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Monocytes control γ/δ T-cell responses by a secreted product

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SUMMARY

Gamma-irradiated *ex vivo* bovine monocytes induce proliferation of γ/δ T cells in the autologous mixed lymphocyte reaction (AMLR), whereas when not irradiated they prevent this response. In contrast, non-irradiated autologous monocytes have no effect on bovine α/β T-cell proliferation in the allogenic MLR suggesting that the regulation is specific for γ/δ T-cell responses. Here, we showed that the inhibition was not mediated by inducing cell death and that the ability of *ex vivo* monocytes to prevent proliferation of γ/δ T cells was not generalized in that γ/δ T cells still responded to mitogenic stimulation. Inhibition of the AMLR by non-irradiated monocytes could not be overcome by addition of interleukin-2 to the cultures or by costimulation with antibodies to WC1, a γ/δ T-cell-specific cell-surface differentiation antigen shown elsewhere by us to be involved in activation of γ/δ T cells. Furthermore, we showed that monocytes inhibited γ/δ T-cell responses via a soluble product since inhibition occurred even when monocytes and γ/δ T cells were separated by membranes of transwells or when supernatants from monocyte cultures were added to AMLR cultures. Maximal secretion of the inhibitory product by the monocytes occurred during the first 6 hr of *in vitro* culture at 37°, rapidly decreased thereafter, and did not occur when monocytes were incubated at 4°. The inhibition was not attributable to nitric oxide, reactive oxygen intermediates, prostaglandin E_2 or transforming growth factor-β (TGF-β) but the ability of monocyte supernatants to mediate inhibition was sensitive to heating at 65°. Lipopolysaccharide and granulocyte–macrophage colony-stimulating factor activation of monocytes temporarily abrogated their ability to inhibit proliferation. In contrast, heat-shocking had no effect on their ability to inhibit. We hypothesize that non-irradiated monocytes produce the inhibitory material *in vivo* in order to regulate γ/δ T-cell responses to self-derived monocyte membrane components, but that when monocytes are altered by infection, transformation, irradiation, or cytokine activation, production of the inhibitor is temporarily suspended allowing stimulation of γ/δ T cells to occur.

INTRODUCTION

There are two major lineages of T cells: α/β T cells, which constitute the majority of T cells in adults and recognize foreign peptides presented by autologous major histocompatibility complex molecules and γ/δ T cells which are prominent in the blood of young ruminants[1] and at epithelial surfaces (see ref. 2 for review). γ/δ T cells have been shown to localize at sites of microbial infections (for review see ref. 3) and to proliferate and secrete cytokines following stimulation with autologous cells.[2,4–6] However, their role in protective immunity and the antigens they recognize, with the exception of heat-shock proteins,[2,4] are largely undefined.

Even less is known about the regulation of γ/δ T-cell responses. Anergized γ/δ T cells are found *in vivo*[9,10] and it has been shown that many γ/δ T cells undergo apoptosis following activation *in vivo*,[10] suggesting that these are mechanisms by which γ/δ T-cell responses are regulated. *In vitro* studies have shown that non-irradiated monocytes can prevent proliferation of bovine γ/δ T cells[7] in response to stimulation by γ-irradiated monocytes in the autologous mixed lymphocyte reaction (AMLR).[7,8] Although the mechanisms responsible for these effects are unknown, it is known that T cells can be anergized as a result of stimulation through the T-cell receptor (TCR) in the absence of costimulation (for review see ref. 11) while other inappropriate signalling sequences have been shown to result in anergy, apoptosis, or arrest in all stages of the cell cycle.[12–14] In addition, several macrophage products have been shown to regulate T-cell responses. These include hydrogen peroxide,[15] nitric oxide,[16] prostaglandins[17] and transforming growth factor-β (TGF-β),[18,19] which act by oxidizing cellular substrates

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Abbreviations: AMLR, autologous mixed lymphocyte reaction; cRPMI, complete RPMI; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFA, immunofluorescence assay; MD-PBMC, monocyte-depleted peripheral blood mononuclear cells; N^GMMLA, N^G-monomethyl-L-arginine.

