice. Considering the consistent observations of increased innate IFN-γ expression in SLC11A1 wild-type mice in existing literature, our data suggest that innate lymphocyte function may be underestimated in common inbred laboratory mice lacking functional SLC11A1.

Materials and Methods

Cells and reagents

Mouse spleen cells were collected by homogenization from TCRα−/− mice (C57BL/6 background) and from wild-type mice. The SLC11A1-deficient mice on the 129Sv background (breeding pair) were obtained from Dr. P. Groz (McGill University, Quebec, QC, Canada) and bred in-house. Wild-type 129/Sv mice were purchased from Charles River Laboratories (129S2/SvPasCrl). Human and bovine PBMCs were isolated from peripheral blood using Histopaque 1077. Studies involving blood from human subjects and animals were carried out in compliance with the Montana State University Institutional Review Board and Institutional Animal Care and Use Committee, respectively. Each experiment was performed with cells from at least three individuals. All cells were cultured at ~2 × 10^6/mL in Xvivo serum-free medium (Lonza). MOLT14 cells, a γδ T cell–like line that respond similarly to γδ T cell agonists (27), were originally obtained from DSMZ and maintained in complete RPMI 1640 medium with 10% Hyclone FBS. The reagents potassium bisperoxo(1,10-phenanthroline) dimer (HB19; BioLegend), CD14 (clone HIB19; eBioscience), and CD16 (clone 3G8; BD Biosciences) were purchased from BD Biosciences. Mouse L cells (fibroblasts) expressing human E-selectin (ELAM cells), as described previously (31). ELAM cells were collected and suspended at 4 × 10^6 cells/well and incubated for 24 h. The coverslip was removed from wells and washed twice with HBSS and twice with PBS. Cells were fixed with ice-cold 100% methanol for 10 min, dried, and stored at −80°C. Coverslips were equilibrated to 25°C, washed with PBS, permeabilized with 0.25% Triton X-100 in PBS for 10 min, blocked with 10% horse serum (HS) in PBS for 30 min, and stained with primary anti-human SLC11A1 Ab (Abnova clone 2G2) diluted 1:300 in 10% HS in PBS for 1 h. Cells were then washed with PBS and stained with secondary anti-mouse Alexa Fluor 594 (Molecular Probes) at 1:1000 in 10% HS in PBS for 30 min. After an additional wash with PBS, cells were stained with FITC-conjugated GD3.8 for 30 min 1:800 in 10% HS in PBS with 0.25% Triton X-100, washed with PBS, and coverslipped. Fluorescence was assessed on a Nikon 80i Eclipse microscope. Total PBMCs and ELAM-selected bovine γδ T cells were used in Western blotting assays. Total bovine PBMCs and human Mono Mac 6 cells were used as positive controls for SLC11A1 experiment, and ELAMs alone were used as negative control cells. Cells pellets were lysed in 20 μl Mammalian Protein Extraction Reagent (Pierce) with Halt Protease and Phosphatase Inhibitor Cocktail (Pierce) per manufacturer’s suggestions on ice for 30 min. Lysates were centrifuged at 8000 × g for 2 min and 6× Laemmli Sample buffer added to lyse supernatant fluids. Samples were boiled for 5 min and loaded onto a prepared 7.5% SDS-PAGE gel, and proteins were transferred to polyvinylidene difluoride membrane. The blot was blocked in 3% BSA in TBST (for 1 h at room temperature). Primary Ab, anti-SLC11A1, was diluted 1:400 in 3% BSA in TBST and left overnight at 4°C while rocking. The blot was washed and then incubated for 1 h at room temperature while rocking with goat anti-mouse IgG-HRP diluted 1:4000 in 3% BSA TBST. The blot was washed again before adding Novex ECL (Invitrogen) and then exposed to radiography film and processed.

