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Bovine WC1$^-$ $\gamma\delta$T cells incubated with IL-15 express the natural cytotoxicity receptor CD335 (NKp46) and produce IFN-γ in response to exogenous IL-12 and IL-18

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Summary
The $\gamma\delta$T cells of ruminants are believed to participate in innate immunity and have been described with regulatory, inflammatory and cytotoxic functions. Here we describe a subset of CD3$^+$ TcR1$^+$ WC1$^-$ $\gamma\delta$T cells expressing CD335 (NKp46), classically associated with CD3$^-$ natural killer (NK) cells, as a consequence of incubation with IL-15. This population, undetectable at the time of collection, developed after 2 week of IL-15 culture from splenic leukocytes (SPL) reaching greater than 50% of the total $\gamma\delta$T cells. However, they did not grow well from peripheral blood leukocytes (PBL). Splenic $\gamma\delta$T cells positively selected by magnetic separation prior to incubation with IL-15 and analyzed by flow cytometry, consistently yielded CD3$^+$ cells expressing CD335. These cells arose from the CD335$^-$ $\gamma\delta$T cell population suggesting that the new population represents up-regulation of CD335 by $\gamma\delta$T cells. CD335 mRNA expression from sorted IL-15-incubated SPL CD335$^+$ $\gamma\delta$T cells or NK cells exceeded that of CD335$^-$ $\gamma\delta$T cells. Incubation with IL-12 and IL-18 increased the number of CD335$^+$ $\gamma\delta$T cells in both the PBL and SPL fractions as compared to controls or IL-12 or IL-18 alone. In addition, CD335$^+$ $\gamma\delta$T cells demonstrated a robust ability to produce IFN-γ in response to exogenous IL-12 and IL-18. Taken as a whole, we describe a new phenotypically distinct bovine $\gamma\delta$T cell sub-population capable of participating in type 1 immune responses.
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Abbreviations: SPL, splenic-derived lymphocytes; PBL, peripheral blood lymphocytes; NCR, natural cytotoxicity receptor; γc, common γ-chain; rHu, recombinant human; DP, double positive CD335$^+$ TcR1$^+$ $\gamma\delta$T cells.

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Introduction

The significance of innate immunity in the initial response to a variety of viral, bacterial and protozoal diseases is becoming increasingly clear [1–4]. Two phenotypically distinct cell types that participate in innate immunity are natural killer (NK) cells and $\gamma\delta$T cells. Both cell types have broad tissue distribution and have been ascribed cytotoxic [5–7] and regulatory functions [8,9]. Classic NK cells lack CD3 which is common to the T cell receptor, but display a variety of lineage-specific markers such as the natural cytotoxicity receptors (NCR) NKp30, NKp44 and CD335 (previously identified as NKp46) (reviewed in Ref. [10]). The NCR belong to a family of NK receptors known as the immunoglobulin superfamily which includes killer cell Ig receptor. In the bovine, $CD3^+$ $\gamma\delta$T cells can be divided into two distinct subsets based on the expression of WC1, a member of the cysteine-rich scavenger receptor family [11–13]. Bovine NK and $\gamma\delta$T cells can be further differentiated by the expression of CD2 and CD8 [11–14].

An interesting property of $\gamma\delta$T cells is tissue tropism: a phenomenon that has been described for mice, humans and cattle [12,15,16]. In mice, cells with different isoforms of the γ- and δ-chain predominate in different tissues such as skin, lymph nodes, intestinal epithelium and spleen [15,17,18]. The accumulation of tissue-specific TCR isoforms can be influenced by IL-15, a member of the common γ-chain (γc) cytokine "superfamily" that also includes IL-2, IL-4, IL-7, IL-9 and IL-21. In the case of IL-15, the cytokine binds a heterotrimeric receptor composed of the unique IL-15Rα chain, and the shared IL-2/15Rβ (CD122) and γc chains [19]. γc cytokines trigger the Janus kinase-signal transducer and activator of transcription (Jak-STAT) pathway, regulating gene transcription. During the generation of $\gamma\delta$T cells, chromatin accessibility at the TCRγ locus is believed to be temporally modulated by signals mediated by the IL-7 receptor [20]. Rearrangement of the TCRγ locus is arrested in IL-$7^{-/-}$ mice [21]. IL-15 has recently been documented to restore Vγ5-expressing cells to the gut of IL-$7^{-/-}$ mice in a manner that was not merely a replacement for IL-7 [22]. IL-15 exerted an effect via STAT5 at a chromatin domain distinct from that controlled by IL-7 with the resulting expansion of a phenotypically unique $\gamma\delta$T cell [22].