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resulting in apoptosis,[20,21] or by inhibiting cellular respiration, calcineurin function or cyclin E-dependent kinase activity, respectively. The goal of studies presented here was to ascertain the manner by which monocytes prevent proliferation of γ/δ T cells in the AMLR. We present evidence that the regulation of γ/δ T-cell responses by *ex vivo* monocytes occurs as a result of induction of unresponsiveness or anergy via a secreted monocyte product that is not reactive oxygen or nitrogen intermediates, TGF-β, or prostaglandin.

MATERIALS AND METHODS

Animals and peripheral blood mononuclear cells

Blood donors were female *Bos taurus* Holsteins, 2 years of age. Cattle were kept in an open holding pen. Blood was collected by venepuncture of the jugular vein either into a solution of heparin or it was defibrinated as described previously.[22] Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation of blood over a solution of Ficoll–Hypaque (Ficoll–Paque, Pharmacia LKB Biotechnology Inc., Piscataway, NJ) using standard techniques. Mononuclear cell populations isolated from defibrinated blood were further depleted of monocytes by incubating in polystyrene tissue culture flasks for 1 hr at 37°. Non-adherent cells contained less than 2% monocytes and are referred to throughout as monocyte-depleted PBMC (MD-PBMC). PBMC from heparinized blood contained 7–15% monocytes.[22]

Culture medium

Cells were generally cultured in complete RPMI (cRPMI) consisting of RPMI-1640 with 10% heat-inactivated fetal calf serum, 2 μM L-glutamine, 60 μg/ml gentamicin and 5×10^{-5} M 2-mercaptoethanol. Where noted, Sigma Hybrimax medium (Sigma Chemical Company, St Louis, MO) with 60 μg/ml gentamicin was employed.

Purification of γ/δ T cells and indirect immunofluorescence

MD-PBMC were enriched to approximately 97 ± 3% γ/δ T cells by negative selection over a mouse T-cell immunocolumn (Biotex Laboratories, Edmonton, Canada) essentially as described by the manufacturer. To remove non-γ/δ T cells, PBMC were incubated with a cocktail of monoclonal antibody (mAb) which included anti-CD4 mAb IL-A12,[23] CACT83B and CACT138A (VMRD, Pullman, WA), anti-CD8 mAb IL-A51,[24] anti-CD2 mAb IL-A42, anti-CD11c mAb IL-A15 and IL-A58 which reacts with bovine immunoglobulin λ and κ light chains.[25] Cells were treated with two rounds of incubation with the mAb cocktail and column passage, using a single column for both passages. Indirect immunofluorescence assays (IFA) using cell-type-specific mAb were analysed by flow cytometry or ultraviolet microscopy as described[23] using the above mAb and IL-A29 which reacts with the bovine γ/δ T-cell WC1 surface protein.[8]

Isolation of monocytes

Monocytes were isolated from PBMC by adherence to plasma-coated gelatine as described.[22] The adherent population contained 70–90% monocytes, as determined by esterase staining. To heat-shock monocytes, they were incubated at 46° for 5 min as described by others.[26] To activate monocytes, they were cultured for either 4 hr or overnight with 20 units/ml of human recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF) (Boehringer, Mannheim, Indianapolis, IN) or 100 μg/ml lipopolysaccharide (LPS) (Sigma). Activated monocytes were washed before addition to the AMLR cultures.

Proliferation assays

AMLR cultures were established as described previously[7] using MD-PBMC or purified γ/δ T cells as responder cells. Stimulator cells were PBMC-isolated from blood that had been collected in heparin and therefore contained monocytes or monocytes purified by plasma-gel adherence. Stimulator cells received 5000 rads of γ-irradiation or were fixed with paraformaldehyde[27] before addition to the AMLR cultures. Control cultures that did not receive stimulator cells were prepared for each experiment to assess background levels of proliferation of responder cells and are referred to throughout as 'medium control'. Proliferation by concanavalin A (Con A) was induced with 1 mg/ml of Con A (Sigma). Where indicated, non-irradiated *ex vivo* monocytes isolated by plasma-gel adherence were added to cultures such that they were equivalent to 10% of the responder cell concentration. Where indicated, recombinant human interleukin-2 (rIL-2) at 10 units/ml was added to AMLR cultures.