Flow cytometric cell analysis

The method for detection of intracellular SLC11A1 by flow cytometry was adapted from Stober et al. (32). For measurement of SLC11A1 and activation markers, human or bovine PBMCs were either resting or stimulated with LPS from Escherichia coli (Sigma-Aldrich), Con A, PMA, ionomycin, or staphylococcal enterotoxin B (SEB; Toxin Technology) at the indicated concentrations and cultured for 24 h. Prior to staining for intracellular IFN-γ, brefeldin A (BFA) (27) was added to ELAM cells. Cells were fixed with 4% paraformaldehyde for 20 min on ice. Cells were washed with PBS with 2% HS (flow buffer) and then permeabilized by incubation in flow buffer with 0.2% saponin from 1 h to overnight. Cells were then stained with the SLC11A1-specific mAb (2G2) or an isotype-matched negative control mAb (2G2) at 1:200 in flow buffer with 0.2% saponin for 1 h at 4°C. 2G2 was developed from a fusion peptide aa 305–351 of human SLC11A1 (which is 96% similar to the same region of bovine SLC11A1), Mouse SLC11A1 has an 80% identity in this region, and the Ab was found not to react with SLC11A1+ mouse cells (from an SLC11A1 wild-type 129/Sv mouse; data not shown). Cells were washed three times with flow buffer with 0.2% saponin and stained with PE- or FITC-labeled anti-mouse IgG, Fab2 secondary Abs (Jackson Immunoresearch Laboratories) for 1 h. Secondary Abs were used alone as negative controls. Cells were washed again twice with flow buffer with 0.2% saponin and once with flow buffer and then incubated with 10% normal mouse sera. Cells were then finally stained with directly labeled Abs to anti-bovine γδ TCR (GD3.8, anti-bovine CD335 (AbD Serotec), or IL2Ra (clone LCCT332A; VMRD) directly conjugated to PE, IFN-γ (AbD Serotec), PE labeled, diluted in flow buffer with 0.2% saponin added after a third flow buffer with 0.2% saponin wash, or directly labeled anti-human CD19 (clone HIB19; eBioscience), CD25 (clone BC96; BioLegend), CD56 (clone MEM-188; BioLegend), CD94 (Immunotech), CD69 (clone FN50; BioLegend), γδ TCR (Immunotech), and IFN-γ (clone 4S.B3; BioLegend). Only cells in the lymphocyte gate, based on forward and side scatter, were analyzed. Each assay was performed with cells from three to five individual donors, and each was repeated on three separate occasions. In addition to isotype-matched Abs and secondary only controls, SLC11A1+ cells were always apparent as internal negative controls. For detection of phosphotyrosine, cells were fixed with 4% PFA at 37°C and permeabilized in ice-cold 100% methanol, washed, and stained with the PE-labeled Phosflow Phospho-tyrosine Ab (BD Biosciences) following the manufacturer’s instructions.

Salamonella enterica serovar Typhimurium infection

A natural calf isolate of Salamonella enterica serovar Typhimurium (S. Typhimurium) or mouse strain SL1344 were cultured at 37°C with rotation for 12 h then subcultured 1:40 for 2 h before use. Human PBMC or Mono Mac 6 cells were collected and suspended at 2 × 10^6 cells/ml in antibiotic-free DMEM and infected with S. Typhimurium at multiplicities of infec-
tion of 0.1–1, and 10 bacterium to cell in suspension for 3 h as described previously (33). Cells were washed twice with fresh DMEM and centrifugation and cultured again in DMEM plus 100 μg/ml gentamicin for 2 h. Cells were washed once again with DMEM and plated at ~2 × 10⁶/ml in DMEM for 18 h. PBLs from human subjects were added to S. Typhimurium–infected Mono Mac 6 cells or cocultured with infected monocyte/macrophages in PBMCs. Infected monocytes in the cultures quickly adhered were larger and contained visible vacuoles. After 18 h, PBLs were assessed by flow cytometry for expression of SLC11A1, CD69, and IFN-γ. Negative controls included treatment with media only or incubation during the infection with 10 μg/ml Salmonella LPS (Sigma-Aldrich) instead of S. Typhimurium. Mouse adherent splenocytes were stimulated with S. Typhimurium in antibiotic-containing medium as described elsewhere (20). Briefly, splenocytes were plated at 2 × 10⁶/ml and adhered to wells overnight. Nonadherent cells were removed, and adherent cells were stimulated with 10⁶ or 10⁷ S. Typhimurium (strain SL1344) and cultured for 8 h. Supernatant fluids were sterile filtered and applied to nonadherent cells in triplicate wells. These cells were cultured 48 h with media only, uninfected supernatant fluids, IL-12 (1–250 ng/ml), or Con A/IL-2 controls, and supernatant fluids were then assessed for IFN-γ expression using a commercial mouse IFN-γ ELISA set (BD OptEIA).

Expression of SLC11A1 in human cells

MOLT 14 cells were maintained at 5 × 10⁶/ml or greater and passaged 24 h prior to electroporation using the Nucleofector system (Lonza). A total of 5 × 10⁶ cells were electroporated using program C-05 with 20 μg plasmid expressing SLC11A1 with a GFP tag (Origene). Cells were rested 24 h, activated, as described above, or using recombinant human IL-1β, IL-12, or IL-18 at 10 ng or 1 μg/ml, and expression of GFP (SLC11A1-GFP) and activation markers CD69, CD25, and IFN-γ were assessed after an additional 24 h. Cell-free supernatant fluids were collected from human PBMCs stimulated with either medium only or LPS for 24 h and used to stimulate SLC11A1-GFP–transfected MOLT14 cells to assess the effects of human cytokines expressed by PBMCs.

Statistical analyses

Statistical significance was calculated using two-way ANOVA, paired one-tailed Student t test, and/or Wilcoxon-matched pairs signed-rank test in Prism 5 (GraphPad), as described in figures and/or figure legends.

Results

SLC11A1 transcript expression in innate lymphocytes

To begin to understand SLC11A1 expression in γδ T cells, we examined SLC11A1 transcripts amplified from purified γδ T cell populations. There was clearly greater SLC11A1 transcript expression in mouse γδ T cells compared with αβ T cells (Fig. 1A). Greater expression of SLC11A1 was also noted in sorted human γδ T cells compared with non-γδ T cells (Fig. 1B). A similar situation was observed in bovine cells upon comparison of sorted γδ T cells to the non-γδ T cell lymphocyte population (data not shown). Thus, SLC11A1 transcripts were detected in γδ T cells to a greater extent than in non-γδ T cell subsets in all three species tested.

