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Regulation of T-cell activation in the lung: alveolar macrophages induce reversible T-cell anergy *in vitro* associated with inhibition of interleukin-2 receptor signal transduction

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SUMMARY

Alveolar macrophages (AM) are recognized as archetypal 'activated' macrophages with respect to their capacity to suppress T-cell responses to antigen or mitogen, and this function has been ascribed an important role in the maintenance of local immunological homeostasis at the delicate blood : air interface. The present study demonstrates that this suppression involves a unique form of T-cell anergy, in which 'AM-suppressed' T cells proceed normally through virtually all phases of the activation sequence including Ca^{2+} flux, T-cell receptor (TCR) modulation, cytokine [including interleukin-2 (IL-2)] secretion and IL-2 receptor (IL-2R) expression. However, the 'suppressed' T cells fail to up-regulate CD2, and do not re-express normal levels of TCR-associated molecules after initial down-modulation; moreover, they are unable to transduce IL-2 signals leading to phosphorylation of IL-2R-associated proteins, and remained locked in G_0/G_1. The induction of this form of anergy is blocked by an NO-synthase inhibitor, and is reversible upon removal of AM from the T cells, which then proliferate in the absence of further stimulation. We hypothesize that this mechanism provides the means to limit the magnitude of local immune responses in this fragile tissue microenvironment, while preserving the capacity for generation of immunological memory against locally encountered antigens via clonal expansion of activated T cells after their subsequent migration to regional lymphoid organs. In an accompanying paper, we demonstrate that a significant proportion of T cells freshly isolated from lung exhibit a comparable surface phenotype.

INTRODUCTION

In response to immunological stimuli T lymphocytes undergo a complex activation process, culminating in proliferation and clonal expansion. There are a large variety of experimental systems described in which modulation of T-cell activation results in the suppression of T-cell proliferation (non-responsiveness), although the precise mechanisms responsible for the induction and maintenance of this state are for the most part unknown. The best characterized model is that of classical T-cell anergy (reviewed in ref. 1), in which inappropriate costimulation during activation results in a reversible state of T-cell hyporesponsiveness which is due to inhibition of interleukin-2 (IL-2) production.

It has been proposed that down-regulation of T-cell activity in primary lymphoid organs via mechanisms such as the induction of anergy plays an important role in the maintenance of overall immunological homeostasis, contributing to such processes as the development of peripheral tolerance to exogenous antigens and control of self-reactive lymphocytes.[2] It has recently become evident that similar T-cell down-regulatory mechanisms are operative in peripheral tissues outside the lymphoid system, one of the most important examples being the respiratory system.

Within the lung, the presence of large numbers of mature immune effector cells, together with virtually continuous exposure to environmental antigens at the sensitive blood–air interface, dictate that the overall level of steady-state T-cell responses must be meticulously controlled. One mechanism by which this appears to occur involves regulation of T-cell activation by alveolar macrophages (AM). The AM from all species tested except the guinea-pig have been shown to suppress the proliferation *in vitro* of T cells in response to mitogenic stimulation (reviewed in ref. 3). In addition, recent experiments demonstrating up-regulation of a variety of aspects of T cell-dependent local immunity in the lung following *in situ* depletion of AM populations have provided direct evidence for the operation of such a regulatory mechanism *in vivo*.[4]

The present study seeks to elucidate the process of AM-induced suppression of T-cell proliferation *in vitro* in the rat model, and to identify underlying mechanisms by which the T

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cells are rendered hyporesponsive following mitogenic and antigenic stimulation. In particular, we have focused on modulation of T-cell surface molecules, the production of cytokines during suppression, and the functional state of the interleukin-2 receptor (IL-2R) with specific regard to the capacity to bind IL-2 during this process.

MATERIAL AND METHODS

Animals

Inbred, specific pathogen-free female WAG rats aged 6–12 weeks (Animal Resource Centre, Murdoch University, Western Australia), maintained under barrier conditions, were employed in the series of experiments described below.

Immunization

WAG rats received $2 \times 100\,\mu g$ injections of ovalbumin–Freund's complete adjuvant (OVA–FCA; OVA, Sigma Chemical Co., St Louis, MO; FCA, Difco Laboratories, Detroit, MI) at the base of the tail. Inguinal and para-aortic lymph nodes (LN) were removed on days 14–16 postpriming.

Cell preparation and culture

Methods for preparation of single cell suspensions from LN and spleens and collection of AM by bronchoalveolar lavage (BAL) were as described in ref. 5. Peritoneal macrophages (PM) were obtained by peritoneal lavage with 20 ml phosphate-buffered saline (PBS) containing 0·2% bovine serum albumin (BSA).

In experiments involving *in vitro* mitogenic T-cell activation, splenic or mesenteric LN T cells were cultured at 2×10^5 per 200 μl in microplates in RPMI-1640 medium supplemented with L-glutamine, 5% fetal calf serum (FCS) and 5·0 μg/ml concanavalin A (Con A), in the presence or absence of AM. For proliferative studies, DNA synthesis was measured at 48 hr (unless otherwise indicated) via incorporation of [^{3}H]thymidine (specific activity 5 Ci/mmol) into DNA, which is expressed below as mean disintegrations per minute (d.p.m.) per culture.[5] For studies involving antigenic activation of sensitized LN T cells, cells were cultured at a density of 4×10^6/ml in microplates in RPM-1640 medium supplemented with L-glutamine, 1% fresh normal rat serum, 2×10^{-5} M 2-mercaptoethanol, 100 mM sodium-pyruvate and 40 μg/ml OVA in the presence or absence of AM; DNA synthesis was measured at 96 hr.

In experiments examining the reversibility of AM-induced suppression, T cells were removed, after gentle resuspension, from AM/T-cell cocultures, washed twice in RPMI-1640 medium and subsequently recultured in fresh medium with or without mitogenic/antigenic restimulation.

Blocking reagents

For experiments involving modulation of AM-induced immunosuppression: anti-transforming growth factor-β (TGFβ) blocking antibody was from R&D Systems (Minneapolis, MN); recombinant IL-1 was kindly donated to Dr U. R. Kees (Institute for Child Health Research, Perth, Western Australia) from Immunex (Seattle, Washington); and *N*G-monomethyl-arginine (MMA) was from Calbiochem (San Diego, CA).