Transwell assays

Transwell cultures were prepared in 24-well tissue culture plates with 0·4 μm nucleopore membrane cell cultures inserts in the wells (Costar, Cambridge, MA). AMLR were established in the lower portion of the well (5×10^6 responder cells/$1{\cdot}25 \times 10^6$ γ-irradiated PBMC as stimulator cells) or in the upper portion of the well (5×10^5 responder cells/$1{\cdot}25 \times 10^5$ stimulator cells). Non-irradiated *ex vivo* monocytes were added to the other portion of the transwells such that the final proportion constituted 10% of the total responder cell number, a concentration shown previously to inhibit the AMLR.[7] Control cultures were also prepared with monocytes added directly to cultures or without autologous monocytes. The cultures were maintained in a humidified atmosphere at 37° in 5% CO_2 in air. After 5 days, aliquots of the cultures were transferred to 96-well microtitre plates and the amount of cell proliferation was measured by incubating the cells overnight with 0·5 μCi of [^{3}H]thymidine per culture well. Preliminary experiments indicated that transfer of the cells after 4 days of culture did not alter the level of proliferation measured (A. Okragly, unpublished observation). The mean ± SD of c.p.m. of [^{3}H]thymidine incorporated in triplicate cultures was determined.

Evaluation of known T-cell suppressor elements

Inhibitors of various monocyte products were added to AMLR cultures with or without non-irradiated *ex vivo* monocytes as follows: superoxide dismutase at a concentration of 100 μg/ml, catalase at 100 μg/ml, N^G-mono-methyl-L-arginine (N^GMMLA) at 500 μM, and indomethacin at 1 μg/ml. TGF-β was neutralized by adding 10 μg/ml of polyclonal anti-TGF-β antibodies (R & D Systems, Minneapolis, MN) to the culture system. Monocyte supernatants were collected after various times and assessed for nitrite, a rapid conversion product of nitric oxide, by the Griess reaction as described.[28]

Indomethacin and anti-TGF-β antiserum were tested in murine systems to ensure activity since neither are species specific.

Evaluation of antibodies to WC1

Affinity-purified mAb IL-A29 was diluted in sterile phosphate-buffered saline (PBS), pH 7·0 to a concentration of 10 μg/ml. One hundred microlitres of the mAb/PBS solution was added per well of 96-well flat bottom microtitre plates. The plates were incubated at 4° at least overnight. To assess binding of the mAb to the well, a standard enzyme-linked immunosorbent assay (ELISA) was done with similarly coated plates.[29] When ready for use, the antibody solution was removed, the wells rinsed with PBS and AMLR cultures established as indicated above.

Transfer of monocyte supernatants

Monocytes were suspended at 5×10^5 cells/ml in cRPMI and incubated in polypropylene tubes for various times at 4° or 37° as indicated. Where not specified, supernatants were generated from cells incubated for 6 hr at 37°. After incubation, monocyte suspensions were centrifuged at 1000 *g* for 10 min, the supernatant collected and transferred without storage to AMLR cultures.