Expression of SLC11A1 protein in innate lymphocytes

Expression of SLC11A1 protein was detected using immunoblotting, immunofluorescence, and flow cytometric assays. The mAb specific for human SLC11A1 (clone 2G2) was found to cross-react with bovine SLC11A1. Bovine γδ T cells were sorted using ELAM cells, as described previously (31). Fig. 2A demonstrates an isolated ELAM cell with multiple attached bovine γδ T cells stained with clone 2G2 and GD3.8 (specific for bovine γδ TCR). These data supported expression of SLC11A1 protein by γδ T cells. An SLC11A1-specific band at ~110 kDa was detected by Western immunoblotting in bovine PBMCs, ELAM-selected γδ T cells (Fig. 2B), and human Mono Mac 6 cells (data not shown). To confirm these findings, we adapted an intracellular staining protocol for SLC11A1 (32) to PBLs and counterstained γδ T cells and other lymphocyte subsets. Bovine lymphocytes were found to be 45–71% SLC11A1* (average 63.88 ± 12.76, n = 4). Within

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** SLC11A1 transcripts were preferentially expressed in γδ T cells. (A) Mouse γδ T cells were sorted from αβ-TCR–deficient mouse spleens on a C57BL/6 background, and αβ T cells were sorted from the spleen of a wild-type mouse. Quantitative PCR was performed to detect SLC11A1 transcripts and normalized to the expression of β-actin. Error bars represent SD from three technical replicates. (B) SLC11A1 expression was preferentially detected in enriched human γδ T cells compared with γδ TCR–, CD19+, CD11b+, and αβ T cell–enriched population isolated from peripheral blood of n = 3 donors; Student t test; ***p < 0.001, **p < 0.01.
of SLC11A1 in murine innate lymphocytes. In contrast to the currently held assumption that SLC11A1 is only expressed in macrophages, SLC11A1 was expressed by most innate lymphocyte subsets.

**SLC11A1* innate lymphocytes are more readily activated**

Because SLC11A1 has been identified as a factor that promotes activation of monocyte/macrophages, we compared the activation profiles of SLC11A1* to SLC11A1− lymphocytes. Because γδ T cells directly responded to agonists, such as LPS and LPS-induced cytokines in PBMCs, we tested whether the differential expression of SLC11A1 was associated with this response. Bovine PBMCs were activated ex vivo for 24 h with very low (nanograms per milliliter) concentrations of Con A or LPS, and the effects on induced IL-2 receptor (IL2Rα, CD25) and IFN-γ expression were measured by flow cytometry. Stimulation did not change the number of cells expressing SLC11A1 (data not shown). Although SLC11A1− cells were activated by >100 ng/ml Con A, SLC11A1* bovine lymphocytes were significantly more prone to activation in response to lower concentrations of Con A (Fig. 3A). Of particular note, only SLC11A1* bovine lymphocytes increased expression of CD25 in response to varying concentrations of LPS, and the response was very consistent even at subnanomolar LPS concentrations (Fig. 3B). On the basis of these results, bovine cells from four different calves were stimulated with LPS (100 ng/ml) or vehicle only (media). After wash, T cells were selected by adherence to ELAM-sorted bovine γδ T cells demonstrated staining of SLC11A1 (Alexa 549) on γδ T cells (labeled with FITC). Original magnification ×100 (B) Bovine γδ T cells were selected by adherence to ELAM cells and compared with PBMCs and ELAM cells alone by Western immunoblotting and staining with anti-human SLC11A1 mAb. (C) Intracellular FACS staining on bovine PBMCs demonstrated SLC11A1 expression in most γδ T cells and in the majority of CD335− cells (± SD). Shown are the average percentages of the indicated marker positive cells that are also SLC11A1* (± SD). (D) Intracellular staining on human cells indicates that SLC11A1 is not expressed in B cells but is expressed in the majority of γδ T cells and NK cells. Shown are the average percentages of the indicated marker positive cells that are also SLC11A1* (± SD). (E) Memory/activated phenotype cells (CD3+,CD45RO+) demonstrated intermediate SLC11A1 staining; average MFI and modes for three populations are shown (± SD). Representative images from at least three experiments averages are calculated based on PBMCs from at least three donors per experiment.
some donors strongly increased expression of IFN-γ only in SLC11A1+ lymphocytes (data not shown), but this result was inconsistent between donors. These data underscore the importance of SLC11A1 expression in innate lymphocytes early in infection as SLC11A1+ cells were more readily activated in response to both mitogen stimulation and in the context of Salmonella infection.

