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Modulation of WC1, a lineage-specific cell surface molecule of γ/δ T cells, augments cellular proliferation

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SUMMARY

WC1, also known as T19, is the only unique γ/δ T-cell differentiation antigen described to date other than the γ/δ T-cell receptor. We present evidence that modulation of WC1 results in augmented proliferation of γ/δ T cells. Immobilized IL-A29, a monoclonal antibody (mAb) specific for WC1, augmented proliferation of γ/δ T cells in the autologous mixed leucocyte reaction (AMLR) as well as proliferation induced by either anti-CD3 or anti-CD5 mAb. In contrast, anti-CD5 mAb did not increase proliferation in the AMLR even though both CD5 and WC1 are members of the scavenger receptor cysteine-rich family of proteins and are expressed by bovine peripheral blood γ/δ T cells. IL-A29 did not induce proliferation when assessed alone or in the presence of either phorbol myristate acetate (PMA) or interleukin-2. IL-A29 also did not induce detectable calcium mobilization when evaluated in the presence of monocytes, PMA, or following cross-linking of IL-A29 with anti-immunoglobulin antibody. We conclude that WC1 is a γ/δ T-cell lineage-specific cell-surface differentiation antigen which is involved in activation of γ/δ T cells using an as yet unidentified pathway.

INTRODUCTION

Many similarities exist between γ/δ and α/β T cells, including their functional attributes as well as the structure of their T-cell receptor (TCR) and its association with CD3, the main signal transducing element.[1–3] However, a number of cell-surface differentiation antigens that have been shown to be crucial for α/β T-cell activation by promoting adhesion and signal transduction are often absent from γ/δ T cells. These include CD4 and CD8 (for review see ref. 4) which act as coreceptors for the α/β TCR, promoting signal transduction by introducing $p56^{lck}$ into the vicinity of the TCR complex and possibly by stabilizing cellular associtions through their interaction with major histocompatibility complex (MHC) molecules on antigen-presenting cells.[5–8] The crucial roles of CD4 and CD8 have been demonstrated by the ability of monoclonal antibodies (mAb) reactive with these molecules to inhibit α/β T-cell responses.[9–12] Other cell surface molecules implicated in T-cell activation, including CD2, CD5 and CD6, are largely pan T-cell markers on mature α/β T cells but are present to variable degrees on γ/δ T cells. CD2 is found on human[13] but not bovine peripheral blood γ/δ T cells;[14] CD5 is expressed by bovine γ/δ cells[14] but only by a subpopulation of human γ/δ T cells;[15] CD6 is only expressed by a minor subpopulation of bovine γ/δ T cells.[16] Regarding function, CD2 facilitates adhesion to antigen-presenting cells by binding lymphocyte function-associated antigen-3 (LFA-3). Cross-linking of CD2 to the TCR/CD3 complex by antibodies enhances T-cell responsiveness.[17] Similar results have been obtained with antibodies to CD6,[18,19] while antibodies to CD5 enhance T-cell activation without being coaggregated with CD3, suggesting CD5 is an intimate part of the TCR-signalling complex.[20] The precise role of CD5 and CD6 in T-cell activation is unclear although antibodies to CD5 and CD6 synergize with phorbol myristate acetate (PMA) to activate T cells[19,21] and they can activate alone in the presence of monocytes.[18,22]

The significance of the absence of these particular cell surface differentiation antigens on γ/δ T cells is unclear at this time since relatively little is known about activation of γ/δ T cells including the nature of most molecules that are recognized by their TCR (see ref. 4). Direct activation of human γ/δ T cells through CD2 has been demonstrated, suggesting similarities with α/β T-cell activation although the details of the activation requirements differed.[23] It is also known that thymus-derived

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Abbreviations: AMLR, autologous mixed leucocyte reaction; Con A, concanavalin A; c.p.m., counts per minute; HBSS, Hanks' balanced salt solution; Indo-1 AM, indo-1 pentaacetoxymethyl ester; IFN, interferon; IL-2, interleukin-2; mAb, monoclonal antibody; MD-PBMC, monocyte-depleted peripheral blood mononuclear cells; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; SEM, standard error of the mean; SRCR, scavenger receptor cysteine-rich; TCR, T-cell receptor.

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γ/δ T cells require p56[lck] for maturation and activation,[24] a molecule largely associated with CD4 and to a lesser extent with CD8 in α/β T cells. However, in contrast to α/β T cells, γ/δ T cells generally are not MHC restricted[25] and non-polymorphic MHC molecules such as Qa and TL have been implicated as recognition elements for activation of γ/δ T cells.[26] Given this in conjunction with the fact that CD4 and CD8 function by interacting with MHC molecules, it is reasonable to speculate that surface molecules quite different from CD4 and CD8 are required by γ/δ T cells to interact with molecules on the surface of stimulatory cells and transduce activation signals. WC1, or T19, is a cell surface differentiation antigen uniquely expressed by γ/δ T cells[14,27] that might play such a role in lieu of 'absent' T-cell differentiation antigens.

WC1/T19 is the only unique γ/δ T-cell differentiation antigen described to date. Based on DNA sequence homology, WC1/T19 is a member of the scavenger receptor cystein-rich (SRCR) family of molecules exemplified by CD5 and CD6.[28] It is a heterodimer of 215000 and 300000 MW[29] with a large extracellular domain and a short intracytoplasmic tail.[28] It has been identified on both sheep and bovine γ/δ T cells by mAb that react with common epitopes[30] and mRNA for a homologous molecule has been identified in human and muring γ/δ T cells.[28] Experiments reported here evaluated the role of WC1 in signal transduction by evaluating the effect which antibodies to WC1 have on γ/δ T-cell responses including induction of proliferation and mobilization of calcium.