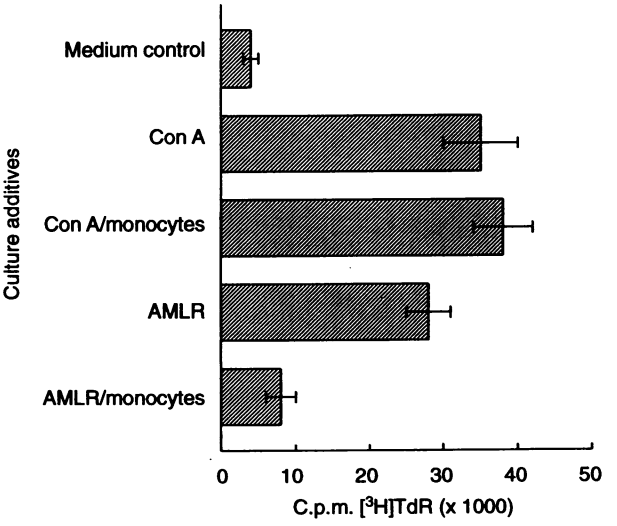

Figure 1. Responses by γ/δ T cells purified by affinity columns to stimulation with Con A or γ-irradiated monocytes in the presence or absence of *ex vivo* monocytes. The decrease in response of the AMLR with monocytes was significant ($P \leq 0{\cdot}004$) as determined by the Mann–Whitney *U* test.

RESULTS

Monocytes prevent proliferation of γ/δ T cell in an AMLR but not in response to Con A stimulation

In AMLR cultures that contained >10% non-irradiated *ex vivo* autologous monocytes, the amount of proliferation of γ/δ T cells as measured by [^{3}H]thymidine incorporation was decreased by at least 50%. After 6 days of culture, the percentage of cells positive for WC1, the bovine γ/δ T-cell lineage-specific cell surface differentiation antigen, was determined by IFA. The proportion was 67% in the control AMLR cultures but only 32% in AMLR cultures that contained non-irradiated monocytes, a percentage similar to that found in resting PBMC. However, in the Con A-stimulated control cultures, there was no significant difference in the amount of [^{3}H]thymidine incorporated regardless of whether or not non-irradiated monocytes were present (data not shown; four experiments performed). Immunofluorescence analysis of cells in the Con A-stimulated cultures indicated that the percentage of cells positive for WC1, was also similar with and without monocytes present: control Con A cultures were 57% γ/δ T cells; Con A cultures with non-irradiated monocytes were 51% γ/δ T cells (the percentage of γ/δ T cells was lower than in the AMLR since α/β T cells also proliferate to Con A). When purified populations of γ/δ T cells were similarly evaluated, again γ/δ T cells proliferated in the presence of the non-irradiated autologous monocytes when they were stimulated with Con A but not when stimulated with irradiated PBMC (Fig. 1). Analysis of cells in monocyte-suppressed AMLR cultures with trypan blue after 1 week indicated that the γ/δ T cells were viable. Together the above results indicated that the γ/δ T cells were prevented by non-irradiated monocytes from responding specifically to the AMLR stimulus but were not induced to undergo programmed cell death or killed by a toxic monocyte product. The addition of human rIL-2, which has T-cell growth factor activity for bovine T cells, did not overcome the suppressive effect of non-irradiated *ex vivo* monocytes on the AMLR (data not shown; two experiments performed). Monoclonal antibody IL-A29 reactive with WC1, a cell-surface marker found on bovine γ/δ T cells and shown elsewhere to be involved in signal transduction,[29] also was unable to overcome the inhibition of the AMLR mediated by non-irradiated autologous monocytes (data not shown).

The unresponsiveness is induced by a secreted monocyte product

Initial experiments showed that non-γ-irradiated paraformaldehyde-fixed *ex vivo* monocytes did not inhibit γ/δ T-cell proliferation (data not shown), but rather stimulated proliferation indicating that the stimulator molecule but not the inhibitory function was mediated by a constitutively expressed membrane molecule.[30] In additional experiments reported here, the non-irradiated monocytes were separated from the responder and stimulator cell populations by a 0·4 μm membrane in transwells. In these cultures the γ/δ T-cell proliferation was decreased, although it was not as pronounced as in cultures with non-irradiated monocytes added directly to the AMLR (Table 1). IFA analysis indicated that control AMLR cultures contained 69% γ/δ T cells, AMLR cultures with non-irradiated monocytes added into the same culture wells contained only 42% γ/δ T cells, and AMLR with non-irradiated monocytes separated by the transwell membrane contained 47% γ/δ T cells (Experiment 1, Table 1).