Lymphokine production

The production of IL-2 was determined using the standard CTLL-2 bioassay. Levels of IL-3/granulocyte–macrophage colony-stimulating factor (GMCSF) were determined using the FDC-P1 bioassay. Titres of IL3/GMCSF in culture supernatants were determined from dose–response curves relative to a positive standard used in each bioassay conducted (standardized preparations of WEHI-3 conditioned medium.[6] For the purposes of these experiments, the positive standard was set to an arbitrary value of 100 U/ml. The production of interferon-γ (IFN-γ) was determined using a rat IFN-γ enzyme-linked immunosorbent assay, described in detail in ref. 7, and standardized against recombinant rat IFN-γ (reagents kindly provided by Dr P. Van der Meide, TNO Primate Centre, Rijswijk, the Netherlands).

Flow cytometry

Flow cytometric analysis of surface marker expression was performed on an EPICS Elite cytometer equipped with standard analytical software. Monoclonal antibodies (mAb) used were anti-rat CD5 (Ox19; pan T-cell),[8] anti-rat CD3,[9] anti-rat T-cell receptor (TCR)α/β chain (R73),[10] anti-rat IL-2Rα (Ox39),[11] anti-rat IL-2Rβ (NDS62),[12] anti-rat CD2 (Ox34)[13] and Ox48[11] an anti-rat mAb which binds a cell surface marker appearing selectively on rat blast cells; a mouse anti-human mAb was employed as a negative control.

The expression of surface CD3, TCRα/β, CD2, Ox48 or IL-2R on T cells was examined via staining with one of the mAb listed above, followed by biotinylated sheep–anti-mouse then streptavidin phycoerythrin, and finally Ox19–fluoroscein isothiocyanate (FITC). For analysis, T cells were positively selected by gating on the pan-T-cell antibody Ox19 (CD5), which is not expressed on rat B cells.

Analysis of DNA content of T cells was performed on day 3 of the cultures. Cells were incubated in a solution of propidium iodide (50 μg/ml), RNAase (40 μg/ml), Triton-X (0·1%), polyethylene glycol 6000 (3% w/v) and 4 mM citrate buffer pH 7·8 for 30 min at 37°. Cells were then left to stand at 4° in the dark for at least 1 hr prior to analysis. Analysis of the DNA content was performed on an EPICS elite cytometer and cell cycle analysis performed using the MODFIT program (Verity Software House, Topsham, ME). The results are expressed as fluorescence intensity on a linear scale in arbitrary units.

IL-2 binding studies

Analysis of the ability of IL-2R-expressing T cells to bind IL-2 was performed by employing an IL-2–biotin conjugate (Amersham, Australia). Cells were incubated for 1 hr with IL-2–biotin (5 μg/ml), washed in PBS, and finally labelled with FITC. The capacity of IL-2R-expressing T cells to bind IL-2 was determined using standard analytical procedures on an EPICS elite cytometer.

Detection of CD28 mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR)

Due to the non-availability of a mAb for CD28 in the rat at the time of this study, we utilized the technique of RT-PCR to detect CD28 mRNA.

Oligonucleotides were designed from their known cDNA sequences (CD28;[14] β-actin[15]). The primers were synthesized in

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house and purified by *n*-butanol extraction. The expected PCR product sizes were: CD28 940 base pairs (bp); β-actin 661 bp.

Total cellular RNA was isolated from cultured cells by standard guanidinium thiocyanate–phenol/chloroform extraction. Isolated RNA was dissolved in diethylpyrocarbonate-treated double-distilled water (DEPC-H_2O). First strand DNA was synthesized by transcribing total RNA using 20 U of AMV reverse transcriptase (Promega) and 2 μg of oligo dT_{15mer} (Promega) as a primer. For PCR reactions cDNA transcripts were amplified through 25–30 cycles of PCR, carried out in a Perkin Elmer thermal cycler (denaturation, 1 min at 94°; annealing, 1 min at 55°; extension, 2 min at 74°). For PCR, 5 μl of first strand cDNA was added to 20 μl of PCR mix containing: 2·5 μl 10× reaction buffer, 2 μl dNTP mix (10 mM, dATP, dCTP, dGTP, dTTP), primers at 10 pmol/μl, 25 mM $MgCl_2$, 1 U of Thermus aquaticus (*Taq*) DNA polymerase (Boehringer Mannheim) and sterile dH_2O. Alternatively, PCR was carried out incorporating ^{32}P-labelled dATP (5 μCi/PCR reaction) into the product. For these experiments, PCR reactions were amplified through 15–20 cycles, using the same conditions as described above with the exception that dATP concentrations in the dNTP mix were limiting. PCR products were separated by electrophoresis on ethidium bromide (EtBr)-stained 1·5% agarose gel and viewed under ultraviolet light, or by autoradiography as appropriate. The amount of mRNA was estimated by image scan analysis (using an Epson scanner) in conjunction with densitometry performed using the image scan analysis software program. Each sample cDNA was amplified by β-actin primers and processed in the same way to allow starting levels of cDNA to be compared.

Western blotting to detect IL-2-induced signal transduction

T cells were cultured in the presence or absence of AM and stimulated with mitogen/antigen as previously described. To remove IL-2 already produced during culture the T cells were washed three times in RPMI-1640 medium supplemented with 5% FCS on day 1 or 2 after initial stimulation. Equivalent cell numbers were then replated in fresh medium and allowed to equilibrate at 37° for at least 3 hr. Recombinant IL-2 (rIL-2) was added to the T-cell cultures for various intervals of time as indicated. IL-2 stimulation was terminated by the addition of ice cold lysis buffer containing 1 mM sodium orthovanadate, 2 mM EDTA, 10 mM iodoacetamide, 25 μg/ml *p*-nitrophenylguanidinebenzoate, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 0·5% Triton-X-100. After addition of the lysis buffer (100 μl/ 10^7 cells), cells were vortexed and left on ice for 15 min. The lysates were cleared by centrifugation at 14 000 ***g*** at 4° for 15 min. An appropriate amount of 3·3× sample buffer was added to the supernatant and the samples were then boiled for 5 min and placed on ice or frozen until required.

Samples were electrophoresed on a 10% sodium dodecyl sulphate–polyacrylamide gel and then transferred to a 0·22 μm nitrocellulose membrane. After blocking at 4° overnight the membranes were probed for phosphotyrosine activity. Briefly, after washing in Tris-buffered saline (20 mM Tris, 150 mM NaCl) with 0·05% Tween and 0·1 mM Na_3VO_4 (TBS–$T_{0·05}$, 0·1 mM Na_3VO_4), the membranes were incubated with 1 μg/ml anti-phosphotyrosine mAb (Upstate Biotechnology Incorp., Lake Placid, NY) for 2 hr at room temperature. Membranes were washed a further three times and then incubated with biotinylated sheep-anti-mouse for 1 hr at room temperature, washed, and finally incubated with streptavidin horseradish peroxidase for 1 hr at room temperature. After final extensive washing the membranes were developed by the enhanced chemiluminescence method (ECL; Amersham, Australia) according to the manufacturer's directions.

