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γδ T Cell Function Varies with the Expressed WC1 Coreceptor[1]

Aric N. Rogers,* Denille G. VanBuren,[†] Emmett E. Hedblom,* Mulualem E. Tilahun,[†] Janice C. Telfer,*[†] and Cynthia L. Baldwin[2]*[†]

WC1 molecules are transmembrane glycoproteins belonging to the scavenger receptor cysteine-rich family and uniquely expressed on γδ T cells. Although participation of WC1^{+} γδ T cells in immune responses is well established, very little is understood regarding the significance of expressing different forms of the WC1 molecule. Two forms previously identified by mAbs, i.e., WC1.1 and WC1.2, are expressed by largely nonoverlapping subpopulations of γδ T cells. In this study it was shown that expression of the WC1.1 coreceptor was the main indicator of proliferation and IFN-γ production in response to autologous and bacterial Ags as well as for IFN-γ production without proliferation in Th1-polarizing, IL-12-containing cultures. Nevertheless, after culture in either Th1-polarizing or neutral conditions, mRNA was present for both T-bet and GATA-3 as well as for IL-12Rβ2 in WC1.1[+] and WC1.2[+] subpopulations, and neither produced IL-4 under any conditions. Although the steady decrease in the proportion of WC1.1^{+} cells, but not WC1.2^{+} cells, within PBMC with animal aging suggested that the two subpopulations may have different roles in immune regulation, cells bearing either WC1.1 or WC1.2 expressed mRNA for regulatory cytokines IL-10 and TGF-β, with TGF-β being constitutively expressed by ex vivo cells. Overall, the results demonstrate that the form of the WC1 coreceptor expressed on γδ T cells divides them into functional subsets according to IFN-γ production and proliferative capacity to specific stimuli as well as with regard to representation within PBMC. Finally, evidence is provided for minor differences in the intracytoplasmic tail sequences of WC1.1 and WC1.2 that may affect signaling. *The Journal of Immunology,* **2005, 174: 3386–3393.**

The γδ T cells bearing the lineage marker WC1 are associated with production of the proinflammatory cytokine IFN-γ (1–3) and are among the earliest cells to accumulate upon intradermal injection of *Mycobacterium bovis* purified protein derivative, preceding the arrival of αβ T cells and macrophages (4). The majority of γδ T cells in the blood of young ruminants express WC1, whereas in other tissues, such as the spleen, the γδ T cells are WC1⁻ (5). Serial analysis of gene expression and microarray analyses suggest that the WC1^{+} γδ T cells of cattle represent the inflammatory population, whereas the WC1⁻ γδ T cells are regulatory cells and share characteristics with myeloid lineage cells (6, 7). This is supported by the observation that animals depleted of WC1^{+} cells by anti-WC1 Abs show a decrease in nonspecific production of IFN-γ and a bias toward a Th2 response, as evidenced by increased IL-4 and decreased IgG2 Abs (8). Thus, WC1^{+} γδ T cells appear to be important mediators of cell-mediated (type 1) immunity. Although a generalized role for γδ T lymphocytes is emerging from these and other studies in ruminant systems (9) as well as nonruminant systems (10), the significance of WC1 expression itself remains enigmatic.

WC1, also known as T19, is a large, type 1, integral membrane glycoprotein belonging to the scavenger receptor cysteine-rich (SRCR)[3] family. Although WC1 proteins have only been found on γδ T cells of ruminants, cDNA inferred to be the murine WC1 homologue was recently identified (11). WC1 molecules are products of a multigene family (12–14), and bovine γδ T cells express three known variants, WC1.1, WC1.2, and WC1.3 (15). WC1.1 and WC1.2 are expressed on predominantly nonoverlapping subsets of bovine γδ T cells, whereas WC1.3 is expressed on a small subpopulation of WC1.1^{+} cells (15). Although only WC1.1 has been fully sequenced, limited sequencing and restriction mapping indicates substantial differences among the three cloned WC1 forms in their extracellular SRCR domains, of which there are 11 (15).

The amino acid sequence of archetypal WC1 (WC1.1) (14) suggests a role in cellular activation based on the potential ITAM [YEDALAEAVYEEL] in the cytoplasmic tail proximal to the transmembrane region. A role in cellular activation is supported by examples of other known SRCR family members present on T cells, namely CD5 and CD6 (16), both of which have roles in activation and regulation of T cell responses (17–20). There is evidence that Ab-mediated signaling through WC1 increases cellular activation (21) as well as cell cycle arrest (22). These differences in purported WC1 function may be the result of cross-linking different forms of the molecule, given that different Abs and cellular systems were used. It has been suggested that the significant extracellular variation in WC1 may represent a new mechanism of pattern recognition and regulation of immune responses by γδ T cells (23). In this study we evaluated functional responses of γδ T cells expressing alternate forms of WC1, their proportional

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[3] Abbreviations used in this paper: SRCR, scavenger receptor cysteine rich; AMLR, autologous MLR; SH2, Src homology 2.

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representation within the PBMC population of animals of varying ages, and the potential signaling motifs of their intracytoplasmic tails.

Materials and Methods

Animals and cells

Blood was obtained from female cattle, ages 1 day to 44 mo, via venipuncture of the jugular vein. Blood was either collected into a solution of heparin or defibrinated as indicated for the assay (24). PBMC were isolated from blood via density gradient centrifugation over Ficoll-Hypaque (Ficoll-Paque; LKB-Pharmacia Biotechnology) by standard techniques. Culture medium consisted of RPMI 1640, 10% heat-inactivated FBS, 2 μM L-glutamine, 60 μg/ml gentamicin, and 5×10^{-5} M 2-ME. PBMC cultured with sonicates of *Leptospira borgpetersenii* serovar Hardjo were obtained from animals vaccinated with Spirovac (Biocore), a whole cell vaccine containing aluminum hydroxide administered in two s.c. doses, 4 wk apart (25).