**FIGURE 3.** SLC11A1 was strongly associated with activation of bovine cells. Bovine PBMCs were activated for 24 h with diminishing concentrations of Con A or LPS and stained for SLC11A1 and CD25 or IFN-γ. (A) SLC11A1+ cells were slightly more prone to activation (CD25 expression) by low doses of Con A, significance as calculated by two-way ANOVA. Representative FACS plots are shown below from high to low ConA concentrations. (B) Upon stimulation with very low doses of LPS, only SLC11A1+ cells were induced to express CD25. SLC11A1+ and SLC11A1− cells were significantly different as calculated by two-way ANOVA. A representative FACS plot is shown. (C) Bovine SLC11A1+ cells expressed significantly more intracellular IFN-γ. Bovine PBMCs were stimulated with 100 ng/ml LPS or 50 ng/ml Con A for 12 h, treated with brefeldin A for 6 h, and then fixed and stained for SLC11A1 and IFN-γ expression. Only SLC11A1+ cells expressed IFN-γ in response to LPS stimulation and were significantly more responsive to Con A (Student t test). These data represent at least three repeat experiments performed with at least three individual calves per experiment. Error bars represent SE. ***p < 0.001, **p < 0.01.

**FIGURE 4.** Human cells expressing SLC11A1 were more readily activated and preferentially expressed IFN-γ. Human PBMCs were isolated and cultured 24 h with the indicated stimuli and then stained for SLC11A1 and CD69, CD25, or IFN-γ. (A) SLC11A1+ human cells demonstrated slightly enhanced expression of CD69 in response simulation of PBMCs with very low concentrations of LPS. (B) A significantly greater percentage (Student t test) of CD69+ cells was evident in the SLC11A1+ lymphocytes in response to 10 ng/ml LPS, combined data from two experiments; n = 7 donors. These data were also found to be significant n = 0.0078 using nonparametric Wilcoxon test. Shown is a representative FACS plot demonstrating the differences between SLC11A1+ and SLC11A1− human cell populations after 10 ng/ml LPS stimulation. (C) PMA/ionomycin treatment resulted in significant changes to IFN-γ expression. Similar to the bovine cells, human SLC11A1− cells did not express IFN-γ, whereas SLC11A1+ cells expressed IFN-γ in response to very low concentrations of PMA/ionomycin. (D) SLC11A1+ innate lymphocytes are significantly more activated in response to bacterial infection. When human monocyte/macrophages were infected with S. Typhimurium, the SLC11A1+ cells in the cultures expressed significantly more CD69 than did the SLC11A1− cells (Student t test). Medium only or brief exposure to Salmonella LPS resulted in minimal changes. Shown are combined data from two experiments. All figures are representative results from at least three experiments performed with PBMCs from at least three donors per experiment. Error bars represent SD. **p < 0.01, *p < 0.05.
PTP inhibition activates lymphocytes

Because SLC11A1 enhances macrophage activation through iron-dependent PTP inhibition (9), we sought to simulate the effect of SLC11A1 expression in lymphocytes by chemically inhibiting PTPs. The capacity for innate lymphocytes to become activated in response to PTP inhibition was determined using the PTP inhibitor bpV(phen) (28, 29). In support of this approach, in vivo inoculation of bpV(phen) in the mouse asthma model promoted Th1-type cytokine response, primarily IFN-γ, expression (34). Addition of bpV(phen) induced activation of lymphocytes from both cattle and humans (Fig. 5). SLC11A1+ cells were more prone to activation by bpV(phen) alone, suggesting a strong association between SLC11A1 expression and the capacity for activation by PTP inhibition. Inhibition of PTPs only slightly activated SLC11A1− bovine cells, suggesting that these cells may lack the capacity for activation by PTP blockade that accompanies SLC11A1 expression (Fig. 5A). In contrast, addition of bpV(phen) resulted in the activation of SLC11A1+ bovine lymphocytes in the absence of LPS but did not alter their activation in the presence of LPS. Human SLC11A1− cells could be activated by low-dose LPS upon addition of bpV(phen), nearly to the level of LPS alone on SLC11A1+ cells (Fig. 5B). This suggested that the lack of response to LPS by SLC11A1− cells was not due to an inability to recognize LPS or LPS-induced cytokines expressed by PBMCs. Addition of bpV(phen) further promoted the activation of human SLC11A1+ cells in response to LPS (Fig. 5B). These observations are similar to the observations of Gomez et al. (9) upon comparison of SLC11A1− and SLC11A1+ (transfected) RAW 264.7 murine macrophages. In contrast to findings with macrophages, there was a greater response by SLC11A1-expressing lymphocytes to this treatment, suggesting an association between SLC11A1 expression and capacity for activation by PTP inhibition in innate lymphocytes.

Role of iron in activation of lymphocytes

Iron is a primary substrate for SLC11A1 (2). PTP inhibition is dependent on iron, and in SLC11A1− RAW264.7 macrophage cells, iron excess inhibited PTP activity to a similar extent as in resting RAW264.7 NRAMP-1 (SLC11A1+) cells, supporting an effect for iron in PTP inhibition (9). A similar experiment was conducted to determine the effects of iron, the SLC11A1 substrate, on innate lymphocyte activation. When human cells were cultured in iron-free media, responses of SLC11A1+ cells were not altered by iron excess, suggesting that activation of these cells by LPS is already maximal and is not affected by excess extracellular iron. However, similar to SLC11A1− RAW264.7 macrophages (9), SLC11A1+ human lymphocytes demonstrated dramatic changes in response to increasing concentrations of ferric citrate (Fig. 5C). Unstimulated SLC11A1+ cells were activated by iron excess, which had a greater effect than did LPS. Furthermore, in the presence of excess iron, SLC11A1+ human lymphocytes responded robustly to LPS stimulation, similar to SLC11A1+ cells. These data suggest human lymphocytes can be activated in the presence of iron, the SLC11A1 substrate. SLC11A1 is associated with retained tyrosine phosphorylation