RESULTS

Suppression of *in vitro* T-cell proliferation by AM

Figure 1a illustrates the influence of increasing numbers of AM on the proliferation of T-cell populations in response to mitogenic and antigenic signalling. For T cells stimulated with the mitogen Con A, the addition of 10^3 AM per culture (equating to 0·6% of the T-cell density) resulted in 50% suppression of T-cell proliferation. OVA antigen-specific T-cell proliferation was even more sensitive to the effects of AM, and 80% suppression of T-cell proliferation was achieved with the same density of AM. Figure 1b illustrates the effects of adding AM at varying time points after the initiation of T-cell activation by mitogen. T-cell activation was initiated at $t = 0$, the AM were added where indicated (open squares), and proliferation was determined as ^{3}H-labelled DNA synthesis 48 hr later, and compared to parallel AM-free (control) cultures. Data shown represent the percentage suppression of proliferation, achieved by addition of AM at the different time points. In addition, control (AM-free) cultures were [^{3}H]-thymidine labelled at various time points to determine relative rates of ^{3}H-labelled DNA synthesis at different stages of the cultures (closed symbols). The results of these experiments

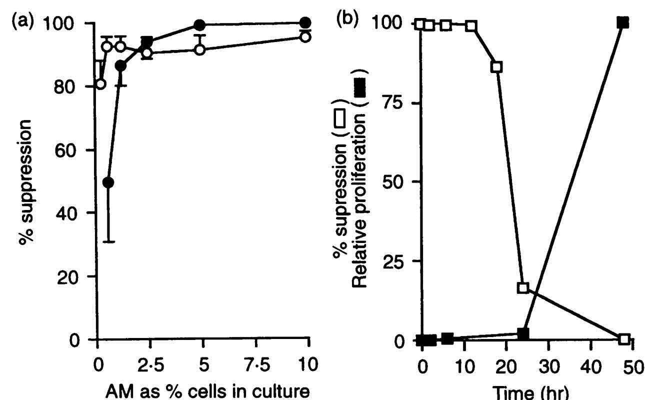

Figure 1. (a) Effect of AM on splenic T-cell proliferative responses to Con A (open circles) and OVA (closed circles). AM were added in increasing numbers to T-cell cultures stimulated with Con A or OVA as detailed in the Materials and Methods. DNA synthesis was measured via [^{3}H]thymidine incorporation. Data are expressed as the mean percentage suppression (± SD), where $n = 20$ for Con A, and $n = 5$ for OVA. (b) Effect of AM, added at various stages of T-cell activation on splenic T cell proliferation, compared to the normal relative rate of proliferation as a function of time. AM were added to T-cell cultures at various times after initial activation, as described in the Materials and Methods. Data are representative of a series of three experiments and are expressed as % suppression of T-cell proliferation (open symbols). In parallel cultures, DNA synthesis was determined at time-points after activation, equivalent to those employed in the AM experiments; data for each time point were normalized against the figure for 48 hr (closed symbols).

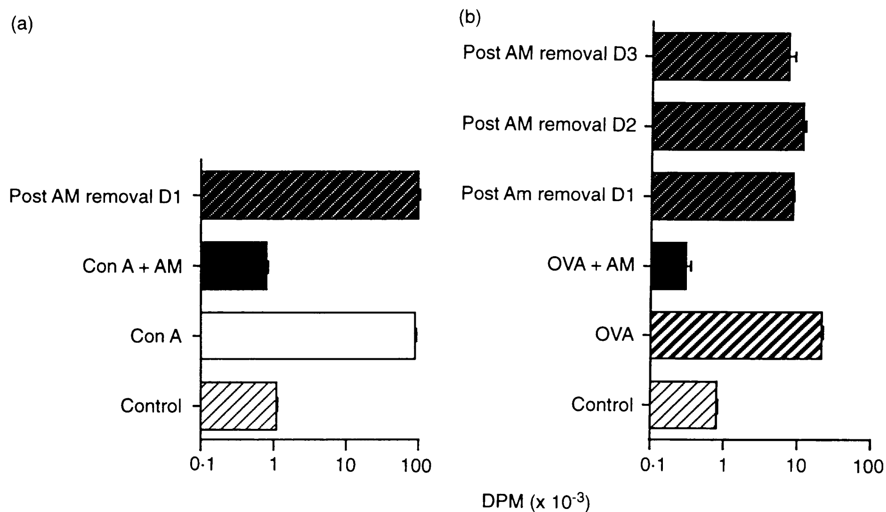

Figure 2. The ability of AM suppressed splenic T cells to regain their proliferative capacity after removal of AM. T-cell cultures were either left unstimulated or were stimulated with Con A or OVA, in the presence or absence of AM. The AM were removed from replicate cultures at various time points, as described in the Materials and Methods. Data are expressed as mean d.p.m. (± SD) and are representative of a series of eight experiments for Con A and five experiments for OVA.

demonstrate that complete suppression of T-cell proliferation can be attained only if AM are added to the T-cell culture within an initial 24-hr 'window'. Once activation has been allowed to proceed to the point at which proliferation of the T cell first becomes detectable (around 24 hr) the addition of AM to the culture has no effect on subsequent proliferation.

Reversibility of AM-induced T-cell suppression

Figure 2a/b examines the reversibility of the AM-induced state of T-cell non-responsiveness in both Con A and antigen-driven activation systems. In these experiments parallel replicate cultures were established consisting of controls (unstimulated T cells plus AM in medium) and T cells plus Con A/OVA, alone or with 10^4 AM (equivalent to 5% total cells in culture). One of the replicate series was assayed for lymphocyte proliferation after 48 hr culture for Con A and 96 hr for OVA. In the other culture series, T cells were removed from the T-cell/AM cocultures after days 1, 2, or 3. The T cells were washed and subsequently re-cultured in fresh stimulant-free medium for a further 48 hr prior to assay for lymphocyte proliferation.

Consistent with the data of Fig. 1, T cells activated with Con A or OVA in the (continual) presence of AM failed to proliferate. When AM were removed from Con A-activated cultures after 24 hr, rapid proliferation ensued, despite the absence of further stimulation. For T cells activated with OVA in the presence of AM, the removal of the AM on days 1, 2, or 3 resulted in the initiation of proliferation without the necessity of restoration of the antigen, which was effectively lost during the AM removal process. Data shown are the mean d.p.m. (± SD) from a representative experiment ($n = 8$ for Con A and $n = 5$ for OVA).