Supernatants from monocytes incubated for 6 hr at 37° also transferred suppression, whereas those incubated at 4° did not, suggesting that secretion requires metabolically active cells (Table 2). In contrast, the monocytes themselves could no longer inhibit after incubation at 37° whereas those incubated at 4° could still do so (Table 2). In further experiments,

Table 1. Evaluation of the ability of non-irradiated monocytes to inhibit the AMLR across transwells

Exp no.*	Medium	Control wells†		Transwells‡	
		AMLR	AMLR + non-irradiated monocytes	AMLR	AMLR + non-irradiated monocytes
1	984 ± 170§	36211 ± 383	2647 ± 1318	31477 ± 6438	5471 ± 1438
2	706 ± 195	63342 ± 9736	4620 ± 599	62455 ± 2984	12514 ± 8563
3	412 ± 259	60960 ± 2726	28281 ± 3922	71884 ± 893	43246 ± 1147

* Experiments 1 and 2 were done with cells from animal #65, experiment 3 with cells from #66.

† In column indicated, non-irradiated monocytes were added directly to wells containing AMLR responder and stimulator cells (γ-irradiated monocytes).

‡ In column indicated non-irradiated monocytes were separated from the AMLR cultures by a transwell membrane.

§ Mean ± SD of c.p.m. of [^{3}H]thymidine incorporation in replicate cultures. The decrease in proliferation in the AMLR when monocytes were added directly or when separated by transwells was significant ($P \leqslant 0{\cdot}005$) as determined by the Mann–Whitney U test.

Table 2. Effect of preincubation of non-irradiated monocytes at 4° or 37° on their ability or their culture supernatants to inhibit the AMLR*

Exp no.	Medium	AMLR	AMLR + non-irradiated monocytes	Non-irradiated monocytes†		Monocyte supernatants‡	
				4°	37°	4°	37°
1	1837 ± 294§	27659 ± 5241	3486 ± 193	2622 ± 542	29538 ± 6442	30776 ± 4520	5891 ± 455
2	471 ± 239	31467 ± 8101	6754 ± 911	7793 ± 1089	29664 ± 985	24871 ± 7421	4338 ± 802
3	423 ± 43	19627 ± 2269	1117 ± 413	768 ± 423	13445 ± 9298	19746 ± 2267	1621 ± 413

* Experiment 1 was performed with cells from animal #65, experiments 2 and 3 with cells from #66.

† Monocytes were preincubated for 6 hr at either 4° or 37° and then transferred to AMLR cultures following washing.

‡ The supernatants from monocytes cultured for 6 hr at either 4° or 37° were transferred to AMLR cultures.

§ Mean ± SD of [^{3}H]thymidine incorporation of replicate cultures. The decreases in proliferation in the AMLR following addition of non-irradiated monocytes, non-irradiated monocytes incubated at 4° or supernatants from the monocytes incubated at 37° were significant ($P \leqslant 0{\cdot}004$) as determined by the Mann–Whitney U test. There was no significant decrease in the proliferation in the AMLR in cultures with addition of monocytes that had been incubated at 37° or supernatants from monocytes incubated at 4° ($P > 0{\cdot}05$).

Table 3. Evaluation of the ability of non-irradiated monocytes to inhibit the AMLR after pre-incubation for various times

Exp no.*	Medium	AMLR	AMLR with non-irradiated monocytes	Time non-irradiated monocytes preincubated at 37° before addition to AMLR cultures (hr)			
				2	4	6	8
1	1837 ± 294†	27659 ± 5241	3486 ± 193	2224 ± 186	1962 ± 589	5801 ± 1732	19346 ± 1694
2	471 ± 239	31467 ± 8101	6754 ± 911	5412 ± 1715	4326 ± 812	3752 ± 473	16643 ± 1941

* Experiment 1 was performed with cells from animal #65, experiment 2 with cells from #66. Monocytes were washed three times following preincubation before addition to AMLR cultures.