Blocking PTP activity increases the phosphorylation states of cellular factors, thus, tyrosine phosphorylation was measured following chemical PTP blockade and in association with SLC11A1 expression in activated cells. The degree of phosphorylation and subsequent PTP activity was strongly dependent on the type of stimulation. A number of stimuli were screened and PMA/ionomycin and SEB clearly induced subsequent PTP activity. These reagents were used on human PBLs with and without chemical PTP blockade (bpV(phen)), and phosphotyrosine was measured by flow cytometry over time. PMA/ionomycin caused staining that diminished by 30 min poststimulation, and this reduction in phosphorylation was clearly blocked by addition of bpV(phen) (Fig. 6A). SEB also

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**FIGURE 5.** In innate lymphocytes demonstrated the capacity to be activated by PTP inhibition and iron excess. (A) SLC11A1− bovine cells were more sensitive to bpV(phen) treatment, which specifically inhibits PTPs (28, 29). Bovine cells were treated with either medium only or 2 ng/ml LPS with increasing concentrations of bpV(phen). Unstimulated SLC11A1− bovine cells were activated by bpV(phen), SLC11A1− bovine cells remained recalcitrant to activation in these conditions, but unstimulated cells demonstrated a very slight but significant increase in CD25 expression with increasing bpV(phen) concentrations. (B) Human lymphocytes increased activation upon stimulation with bpV(phen), regardless of SLC11A1 expression or addition of LPS. The low-level LPS activation of human SLC11A1+ cells was enhanced by bpV(phen) addition. (C) SLC11A1− human cells cultured in DMEM, a minimal protein-free medium with a low level of iron, were activated by LPS in iron excess. SLC11A1+ cells demonstrated a lower background staining with CD69 and a more enhanced response to LPS stimulation that was not altered by iron excess. Unstimulated SLC11A1− human lymphocytes cells were activated by iron excess, which had a slightly greater effect than did LPS. LPS-stimulated SLC11A1− cells were robustly activated by the addition of iron to the media. Error bars represent SD. ***p < 0.001, **p < 0.05; significance differences from untreated cells in the same group using Student t test. These are representative experiments from three independent experiments with at least three donor individuals per experiment.
SLC11A1+ cells retain tyrosine phosphorylation compared with SLC11A1− cells, similar to treatment with bpV(phen). Stimulated cells demonstrated reduced staining with a phosphotyrosine-specific Ab after intervals of stimulation up to 4 h. Cells stimulated with PMA/ionomycin (A) had reduced staining after 30 min, which was maintained whereas cells stimulated with SEB (B) had maximal loss of staining by 2 h poststimulation. Inclusion of bpV(phen) in the media facilitated retention of tyrosine phosphorylation, which was dependent on the type of stimulation. SLC11A1 is expected to block PTP function; thus, SLC11A1+ lymphocytes retained phosphorylation compared with SLC11A1− cells. (C) Representative plots demonstrate costaining of the SLC11A1+ and SLC11A1− lymphocyte populations for tyrosine phosphorylation. SLC11A1+ cells retained phosphorylation after 1 h with both stimuli, compared with SLC11A1− cells. (D) The retention of phosphorylation was apparent 1 h after PMA/ionomycin staining and at all intervals following stimulation with SEB. Representative data from at least two independent experiments performed with three donor individuals each. Error bars represent SD. Student t test. **p < 0.01, *p < 0.05.

resulted in phosphorylation that diminished more gradually over time. In the case of SEB, the addition of bpV(phen) robustly increased the staining of phosphotyrosine. Thus, consistent with published the descriptions of bpV(phen) (28, 29), this reagent effectively blocks PTPs in lymphocytes, and appropriately represents the expected effects of SLC11A1 expression.

Similar to chemical blockade of PTPs, enhanced tyrosine phosphorylation was evident in SLC11A1+ lymphocytes compared with those lacking SLC11A1. Costaining for SLC11A1 and phosphotyrosines demonstrated that SLC11A1-expressing lymphocytes retained phosphorylation 1 h after PMA/ionomycin stimulation and at all intervals tested following SEB stimulation (Fig. 6C, 6D). Consistent with its presumed function in monocytes, SLC11A1 expressed in lymphocytes is associated with retained phosphorylation following activation and is, thus, a likely mechanism of augmented activation in innate lymphocytes.