In vitro cultures

PBMC were cultured at 2.5×10^6 cells/ml, except for Con A-stimulated cultures, which were seeded at 6.25×10^5 cells/ml. All cultures were conducted at 37°C in 5% CO_2 in air. PBMC isolated from heparinized blood were used in cultures stimulated with PMA and ionomycin (1 μg/ml each; Sigma-Aldrich), Con A (5 μg/ml; Sigma-Aldrich), anti-CD3 mAb (protein A-purified mAb Veterinary Medical Research & Development (VMRD) at 10 μg/ml used to precoat plates as previously described by Hanby-Flarida et al. (21)), IL-12 (250 U/ml human rIL-12) as described previously (26) (BD Pharmingen), or a sonicate of leptospira (0.5 μg/ml protein *L. borgpetersenii* serovar Hardjo-bovis clone RZ33) (25). PMA-, ionomycin-, Con A-, and IL-12-stimulated cultures were incubated for 4 days, whereas the leptospira sonicate cultures were incubated for 6 days before analysis unless indicated otherwise. For autologous MLR (AMLR) cultures, PBMC were depleted of monocytes by isolation from defibrinated blood and were seeded at 2.5×10^6 cells/ml. These monocyte-depleted PBMC were stimulated with gamma-irradiated (5000 rad) monocyte-containing PBMC at a density of 6.5×10^5 cells/ml for 6 days. For all test cultures, control cultures were established that were identical except for exclusion of the Ags, cytokine, mitogen, or gamma-irradiated stimulator cells and are referred to as medium cultures. In some experiments, cells were loaded with CFSE (Molecular Probes) before culture using 3 μM CFSE and 1.2 μM Pluronic F127, as described previously (27), to assess cell division. Cellular responses analyzed included assessment of cell division by CFSE intensity, enumeration of subpopulations, expression of CD25, production of IFN-γ by flow cytometry, and evaluation of mRNA transcript production as described below.

Immunofluorescence and flow cytometric analyses

Cells in culture were stained for cell surface differentiation molecules, fixed in 1% paraformaldehyde, and analyzed via flow cytometry for two- or three-color fluorescence (FACSCalibur; BD Biosciences) using the following primary mAb: BAG25A and BAQ159A for WC1.1 (VMRD), CACTB32A for WC1.2 (VMRD), CACT21A for WC1.3 (a gift from Dr. W. C. Davis, Pullman, WA) (15), IL-A29 (28) and CC15, which are pan-specific for all WC1 molecules (Serotec), GB21A for δ-TCR (VMRD), and UC-2C2 for CD25 (a gift from Dr. B. Taylor, Davis, CA) (29). Secondary Abs used for indirect staining were isotype-specific polyclonal goat anti-mouse Ig conjugated with PE or FITC (Southern Biotechnology Associates). In staining procedures that used secondary Abs, unrelated isotype-matched primary mAb were used with matching secondary Abs and used as negative controls (Southern Biotechnology Associates and BD Pharmingen). Where indicated, analyses were conducted using CACTB32A directly labeled with zenon Alexa Fluor 488 (Molecular Probes). Biotinylated anti-IFN-γ mAb 7B6 (a gift from J. J. Letesson, Leeven, Belgium) (30) and streptavidin-PerCP were used for intracellular staining essentially as previously described (25). Biotinylated isotype-matched (IgG1) mAb was used as a control primary Ab in these experiments. Viable cells were gated based on scattering characteristics. Proliferation of cells stained with the phenotypic markers described above was assessed by logarithmic plots of CFSE intensity. Analysis of flow cytometric data was conducted using CellQuest (BD Biosciences).

Enumeration of cells within WC1 subpopulations in cultures

To assess a relative change in the proportion of cells expressing a particular form of WC1 after culture, cells were stained for the expression of WC1.1 or WC1.2 by indirect immunofluorescence, and the percentage of each was

determined. The total percentage of $WC1^+$ cells was defined as the sum of cells bearing either WC1.1 or WC1.2 based on pilot studies, confirming this was the case using a pan-specific anti-WC1 mAb. Changes in representation of $WC1.1^+$ or $WC1.2^+$ cells as a result of culture were calculated as follows: % change (Δ) in $WC1.1^+$ cells = ([% $WC1.1^+$/% total $WC1^+$]$_{stimulated}$ − [%$WC1.1^+$/% total $WC1^+$]$_{control}$) × 100%; % Δ $WC1.2^+$ cells = ([% $WC1.2^+$/% total $WC1^+$]$_{stimulated}$ − [% $WC1.2^+$/% total $WC1^+$]$_{control}$) × 100%.

Sorting of cells

Cells were sorted after 48 h in Con A- or IL-12-stimulated cultures using magnetic bead cell sorting (Miltenyi Biotec). This was performed essentially as previously described (31) in that cells were positively purified by reacting PBMC with primary mAb, followed by goat anti-mouse IgG-conjugated magnetic microbeads in a suspension of PBS with 5% heat-inactivated horse serum. The exception was that the entire isolation procedure was conducted at 0–4°C, and cells were filtered through a 30-μm pore size mesh (Miltenyi Biotec) immediately before application to the column. In pilot experiments, cells that had been cultured with Con A for 48 h were resuspended by pipetting, filtered, and then stained to assess the proportion of cells in subpopulations with and without filtering. This ensured mesh filtration removed cellular aggregates without biasing results. $WC1.1^+$ cells were positively selected over magnetic bead columns after staining with mAb BAQ159A and enriched to 97.3%, on the average, whereas $WC1.2^+$ cells were positively selected after staining with mAb CACTB32A and enriched, on the average, to 98.4%. Ex vivo PBMC were stained in the same way, but were sorted via flow cytometry (FACSAria; BD Biosciences) to 96.8% (WC1.1) and 98.3% (WC1.2) purity.