Previously, we used IL-15 to expand NK cells from peripheral blood (peripheral blood leukocytes—PBL) and homogenized spleen aspirates (splenic-derived leukocytes—SPL) [23]. The expanded cell preparations contained a percentage of cells expressing CD335 that was similar to preparations obtained by depletion of $CD3^+$ cells, B cells and monocytes and were used as an enriched NK cell preparation for studies involving inflammatory cytokines. However, the recent description of NCR on purified or cloned $\gamma\delta$T cells incubated with IL-15 [24] caused us to revisit the procedure. Here we describe the up-regulation of $CD335^+$ by a subpopulation of $\gamma\delta$T cells as a consequence of culturing with IL-15 and document the production of the type 1 regulatory molecule IFN-γ by the newly described subpopulation.

Materials and methods

Source of leukocytes

Nine Holstein–Friesian calves were obtained at 8–12 weeks of age and maintained according to the American Association for Laboratory Animal Care procedures with an acceptable bovine ration, water and mineral block provided *ad libitum*. The spleen of each animal was surgically marsupialized to facilitate aspiration of spleen cells [25]. The procedure has proven to be a means of acquiring sequential samples from a single animal without demonstrable consequences to splenic phenotypic ratios or basal levels of cellular activity [26]. Peripheral blood was aseptically collected in evacuated bottles containing acid citrate dextrose (ACD), pH 7.3. Splenic aspirates were aseptically collected in syringes containing ACD under local anesthesia and processed into a single-cell suspension using a tissue homogenizer. Splenic cell suspensions or peripheral blood were layered onto Hypaque–Ficoll (1.086 g/L) (Accu-Paque, Accurate Chemicals, Westbury, NY) and centrifuged for 30 min at 1500*g* at 4 °C. Cells were collected from the interface and washed in 50 mL Dulbecco's modified eagle's medium (DMEM), pH 7.35, for 7 min at 1500*g* at 4 °C. The cells were suspended with DMEM and washed twice at 400*g* for 7 min and 4 °C to remove platelets. The final pellets were suspended in Iscove's medium (Invitrogen, Carlsbad, CA) containing 25 mM Hepes, 2 mM glutamine, 10 μg/mL gentamycin, 50 μM mercaptoethanol, and 15% essentially endotoxin-free fetal bovine serum (FBS) (<0.06 EU/mL as assayed by Limulus amoebocyte lysate gelation) (Hyclone, Logan, UT).

Cell culture

For experiments using whole cell preparations, PBL or SPL at 1×10^6/mL in Iscove's/FBS containing 20 ng/mL recombinant human (rHu) IL-15 (R&D Systems, Minneapolis, MN) were incubated for 14 days at 37 °C and 5% CO_2 as previously described [23]. Prior to functional studies, cultured cells were collected and suspended to a concentration of 1×10^7 cells/mL. The cell purity of isolated or enriched cells was determined by one- or two-color flow cytometry. For some studies, cells were cultured overnight in combinations of 20 ng/ml rHu IL-12 (R&D Systems) and 20 pg/ml rHu IL-18 (Medical & Biological Labs, Woburn, MA) as previously described [23] or in combination with exogenously added 50 U/ml recombinant bovine IFN-γ generously provided by Drs. D. Godson and L. Babiuk, Vaccine and Infectious Diseases Organization, VIDO, Saskatoon, Saskachewan, Canada). Identification of IFN-γ producing cells was accomplished by internal label FACS as previously described [23]. Briefly, overnight cytokine-stimulated cells were incubated for 4 h with Golgi Plug (Becton Dickinson, San Jose, CA), collected and stained for surface determinants. The cells were fixed and permeabilized with Cytofix/CytoPerm™ (Becton Dickinson) for internal staining of accumulated IFN-γ. Permeabilized cells were incubated with anti-bovine IFN-γ-FITC (MCA1783-F) (Serotec, Raleigh, NC) and washed with Perm/Wash™ solution (Becton Dickinson).