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Role of caveolin-3 in lymphocyte activation

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ABSTRACT

Aims: Caveolins are structural proteins clustered in lipid-rich regions of plasma membrane involved in coordinating signal transduction in various organ systems. While caveolin-1 (Cav-1) has been shown to regulate lymphocyte activation, the role of caveolin-3 (Cav-3) in immune system signaling has not been investigated. We tested the hypothesis that Cav-3 modulates lymphocyte activation.
Main methods: Lymphocyte/leukocyte subpopulations from WT and Cav-3 mice were profiled with flow cytometry. Cytokine production in quiescent and activated splenocytes from WT and Cav-3 mice was assessed with ELISA.
Key findings: Levels of T-cells, monocytes, and natural killer cells were not different between WT and KO mice, however KO mice had lower B-cell population-percentage. Functionally, activated lymphocytes from Cav-3 KO mice demonstrated significantly reduced expression of IL-2 compared to WT, while expression of TNFα, IL-6, and IL-10 was not different. Finally, expression of IL-17 was significantly reduced in T-helper cells from KO mice, while IFNγ was not, suggesting that Cav-3 is a determinant in the development of the Th-17 subpopulation.
Significance: This study is the first to demonstrate that Cav-3 may be a novel participant in B-cell expression, T-cell cytokine production and activation of inflammation.

Introduction

Membrane lipid rafts (MLRs) are discreet microdomains of the cell membrane that concentrate and localize cellular signaling molecules by providing a lipid-rich (i.e., sphingomyelin, glycosphingolipids, and cholesterol) platform for protein anchoring. By providing a stable binding environment for protein–protein interactions, MLRs promote a variety of physiological functions such as cell surface signaling [1–3], endocytosis [4], calcium homeostasis [5–7] and intracellular cholesterol transport [8]. Caveolae, morphologic invaginations of the cell membrane, are a subset of MLRs rich in the structural/scaffolding proteins caveolins [9], a family of proteins approximately 17–24 kDa in size that exist in three isoforms (Cav-1, -2, and -3). Cav-1 is essential for formation of caveolae in endothelial cells, fibroblasts, and pneumocytes [10], whereas Cav-2 plays an unclear but likely supportive role by forming hetero-oligomers with Cav-1 [10,11]. Cav-3 KO mice are viable but subject to skeletal and cardiac myopathies [12,13], and our laboratory and others have demonstrated a critical role for caveolin in protection from ischemia–reperfusion injury [14–17].

MLR-mediated signal transduction is an important element in the activation of the immune system [18–22] and inflammatory response [23–27]; however, this previous work has largely focused on the more ubiquitously expressed Cav-1 isoform. In contrast, the role of Cav-3 in immune system signaling has been relatively under-investigated. Therefore, in the present study we utilized global Cav-3 KO mice to define the role of Cav-3 in immune system signaling by testing the hypothesis that Cav-3 participates in physiologic T-cell activation.

Materials and methods

Animals

Animals (total n = 35) were treated in compliance with the Guide for the Care and Use of Laboratory Animals (National Academy of Science). All protocols were approved by the Veterans Affairs San Diego Healthcare System Institutional Animal Care and Use Committee. Animals were kept on a 12-h light/dark cycle in a temperature-controlled room with ad libitum access to food and water. The genotype of Cav-3 KO mice was confirmed by PCR.

Lymphocyte isolation

Eight- to ten-week-old Cav-3 KO (13) mice (n = 19) or age-matched C57BL/6 wild type (WT) controls (n = 16) were euthanized and spleens harvested and macerated through a 70 μm cell strainer (Fisher Scientific). Residual red blood cells were lysed with 5 mL ammonium-chloride-potassium (ACK) lysis buffer (Life Technologies)

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for 5 min at room temperature. The lymphocytes were then washed and pelleted twice before being resuspended in RPMI media (Invitrogen) supplemented with 10% fetal calf serum (FCS, Gibco), 2 mM glutamine (Sigma-Aldrich), 50 U/mL penicillin (Sigma-Aldrich), 50 μg/mL streptomycin (Sigma-Aldrich), 0.6 mM sodium pyruvate (Sigma-Aldrich), 1 mM HEPES (Sigma-Aldrich), and 0.055 mM β-mercaptoethanol (Sigma-Aldrich).

Flow cytometry

Rat anti-mouse CD3 FITC antibody (561798, BD Pharmingen), CD14 FITC (11-0141-82, eBioscience), CD16 FITC (11-0161-82, eBioscience), CD19 FITC (11-0193-82, eBioscience), and Rat IgG FITC control (556923, BD Pharmingen) were used to stain splenocytes for flow cytometric analysis. 3×10^6 splenocytes were pelleted and resuspended in the 1:100 dilution of antibodies and 2% FCS:PBS (Gibco), then incubated for 30 min on ice. The stained cells were washed and pelleted 3 times with 2% FCS: PBS. Finally cells were fixed in 1% paraformaldehyde:PBS (Fisher Scientific) before flow analysis in a Becton Dickinson FACSCalibur flow cytometer (Flow Cytometry Research Core at the VA San Diego).

T-cell activation

12 well plates were coated with 5 μg/mL rat anti-mouse CD3 antibody (16-0032-85, eBioscience) and 5 μg/mL CD28 (16-0281-85, eBioscience) in PBS (Invitrogen) overnight at 4 °C. 10 μg/mL rat Ig (16-4301-85, eBioscience) in PBS was used as a control. After antibody coating, the plates were washed once with PBS. 4×10^6 freshly isolated lymphocytes were then plated into wells with RPMI media (Gibco). Lymphocytes were then cultured at 36 °C for 48 h.