**Expression of SLC11A1 enhances lymphocyte activation**

To determine whether SLC11A1 augments activation of innate lymphocytes, human MOLT14 cells were transfected with SLC11A1, and their activation was assessed. MOLT14 cells were SLC11A1− as determined by intracellular flow cytometry, were generally recalcitrant to activation, and not readily activated by LPS compared with human peripheral blood SLC11A1+ lymphocytes. These cells were transfected with the SLC11A1 gene tagged with GFP and activated for 24 h, and the activation phenotype of SLC11A1-GFP positive or negative cells was assessed by counterstaining with Abs specific for CD69, CD25, and intracellular IFN-γ. Transfected MOLT14s were stimulated with various cytokines and agonists. Unstimulated cells expressing SLC11A1-GFP expressed significantly more baseline CD69, and CD69 expression was not further induced by IL-1, IL-12, IL-18 (alone or in various combinations), or LPS (data not shown). Both Con A/IL-2 and PMA/ionomycin stimulation increased the expression of CD69 and SLC11A1-GFP+ cells expressed significantly more CD69 in response to Con A/IL-2 (Fig. 7A). There was no difference in CD69 expression between SLC11A1-GFP-positive and −negative cells in response to PMA/ionomycin (data not shown). Transfected MOLT14 cells were also stimulated with conditioned supernatant fluids from unstimulated or LPS-stimulated human PBMCs, collected after 24 h culture, to present the transfected MOLT14 cells with the same cytokine milieu as in other in vitro assays. Conditioned medium from human PBMCs (n = 5 different donors) increased expression of CD69, but the effect was greater with supernatant fluids from LPS-stimulated PBMCs (Fig. 7B). After stimulation with these conditioned media, expression of CD69 was significantly greater on SLC11A1-GFP+ cells (Fig. 7B). Only supernatant fluids from human PBMCs induced expression of IFN-γ by MOLT14 cells, and the effect was significantly greater in SLC11A1-GFP+ cells. Cells transfected with control GFP plasmid did not demonstrate altered CD69 or IFN-γ fluorescence in response to any of these agonists (data not shown). MOLT14 cells increased activation in response to bpV(phen), but demonstrated only slight increases in CD69 expression in response to LPS in iron excess in DMEM (data not shown), unlike human SLC11A1− lymphocytes (Fig. 5C). Thus, these cells have the capacity to become activated by PTP blockade but may be less sensitive to passive iron sensing for promoting activation and/or are less tolerant of minimal protein-free medium. These data demonstrate enhanced activation of SLC11A1-GFP expressing human MOLT14 cells and underscore the potential importance of SLC11A1 expression for maximal activation of innate lymphocytes.
SLC11A1 expression in nonadherent splenocytes results in increased IFN-γ expression

Enhanced expression of IFN-γ has long been associated with SLC11A1 expression. Ramarathinam et al. (19, 20) demonstrated that stimulation of SLC11A1+/+ adherent splenocytes (macrophages) with Salmonella resulted in greater IFN-γ expression from non-adherent spleen-derived cells than did similar treatment of SLC11A1−/− adherent cells. The predominant cellular source of IFN-γ in nonadherent mouse splenocytes was NK cells (20). SLC11A1 expression in innate lymphocytes was not directly investigated; however, isolated nonadherent spleen cells (mostly lymphocytes) from SLC11A1+/+ mice consistently expressed significantly greater IFN-γ in response to supernatant fluids than did nonadherent cells from SLC11A1−/− mice (19, 20). Because SLC11A1 has been assumed to be expressed only in monocytes, the significance of this nonadherent cell-dependent response was not discussed. We performed a similar experiment using splenocytes from 129/Sv and congenic 129/Sv (SLC11A1−/−) mice. Adherent cells from both strains were either mock treated or treated with 10^6 or 10^7 S. Typhimurium (strain SL1344) for 8 h. Supernatant fluids were collected, sterile filtered, and applied to nonadherent splenocytes derived from the two strains. Fig. 8A demonstrates that, similar to the earlier findings (19, 20), nonadherent cells from wild-type mice consistently expressed greater IFN-γ when compared with nonadherent cells from 129/Sv (SLC11A1−/−) mice. The difference was significant only following infection with the lower dose of

FIGURE 7. Expression of SLC11A1 rendered MOLT14 human γδ T cells more prone to activation. MOLT14 cells were electroporated with a plasmid expressing SLC11A1-GFP and stimulated for 24 h with various agonists. (A) Cells expressing SLC11A1-GFP expressed significantly more CD69, both resting and following activation with Con A/IL-2 (100 ng/ml). (B) SLC11A1-GFP+ cells expressed significantly more CD69 in response to conditioned media. Media from unstimulated and LPS-stimulated human PBMCs (from n = 5 donors) collected after 24 h were used to stimulate SLC11A1-GFP+ transfectected MOLT14 cells, resulting in increased expression of CD69, in the LPS-conditioned media in particular. (C) SLC11A1-GFP+ cells expressed significantly more IFN-γ in response to both types of conditioned media. These results represent pooled data from three independent experiments. Error bars represent SD, Student t test. ***p < 0.001, **p < 0.01, *p < 0.05.