MATERIALS AND METHODS

Animals and cells

Blood donors were female *Bos taurus* Holsteins, 1 to 3 years of age. Blood was collected by venepuncture of the jugular vein either into heparin or it was defibrinated as described previously.[31] Peripheral blood mononuclear cells (PBMC) were separated from blood by density gradient centrifugation over Ficoll–hypaque (Ficoll-Paque, LKB-Pharmacia Biotechnology Inc., Piscataway, NJ) using standard techniques. Cells were suspended in tissue culture medium consisting of RPMI-1640, 10% heat-inactivated fetal calf serum (FCS), 2 μM L-glutamine, 60 μg/ml gentamicin and 5×10^{-5} M 2-mercaptoethanol. PBMC from defibrinated blood, which have fewer monocytes than those isolated from heparinized blood, were further depleted of monocytes by plastic adherence.[31] The plastic non-adherent population is referred to throughout as monocyte-depleted PBMC (MD-PBMC), whereas mononuclear cells isolated from heparinized blood contained 7–15% monocytes[31,32] and are referred to simply as PBMC.

Purification of γ/δ T cells

Cell composition was assessed by indirect immunofluorescence and analysed by flow cytometry or ultraviolet microscopy as previously described.[33,34] *Ex vivo* MD-PBMC were negatively-enriched for γ/δ T cells by affinity column purification using T-cell immunocolumns (Biotex Laboratories, Edmonton, Alta, Canada) according to manufacturer's instructions but with the following modifiecations. The cells were first incubated in a cocktail of monoclonal antibodies (mAb) that included IL-A12,[9] CACT138A and CACT83B that react with bovine CD4 (VMRD Inc., Pullman, WA); IL-A51 that reacts with bovine CD8;[10] IL-A15 that reacts with the monocyte marker CD11c; and IL-A58 that reacts with light chains of bovine immunoglobulin.[35] After passage over the column, the non-adherent cells were again incubated with the mAb cocktail and repassaged over the same column. A portion of the non-adherent cells was cultured with human recombinant interleukin-2 (IL-2) (10 units/ml) overnight at 37° in 5% CO_2 and the proportion of γ/δ T cells was determined the next day by reactivity with IL-A29 and other cell type-specific mAb listed above. The column-purified populations had between 93% and 97% IL-A29$^+$ cells.

γ/δ T-cell lines

Bulk autologous mixed leucocyte reactions (AMLR) were established in 24-well tissue culture plates by increasing the constituents tenfold from the AMLR established in 96-well plates[32] (see below). After 7 days of culture, cells were recultured at $1{\cdot}25 \times 10^6$ cells/ml with 10 units/ml of human recombinant IL-2 (Boehringer Mannheim Biochemicals, Indianapolis, IN). Cells were restimulated by addition of fresh IL-2 every 5 days. The restimulated γ/δ T-cell lines maintained specificity for reactivity in the AMLR and consisted of approximately 84% WC1$^+$ cells, 10% CD8$^+$ cells and 6% CD4$^+$ cells as assessed by immunofluorescence.

Proliferation assays

AMLR cultures were established in 96-well flat bottom microtitre plates as described previously[32] using 5×10^5 cells/well of MD-PBMC, affinity-column-enriched populations of γ/δ T cells at 10^5 cells/well or a γ/δ T-cell line at $6{\cdot}3 \times 10^4$ cells/well as responder cells. Stimulator cells were $1{\cdot}25 \times 10^5$ PBMC/well that had receved 5000 rads of γ/irradiation from a ^{137}Cs source prior to addition to the AMLR except where it is indicated that they were fixed with 1% paraformaldehyde.[36] Control cultures included responder cells with medium only to assess background proliferation. Triplicate cultures were established for each treatment and the amount of cell proliferation was measured by [^{3}H]TdR. The mean ± SEM of counts per minute (c.p.m.) of incorporated [^{3}H]TdR in replicate cultures was calculated for each treatment. Culture time was 6 days unless indicated otherwise.

PMA, ionomycin nd concanavalin A (Con A) (Sigma Chemical Co., St. Louis, MO) were dissolved according to the manufacturer's instructions, diluted in phosphate-buffered saline (PBS) and filter sterilized for use in proliferation assays. For proliferation, Con A was used at 1 μg/ml, ionomycin at 50 μg/ml and PMA was evaluated over a range of concentrations as indicated in the Results. They were used alone or in combination with one another or immobilized antibodies. Controls included cells with medium only to assess background proliferation. Responder cell populations included ex vivo MD-PBMC used at 5×10^5 cells/well, γ/δ T-cell populations purified by affinity columns used at 10^5 cells/well or a γ/δ T-cell line used at 6.3×10^4 cells/well and proliferation was assessed by [^{3}H]TdR as described above.

Cytokine measurements

Supernatants from AMLR cultures were assessed for IL-2 by evaluating their ability to induce proliferation of a bovine IL-2-dependent cell line during a 48-hr culture period. Proliferation of the IL-2-dependent cell line was assessed by incorporation of [^{3}H]TdR. Human recombinant IL-2 was used as a positive control. The presence of bovine IL-2 was assessed by adding

polyclonal antibody that neutralizes bovine IL-2 (courtesy of American Cyanamide, Princeton, NJ). Triplicate cultures were established for each treatment and the mean ± SEM of c.p.m./well of [^{3}H]TdR determined. Interferon-γ (IFN-γ) was measured in a cell protection assay similar to that described previously using Madin–Darby bovine kidney cells as indicators and vesicular stomatitis virus.[37] Human IFN-α (NIAID Repository, Biotech Research, Rockville, MD) was used as a positive control and to establish a standard curve relative to which the interferon activity in supernatants was expressed. IFN-γ activity was confirmed by acidification of AMLR culture supernatants and re-testing.[37]

Immobilization of antibodies

Murine mAb IL-A29 (IgG1) which reacts with WC1, IL-A25 (IgG1) which reacts with bovine monocytes, and BLT-1 (IgG2a) which reacts with bovine CD5 were purified by adherence to protein A/G, while anti-bovine CD3 mAb MM1A (IgG1) was purchased as clarified mAb (VMDR). Antibodies were diluted in sterile PBS to a concentration of 10 μg antibody/ml unless otherwise indicated. One hundred microlitres was added per well of 96-well flat bottom microtitre plates, incubated at 4° at least overnight, after which excess antibody was removed and the plates were used for proliferation assays. A standard enzyme-linked immunosorbent assay was performed to assess binding of mAb in similarly coated plates.