Effect of AM on lymphocyte cell cycle progression

Analysis of DNA content was determined on day 3 after initiation of the cultures and the results indicated that while a significant proportion of the normal Con A-stimulated T cells progressed to the S/G_2M phases of the cell cycle, T cells stimulated with Con A in the presence of AM did not. Figure 3 shows a representative experiment: 97·4% of the unstimulated cells were in G_0/G_1; for Con A-stimulated cells, 39% were in G_0/G_1, 51·4% were in S phase and 9·7% were in G_2/M; for Con A-stimulated cells in the presence of AM, 93·9% were in G_0/G_1; for cells initially stimulated with Con A in the presence of AM and subsequently removed after day 1, 70·6% were in G_0/G_1 and 29·4% were in S phase.

Surface expression of TCR-associated molecules during *in vitro* activation in the presence of AM

Figure 4 examines surface expression of CD3 on T cells ($CD5^+$ cells) at the 24-hr and 48-hr time-points following initiation of the T-cell cultures. CD5 expression was observed to decrease by small but variable amounts following activation; however, these changes were always restricted within the normal positive range and thus did not restrict the use of this mAb as a means to identify positively T cells during these experiments. The intensity of expression of TCR-associated surface molecules is shown as mean fluorescence intensity (MFI) and was determined relative to the normalized control cell MFI for each experiment.

In these experiments, parallel replicate cultures were established containing 2×10^5/ml splenocytes plus medium alone, unstimulated lymphocyte plus AM, lymphocytes plus 5 μg Con A alone or with 10^4 AM (equivalent to the addition of 5% AM). Cultures were analysed for TCR surface expression at 24 and 48 hr, with parallel replicate cultures being subsequently assayed at 48 hr to verify that lymphocyte proliferation was indeed completely inhibited.

Data are expressed as mean % T cells (± SD) expressing CD3 in conjunction with the mean corresponding value for the relative MFI of CD3 expression ($n = 15$). In the cultures containing Con A alone vigorous T-cell proliferation occurred, and CD3 expression was down-modulated markedly on day 1

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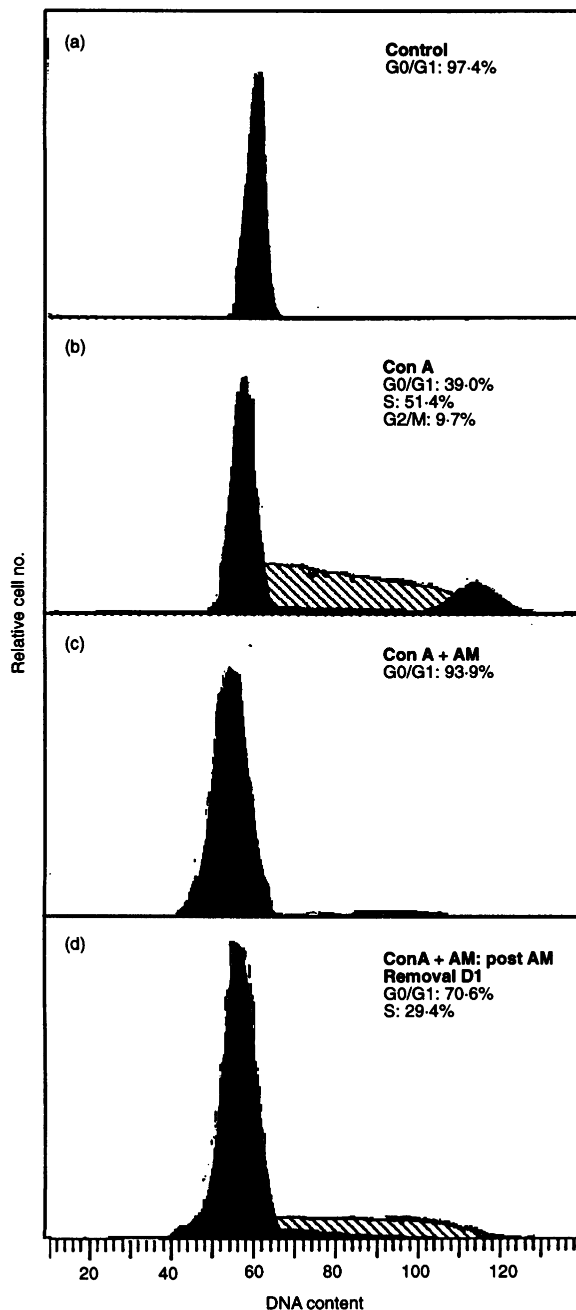

Figure 3. Cell cycle analysis of splenic T cells stimulated with Con A in the presence of AM. T-cell cultures were stained with PI and analysed by flow cytometry as detailed in the Materials and Methods. (a) Unstimulated cells; (b) cells stimulated with Con A; (c) cells stimulated with Con A plus AM added at 5% of the final T-cell concentration; (d) cells stimulated with Con A plus AM for 24 hr, washed and recultured in control medium. Data are representative of a series of four experiments and are expressed as the relative cell number (x axis) versus the fluorescence intensity (y axis) in a linear scale in arbitrary units. The percentage of cells in the respective stages of the cell cycle are shown in each panel.

compared to control cultures containing no mitogen (for activated and control T cells respectively: 65·5% T cells expressing CD3 at MFI 0·4 versus 98·2% at MFI 1·0). Re-expression of CD3 on the activated T cells was evident by

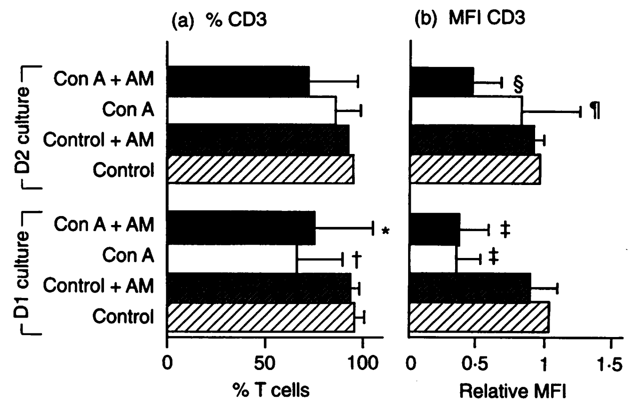

Figure 4. Effect of AM on CD3 expression of splenic T cells activated with Con A. T-cell cultures were either left unstimulated (control) or were activated with Con A in the presence or absence of AM. After 24 and 48 hr the cultures were stained for CD5 and CD3 and analysed by flow cytometry as described in the Materials and Methods. Data from 15 experiments are expressed as (a) mean percentage $CD5^+$ T cells ($\pm$SD) expressing CD3 and (b) mean relative MFI ($\pm$SD) of CD3 expression on $CD5^+$ T cells (normalized to splenic T-cell control). $*P < 0·05$ and $\dagger P < 0·001$ compared to control cultures by t-test; $\ddagger P < 0·001$ and $\S P < 0·01$ compared to control cultures by paired t-test; no significant difference compared to control cultures by paired t-test. ¶ no significant difference compared to control cultures by paired t-test.

day 2 (for activated and control T cells respectively: 85·5% at MFI 0·9 versus 97·5% at MFI 1·0 on day 2).