RNA isolation and PCR

RNA was isolated from either ex vivo PBMC or after 48 h of culture using TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions. Total RNA was subjected to DNase digestion and then used for cDNA synthesis using a commercial RT kit (Promega). PCR was conducted using primer pairs described below along with PCR reagents (Promega) to yield a final concentration of 2 mM $MgCl_2$. Cycling conditions were 30 s at 95°C, 1 min at 52°C, and 45 s at 72°C for 30 cycles. PCR products were analyzed in 1.2% Tris-acetic acid-EDTA agarose gels, cloned into the pCR2.1 vector (Invitrogen Life Technologies), and sequenced commercially. The following primers were used in semiquantitative PCR with the PCR product length in parentheses and GenBank accession numbers in brackets: bovine *GAPDH* [U85042]: sense primer, 5′-GTCATCATCTCTGCACCTTCT-3′; antisense primer, 5′-ACCACCTTCT TGATCTCATCAT-3′ (430 bp); bovine *GATA-3* [TC211018]: sense primer, 5′-CCAGACCAGAAACCGAAAAA-3′; antisense primer, 5′-ACCATACT GGAAGGGTGGTG-3′ (234 bp); bovine *IFN-γ* [NM_174086]: sense primer, 5′-TAAGGGTGGGCCTCTCTTCT-3′; antisense primer, 5′-AAATATTG CAGGCAGGAGGA-3′ (387 bp); bovine *IL-4* [NM_173921]: sense primer, 5′-CAGTGCTGGTCTGCTTACTG-3′; antisense primer, 5′-CAAGAG GTCTTTCAGCGTAC-3′ (338 bp); bovine *IL-10* [U00799]: sense primer, 5′-GTGAACTCACTGGGGGAGAA-3′; antisense primer, 5′-CCCTCTCTT GGAGCTCACTG-3′ (112 bp); bovine *IL-12R-β2*: sense primer, 5′-CTGGAATATGGTTAATGTCAC-3′; antisense primer, 5′-GAGTTAGCT GCAGACACAG-3′ (408 bp); bovine *T-bet* [TC216567]: sense primer, 5′-CCTGGCACCCAACTGTCAACT-3′; antisense primer, 5′-GAAA CTCGGCCTCATAGCTG-3′ (172 bp); bovine *TGF-β1* [M36271]: sense primer, 5′-CCTTACATCTGGAGCCTGGA-3′; antisense primer, 5′-GTTGGACAACTGCTCCACCT-3′ (165 bp); and bovine *WC1* [X63723]: sense primer, 5′-TTCTCTGCCTTATCCTGGGGT-3′ or 5′-CGCTTCTCT TCCTGGTCC-3′; antisense primer, 5′-GGCAGTTAGAATATCTGAG GCTTCATCTC-3′. Bovine *IL-12R-β2* primers were based on sequences from White et al. (26), bovine *T-bet* was from the TIGR *Bos taurus* Gene Index ⟨http://tigrblast.tigr.org/tgi/⟩, and sequences coding for the transmembrane and cytoplasmic tail of bovine WC1 were based on data from Wijngaard et al. (15) and our pilot studies in this report. Analysis of sequences was performed using BioEdit version 5.0.6 ⟨http://www.mbio.ncsu.edu/BioEdit/bioedit.html⟩.

Statistical analyses

Statistical analyses were conducted using MINITAB (Minitab) and Microsoft Excel data analysis software. Where appropriate, the two-tailed Student's *t* test was used to detect significant differences.
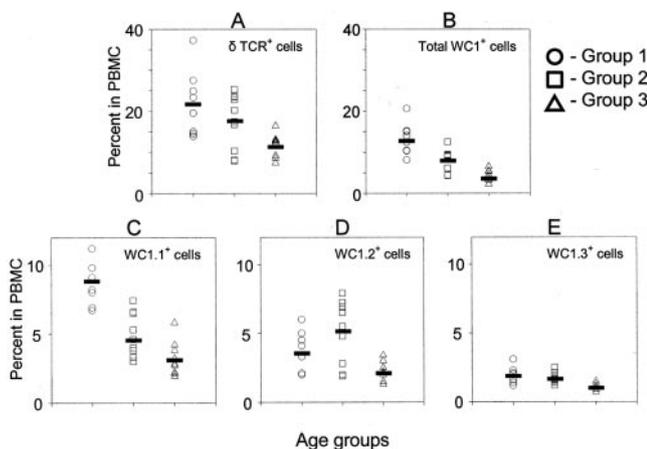
Results

Representation of WC1 variants on cells in animals of various ages

It has been established that the proportion of γδ TCR$^+$ cells bearing the lineage-specific determinant WC1 decreases within the PBMC population with age (32). In this study the relative proportions of total γδ T cells and those bearing variants of WC1 were evaluated for animals in three age groups representing newborns (0–2 mo), adolescents (15–21 mo), and adults (27–44 mo; Fig. 1). The groupings were based on preliminary data analysis, which indicated that large changes in the percentage of WC1$^+$ γδ T cells within PBMC occurred within the first few months of life, whereas considerably less variation occurred after 6 mo, and only small changes occurred in animals over 2 years of age. For all the lymphocyte populations represented in Fig. 1, the differences in percentages between the newborn and adult age groups were statistically significant ($p \leq 0.05$, by t test), except for WC1.2$^+$ cells ($p = 0.058$; Fig. 1D). When comparing the newborn and intermediate age groups, the mean decreases in percentage of total γδ T cells, WC1$^+$ γδ T cells, and WC1.1$^+$ γδ T cells were very similar (4.4, 4.7, and 4.2%, respectively), demonstrating that most of the decrease in γδ T cells during the first 2 years after birth was due specifically to a decrease in the WC1.1$^+$ cells. In contrast, the relative representative of WC1.2$^+$ cells did not decrease. Although the difference was not significant, the mean representation of WC1.2$^+$ cells was greater in the adolescent group compared with the newborn group.

Proliferation of cells in response to stimulation varies according to the form of WC1 expressed
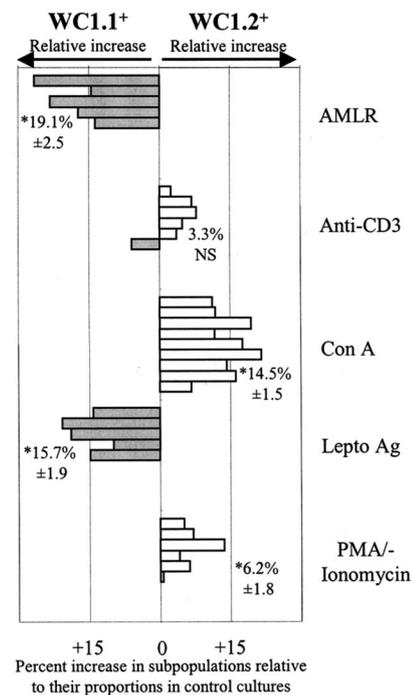
Given that WC1.1 and WC1.2 are mainly expressed on nonoverlapping subpopulations showing disparate patterns of change with animal aging, we hypothesized that these cells also differed functionally. Thus, their responses to several stimuli known to induce