Calcium measurements

Optimal loading of lymphocytes with Indo-1 pentaacetoxymethyl ester (Indo-1AM; Molecular Probes, Junction City, OR) was established by titrating the Indo-1AM from 100 μM to 0.3 μM and assessing intracellular calcium concentrations by spectrofluorimetry in preliminary experiments and evaluating calcium mobilization by flow cytometry following perturbation with 1 μg ionomycin (Sigma), essentially as described elsewhere.[38,39] Optimal results were obtained by loading cells with 3 μM of Indo-1 AM for 45 min at 37°, following which they were pelleted by centrifugation and resuspended in Hanks' balanced salt solution (HBSS). Intracellular calcium mobilization was assessed by comparing the results of experiments conducted with cells suspended in HBSS containing calcium with those utilizing cells suspended in calcium-free HBSS (HBSS without Ca^{2+} or Mg^{2+} and with 10 mM EDTA). Cells were prewarmed to 37° for 5 min before analysis and maintained at 37° during analysis. Analyses were performed on the Coulter Epics Elite Cytometer (Coulter, Hialeah, FL) equipped with a Coherent Innova 305 water-cooled argon laser (Coherent, Palo Alto, CA). Using forward versus 90° light scatter characteristics, lymphocytes were gated from myeloid cells and debris. Indo-1 AM-labelled cells were excited at 351–364 nm and analysed at a rate of 400 to 600 cells/s to detect bound and free calcium levels at wavelengths of 405 and 495 nm, respectively. Baseline data were established for 1 min before addition of antibodies or PMA after which collection continued for an additional 7–9 min. Data were represented in a histogram display of ratio (405 nm/495 nm) versus time. Data were stored in list mode and extended analysis was performed using MultiTime software (Phoenix Flow Systems, San Diego, CA). Cell numbers were normalized per unit time. Monoclonal antibodies were added so that the final concentration was 10 μg/ml. Cross-linking antibodies were F(ab)$_2$ anti-mouse immunoglobulin and added to a final concentration of 25 μg/ml. PMA was added at 2·5 μg/ml. In all experiments where no calcium mobilization occurred following addition of antibodies and/or PMA, ionomycin was added afterwards to confirm thatthe cells were capable of mobilizing calcium in response to an appropriate stimulus (data not shown in results).

RESULTS

Effect of WC1 perturbation on responses in the AMLR

Although the *in vivo* significance of the AMLR is unknown, the proliferating cells in *in vitro* cultures are largely or entirely γ/δ T cells.[14,32] The AMLR is readily initiated by culturing autologous γ-irradiated monocytes with peripheral blood lymphocytes. To determine if the γ/δ T-cell lineage-specific glycoprotein WC1 plays a role in γ/δ T-cell activation, mAb IL-A29 reactive with WC1 was immobilized to culture wells containing AMLR cultures. The addition of immobilized IL-A29 but not the isotype-matched control mAb IL-A25 increased proliferation of γ/δ T cells in the AMLR when responder cell populations consisted of either *ex vivo* MD-PBMC, purified *ex vivo* γ/δ T-cell populations or a γ/δ T-cell line (Fig. 1). Immobilized mAb IL-A29 alone, however, did not directly stimulate proliferation of γ/δ T cells (Fig. 1a, b) even when IL-2 was present (see Fig. 1c). Augmentation of proliferation by IL-A29 was most readily observed when the proliferation in the AMLR was suboptimal as represented in Fig. 1a. In other experiments employing *ex vivo* MD-PBMC in which the AMLR proliferation approached maximal levels, the degree of enhancement by IL-A29 was similar to that represented in Fig. 1b, c, i.e. approximately 60%. Since both CD5 and WC1 are proposed to be members of the SRCR family and expressed on the surface of bovine peripheral blood γ/δ T cells, anti-CD5 mAb BLT-1, although a different isotype than IL-A29, was evaluated for its ability to augment proliferation in the AMLR as a control. In contrast to IL-A29, BLT-1 had no effect on proliferation in the AMLR (data not shown). Augmentation of γ/δ T-cell proliferation in the AMLR cultures by immobilized mAb IL-A29 did not result in alteration of the kinetics of the proliferative response, i.e. the peak day for proliferation still occurred after 6 days of culture. Similarly, evaluation of IL-2 and interferon γ (IFN-γ) production by biological assays suggested that secretion of cytokines was not increased in the presence of immobilized IL-A29 on the day peak production occurred in control cultures (day 5 for IL-2 and day 4 for IFN-γ) (Fig. 2). Rather, costimulation by IL-A29 appeared to delay the maximal production.

Ability of IL-A29 to augment anti-CD3 mAb-induced proliferation

While the above experiments indicated that immobilized anti-WC1 antibodies augment stimulatory signals provided in the AMLR, there is no formal proof that stimulation of γ/δ T-cell proliferation occurs via the TCR. Therefore we performed further experiments to evaluate the ability of immobilized IL-A29 to augment proliferation induced through the TCR complex by mAb reactive with CD3. The results indicate that immobilized IL-A29 also synergized with suboptimal concentrations of anti-CD3 mAb to enhance proliferation (Fig. 3).