Consistent with the data of Fig. 1, the cultures containing 10^4 AM failed completely to proliferate, but nevertheless the T cells exhibited CD3 down-modulation which was comparable to that occurring in non-suppressed (AM-free) cultures. However, in the presence of AM, the T cells failed to re-express normal levels of CD3 and remained $CD3^-$ to CD3-low. Modulation of surface TCR$\alpha\beta$ was also examined under similar circumstances, and the results obtained were comparable to those for CD3 (data not shown).

Modulation of CD2 expression after *in vitro* activation in the presence of AM

These experiments followed the same format as Fig. 4. The percentage of T cells expressing the CD2 antigen after activation with Con A in the presence or absence of AM did not alter significantly from control T cells after 24 hr, and was observed to be only slightly reduced after 48 hr in cultures containing AM (data not shown). Analysis of the intensity of CD2 expression demonstrated that while relevant MFI figures were significantly increased in proliferating cultures (a normal consequence of T-cell activation) at both the 24-hr and 48-hr time-points, the presence of AM clearly inhibited this increase in the level of CD2 expressed on T cells postactivation. However, it was additionally observed that if the addition of AM to the Con A-stimulated cultures was delayed for 24 hr, the surface expression of CD2 was also down-modulated, despite the fact that AM added to the culture at this time-point did not exert any inhibitory effect on the proliferation of the T cells (see Fig. 5c for a representative experiment ($n = 3$); see also Fig. 1b above). This suggests that AM-induced changes in CD2

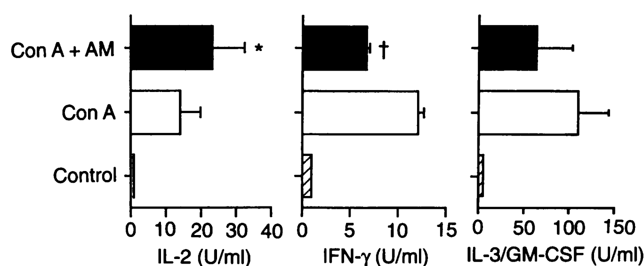

Figure 5. Effect of AM on lymphokine production by Con A-activated splenic T cells. Splenic T-cell cultures were activated with Con A in the presence or absence of AM, and 24-hr culture supernatants were assayed for the presence of IL-2, IL-3/GMCSF, and IFN-γ as described in the Materials and Methods. Data are expressed as mean levels ($\pm$SD) detected in the culture supernatants of (a) IL-2 ($n = 10$), (b) IFN-γ ($n = 10$) and (c) IL-3 GMCSF ($n = 3$). *$P < 0{\cdot}02$ and †$P < 0{\cdot}01$ by Student's t-test for T cells stimulated in the presence versus the absence of AM.

expression do not play a primary role in the mechanism of suppression.

IL-2R expression and IL-2 binding in the presence of AM

As reported previously,[19] expression of the α and β chains of the IL-2 receptor were up-regulated to the same degree in proliferating (AM-free) CON A-stimulated cultures, and in cultures where proliferation was completely inhibited by the presence of AM (data now shown). In addition, binding of the biotinylated recombinant IL-2 was comparable in both sets of cultures (data not shown).

Expression of the activation marker Ox48 in the presence of AM

The surface antigen recognized by OX48 is considered to be a 'late' activation antigen expressed selectively by rat T-cell blasts.[11] In rapidly proliferating cultures (day 2 postactivation in the absence of AM), on average $37{\cdot}3 \pm 9{\cdot}1\%$ T cells expressed Ox48 compared to $4{\cdot}9 \pm 2{\cdot}1\%$ in the presence of AM.

Effect of AM on levels of CD28 mRNA

Semi-quantitative RT-PCR was utilized for the detection of CD28 mRNA in T cells activated with Con A and cultured in the presence or absence of AM for 24 hr. β-actin mRNA expression was also measured to compare cDNA starting levels for each sample, and a negative control was incorporated in each experiment. The level of CD28 mRNA in each sample was estimated by densitometry and normalized against the β-actin product. After normalization in each experiment ($n = 5$), the levels of CD28 mRNA between groups were compared by paired t-test. These data indicated that levels of CD28 mRNA expression following mitogenic activation of T cells in the presence of AM ($0{\cdot}86 \pm 0{\cdot}048$) resulted in slightly reduced levels of detectable CD28 mRNA, which were not significantly different from activated controls (normalized to 1·0).

Effect of EM on T cell cytokine production

Measurement of the concentration of bioactive IL-2 in the supernatant of T-cell cultures at the 24-hr time-point (Fig. 5) indicated the presence of high levels of IL-2 in the cultures containing AM, despite the failure of the lymphocytes to proliferate. The slightly lower levels of IL-2 in cultures free from AM is most likely a reflection of the consumption of IL-2 during T-cell proliferation.

The production of IFN-γ and IL3/GM-CSF was reduced by 40–50% in T-cell cultures activated with Con A in the presence of AM, but production levels were still high, despite the complete inhibition of proliferation.