† Mean ± SD of c.p.m. of [^{3}H]thymidine incorporation in replicate cultures. The decrease in proliferation measured in the AMLR when freshly-isolated non-irradiated monocytes or pre-incubated non-irradiated monocytes (all time lengths) were added was significant ($P \leqslant 0{\cdot}01$) as determined by the Mann–Whitney U test.

monocytes pre-incubated at 37° for various periods of time before addition to AMLR cultures began to lose their ability to inhibit the proliferation of the AMLR cultures after 6 hr of preincubation, although partial inhibition could still be mediated by monocytes preincubated for as long as 8 hr (Table 3). These results suggested that the inhibitory element was largely secreted during the first 8 hr at 37°. Culture supernatants from monocytes incubated in Teflon tubes, to

Table 4. Effect of various inhibitors on non-irradiated-monocyte-mediated suppression of the AMLR

Exp no.*	Medium	AMLR	AMLR with non-irradiated monocytes (inhibitors added)†				
			None	SOD	Cat	NMMLA	Indo
1	246 ± 93‡	35006 ± 4074	1149 ± 662	1437 ± 543	608 ± 290	680 ± 299	715 ± 143
2	142 ± 35	16860 ± 4318	238 ± 26	373 ± 75	179 ± 90	400 ± 65	395 ± 84

* Experiment 1 was performed with cells from animal #65, experiment 2 with cells from #66.

† Cat, catalase; Indo, indomethacin; NMMLA, N^G-monomethyl-L-arginine; SOD, superoxide dismutase.

‡ Mean ± SD of c.p.m. of [^{3}H]thymidine incorporation in replicate cultures. The decrease in proliferation in the AMLR with monocytes, regardless of the presence of inhibitors, was significant ($P \leqslant 0{\cdot}001$) as determined by the Mann–Whitney U test.

which they do not adhere, for 6 hr at 37° also inhibited the AMLR (medium 536 ± 30, AMLR 26698 ± 661, 'Teflon tube' supernatant added to the AMLR 12585 ± 1986) indicating that adherence and/or activation of monocytes by adherence is not required. Finally, significant suppression was also mediated by supernatants of monocytes cultured in protein-free/serum-free medium, i.e. Sigma Hybrimax medium (data not shown), indicating it is not produced as a result of monocyte activation by serum constituents.

Addition of inhibitors of known T-cell suppressor molecules produced by mononuclear phagocytes

When superoxide dismutase and catalase, which degrade superoxide anion and hydrogen peroxide, respectively; N^GMMLA, which inhibits formation of nitric oxide; or indomethacin, which prevents prostaglandin E_2 production, were added to AMLR cultures containing non-irradiated *ex vivo* monocytes no diminution in the ability of the monocytes to inhibit γ/δ T-cell proliferation was observed (Table 4). Proliferation occurred in control AMLR cultures in the presence of these inhibitors (data not shown) indicating that the non-responsiveness of the γ/δ T cells was not due to toxicity of the inhibitors. In addition, no nitrite, an indicator of nitric oxide production, was detected above background levels in supernatants collected after 6, 24, 48, or 72 hr from AMLR cultures containing non-irradiated monocytes (data not shown).

The ability of supernatants to inhibit proliferation was destroyed when incubated at 65° or 100° for 5 min, suggesting it was proteinaceous in nature. Further experiments were conducted to determine if it was TGF-β, since this is a protein secreted by macrophages and which inhibits T-cell proliferation. Neutralizing antibodies to TGF-β, added to cultures at levels shown to be effective in other systems, did not prevent the inhibition (data not shown). Further attempts to characterize or purify the inhibitory element were not made at this time, since the activity was labile when stored at 4° overnight or frozen at either −20° or −70°, perhaps due to the low concentration of the active element in culture supernatants.