proliferation of WC1$^+$ γδ T cells were evaluated. Subsequent to stimulation, the relative proportion of WC1.1$^+$ and WC1.2$^+$ cells was compared with that in medium control cultures for multiple experiments using PBMC from several different animals (Fig. 2). This method of comparison represents the sum total of cells within the WC1.1$^+$ and WC1.2$^+$ γδ T cell subpopulations that proliferated or survived without proliferation and excluded changes by other subpopulations of leukocytes found in the cultures. Compared with their relative representation in medium control cultures, representation of WC1.1$^+$ cells in AMLR cultures or those with leptospira Ag was significantly greater than that of WC1.2$^+$ cells. In contrast, Con A stimulation resulted in a greater relative representation of WC1.2$^+$ cells. Plate-bound anti-CD3 stimulation, which does not induce substantial proliferation of bovine γδ T cells (30), did not result in significant changes in the representation of either population relative to the other, whereas PMA/ionomycin favored only marginal increases in representation of WC1.2$^+$ cells. The change in representation of WC1.3$^+$ cells, a form of WC1 coexpressed on a subset of WC1.1$^+$ cells, followed the pattern of WC1.1$^+$ cells (data not shown).

To more clearly resolve the reason for the changes in relative representation of WC1.1$^+$ and WC1.2$^+$ cells in cultures with various stimuli, PBMC were loaded with CFSE and proliferation by the subpopulations determined using two-color flow cytometric analysis (Fig. 3). Although Con A stimulation induced proliferation of both WC1 subpopulations, the response by WC1.1$^+$ cells was consistently less vigorous than that of the WC1.2$^+$ cells (Fig.

FIGURE 1. Analysis of various γδ T cell populations within PBMC. Indirect immunofluorescence staining for surface expression of TCR δ-chain (*A*; mAb GB21A), all WC1 molecules (*B*; mAb IL-A29), WC1.1 (*C*; mAb BAQ159A), WC1.2 (*D*; mAb CACTB32A), and WC1.3 (*E*; mAb CACT21A) on PBMC from 30 animals is shown. Animals were subdivided by age into three groups, with similar numbers of animals per group: group 1 (○), 1 day to 2 mo; group 2 (□), 15–21 mo; and group 3 (△), 27–44 mo. The mean and SE for each age group in order are as follows for data represented in each panel: *A*) 22.0 ± 2.8, 17.6 ± 2.1, and 11.3 ± 1.1%; *B*) 13.3 ± 1.4, 8.6 ± 0.9, and 4.2 ± 0.5%; *C*) 9.0 ± 0.7, 4.8 ± 0.5, and 3.0 ± 0.5%; *D*) 3.6 ± 0.5, 5.1 ± 0.7, and 2.1 ± 0.2%; and *E*) 2.0 ± 0.2, 1.7 ± 0.1, and 1.1 ± 0.1%. Significant differences between groups 1 and 3 were found for data in *A*, *B*, *C*, and *E* by Student's t test ($p \leq 0.05$).

FIGURE 2. Proliferation of WC1$^+$ γδ T cell subsets in response to various stimuli. After culture of PBMC with the indicated stimuli, cells were stained by indirect immunofluorescence, and the percentages of cells bearing WC1.1 or WC1.2 relative to total WC1$^+$ cells were compared with those percentages in the unstimulated medium controls (see *Materials and Methods* for calculation). Bars represent results from separate experiments, and each was assessed using PBMC from three to six different animals (▨, relative representational increases of WC1.1$^+$ cells; □, relative representational increases in WC1.2$^+$ cells in stimulated cultures compared with unstimulated cultures). The mean and SEM of the relative increase in representation are indicated for each culture condition, and significance ($p \leq 0.05$) indicated by an asterisk.
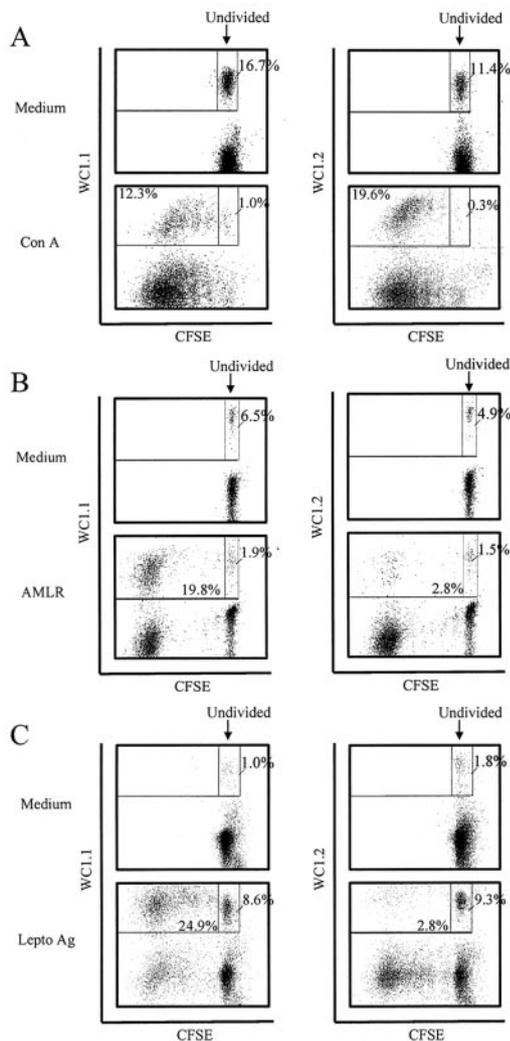
FIGURE 3. Proliferation of cultured WC1.1$^+$ and WC1.2$^+$ γδ T cells analyzed by CFSE. CFSE-loaded PBMC stimulated with Con A (*A*), AMLR (*B*), or leptospira Ag (*C*) and their accompanying medium control cultures were stained for cell surface phenotype WC1.1 or WC1.2 by indirect immunofluorescence (ordinate) and analyzed for cell division (abscissa) by flow cytometry. Results are presented as dot plots of 10,000 cells in each panel and are representative of experiments performed with PBMC from three different animals and typify responses represented by data reported in Fig. 2. Cells above the horizontal line were positive for the cell surface marker indicated, and dividing cells were those with decreased CFSE intensity. The percentage of cells in various regions is indicated. In some experiments CFSE loading resulted in greater fragility of the γδ T cells in medium cultures as culture time increased, as shown in *C*.