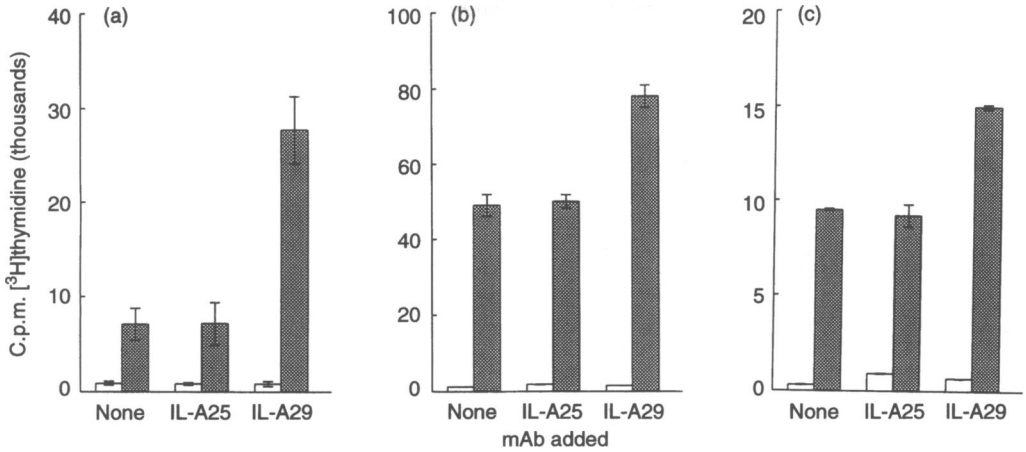

Figure 1. The AMLR was evaluated using (a) *ex vivo* MD-PBMC as responder cells (data represent mean and SEM of replicate cultures from one of four experiments); (b) *ex vivo* γ/δ T cells isolated by affinity columns as responder cells (data represent mean and SEM from replicate cultures representative of two experiments performed); or (c) a γ/δ T-cell line as responder cells with recombinant IL-2 (10 units/ml) included in all cultures (data represent the mean and SEM from triplicate cultures). AMLR cultures had γ-irradiated stimulator cells (solid bars) whereas medium control cultures had no γ-irradiated stimulator cells (open bars). The mAb added to the AMLR and medium control cultures is indicated on the abscissa. IL-A25 was included as an isotype control. Proliferation was measured by [^{3}H]TdR incorporation after 6 days of culture except for the γ/δ T-cell line in (c) which was evaluated after 3 days.

Ability of IL-A29 to synergize with PMA or ionomycin to induce proliferation

Since antibodies to a number of cell surface differentiation antigens stimulate proliferation of T cells when combined with protein kinase C activation by phorbol esters, and preceding experiments indicated that IL-A29-mediated signalling alone was not sufficient to stimulate proliferation, we evaluated the ability of IL-A29 to synergize with PMA to induce proliferation. We have shown elsewhere that PMA maximally synergizes with ionomycin when used at concentrations ranging from 50 pg/ml up to 5 μg/ml.[40] In experiments performed here, immobilized IL-A29 did not synergize with PMA at any of those concentrations to stimulate proliferation of either MD-PBMC (data not shown) or γ/δ T cells (Table 1), nor did IL-A29 synergize with ionomycin (Table 1). In contrast, anti-CD5 mAb BLT-1 synergizes with PMA[40] indicating that PKC activation in bovine lymphocytes can be augmented by

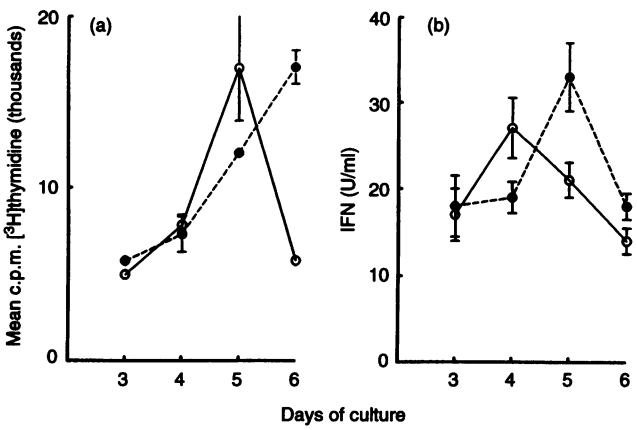

Figure 2. The effect of immobilized IL-A29 on cytokine production in the AMLR was evaluated. Supernatants from AMLR cultures employing γ/δ T cells as responders without mAb IL-A29 (solid line) or with immobilized mAb IL-A29 (broken line) present were evaluated for their ability to induce proliferation of an IL-2-dependent cell line as measured by incorporation of [^{3}H]TdR (a) or for their IFN-γ activity as assessed by a viral cytotoxicity assay (b). Results are representative of three experiments performed. IFN-γ was confirmed by acidification of AMLR supernatants which decreased the ability of the supernatants to protect Madin–Darby bovine kidney cells from a lethal vesticular stomatitis virus challenge by ⩾ 68% but had no effect on the protective activity of human recombinant IFN-α in control cultures. Similarly, polyclonal antibodies reactive with bovine IL-2 reduced the ability of AMLR supernatants to stimulate proliferation of the IL-2-dependent cell line by ⩾ 71% but had no effect on human recombinant IL-2-induced proliferation, confirming the presence of bovine IL-2. Paraformaldehyde-fixed stimulator cells were employed in the AMLR cultures and did not secrete detectable levels of either IL-2 or IFN-γ.

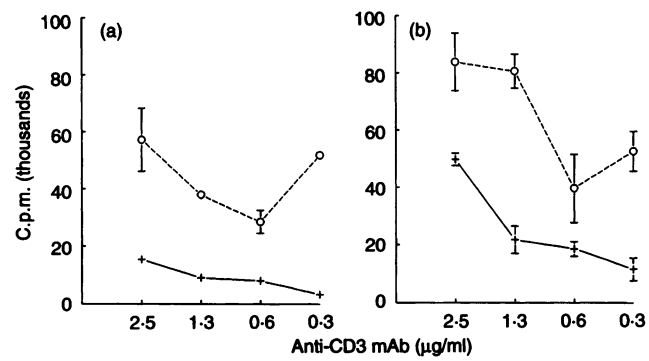

Figure 3. Proliferation induced by suboptimal concentrations of anti-CD3 mAb either alone (solid line) or with immobilized IL-A29 present (broken line) was evaluated using a γ/δ T-cell line (a) or *ex vivo* MD-PBMC (b). The concentration of IL-A29 used to coat wells was kept constant at 10 μg/ml while the concentration of anti-CD3 varied as indicated on the abscissa. Proliferation was determined after 72 hr by [^{3}H]TdR incorporation and data represent mean c.p.m. and SEM of three replicate cultures for each experiment presented. Background proliferation of cells in cultures containing medium alone or with IL-A29 mAb only present were (a) ⩽ 2200 ± 300 c.p.m. and (b) ⩽ 3200 ± 1600 c.p.m.