Cytokine detection

Supernatants were isolated after T-cells were stimulated by antibody and diluted 1:100 or 1:1000 final concentration for assay by enzyme-linked immunosorbent assay (ELISA). ELISAs (Life Biosciences) for IL-2, IL-17, IL-6, IL-10, INFγ, and TNFα were utilized according to the manufacturer's protocols. Data was acquired via Tecan Plate Reader Infinite M200.

Statistics

All data were analyzed via Mann Whitney U test. Significance was set at $p < 0.05$. All data are presented as mean ± SEM. All statistical analysis was performed using Prism 6 (GraphPad Software, Inc.).

Results

Lymphocyte populations

Lymphocyte/leukocyte subpopulations of T-cells, B-cells, monocytes and natural killer cells were determined in WT and Cav-3 KO mice using flow cytometry (Fig. 1). We observed a decrease in the population distribution of B-cells in Cav-3 KO mice relative to WT mice; however, T-cell, monocyte, and natural killer cell populations were not altered.

T-cell activation and cytokine production

We sought to evaluate the cytokine production from splenic lymphocytes harvested from WT and Cav-3 KO mice after T-cell activation (Fig. 2) using stimulating anti-CD3 and anti-CD28 antibodies. Cytokine levels from supernatants of cells treated with stimulating antibodies were compared with those of nonspecific control antibody. For each experimental set, the cytokine levels were normalized to the average WT level to reduce variation between experiments. We found that IL-2 and IL-17 production was significantly reduced in Cav-3 KO mice relative to WT. No differences in IFNγ, TNFα, IL-6, and IL-10 levels were observed.

Discussion

The potential role for Cav-3 protein in the immune response has never been investigated. In our experiments we observed that levels of T-cells, monocytes, and natural killer cells were not different between WT and KO mice, however KO mice had significantly fewer B-cells. Additionally, we observed that production of the activating cytokine IL-2 after T-cell stimulation with anti-CD3 and anti-CD28 antibodies was reduced. This suggests that Cav-3 signaling is at least partially involved in the production of this cytokine. However, we did not find that production of the Th-1 specific cytokine (IFNγ), the proinflammatory cytokines (TNFα, IL-6), nor the inhibitory cytokine (IL-10) was significantly affected by the lack of Cav-3 expression. Taken together this suggests that Cav-3 may be important for initial T-cell activation by elaboration of IL-2, and that either lowered IL-2 levels and/or Cav-3 itself, may also be important for subsequent production of the inflammatory mediator IL-17. How this relates to the overall pro- or anti-inflammatory state remains to be elucidated, as other cytokine levels we measured appeared to be unaffected by the lack of Cav-3 expression, even in the face of reduced baseline IL-2 production.

Previous work regarding a role for MLR and caveolin mediated signal transduction in activation of the immune system and the inflammatory response has focused on the Cav-1 isoform, demonstrating a role in T-cell activation by upregulation of CD86 on antigen presenting cells [18], and the T-cell recall-response to antigen [19]. Tomassian et al. [28] demonstrated that Cav-1 is essential to coordinate an appropriate T-cell response to antigen, while Fu et al. [22] observed that Cav-1 is necessary for the transformation of quiescent monocytes to active macrophages. Upregulation of Cav-1 has been observed in adult T-cell leukemia cells [20] and macrophages undergoing apoptosis [21], and Cav-1 has been shown to play a critical role in induction of inflammatory pathways via interleukin-1β (IL-1β) signaling and NFκB activation [25]. In the respiratory system, alveolar macrophages with an inflammatory phenotype were associated with enhanced expression of Cav-1 [24]. Cav-1 knockout mice (KO) showed reduced LPS driven lung inflammation [25] and reduced activation, adhesion, and transendothelial migration of neutrophils [27]. Finally, Cav-1 KO mice demonstrated reduced survival in an ischemic-bowel model of sepsis, associated with an enhanced expression of TNFα and IL-6 [26].

Although Cav-1 appears to play a prominent role in immune system signaling, there is limited information regarding a role for Cav-3. Cav-3 has been detected in bovine lymphocytes [29], but to date the specific role of Cav-3 in lymphocyte activation had not been investigated. Cav-3 expression was thought to be restricted to skeletal, cardiac, and some smooth muscle [30]. However we have recently demonstrated that Cav-3 is necessary in coordinating microglial activation in the brain [31], and that Cav-3 KO mice have an enhanced neuroinflammatory response to traumatic brain injury [32], suggesting a regulatory role for Cav-3 in immunomodulation in non-muscle cell types. Our current results suggest that Cav-3 may be involved in lymphocyte differentiation, proliferation and/or apoptosis. We observed that the B-cell population appears to be reduced in Cav-3 KO mice. This could either be due to a disorder in B-cell development and/or an increase in B-cell apoptosis in mice lacking Cav-3. Although we have previously demonstrated that Cav-3 plays an anti-apoptotic role in cardiac myocytes [33] via induction of pro-survival Akt signaling, given the complexities of immune cell differentiation, activation and cytokine signaling heterogeneity between multiple cell types, the mechanism for the observed decrease in B-cells is likely multifactorial, and at present, it is unclear whether the reduction in the B-cell population was due to decreased differentiation/proliferation or increased apoptosis. Similarly, the reduction in IL-17 could be due to a defect in