3*A*). Even in cultures in which cells bearing WC1.1 were the majority of ex vivo WC1$^+$ cells, culture with Con A favored the expansion of cells expressing WC1.2, so that by the end of the culture period they predominated. In contrast, AMLR and leptospira Ag stimulation resulted in almost exclusive proliferation by WC1.1$^+$ cells (Fig. 3, *B* and *C*).

Because changes in the proportion of cells bearing either WC1.1 or WC1.2 could theoretically be due to changes in receptor expression, WC1.1$^+$ or WC1.2$^+$ cells were sorted by MACS and evaluated ex vivo as well as after culture with Con A or medium for 4 days. Although the cells responded to the Con A stimulations, as assessed by CFSE-loading, there was no change in the expressed form of WC1 on the cells, i.e., WC1.1$^+$ cells did not acquire WC1.2 expression and vice versa, as assessed by immu-

nofluorescence staining of the cells after culture (data not shown). However, the sorted cells did have transcripts coding for the intracytoplasmic tail sequences of both forms (data not shown), suggesting that surface expression may be controlled at the level of translation and not transcription.

Variant of WC1 expressed corresponds to IFN-γ production

WC1$^+$ γδ T cells separated into subpopulations according to the variant of WC1 expressed reflected their relative abilities to proliferate in response to various stimuli. This suggested that the subpopulations might also differ in their cytokine profiles. Results of previous studies showed that γδ T cells in AMLR and leptospira Ag-stimulated cultures produced IFN-γ, and moreover, that almost all bovine γδ T cells producing IFN-γ were WC1$^+$ (3, 33). Three-color flow cytometry was used to evaluate IFN-γ production within the WC1.1$^+$ and WC1.2$^+$ populations (Fig. 4). After 6 days of culture with leptospira Ag, both WC1.1$^+$ and WC1.2$^+$ cells were present in the cultures in substantial proportions (Fig. 4A, *left panel*). However, almost all the WC1$^+$ cells producing IFN-γ expressed WC1.1 and not WC1.2 (Fig. 4A, *right panel*). The frequencies of WC1.1 single-positive and WC1.1/WC1.2 double-positive cells among the IFN-γ$^+$ population (Fig. 4A, *right panel*) were the same as among the total cells in culture (Fig. 4A, *left panel*). The potential for WC1.1$^+$ and WC1.2$^+$ cells to secrete IFN-γ was also evaluated using Th1-polarizing conditions. A previous study demonstrated that culture with IL-12 induced IFN-γ production in the absence of proliferation by bovine PBMC (26, 33). Again, the IFN-γ-producing WC1$^+$ cells were largely WC1.1$^+$, with a minor population of IFN-γ-producing WC1.2$^+$ cells (Fig. 4B, *right panel*). However, representation of WC1.2$^+$ cells within the IFN-γ$^+$ population was small, considering their much larger representation within the culture (Fig. 4B, *left panel*). Unlike in IL-12 cultures, where proliferation does not take place, in all experiments performed with leptospira Ag-stimulated cultures the intensity of WC1.1 staining on the IFN-γ$^+$ cells was intermediate to low, suggesting either that the level of WC1 may decrease with cell division or that the WC1.1-highly expressing
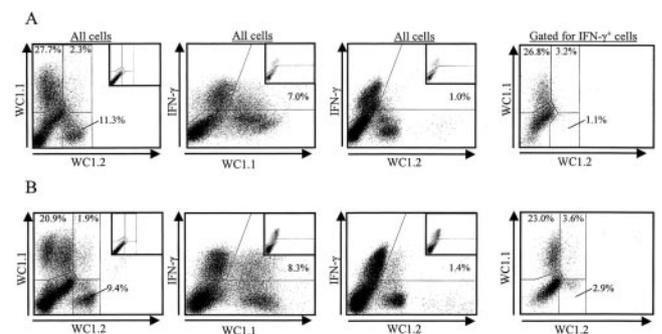
FIGURE 4. IFN-γ production by WC1.1$^+$ and WC1.2$^+$ γδ T cells in response to various stimuli. PBMC in leptospira Ag-stimulated (*A*) and IL-12-stimulated (*B*) cultures, stained via direct (zenon Alexa Fluor 499-labeled anti-WC1.2 mAb CACTB32A) and indirect (anti-WC1.1 mAb BAG25A with PE-conjugated isotype-specific secondary and biotin-labeled anti-IFN-γ mAb with streptavidin-PerCP) immunofluorescence, were analyzed via flow cytometry for the expression of WC1.1 and WC1.2 (50,000 events; *left-most panels*). The *middle panels* show IFN-γ staining vs WC1.1 (*middle-left*) or vs WC1.2 (*middle-right*). The *right-most panels* show the same cells as in the *left panels*, except that they include only those cells gated as IFN-γ$^+$. The percentage of cells positive for WC1.1 or WC1.2 is provided relative to the total number of cells for the panel. *Insets* located in the *upper right corner* show the same plot without primary Abs (controls) or, in the case of IFN-γ, without staining for WC1. Results are representative of three experiments performed for each culture condition.

cells tend not to divide and produce IFN-γ in response to leptospira Ag.

CD25 expression on cells expressing different WC1 variants

IL-12-dependent and -independent IFN-γ production is known to be regulated in part by IL-2 (34), which is required for sustained IFN-γ production (35). Thus, cells from the various cultures were evaluated for CD25 expression. The length of culture was varied inversely according to the strength of the stimulus, except for medium cultures that were evaluated along with Con A cultures. A very low level of CD25 was constitutively expressed on cells cultured in medium alone, with identical profiles found for WC1.1+ and WC1.2+ cells (Fig. 5, *column 1*). CD25 expression substantially increased on both WC1.1+ and WC1.2+ cells after only 1 day of culture with Con A relative to that on cells cultured with medium (Fig. 5, *column 2*), with the mean fluorescence intensity of CD25 being 2-fold greater on WC1.2+ cells than on WC1.1+ cells in Con A cultures. This reflected their levels of proliferation (see Fig. 3A), but not IFN-γ production, because Con A does not induce appreciable IFN-γ in WC1+ cells (33). IL-12-stimulated cultures showed almost 3-fold more CD25 expression on WC1.1+ cells than on WC1.2+ cells (Fig. 5, *column 3*), correlating with their production of IFN-γ (refer to Fig. 4) even in the absence of cell division (<1% of CFSE-loaded WC1+ cells divided after 3 days of culture with IL-12; data not shown). In Ag-stimulated cultures, the mean fluorescence intensity of CD25 staining was 2-fold higher on WC1.1+ cells compared with WC1.2+ cells. Therefore, IFN-γ production or cell division positively correlated with CD25 expression, although the lack of IFN-γ production could not be attributed to the lack of CD25 as seen in Con A cultures.