Table 1. Effect of IL-A29 on PMA and ionomycin-induced proliferative responses by γ/δ T cells*

PMA added (μg/ml)	Other additives			
	None	IL-A29	Ionomycin	Ionomycin + IL-A29
50	120 (54)	712 (46)	30 993 (4154)	ND
5	754 (118)	326 (60)	53 358 (3220)	ND
5×10^{-1}	407 (199)	440 (59)	41 033 (5420)	ND
5×10^{-2}	363 (28)	201 (42)	8908 (2889)	ND
5×10^{-3}	465 (267)	371 (194)	1322 (563)	ND
5×10^{-4}	309 (50)	375 (170)	1793 (360)	ND
5×10^{-5}	393 (124)	297 (30)	1253 (253)	ND
0	226 (72)	346 (96)	638 (607)	553 (380)

* γ/δ T cells isolated by affinity column purification were evaluated for proliferation as ascertained by [^{3}H]TdR incorporation after 72 hr of culture in the presence of PMA, ionomycin and/or immobilized mAb IL-A29. Data represent mean c.p.m. and SEM (in parentheses) of replicate cultures. Control cultures of γ/δ T cells stimulated with Con A had 59 300 ± 3500 c.p.m.; ND, not done.

stimulation through cell surface molecules belonging to the SRCR family, similar to that described in other species.[21]

In additional experiments we found that IL-A29 augmented both the low level γ/δ T-cell proliferation and the higher level of MD-PBMC proliferation induced by BLT-1 alone (Fig. 4). The large difference in the levels of proliferation induced by BLT-1 between cultures of γ/δ T cells and MD-PBMC may reflect both the lower number of cells employed in the γ/δ T-cell cultures as well as the lower expression of CD5 by γ/δ T cells relative to that by CD5$^+$ α/β T cells found within MD-PBMC populations.[41] The later cells also may have produced cytokines which additionally assisted γ/δ T-cell proliferation in the MD-PBMC cultures. There was no inherent difference in the potential for proliferation since the response to Con A was of similar magnitude for the two types of responder cell populations (data not shown).

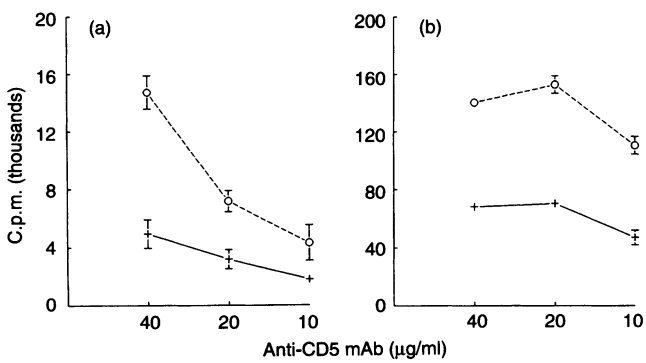

Figure 4. Ex vivo γ/δ T cells isolated by affinity column purification (a) or MD-PBMC (b) were used as indicator cells to evaluate proliferation induced by anti-CD5 mAb alone (solid line) and in conjunction with IL-A29 (broken line). The concentration of IL-A29 used to coat wells was kept constant at 10 μg/ml while the concentration of anti-CD5 mAb varied as indicated on the abscissa. Proliferation was determined after 72 by [^{3}H]TdR incorporation and data represent mean c.p.m. and SEM of three replicate cultures for each experiment presented. Background proliferation of cells in cultures containing medium alone or with IL-A29 mAb only present was (a) $\leqslant$ 2200 ± 700 c.p.m. and (b) $\leqslant$ 41 900 ± 2800 c.p.m.

Calcium mobilization

Monoclonal antibodies (mAb) to cell surface differentiation molecules that induce proliferation, including those to CD3 and CD5, also induce calcium mobilization (for review see ref. 42). Moreover, it has been demonstrated that while antibodies to CD28 have no direct calcium mobilizing activity, when combined with PMA they do so.[43] Thus, experiments were conducted here to evaluate the ability of IL-A29 to induce a calcium flux following cross-linking with anti-antibodies or in the presence of PMA. Initial experiments employing ionomycin to induce calcium flux in the presence or absence of extracellular calcium yielded a pattern similar to that reported with lymphocytes from other species (Fig. 5). The proportion of the response representing intracellular calcium mobilization was ascertained by comparing the result obtained with cells in medium continaing extracellular calcium (Fig. 5a) with that obtained with cells in calcium-free medium (Fig. 5b). Further experiments indicated that anti-CD3 mAb MM1A also induced calcium mobilization (Fig. 5c) although the response was slower and more sustained, as expected, than that measured with ionomycin in calcium-free medium (Fig. 5b). In contrast, mAb IL-A29 had no ability to induce a calcium flux either directly (Fig. 5d) or following cross-linking with anti-mouse antibodies (Fig. 5e). The anti-mouse antibodies used for cross-linking IL-A29 were shown to be effective cross-linkers in experiments utilizing Jurkat cells (data not shown). In the presence of PMA, mAb IL-A29 also did not have the ability to mobilize calcium (Fig. 5f).

DISCUSSION

Since γ/δ T cells often lack important adhesion and signal transducing molecules expressed by α/β T cells,[14,15] it is predictable that γ/δ T cells have other cell surface molecules that fulfil similar or analogous functions. Data presented here suggest a role for WC1 in signal transduction for activation of γ/δ T cells. Immobilized IL-A29, a mAb reactive with WC1, augmented proliferation of γ/δ T cells in several experimental systems. Because augmentation of anti-CD3-induced proliferation by IL-A29 did not require coaggregation of WC1 to the CD3/TCR complex, by analogy with reports for anti-CD5 mAb[20] WC1 may be part of the TCR complex. Moreover, it might be expected that WC1 would be most like CD5 and/or CD6, since based on DNA homology WC1 is a member of the SRCR family to which CD5 and CD6 also belong.[28]