Several different mediators contribute to AM-induced suppression

AM have previously been shown to secrete a variety of mediators which are capable of suppressing T-cell proliferation, as discussed previously. The series of experiments described below were designed to determine systematically the relative contributions of several of these mediators, namely, IL-1R antagonist, nitric oxide, and TGF-β, within our *in vitro* system of AM-induced T-cell suppression. To achieve this, a series of parallel replicate AM : T-cell cocultures was established to enable a comparison between AM-induced suppression under standard conditions, and in the presence of saturating amounts

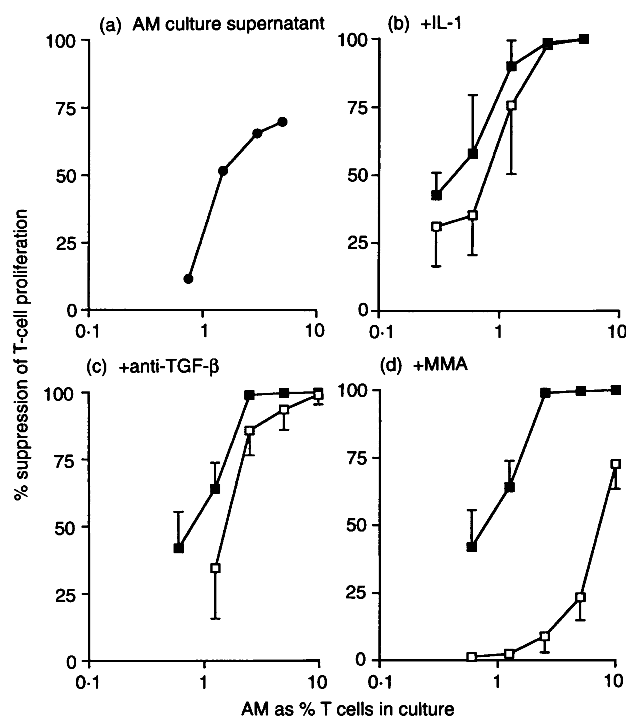

Figure 6. The inhibition of AM-induced splenic T-cell suppression. AM were added in increasing numbers to T-cell cultures activated with Con A. Parallel cultures containing (b) IL-1 (100 U/ml), (c) anti-TGF-β (100 ng/ml) and (d) MMA (0·5 mM) were also established; respective concentrations were determined as optimal in preliminary trials. Also, as shown in (a), 24 hr AM/T-cell/Con A culture supernatants were added at 1 : 1 (v : v) to one series (that from a culture containing 10% AM resulting in an 'equivalent' of 5% in the secondary culture, etc.). Cells were cultured for 2 days and DNA synthesis was measured via [^{3}H]thymidine incorporation. Data are expressed as the mean percentage suppression ($\pm$SD) with and without the added treatments for each series ($n \geqslant 3$).

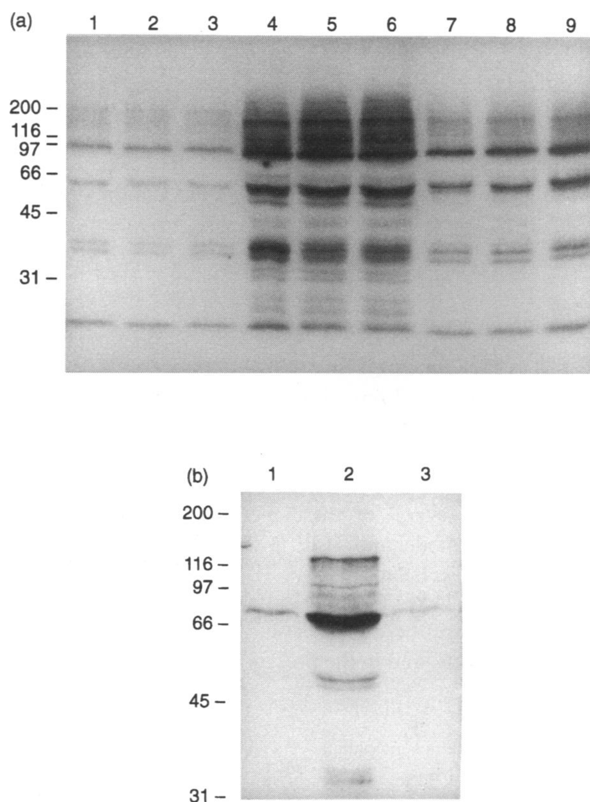

Figure 7. Effect of AM on protein tyrosine phosphorylation following Con A activation of splenic T cells. T-cell cultures were either left unstimulated or were activated with Con A in the presence or absence of AM, added at 5% final T-cell concentration. T cells were washed, recultured and exposed to IL-2 (100 U/ml^{-1}) for various times. Protein lysates were prepared from equivalent cell numbers, and Western blotting with anti-phosphotyrosine mAb was carried out as described in the Materials and Methods. Lanes 1–3, unstimulated cells; lanes 4–6, Con A-stimulated cells; lanes 7–9, Con A-stimulated cells plus AM; lanes 1, 4 and 7, no IL-2 added; lanes 2, 5 and 8, IL-2 added for 2 min; lanes 3, 6 and 9, IL-2 added for 10 min. Results are representative of a series of five experiments.

of either IL-1, the NO-synthetase inhibitor MMA, or anti-TGF-β (Fig. 6b–d). Dose–response curves were established for both the concentration of the blocking agent used and the number of AM added to culture. We also analysed the capacity of AM-derived supernatant (24 hr) to induce suppression of T-cell proliferation (Fig. 6a).

Supernatants derived from overnight AM/T-cell cocultures were still able to suppress T-cell proliferation, confirming a role for diffusable inhibitor(s); however, supernatants were less effective than an equivalent number of fresh AM used directly (panel A). Both IL-1R antagonist and TGF-β appear to play detectable but minor roles in the T-cell suppression observed in our system. Figures 6b and c indicate that the addition of IL-1 and anti-TGF-β respectively results in a small reduction in the ability of AM to suppress T-cell proliferation, which is observed at the lower end of the AM dose curve. Similar findings were also observed after the addition of indomethacin to the T cell : AM coculture, suggesting a relatively minor role for prostaglandin in the T-cell suppression observed (data not shown). In contrast, the addition of MMA (blocking suppression that can be attributed to the action of NO) resulted in a substantial reduction in the ability of AM to suppress T-cell proliferation, throughout the AM dose curve.

IL-2-induced phosphorylation of intracellular proteins

Several cellular proteins are phosphorylated by tyrosine kinases as a result of IL-2 binding to its functional high-affinity receptor. Preliminary results on the effect of AM on activation-induced tyrosine phosphorylation are shown in Fig. 7. The findings indicate that under normal conditions of T-cell activation (free from AM) several cellular proteins have undergone tyrosine phosphorylation in comparison to the control (resting) culture. The high degree of phosphorylation observed at time 0 (no recombinant IL-2 added) reflects the mitogen stimulation *per se* combined with the continuous production of IL-2 by the T cells in log phase (after activation) and the concomitant binding of this IL-2 to its receptor, resulting in signal transduction phosphorylation events. It is thus difficult to ascertain with any certainty which proteins are phosphorylated directly as a result of IL-2 stimulation and thus by extension those that are not. However, T cells that have been rendered non-responsive as a result of AM suppression demonstrate reduced phosphorylation of several of these proteins at all time points examined. Proteins of approximate molecular weights 120 000, 110 000, 105 000, 100 000, 85 000, 75 000, 50 000 and 34 000 were less prominant in T-cell populations activated in the presence of AM. Since several of these proteins are of similar size to proteins known to be associated with IL-2 signal transduction, these are potential candidates for the mechanism via which suppression is achieved. Similar findings were also observed for OVA stimulated T-cell (data not shown).