Evaluation of mRNA expression in WC1 subpopulations

Additional analyses assessed whether IFN-γ production correlated with the level of expression of *IL-12Rβ2*, which codes for a subunit of the high affinity IL-12R, or with transcription factors known to regulate IFN-γ. Cells were sorted without stimulation or after 48 h of stimulation with Con A or IL-12 into WC1.1+ or WC1.2+ populations, and transcription levels were evaluated us-

ing semiquantitative RT-PCR. In all three experiments conducted using cells from two different animals, *IFN-γ* expression was higher in WC1.1+ cells compared with WC1.2+ cells (Fig. 6). However, only marginally higher *IL-12Rβ2* expression was found in WC1.1+ cells compared with WC1.2+ cells. Similarly, although neither WC1.1+ nor WC1.2+ populations had increased *IFN-γ* transcript levels when stimulated with Con A, both expressed transcripts for *IL-12Rβ2,* indicating that gene expression for the high affinity IL-12R subunit alone does not determine IFN-γ production by these cells. Because the transcription factor T-bet positively regulates responses to IL-12 in CD4 T cells, whereas GATA-3 negatively regulates them and promotes Th2 cytokine production (36, 37), sorted populations were analyzed for transcript expression of their genes. *GATA-3* was constitutively transcribed at low levels without activation as well as by both populations after activation with IL-12 or Con A, although WC1.2+ cells expressed higher levels of *GATA-3* in Con A cultures, probably due to higher levels of activation of these cells compared with Con A-stimulated WC1.1+ cells (refer to Fig. 3A and Fig. 5, *column 2*). T-bet was not expressed in resting cells, but was expressed at similar levels in both populations after stimulation. Although T-bet and GATA-3 are generally associated with opposing cytokine responses, coexpression of *T-bet* and *GATA-3* transcripts in activated γδ T cells has previously been demonstrated (38), consistent with the results reported in this study. Moreover, *GATA-3* expression was not accompanied by *IL-4* expression, even though transcript expression of *IL-4* was present in the total PBMC population stimulated with Con A (Fig. 6).

Due to the increase in the percentage of WC1.2+ cells among PBMC in animals 15–21 mo of age compared with that in newborns, we hypothesized that this subpopulation might have a regulatory function, perhaps responsible for the accompanying decrease in the inflammatory WC1.1+ cells. To address this, sorted WC1 populations were evaluated for the expression of genes coding for the regulatory cytokines IL-10 and TGF-β (for review, see Ref. 39). We found *IL-10* expression in both WC1 subpopulations when cultured with IL-12, but minimally or not at all when cultured with Con A. Modestly higher *IL-10* expression was seen in WC1.1+ cells cultured with IL-12 compared with WC1.2+ cells, correlating with the higher activation levels as assessed by IFN-γ
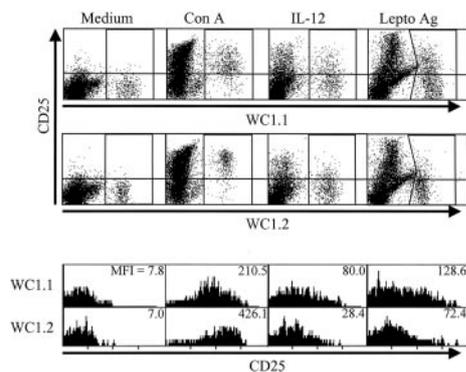
FIGURE 5. CD25 expression on WC1 subsets after culture with various stimuli. WC1+ subsets within PBMC were stained for CD25 expression after culture with medium or Con A (24 h), IL-12 (3 days), or leptospira Ag (5 days). Culture times were different depending upon the strength of the stimulus, except for medium control cultures, which were stained at 24 h because thereafter the cells may begin to die due to lack of stimulus. The *top two rows* represent the dot plots for two-color staining of CD25 vs the indicated form of WC1, whereas the *bottom panels* are histograms of the same cultures representing WC1.1+ (*top row*) or WC1.2+ (*bottom row*) cells only, with mean fluorescence intensity (MFI) shown (isotype control staining has an MFI of 2.9). Results are representative of three experiments performed.
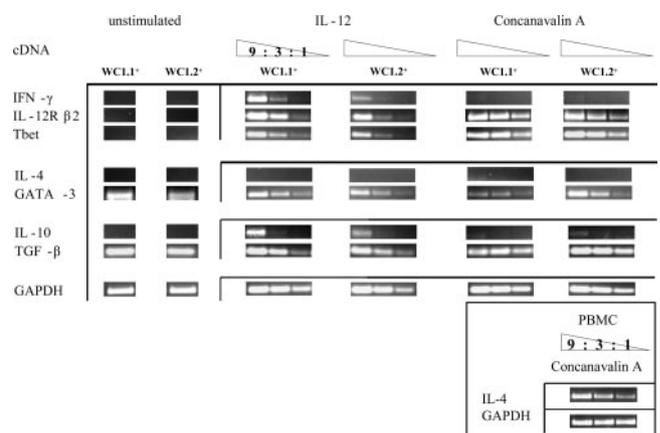
FIGURE 6. RT-PCR analysis of RNA from WC1.1+ and WC1.2+ cells. WC1.1+ and WC1.2+ cells were purified from PBMC ex vivo or after 2 days of culture with IL-12 or Con A and analyzed by RT-PCR. Three-fold serial dilutions of cDNA were used to PCR-amplify transcripts from stimulated cells, whereas only the highest concentration of cDNA was used for ex vivo cells.

production (see Fig. 4*B*) and CD25 expression (see Fig. 5). Interestingly, TGF-β was constitutively expressed with no apparent differences between the subpopulations whether stimulated or ex vivo (Fig. 6), suggesting that both subpopulations may have a regulatory role.