Effect of heat stress and activation on production of the inhibitory element

Further experiments were performed to define the conditions necessary to prevent or, alternatively, amplify secretion of the inhibitory element. Stressing monocytes by warming to 42° for 5 min, a protocol shown by others to induce expression of a heat-shock response,[26] did not destroy their ability to inhibit the AMLR (Table 5). IFA of cells in AMLR cultures indicated that control cultures contained 72% γ/δ T cells, AMLR

Table 5. Effect of activation of non-irradiated monocytes or heat-shocking on their ability to inhibit the AMLR*

Time treated non-irradiated monocytes incubated prior to addition to AMLR	Medium	AMLR	Treatment given to non-irradiated monocytes before addition to AMLR			
			None	LPS	GM-CSF	Heat
4 hr	902 ± 169†	21391 ± 1166	6092 ± 1087	19922 ± 3118	14631 ± 1676	—
24 hr	1387 ± 385	27357 ± 5023	6808 ± 975	6771 ± 1753	2486 ± 851	—
5 min	142 ± 35	16860 ± 4318	238 ± 26	—	—	559 ± 43

* This is representative of two experiments performed with cells from animal #66.

† Mean ± SD of [^{3}H]thymidine incorporation of replicate cultures. The decrease in proliferation in the AMLR cultures when treated monocyte that had been heat-shocked or cultured for 24 hr with LPS or GM-CSF (but not those cultured for 4 hr) were added was significant ($P \leqslant 0{\cdot}004$), as determined by the Mann–Whitney U test.

cultures with non-irradiated monocytes contained 29% γ/δ T cells, and AMLR cultures with heat-shocked monocytes contained 31% γ/δ T cells. (The percentage of γ/δ T cells found in MD-PBMC ranged between 27% and 40% before culture in the AMLR.) In contrast, monocytes activated with either LPS or GM-CSF for 4 hr before addition to AMLR cultures had a diminished ability to inhibit the AMLR although they regained the ability to do so after overnight incubation even though the activators were present during the overnight culture (Table 5). We cannot discount the fact that some carry-over of the reagents may have occurred.

DISCUSSION

Our data indicate that non-irradiated *ex vivo* monocytes prevent γ/δ T-cell proliferation in response to the monocyte stimulatory molecule in the AMLR via a secreted product that results in unresponsiveness of γ/δ T cells but not cell death. The state of unresponsiveness is not generalized since the cells respond to Con A stimulation. We hypothesize that the monocyte inhibitory/suppressor element is active in non-irradiated monocytes *in vivo* thereby preventing γ/δ T-cell responses to the constitutively expressed stimulatory molecule (see Fig. 2). Further, we propose that when monocytes are altered, e.g. by infection with intracellular pathogens, following phagocytosis or by activation with cytokines, the production of the inhibitory element would be abrogated thus allowing γ/δ T-cell proliferation in response to monocyte stimulatory molecules to proceed. Such responses to constitutively expressed stimulatory molecules would contribute to the first line of immunological defence, suggested by other to be the role of γ/δ T cells.[31] This later proposal is supported by data presented here which demonstrated that LPS and GM-CSF activation of monocytes temporarily suspended their ability to inhibit proliferation and that the inhibitory element is not reactive oxygen or nitrogen intermediates, TGF-β, or prostaglandins, which are all products of activated macrophages.

Although heat-shock treatment affects the metabolism of cells by initiating synthesis of several proteins[32] and might also have been expected to be an additional signal for preventing the production of the regulatory element, it did not do so. However, as discussed above, γ-irradiation does so.[7] Gamma-irradiation is known to alter expression of genes[33] and cause double-strand breaks in the DNA[34] which may inhibit the transcription of the mRNA coding for the inhibitory element. Gamma-irradiation also stimulates phosphorylation of tyrosine substrates[35] by kinases that include p53, the 'tumor suppressor' nuclear protein.[36] Phosphorylation events may disrupt the signalling pathway for production of the inhibitory element or its activity may be regulated directly by phosphorylation. Similar regulation of the inhibitory element by phosphorylation may also occur in macrophages following activation by cytokines, a process known to involve a number of phosphorylation events.