FIGURE 8. Nonadherent splenocytes from wild-type mice express greater IFN-γ than do cells from congenic SLC11A1-deficient mice. Adherent cells from 129/Sv and 129/Sv (SLC11A1−/−) mice were infected with two doses of Salmonella for 8 h. Supernatant fluids from these cultures were sterile filtered and applied to nonadherent cells from the two mouse strains. Forty-eight hours later, IFN-γ expression was assessed by ELISA. (A) Nonadherent cells from wild-type mice consistently expressed greater IFN-γ when compared with nonadherent cells from 129/Sv (SLC11A1−/−) mice. The difference was significant only following infection with the lower dose of

Treatment of adherent cells: mock 10^6 10^7 mock 10^6 10^7
Source of adherent cells: 129/Sv(SLC11A1−/−) 129/Sv

IFN-γ (pg/ml)

0 200 400 600 800

B

IFN-γ (pg/ml)

0 200 400 600 800

IL-12 ConA/IL-2

FIGURE 8. Nonadherent splenocytes from wild-type mice express greater IFN-γ than do cells from congenic SLC11A1-deficient mice. Adherent cells from 129/Sv and 129/Sv (SLC11A1−/−) mice were infected with two doses of Salmonella for 8 h. Supernatant fluids from these cultures were sterile filtered and applied to nonadherent cells from the two mouse strains. Forty-eight hours later, IFN-γ expression was assessed by ELISA. (A) Nonadherent cells from wild-type mice consistently expressed greater IFN-γ when compared with nonadherent cells from 129/Sv (SLC11A1−/−) mice. The difference was significant only following infection with the lower dose of Salmonella. (B) Stimulation with IL-12 or Con A/IL-2 did not replicate the differences between the nonadherent cell types. Representative data are shown with significance based on triplicate wells; the experiment was performed three times. Students t test. ***p < 0.001, **p < 0.01, *p < 0.05.
Salmonella, consistent with findings after Salmonella infection in human cells. Differences between mouse strains were not detected in response to IL-12 alone or TCR agonist Con A and IL-2 (Fig. 8B). Thus, stimulation of adherent splenocytes, regardless of mouse strain source, resulted in cytokines that had a significantly greater effect on SLC11A1-expressing nonadherent cells. These findings indicate that the presence of SLC11A1 in innate lymphocytes promotes their optimal activation and early/innate IFN-γ expression and its absence from both monocytes and innate lymphocytes should be considered when using inbred mouse strains.

**Discussion**

Human and bovine γδ T cells, NK, and NK-like cells cattle expressed SLC11A1 protein. Mouse γδ T cells expressed much higher SLC11A1 transcripts than did mouse αβ T cells, suggesting a similar protein expression pattern in SLC11A1+/+ mice is likely. Thus, contrary to current belief, SLC11A1 is expressed in innate lymphocytes as well as macrophages. Expression of SLC11A1 transcripts in total human PBLS has been previously noted (35) but not extensively characterized. Bovine and human lymphocytes expressing SLC11A1 were more sensitive to very low concentrations of stimulants, and only bovine cells expressing SLC11A1 were activated in response to low levels of LPS. Expression of SLC11A1 was associated with retention of tyrosine phosphorylation, supporting a role for SLC11A1 in inhibition of PTPs similar to its role in macrophages (9). Nonadherent splenocytes from wild-type mice consistently expressed more IFN-γ than did cells from SLC11A1-deficient mice in response to 8-h conditioned media from Salmonella-infected adherent cells from either source (19, 20). In this setting, the source of IFN-γ was determined to be largely NK cells, but the potential contribution of γδ T cells was not assessed (20). These data support a role for SLC11A1 in optimal activation and innate IFN-γ expression in innate lymphocytes.

Our findings indicate that SLC11A1+/+ innate lymphocytes express greater IFN-γ. Functional SLC11A1 expression in vivo has been repeatedly associated with increased expression of IFN-γ. In an early characterization of the effects of SLC11A1 on vaccination efficiencies, an increase of IL-2 and IFN-γ in SLC11A1+/+ (wild-type) mice, compared with SLC11A1-deficient congenic mice, was observed (15). IFN-γ expression was significantly increased in both Salmonella-infected and DSS-treated (to induce colitis) SLC11A1+/+ mice, compared with congenic SLC11A1-deficient mice (16, 17). IFN-γ was essential for the protection of SLC11A1+/+ mice from persistent S. Typhimurium infection (18). Enhanced IFN-γ expression was also detected in SLC11A1+/+ mice in a study defining the contribution of this gene to diabetes phenotypes (14). Because of the assumption that SLC11A1 is only expressed in macrophages, the increase in IFN-γ expression has largely been attributed to enhanced macrophage function and IL-12 expression in SLC11A1 wild-type animals. However, IFN-γ expression was detected in the absence of IL-12 transcripts in the SLC11A1+/+ colitis model, suggesting an alternate pathway (16). Considering that γδ T cells and NK cells are critical sources of innate IFN-γ and other cytokines in infectious and inflammatory conditions, the expression of SLC11A1 that augments their activation is a likely mechanism for increased IFN-γ expression in SLC11A1 wild-type mice.