Evaluation of WC1 tail sequences

Although cDNA coding for the archetypal WC1 form, WC1.1, has been completely sequenced (14), that for WC1.2 and WC1.3 has not. Therefore, we cloned and sequenced cDNA derived from transcripts corresponding to the cytoplasmic tails of WC1.2 and WC1.3 (Fig. 7). The sequences were compared to determine whether there was variation in or adjacent to potential phosphorylation sites that might account for the differences in the responses of WC1.1$^+$ and WC1.2$^+$ subpopulations. All three tail sequences contained five tyrosines predicted from the cDNA sequence as well as the YEEL motif associated with binding Src homology 2 (SH2) domain-containing proteins. Additionally, all forms contained a proline-rich motif ([PXPXXPXP] in WC1.1 and WC1.2; [PXPXXPP] in WC1.3), associated with binding 3 domain-containing proteins and intracellular signaling (40). None of the tails had sites of significant homology to the CD4 or CD8 CXC motif necessary for Lck binding (41). Additionally, none of the sequences had a motif analogous to the VVYKKL of CD5, another SRCR family member; this motif is purported to be the docking motif for the second SH2 domain of SHP-1 in the killer inhibitory receptor (20), providing a mechanism by which CD5 inhibits lymphocyte responses.

Sequence differences among WC1 forms were found in the predicted amino acids upstream of the fourth tyrosine residue. Translation of cDNA corresponding to WC1.1 had a [DENY] sequence, whereas WC1.2 and WC1.3 contained [AENY] and [GEDY], respectively. The presence of aspartic acid three nucleotides 5' to those sequences in WC1.1, but not in WC1.2 or WC1.3, is reminiscent of the aspartic acid seen three nucleotides upstream of tyrosines in CD5, the autophosphorylation sites of the tyrosine kinases Lck and Fyn, and CD3γ, -δ, -ε, and -ζ. Thus, differences in the amino acids upstream could affect the efficiency and kinetics of phosphorylation of this tyrosine and, hence, signaling events.
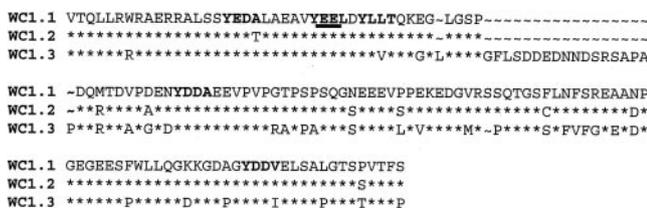
Discussion

Given that there are two major forms of WC1 expressed by largely nonoverlapping populations of ex vivo γδ T cells of ruminants, we hypothesized that WC1$^+$ γδ T cells are divided accordingly into functional subsets. The expression of different WC1 forms may serve to extend the repertoire of γδ T cells, because the TCR of γδ

T cells can be invariant within a tissue (10). If the hypothesis is correct, it might also provide an explanation for the disparity in results regarding the effect of modulation of WC1 on γδ T cell function using different cell systems (21, 22). The results presented in this study support the hypothesis, in that three main differences were found between WC1.1$^+$ and WC1.2$^+$ cells. They had different proliferation potentials to various stimuli, only WC1.1$^+$ cells showed a penchant for producing the inflammatory cytokine IFN-γ, and although WC1.2$^+$ cells initially increased in proportion in PBMC of young animals, WC1.1$^+$ cells decreased steadily with age. Although a previous study showed that IFN-γ$^+$ γδ T cells in cultures stimulated with leptospira Ags were WC1$^+$, it did not exclude the possibility that some WC1$^-$ γδ T cells may also produce IFN-γ given the appropriate stimulus, although the findings of Jutila et al. (6, 7) suggest that this is not likely to be the case.

The question of how alternate WC1 expression is involved in varying γδ T cell responses, especially with regard to IFN-γ production, remains to be answered. Although extracellular differences in WC1.1 and WC1.2, shown previously (15), might result in binding of alternate ligands on cells that direct immune responses, differences in cytoplasmic tail sequences may also affect intracellular signaling. In this study it was shown that transcripts corresponding to WC1 cytoplasmic tails showed potential SH2-binding sites with subtle sequence variations that could affect signaling. The alternative paradigm, that the binding of the different WC1 molecules to ligands on other cell types, such as dendritic cells or macrophages, results in differential functional development of these WC1 subpopulations can be argued from evidence in other systems. It has been established that both dendritic cells and macrophages have subpopulations with different cytokine secretion profiles that affect αβ T cell development into Th1 and Th2 functional subpopulations (42) as well as activation of human Vδ1 γδ T cells (43). It is possible that these APCs or other functionally differentiated cells also have different ligands for WC1 forms on their surface. Although we do not know the ligand for WC1, nor has a role for classical APCs been conclusively established for γδ T cells, it is reasonable to speculate that the ligand for WC1 may be on such cells or on other cells, such as keratinocytes, that have been shown to direct γδ T cell responses (44).

It is particularly interesting that the WC1.1$^+$ cells are the major IFN-γ producers, because the two subpopulations do not differ in many other characteristics that predict IFN-γ production. That is, they both have transcripts for the high affinity IL-12R, IL-12Rβ2, as well as for T-bet and GATA-3, transcription factors that polarize CD4 T cells into Th1 and Th2 functional subpopulations, respectively (36, 37). Although IFN-γ drives the initial development of Th1 CD4 T cells before the expression of IL-12Rβ2 (45), WC1.2$^+$ cells would have had access to IFN-γ in the cultures evaluated in this study because it is produced in high quantities in IL-12-stimulated cultures of bovine PBMC (33). Although the simultaneous transcript expression of T-bet and GATA-3 is contrary to that which occurs in CD4 T cells, simultaneous gene expression has been reported previously for γδ T cells (38). In that study both *T-bet* and *GATA-3* transcripts were made in activated murine γδ T cells, but only T-bet protein was found in Western blots of nuclear extracts from the same cells. It was concluded that GATA-3 transcription was not sufficient to cross-regulate T-bet-mediated IFN-γ production in activated γδ T cells. A similar event may be occurring in this study in WC1.1$^+$ cells. The regulatory mechanism for IFN-γ production in the WC1 subpopulations could also be related to activation of signal transducing molecules, such as STAT4. STAT4 is required for IFN-γ production in response to IL-12 and