We also showed that paraformaldehyde-fixed monocytes do not have the ability to inhibit proliferation of γ/δ T cells. It has been shown by others that similarly fixed cells secrete pre-formed molecules.[27] Thus, the lack of secretion of paraformaldehyde-fixed monocytes may indicate that the inhibitory element is not stored in large quantities within the monocyte but must be actively synthesized. Other experiments indicated that monocytes had to be metabolically active, i.e. incubated at 37°, to produce the inhibitory element but that production decreased over time during *in vitro* culture. This suggests that other signals are necessary to maintain either its production and/or secretion for prolonged periods of time. Such signals might be provided by endothelial cells of blood vessels or lymphoid organs through which the monocytes would normally circulate or contact *in vivo*.

Unlike α/β T-cell anergy induced by TCR stimulation in the absence of co-stimulatory activity[37] which disrupts IL-2 gene transcription and which can be overcome with exogenous IL-2,[11] inhibition of γ/δ T-cell responses by the monocyte inhibition element evaluated here was not abrogated by addition of IL-2. Furthermore, it was not overcome by supplemental signalling through the accessory molecule WC1, a lineage-specific differentiation antigen of γ/δ T cells.[8] Other studies have shown that γ/δ T cells anergized *in vivo* also cannot be restored to responsiveness by IL-2[10] and in other instances IL-2 has been shown to be the cause of γ/δ T-cell anergy, occurring when IL-2 is received prior to TCR stimulation.[38] Our current efforts are focused on evaluating the nature of the γ/δ T-cell unresponsiveness induced by the inhibitory element as well as on characterizing the monocyte product responsible for it.

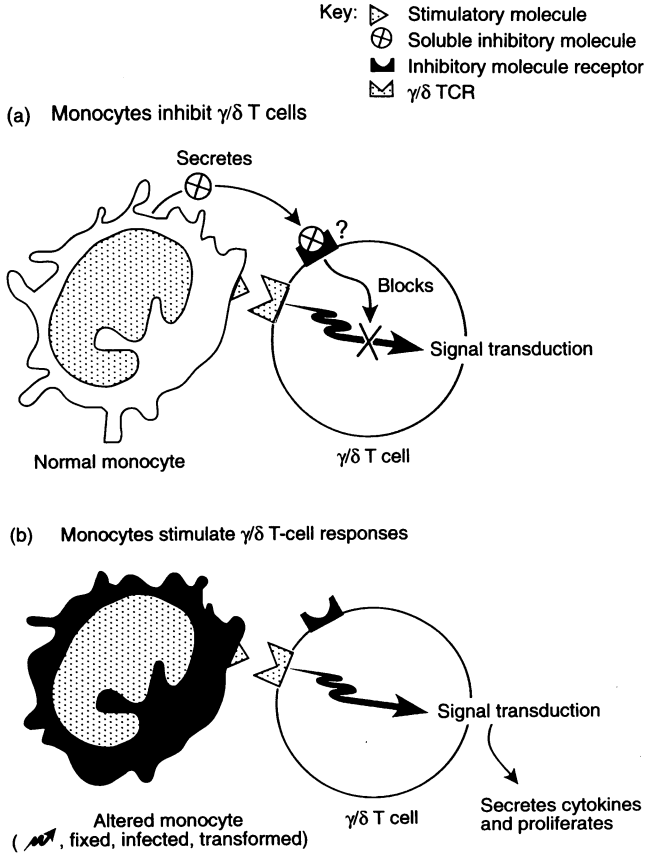

Figure 2. Diagrammatic model of our hypothesis regarding the expression of the monocyte molecule that stimulates bovine γ/δ T-cell proliferation (a and b) and monocyte regulation of the proliferative response by 'unaltered' monocytes (a) but not by 'altered' monocytes (b).

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