However, because bovine γ/δ T cells have CD5,[14] WC1 is unlikely to have completely redundant properties with CD5. Results presented here supported this supposition since IL-A29 augmented anti-CD5-induced proliferation, suggesting that the two mAb stimulate complementary pathways. Furthermore, anti-CD5 mAb did not augment proliferation in the AMLR. Functional aspects of WC1 also differed from those of CD6. Unlike IL-A29, mAb reactive with CD6 have been shown to synergize with PMA to induce proliferation of lymphocytes from other animal species.[19] In addition, anti-bovine CD6

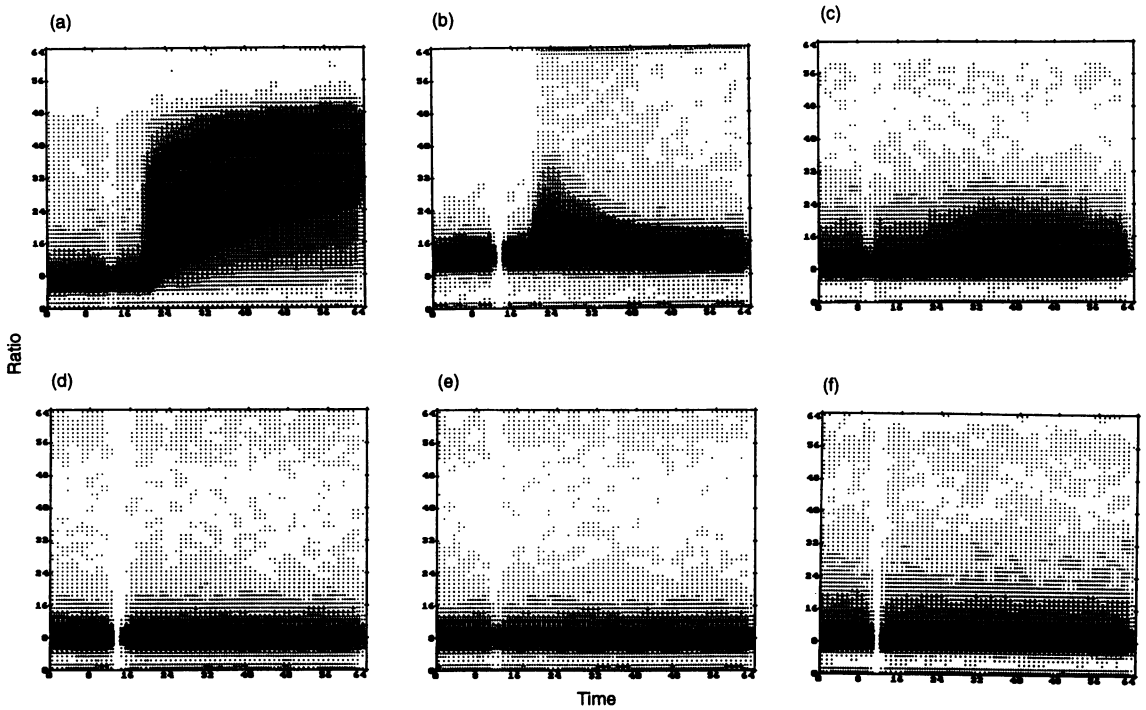

Figure 5. Effect of IL-A29 on calcium mobilization was measured by flow cytometry evaluating the ratio of violet : blue fluorescence over a 10-min assay period (the units of 0–64 are arbitrary increments set by the Phoenix software program[39]). In all assays, the point at which the stimulus (ionomycin, antibodies and/or PMA) was added can be visualized between increments designated 8 and 16 as the interval during which no cells or fewer cells were collected. PBMC containing approximately 10% monocytes were suspended in HBSS that contained calcium for panels a and c–f, while the experiment represented in panel b had no extracellular calcium. Stimuli added were (a) ionomycin, (b) ionomycin with cells in calcium-free medium, (c) anti-CD3 mAb MM1A, (d) mAb IL-A29, (e) continuation of cells in panel (d) with addition of $F(ab)_2$ fragments of a cross-linking anti-mouse immunoglobulin light chain antibody, and (f) mAb IL-A29 plus PMA. The results are depicted as a scattergram with increasing cell number represented by increasing number of dots.

mAb have been shown to stimulate proliferation of bovine lymphocytes if monocytes are present.[33] Unfortunately, it is not possible to do comparable experiments with IL-A29 due to the direct effects which bovine monocytes have on γ/δ T-cell proliferation (see ref. 32).

It might be expected that WC1 would stimulate pathways similar to those induced by perturbation of CD2, since unlike human γ/δ T cells, bovine peripheral blood γ/δ T cells lack CD2.[14] Furthermore, a molecule closely related to WC1 has been shown to be involved in adhesion,[44] a functional characteristic of CD2. A mAb to a single epitope of CD2 has been shown, however, to stimulate proliferation of human γ/δ T-cell clones in the absence of monocytes,[23] results which differ from those obtained in our studies with IL-A29. Such differences are not likely to be attributable to an inherent difference in the signalling capacity of CD2 or CD2-like molecules in cattle, since we have shown previously that anti-CD2 mAb conjugated to a substrate stimulate proliferation of bovine PBMC similar to that observed with human cells.[33]

Finally, WC1 did not have the characteristics of the important T-cell accessory molecules CD4 and CD8 or of the costimulatory molecules exemplified by CD28. Antibodies to CD4 and CD8 do not augment proliferation as IL-A29 did but, rather, as we have reported previously, anti-bovine CD4 mAb inhibit proliferation of bovine lymphocytes[9] similarly to that reported for other species.[10–12] The mAb reactive with CD28 also can inhibit proliferation of T-cell clones, while in the presence of PMA they induce calcium mobilization and proliferation of T cells (see ref. 43) which IL-A29 did not do.

In making the above comparison between IL-A29 and mAb to other cell surface differentiation antigens, i.e., CD2, CD4, CD5, CD6 and CD28, it should be noted, that differences in functional responses may occur even among mAb to the same cell-surface molecule depending upon the epitope of the molecule recognized or the isotype of the mAb. In addition, differences in functional effects occur even when the antibody isotype and antigen epitope recognized are identical as shown by studies with anti-CD2 mAb.[23] While several isoforms of WC1 are present on γ/δ T cells, IL-A29 reacts with an epitope expressed by many isoforms.[28,30] Perhaps different responses would be induced by mAb reactive with different epitopes of WC1.