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WC1.1  VTQLLRWRAERRALSSYEDALAEAVYEELDYLLTQKEG~LGSP----------------
WC1.2  ****************************T****************~****_____
WC1.3  ******R*****************************V***G*L****GFLSDDEDNNDSRSAPA

WC1.1  ~DQMTDVPDENYDDAEEVPVPGTPSPSQGNEEEVPPEKEDGVRSSQTGSFLNFSREAANP
WC1.2  ~**R****A***************************S****S***************C*******D*
WC1.3  P**R**A*G*D**********RA*PA**S****L*V****M*~P****S*FVFG*E*D*

WC1.1  GEGEESFWLLQGKKGDAGYDDVELSALGTSPVTFS
WC1.2  ****************************S****
WC1.3  ******P*****D***P****I****P***T****P
```

FIGURE 7. Sequence of cytoplasmic tails of WC1.1, WC1.2, and WC1.3. More than 30 cDNA sequences corresponding to the intracytoplasmic tails of known forms of WC1 were generated from ex vivo and cultured cells. Tail sequences were identified according to the partially complete genetic analysis of cloned WC1 (14). A single amino acid difference from the putative Wijngaard clone (14) is noted for WC1.2 (V136 to S136). The locations of tyrosine residues and potential SH2-binding sites are shown in bold. The sequence in italics shows the position of the potential SH3-binding site [PVPGTPSP].

presented Ags even when IL-12Rβ2 and T-bet are already expressed by the cell (46). Although speculative, WC1 cytoplasmic tails may act as docking sites for the SH2 domains of STAT4. Thus, subtle differences among these tail sequences could affect the ability of STAT4 to dock and thereby regulate the production of IFN-γ by WC1⁺ γδ T cells.

Although our results indicate that IFN-γ production potential is correlated with WC1.1 expression among γδ T cells, WC1.2⁺ cells were not found to be the Th2 counterpart, a logical prediction from the data. WC1.2⁺ cells did not express detectable *IL-4* mRNA when stimulated with Con A, even though its expression was detected in total PBMC in the same cultures. These transcripts are presumably products of CD4 T cells, as has been shown in previous studies in response to Con A stimulation (1). Although it is, of course, possible that other conditions might induce the expression of IL-4 in WC1.2⁺ cells, a Th1/Th2 functional polarization is also not supported by their different proportional representations in PBMC of animals in various age groups. That is, there is no precedent for a rise in Th2 cytokine-producing αβ T cells with age with a concomitant decrease in Th1 cells.

The increased representation of WC1.2⁺ cells within the PBMC population corresponded with a sharp decrease in WC1.1⁺ cells as well as with development of αβ T cell memory, as shown by an increase in CD4⁺ CD62L^low cells in bovine blood beginning by 2 mo of age.[4,5] However, both WC1.1⁺ and WC1.2⁺ subsets constitutively express high levels of CD62L while in circulation; thus, the two populations are not readily distinguished as effector memory cells vs naive cells. Also both WC1.1 and WC1.2 constitutively express CD45RO, and *CTLA-4* is expressed, but only after activation (see Footnote 4). Thus, these evaluations do not point to obvious explanations regarding the roles of the two subpopulations. However, because the decrease in WC1.1⁺ cells correlated with the acquisition of immunological experience by αβ T cells and thus a diminished need for WC1.1⁺ γδ T cells, we postulated that the rise in WC1.2⁺ cells might be consistent with a regulatory role akin to that which occurs for CD4⁺CD25⁺ αβ T cells. γδ T cells have been shown to have regulatory roles (reviewed in Refs. 47 and 48). For example, the presence of regulatory γδ T cells has been shown to minimize inflammation-associated tissue damage during infection (49, 50), and depletion of WC1⁺ γδ T cells from cattle results in increased Ab responses (51), whereas depletion from PBMC results in increased CD4 proliferation in response to Ag in vitro (52).

Of the four categories of regulatory αβ T cells identified, all produce IL-10 and TGF-β, with Th3 regulatory cells producing low IL-10 and high TGF-β (39). Both WC1⁺ γδ T cell subpopulations most closely match the Th3 cells according to mRNA transcript analyses, with no obvious difference in either *IL-10* or *TGF-β* expression levels between WC1.1⁺ and WC1.2⁺ cells. The simultaneous expression of *IFN-γ* and *IL-10* under Th1-polarizing conditions by WC1.1⁺ cells is similar to the results of Hseih et al. (50), who showed involvement of IFN-γ/IL-10-producing murine γδ T cells in *Listeria* infections and suggested that IL-10 is a regulator of Th1 responses regardless of whether a humoral Th2 response occurs. Although CD25, a marker for regulatory CD4 T cells, has been reported to be constitutively expressed at low levels on resting ex vivo γδ T cells (53), higher constitutive expression was not detected

on WC1.2⁺ cells compared with WC1.1⁺ cells in the group of animals examined in this study. Thus, although no striking differences were observed with regard to regulatory cytokines or constitutive CD25 expression, both WC1.1⁺ and WC1.2⁺ subpopulations could have a regulatory role in cattle through TGF-β production. TGF-β induces the conversion of naive CD4⁺ T cells into CD4⁺CD25⁺ T regulatory cells upon simultaneous stimulation through the TCR (54), and TGF-β produced by γδ T cells has been shown to inhibit CTL and NK tumoricidal activity (55).

In summary, WC1.1⁺ cells were shown to be the primary source of γδ-derived IFN-γ, consistent with previous, more general, reports demonstrating the potential of murine and bovine γδ T cells to secrete IFN-γ (25, 56). WC1.1⁺ cells may foster inflammatory responses in young animals by the direct effect of their IFN-γ on macrophages as well as the development of Th1 responses by Ag-stimulated CD4⁺ and CD8⁺ αβ T cells. Selective activation of a cell population prone to secrete IFN-γ during vaccination may provide a means to consistently stimulate type 1 immune responses and would be especially useful in vaccine design for intracellular bacterial and protozoan pathogens and viruses. To date, the induction of strong type 1 or cell-mediated responses usually requires live attenuated vaccines. Although advances are being made in the stimulation of dendritic cells and macrophages through TLRs to direct the immune system toward such a response, stimulation through appropriate WC1 coreceptors along with TCR may provide an alternative adjuvant effect.

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