The immunosuppressive effect of peritoneal macrophages

It has previously been demonstrated that activated macrophages from sites other than the lung can inhibit T-cell proliferation. Accordingly, parallel experiments were performed in several of the systems described above using peritoneal macrophages (PM) in addition to AM populations. We examined the dose–response curve for suppression, modulation of cell surface receptor expression, cytokine production, and reversibility of T-cell suppression for both of these macrophage populations tested in parallel. Data indicated that the only significant differences between the two macrophage populations were quantitative, manifested as a requirement for approximately twice the number of PM, compared to AM, to achieve 50% suppression of T-cell proliferation; and main features of the characteristics of PM-suppressed T cells were identical to those observed with AM (data not shown).

DISCUSSION

Based on evidence obtained from a variety of experimental systems, it has been proposed that the maintenance of immunological homeostasis in the lung and airways is achieved primarily via endogenous immunosuppressive mechanisms which regulate the local induction of T-cell responses (reviewed in ref. 3).

The present study, which details the effect of AM on *in vitro* T-cell activation/proliferation, suggests that AM play an important immunoregulatory role by inducing a state of reversible T-cell non-responsiveness during local antigenic stimulation. Support for an analogous role for AM *in vivo* is provided by recent experiments involving *in situ* depletion of AM in rats and mice, a manoeuvre which creates a state of immunological hyperreactivity to antigenic challenge via aerosol.[4]

The existence of a relationship between the immunosuppressive function(s) of AM and T-cell reactivity in human lung disease is yet to be conclusively demonstrated, but is increasingly supported by evidence from studies on AM function in sarcoidosis, and to a lesser extent in asthma and chronic lower respiratory tract infection (reviewed in ref. 3). This existing evidence prompted the present more detailed *in vitro* studies on the underlying mechanisms.

This study focused on events associated with T-cell activation/proliferation in the presence of AM after *in vitro* antigenic and mitogenic stimulation. The salient findings are as follows. First, the immunosuppressive activity of AM is independent of the nature of the activation signal (T-cell proliferation can be completely inhibited in both antigen and mitogen-driven systems). However, following T-cell stimulation there appears to be a finite temporal window during which AM are able to exert their immunosuppressive effects, namely, prior to the entrance of T cells in S-phase. Furthermore, the inhibition of T-cell proliferation resulting from AM-mediated suppression is reversible. Thus, following separation from AM, T cells undergo normal progression through the cell cycle without the requirement for further exposure to antigen. This implies that if initial activation occurs within the lung in the presence of AM, the 'suppressed' T cells retain their capacity for proliferation, providing they migrate from the lung mileu, for example to draining lymph nodes. This provides a potential means for preserving capacity to generate T-cell memory against antigens commonly encountered in the lung, while still maintaining tight control of potentially tissue-damaging T-cell activation at the original sites of antigen exposure.

Analysis of the nature of AM-mediated suppression of T-cell populations focused initially on the status of cell surface function-associated molecules. Receptor modulation following mitogenic stimulation was studied by quantifying both the percentage of T cells expressing the relevant receptor, and intensity of expression. The down-modulation of the TCR-associated molecules CD3 and TCR$\alpha\beta$ (a normal consequence of T-cell activation),[16] occurred to a comparable degree in the presence and absence of AM. Failure of T cells that are activated in the presence of AM to re-express CD3 is analogous to T cells which have a continuously engaged TCR. It is of interest to note that the latter cells, although expressing IL-2R, are non-responsive to IL-2-driven proliferation, and as a consequence are unable to progress through the cell cycle.[17,18]

Comparable levels of up-regulation of both the IL-2α and β receptors was also observed on T cells activated in the presence or absence of AM. Collectively these results suggest that at least initially, the process of activation *per se* proceeds normally under the immunosuppressive influence of the AM. In contrast, the expression of the Ox48 antigen, a 'late' activation antigen normally appearing on blast cells, was significantly reduced on those T cells stimulated in the presence of AM.

Failure of the T cells to proliferate in the presence of AM is not due to a lack of available IL-2, which was present at levels exceeding that of control cultures. This finding is consistent with earlier results demonstrating that the addition of excessive amounts of recombinant IL-2 to cultures containing AM does not facilitate T-cell proliferation.[19] These findings distinguish the present phenomenon from that of classical T-cell anergy, in which T-cell hyporesponsiveness is associated with a failure to produce IL-2, and which moreover is reversible upon introduction of exogenous IL-2 into the culture system (reviewed in ref. 1). Furthermore, although slightly decreased, the presence of CD28 mRNA in our experimental system is clearly evident in T cells that have been rendered 'non-responsive' by AM. This is in contrast to a recent report which suggests that the loss of CD28 mRNA is associated with the induction of classical T-cell anergy.[20]

The secretion of both IL-3/GM-CSF and IFN-γ is significantly reduced in T-cell cultures containing AM, but the absolute levels of secretion of these cytokines nevertheless remain high. The significance of the different quantitative effects of AM on production of the different cytokines is unclear, but has also been reported in other *in vitro* T-cell suppression systems.[21]

In conjunction with the data demonstrating comparable levels of IL-2R expression on T cells activated in the presence or absence of AM, we have further shown a comparable ability of these IL-2R expressing activated T cells to bind biotinylated IL-2. Despite its importance in the T-cell activation process there is still relatively little known concerning the precise mechanisms of IL-2-induced signal transduction. Although the IL-2R chains themselves do not possess an intrinsic kinase domain (reviewed in ref. 22), the mechanism used by the IL-2R to transduce signals and initiate cell growth is proposed to be dependent upon the activation of associated protein tyrosine kinases and hence tyrosine phosphorylation of multiple cellular proteins. Consistent with this model, the tyrosine phosphorylation of a number of proteins was seen to occur as a result of normal T-cell activation (and IL-2R signalling) in our system. However, the altered tyrosine phosphorylation of proteins observed in AM-suppressed T-cell cultures implies that interference in activation-induced signal transduction events may be an important part of the mechanism via which AM exert their cytostatic effects. In order to establish the contribution of defective IL-2R signalling to the altered tyrosine phosphorylation patterns observed, we are currently using techniques of immunoprecipitation of cell lysates with a mixture of anti-IL-2Rα and β mAb, followed by immunoblotting with anti-phosphotyrosine mAb. Our preliminary findings indicate that alterations in phosphorylation of IL-2R-associated proteins are an integral part of the changes observed in whole cell lysates.