It is possible that the effects we have observed with antibodies to WC1 could be due to alterations in the cytoskeleton as a result of the interaction of γ/δ T cells with immobilized IL-A29. Alterations of this nature may result in aggregation of other receptors involved directly in signalling with the TCR/CD3 complex, as suggested previously by Ratcliffe *et al.*[45] Such an aggregation might subsequently result in heightened responses to other signals. If this is the mechanism by which the effects of IL-A29 are mediated it may

be unique to the WC1 molecule since anti-CD5 mAb did not have this effect in the AMLR. Presently we can conclude that WC1 is a γ/δ T-cell lineage-specific cell-surface differentiation antigen which augments activation of these cells using an as yet to be identified signalling pathway.

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REFERENCES

1. Bank I., DePinho R.A., Brenner M.B., Cassimeria J., Alt F.W. & Chess L. (1986) A functional T3 molecule associated with a novel heterodimer on the surface of immature human thymocytes. *Nature* **322,** 179.
2. VanNeerven J., Coligan J.E. & Koning F. (1990) Structural comparison of α/β and γ/δ T cell receptor-CD3 complexes reveals identical subunit interactions but distinct cross-linking patterns of T cell receptor chains. *Eur J Immunol* **20,** 2105.
3. Borst J., van de Griend R.J., Oostveen J.W. *et al.* (1987) A T-cell receptor γ/CD3 complex found on cloned functional lymphocytes. *Nature* **325,** 683.
4. Haas, W., Pereira P. & Tonegawa S. (1993) Gamma/delta cells. *Annu Rev Immunol* **11,** 637.
5. Gay D., Maddon P., Sekaly R. *et al.* (1987) Functional interaction between human T-cell protein CD4 and the major histocompatibility complex HLA-DR antigen. *Nature* **328,** 626.
6. Newell M.K., Haughn L.J., Maroun C.R. & Julius M.H. (1990) Death of mature T cells by separate ligation of CD4 and the T-cell receptor for antigen. *Nature* **347,** 286.
7. Rudd C.E., Trevillyan J.M., Dasgupta J.D. & Schlossman S.F. (1988) The CD4 receptor is complexed to a T-cell-specific tyrosine kinase (pp58) in detergent lysates from human T lymphocytes. *Proc Natl Acad Sci USA* **85,** 5190.
8. Veillette, A., Bookman M.A., Horak E.M., Samelson L.E. & Bolen J.B. (1989) Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase $p56^{lck}$. *Nature* **338,** 257.
9. Baldwin, C.L., Teale, A.J., Naessens J., Goddeeris B.M., MacHugh N.D. & Morrison W.I. (1986) Characterization of a subset of bovine T lymphocytes that express BoT4 by monoclonal antibodies and function: similarity to lymphocytes defined by human T4 and murine L3T4. *J Immunol* **136,** 4385.
10. Ellis, J.A., Baldwin C.L., MacHugh N.D. *et al.* (1986) Characterization by a monoclonal antibody and functional analysis of a subset of bovine T lymphocytes that express BoT8, a molecule analogous to human CD8. *Immunology* **58,** 351.
11. Shimonkevitz R., Luescher B., Cerrottini J.-C. & Robson MacDonald H. (1985) Clonal analysis of cytolytic T lymphocyte-mediated lysis of target cells with inducible antigen expression: correlation between antigen density and requirement for Lyt-2/3 function. *J Immunol* **135,** 892.
12. Wilde D.B., Marrack P., Kappler J., Dialynas D.P. & Fitch F.W. (1983) Evidence implicating L3T4 in class II MHC antigen reactivity; monoclonal antibody GK 1.5 (anti-L3Ta) blocks class II MHC antigen-specific proliferation, release of lymphokines and binding by cloned murine helper T lymphocyte lines. *J Immunol* **131,** 2178.
13. Inghirami G., Zhu B.Y., Chess L. & Knowles D.M. (1990) Flow cytometric and immunohistochemical characterization of the gamma/delta T lymphocyte population in normal human lymphoid tissue and peripheral blood. *Am J Pathol* **136,** 357.
14. Clevers H., MacHugh N.D., Bensaid A. *et al.* (1990) Identification of a bovine surface antigen uniquely expressed on $CD4^-CD8^-$ T cell receptor γ/δ T lymphocytes. *Eur J Immunol* **20,** 809.
15. Spour E.F., Leemhuis T., Jenss L., Diamond R., Fillak D. & Jansen J. (1990) Characterization of normal human $CD3^+$ $CD5^-$ and γ/δ T cell receptor positive T lymphocytes. *Clin Exp Immunol* **80,** 114.
16. Baldwin C.L., Morrison W.I. & Naessens J. (1987) Differentiation antigens and functional characteristics expressed by bovine leukocytes. In: *Comparative Aspects of Differentiation Antigens in Lympho-haemopoietic Tissues.* (eds Z. Trnka & M. Miyasaka), p. 455. Marcel Dekker, Inc., New York.
17. Yang S.Y., Chouaib S. & Dupont B. (1986) A common pathway for T lymphocyte activation involving both the CD3-Ti complex and CD2 sheep erythrocyte receptor determinants. *J Immunol* **137,** 1097.
18. Gangemi R.M., Swack J.A., Gaviria D.M. & Romain P.L. (1989) Anti-T12, an anti-CD6 monoclonal antibody, can activate human T lymphocytes. *J Immunol* **143,** 2439.
19. Morimoto C., Rudd C.E., Letvin N.L., Hagan M. & Schlossman S.F. (1988) 2H1-a novel antigen involved in T lymphocyte triggering. *J Immunol* **140,** 2165.
20. Ledbetter JA., Martin P.J., Spooner C.D. *et al.* (1985) Antibodies to Tp67 and Tp44 augment and sustain proliferative responses of activated cells. *J Immunol* **135,** 2331.
21. Vandenberghe P. & Ceuppens J.L. (1991) Immobilized anti-CD5 together with prolonged activation of protein kinase C induce interleukin 2-dependent T cell growth: evidence for signal transduction through CD5. *Eur J Immunol* **21,** 251.
22. Alberola, I.J., Places L., Cantrell D.A., Vives J. & Lianzano F. (1992) Intracellular events involved in CD5-induced human T cell activation and proliferation. *J Immunol* **148,** 1287.
23. Wesselborg S., Janssen O., Pechold K. & Kabelitz D. (1991) Selective activation of γ/δ^+ T cell clones by single anti-CD2 antibodies. *J Exp Med* **173,** 297.
24. Penninger J., Kishihara K., Molina T. *et al.* (1993) Requirement for tyrosine kinase $p56^{lck}$ for thymic development of transgenic γ/δ T cells. *Science* **260,** 358.
25. Strominger J.L. (1989) The γ/δ T cell receptor and Class Ib MHC-related proteins: enigmatic molecules of immune recognition. *Cell* **57,** 895.
26. Porcelli S., Brenner M.B., Greenstein J.L., Balk S.P., Terhorst C. & Belicher P.A. (1989) Recognition of cluster differentiation 1 antigens by human $CD4^-CD8^-$ cytolytic T lymphocytes. *Nature* **341,** 447.
27. Mackay C.R., Maddox J.F. & Brandon M.R. (1986) Three distinct subpopulations of sheep lymphocytes. *Eur J Immunol* **16,** 19.
28. Wijngaard P.L.J., Metzelaar M.J., MacHuch N.D., Morrison W.I. & Clevers H.C. (1992) Molecular characterization of the WC1 antigen expressed specifically on bovine $CD4^-CD8^-$ γ/δ T lymphocytes. *J Immunol* **149,** 3273–3277.
29. Mackay C.R., Beya M.F. & Matzinger P. (1989) γ/δ T cells express a unique surface molecule appearing late during thymic development. *Eur J Immunol* **19,** 1477.
30. Davis W.C., Hamilton M.J., Park Y., Larsen R.A. & Wyatt C.R. (1990) Ruminant leukocyte differentiation molecules. In: *MHC, Differentiation antigens and Cytokines in Animals and Birds. Monographs in Animal Immunology* (ed O. Barta), p. 47. BAR-LAB Inc., Blacksburg.
31. Goddeeris B.M., Baldwin C.L., Ole-Moiyoi O. & Morrison W.I. (1986) Improved methods for purification and depletion of monocytes from bovine peripheral blood mononuclear cells. Functional evaluation of monocytes in response to lectins. *J Immunol Meth* **89,** 165.