Early characterizations of γδ T cells in other models have described them as important early sources of IFN-γ in SLC11A1 wild-type animals but not in SLC11A1−/− mice. The cellular source of early/innate IFN-γ appears to be strongly dependent on the specific animal model studied (36–44). Two groups effectively showed that although IFN-γ was very important for protection from Mycobacterium infection in C57BL/6 mice, γδ T cells were dispensable and not a source of IFN-γ (45, 46). This finding is in contrast to results concerning the function of γδ T cells in human and bovine Mycobacterium infection, where they are assumed to produce IFN-γ and contribute to protection from disease (47–51). Similarly, γδ T cells were found to be unnecessary for protection against Leishmania infection in C57BL/6 mice (52), but are a major source of IFN-γ in human leishmaniasis (53). γδ T cell function has, however, been only minimally assessed in SLC11A1+/+ mice. γδ T cells were enhanced in these mice following Salmonella infection, but their function was not described previously (54). In Salmonella-resistant (Ity', SLC11A1+/+) mice, the absence of γδ T cells did not affect the long-term outcome of Salmonella-induced murine typhoid, but the absence of αβ T cells did (55). When both γδ and αβ T cells were deleted, mice had 10-fold higher bacterial counts in tissues than in the absence of αβ T cells alone. This datum suggests that in SLC11A1-competent mice, γδ T cells are critical for the appropriate development of effective adaptive immunity. Alternatively, descriptions of γδ T cell function in this systemic infection is potentially unlike a mucosally localized infection at which site γδ T cells are concentrated and stimulated. When Salmonella enterocolitis was compared in congenic SLC11A1+/+ and SLC11A1−/− mice, gene expression patterns in ceca suggested strongly enhanced IFN-γ expression in SLC11A1+/+ mice 1 d postinfection (17). IFN-γ was essential for the protection of SLC11A1+/+ mice from persistent S. Typhimurium infection (18). The likely early source of this cytokine is enhanced stimulation of innate lymphocytes (20). Expression of SLC11A1 and its importance for optimal activation and IFN-γ expression from γδ T cells and other innate lymphocytes may offer an explanation for these pervasive differences between SLC11A1+/+ and SLC11A1−/− animals and findings in humans.

The role for γδ T cells in asthma is controversial and also appears to be strongly dependent on the specific animal model used. γδ T cells have been shown to contribute to asthma pathogenesis through production of TH2 cytokines, IgE, and promotion of inflammatory cell infiltration in both SLC11A1-deficient BALB/c and C57BL/6 mice (56, 57). Other groups have observed that airway responsiveness increases in the absence of γδ T cells in C57BL/6 mice, again suggesting a regulatory role (58, 59). In an asthma model in SLC11A1-competent rats, CD8+ γδ T cells were critical for production of IFN-γ, which diminished expression of TH2-type cytokines and provided protection from late allergic airway responses (60). Our data support the potential for inefficient expression of IFN-γ by γδ T cells in SLC11A1-deficient mice. Consistent with a critical role for SLC11A1 in promoting TH1-type responses in asthma, SLC11A1+/+ wild-type mice have decreased IgE and decreased IL-4, compared with congenic SLC11A1-deficient controls (61). Specific inhibition of PTP activity, which functionally mimics SLC11A1 activity in all cells, in an asthma model resulted in a significant reduction in asthma-related symptoms paralleled by increases in IFN-γ (34). Similarly, iron deficiency was shown to strongly decrease the expression of IFN-γ in C57BL/6 mice (62). In the same strain, oral doses of iron-saturated bovine lactoferrin promoted IFN-γ expression that resulted in enhanced chemotherapeutic protection from tumors (63). Thus, the activation of SLC11A1−/− lymphocytes and their capacity to express IFN-γ in SLC11A1−/− mice may be more a result of passive iron sensing, similar to the effects of ferric citrate on SLC11A1−/− human lymphocytes. Expression of SLC11A1 may be important for optimal production of IFN-γ by innate lymphocytes. The significance of γδ T cells in asthma and other disease states affected by IFN-γ expression may remain enigmatic until SLC11A1 expression in innate lymphocytes is considered.
The expression of SLC11A1 in innate lymphocytes should also be considered in the context of antitumor responses. IFN-γ expression from innate lymphocytes is critical for protecting against tumors (64, 65). Stimulation of dendritic cells is thought to be necessary for the optimal expression of IFN-γ from NK cells (66) and is the intent of current cancer vaccine and therapeutic studies (66, 67). Because these therapies are largely tested in SLC11A1−/− mouse tumor models, it would be of interest to study the contribution of SLC11A1 in NK expression of IFN-γ to better translate these therapies to humans.

Our data suggest that innate lymphocyte activation is attenuated in the absence of SLC11A1 expression. The role of SLC11A1 in inflammatory processes in mouse models of disease has only begun to be explored. We detected differences in phenotype based on SLC11A1 expression in human, mouse, and bovine innate lymphocytes. Revisiting the mechanisms of innate lymphocyte activation in mouse models of bacterial disease, inflammatory, and tumor models in SLC11A1 wild-type strains is warranted. Such analyses are likely to increase the relevance and applicability of results determined in mouse models to human health. The capacity of human innate lymphocytes to be activated through PTP inhibition and by increasing iron suggests that iron sufficiency could be an important clinical consideration for optimally activating these cells. SLC11A1 expression in innate lymphocytes and its importance in expression of IFN-γ may be a solution to a longstanding chicken-and-egg conundrum concerning the production of IL-12 and IFN-γ early in infection, which is largely based on data generated using SLC11A1-deficient mouse strains (23).

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Disclosures

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