32. GODDEERIS, B.M., MORRISON W.I., NAESSENS J. & MAGONDU J.G. (1987) The bovine autologous mixed leucocyte reaction: a proliferative response of non-T cells under the control of monocytes. *Immunobiology* **176,** 47.
33. BALDWIN C.L., MACHUCH N.D., ELLIS J.A., NAESSENS J., NEWSON J. & MORRISON W.I. (1988) Monoclonal antibodies which react with bovine T-lymphocyte antigens and induce blastogenesis: tissue distribution and functional characteristics of the target antigens. *Immunology* **63,** 439.
34. LALOR P.A., MORRISON W.I., GODDEERIS B.M., JACK R.M. & BLACK S.J. (1986) Monoclonal antibodies identify phenotypically and functionally distinct cell types in the bovine lymphoid system. *Vet Immunol Immunopathol* **13,** 121.
35. WILLIAMS D.J., NEWSON J. & NAESSENS J. (1990) Quantitation of bovine immunoglobulin isotypes and allotypes using monoclonal antibodies. *Vet Immunol Immunopathol* **24,** 267.
36. SUTTLES J., CARRUTH L.M. & MIZEL S.B. (1990) Detection of IL-1α and IL-1β in the supernatants of paraformaldehyde treated human monocytes. Evidence against a membrane form of IL-1. *J Immunol* **144,** 170.
37. RUBINSTEIN S., FAILLETTI P.C. & PESTKA S. (1981) Convenient assays for interferons. *J Virol* **37,** 755.
38. RABINOVITCH P.S., JUNE C.H., GROSSMANN A. & Ledbetter J.A. (1986) Heterogeneity among T cells in intracellular free calcium responses after mitogen stimulation with PHA or anti-CD3. Simultaneous use of indo-1 and immunofluorescence with flow cytometry. *J Immunol* **137,** 952.
39. JUNE C.H. & RABINOVITCH P.S. (1991) Measurement of intracellular ions by flow cytometry. In: *Current Protocols in Immunology* (eds J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach & W. Strober), p. 5.5.1. John Wiley and Sons, New York.
40. YANG T.J., BALDWIN C.L., HANBY-FLARIDA M., MATHER J.F. & RABINOVSKY E.D. (1995) Monoclonal antibody BLT-1, specific for the bovine homologue of CD5, reacts with the majority of mature T cells, a subpopulation of B cells and stimulates T cell proliferation. *Dev Comp Immunol* **19,** 337.
41. HOWARD C.J., SOPP P., PARSONS K.R. & FINCH J. (1989) In vivo depletion of BoT4 (CD4) and of non-T4/T8 lymphocyte subsets in cattle with monoclonal antibodies. *Eur J Immunol* **19,** 757.
42. JUNE C.H. & RABINOVITCH P.S. (1988) Flow cytometric measurements of cellular ionized calcium concentration. *Pathol Immunopathol Res* **7,** 409.
43. JUNE C.H., LEDBETTER J.A., LINSLEY P.S. & THOMPSON C.B. (1990) Role of CD28 receptor in T-cell activation. *Immunol Today* **11,** 211.
44. WALCHECK B., WATTS G. & JUTILA M.A. (1993) Bovine γ/δ T cells bind E-selectin via a novel glycoprotein receptor: first characterization of a lymphocyte/E-selectin interacton in an animal model. *J Exp Med* **178,** 853.
45. RATCLIFFE M.J.H., COGGESHALL K.M., NEWELL M.K. & Julius M.H. (1992) T cell receptor aggregation, but not dimerization, induces increased cytosolic calcium concentrations and reveals a lack of stable association between CD4 and the T cell receptor. *J Immunol* **148,** 1643.