The recent literature suggests that the IL-2γ chain potentially plays a role in the suppression events described above. The IL-2γ chain has recently been shown to be essential for IL-2 binding and ligand internalization, and also plays an integral role (along with IL-2Rβ) in IL-2-induced signal transduction events.[23] Furthermore, it has recently been demonstrated that clonal anergy can be prevented by signalling through the γ chain of the IL-2R.[24] Unfortunately, due to the lack of appropriate reagents for rat, we have not yet been able to obtain any direct information regarding the IL-2γ chain in

this system: this issue will be addressed in following experiments in other species for which reagents are available.

In the present model it appears that the production of nitric oxide by AM is the major contributing factor responsible for the inhibition of T-cell proliferation. Consistent with this finding and the data described above is the recent report indicating that nitric oxide-mediated T-cell suppression does not affect IL-2 secretion in the rat.[25]

In summary, the present results suggest the operation of a sophisticated control mechanism by which AM impose tight regulation over the T-cell dependent immune response within the lung, limiting the expression of effector functions to a 'single hit' by inhibiting local clonal expansion of the activated T cells. However, the restoration of T-cell proliferative capacity following separation from AM implies that such suppression may occur *in situ* within the lung without disruption of the generation of T-cell 'memory' to antigens encountered locally, which could proceed normally following migration of the T cells from the suppressive lung environment into regional lymph nodes. Furthermore, an extension of these observations to the suppressive nature of the mature resident PM population suggests that similar control mechanisms may also be operating within other organ systems.

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REFERENCES

1. Schwartz R.H. (1990) A cell culture model for T lymphocyte clonal anergy. *Science* **248,** 1349.
2. Schwartz R.H. (1989) Acquisition of immunological self tolerance. *Cell* **57,** 1073.
3. Holt P.G. (1993) Regulation of antigen-presenting function(s) in lung and airway tissues. *Eur Respir J* **6,** 120.
4. Thepen T., McMenamin C., Oliver J., Kraal G. & Holt P.G. (1991) Regulation of immune response to inhaled antigen by alveolar macrophages: differential effects of *in vivo* alveolar macrophage elimination on the induction of tolerance vs. immunity. *Eur J Immunol* **21,** 2845.
5. Holt P.G., Schon-Hegrad M.A. & Oliver J. (1968) MHC class II antigen-bearing dendritic cells in pulmonary tissues of the rat. Regulation of antigen presentation activity by endogenous macrophage populations. *J Exp Med* **167,** 262.
6. Kelso A. (1990) Frequency analysis of lymphokine secreting CD4+ and CD8+ T cells activated in a graft versus host reaction. *J Immunol* **145,** 2167.
7. Van der Meide P. & Borman T.H. (1990) A sensitive two site enzyme immunoassay for the detection of rat gamma IFN in biological fluids. *J Interferon Res* **10,** 183.
8. Mason D.W., Arthur R.P., Dallman M.J., Green J.R., Spickett G.P. & Thomas M.L. (1983) Functions of rat T-lymphocyte subsets isolated by means of monoclonal antibodies. *Immunol Rev* **74,** 57.
9. Nicolls M.R., Aversa G.G., Pearce N.W. *et al.* (1993) Induction of long-term specific tolerance to allografts in rats by therapy with an anti-CD3-like monoclonal antibody. *Transplantation* **55,** 459.
10. Hünig T., Wallny H.J., Hartley J.K., Lawetzky A. & Tiefenthaler G. (1989) A monoclonal antibody to a constant determinant of the rat T cell antigen receptor that induces T cell activation. Differential reactivity with subsets of immature and mature T lymphocytes. *J Exp Med* **169,** 73.
11. Paterson D.J., Jefferies W.A., Green J.R. *et al.* (1987) Antigens of activated rat T lymphocytes including a molecule of 50,000 Mw detected only on CD4 positive T blasts. *Mol Immunol* **24,** 1281.
12. Tellides G., Dallman M.J. & Morris P.J. (1989) Mechanism of action of interleukin-2 receptor (IL-2R) monoclonal antibody (MAb) therapy: target cell depletion or inhibition of function? *Transplant Proc* **21,** 429.
13. Williams A., Barclay A.N., Clark S.J., Paterson D.J. & Willis A.C. (1987) Similarities in sequences and cellular expression between rat CD2 and CD4 antigens *J Exp Med* **165,** 368.
14. Clark G.J. & Dallman M.J. (1992) Identification of a cDNA encoding the rat CD28 homologue. *Immunogenetics* **35,** 54.
15. Ehlers S. & Smith K.A. (1991) Differentiation of T cell lymphokine gene expression: the *in vitro* acquisition of T cell memory. *J Exp Med* **173,** 25.
16. Cantrell D.A., Verbi W., Davies A., Parker P. & Crumpton M.J. (1988) Evidence that protein kinase C differentially regulates the human T lymphocyte CD2 and CD3 surface antigens. *Eur J Immunol* **18,** 1391.
17. Nau G.J., Moldwin R.L., Lancki D.W., Kim D. & Fitch F.W. (1987) Inhibition of IL-2 driven proliferation of murine T lymphocyte clones by supraoptimal levels of immobilised anti-T cell receptor monoclonal antibodies. *J Immunol* **139,** 114.
18. Ceredig R. & Corradin G. (1986) High antigen concentration inhibits T cell proliferation but not interleukin 2 production: examination of limiting dilution microcultures and T cell clones. *Eur J Immunol* **16,** 30.
19. Upham J., Strickland D., Bilyk N. & Holt P.G. (1995) Alveolar macrophages from humans and rodents selectively inhibit T-cell proliferation but permit T-cell activation and cytokine secretion. *Immunology* **84,** 142.
20. Lake R.A., O'Heir R.E., Verhoef A. & Lamf J.R. (1993) CD28 rapidly decays when activated T cells are functionally anergised with specific peptide. *Int Immunol* **5,** 461.
21. O'Hehir R.E., Yssel H., Verma S., deVries J.E., Spits H. & Lamb J.R. (1991) Clonal analysis of differential lymphokine production in peptide and superantigen induced T-cell anergy. *Int Immunol* **3,** 819.
22. Ullrich A. & Schlessinger J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61,** 203.
23. Taniguchi T. & Minami Y. (1993) The IL-2/IL-2 receptor system: a current overview. *Cell* **73,** 5.
24. Boussiotis V.A., Barber D.L., Nakarai T. *et al.* (1994) Prevention of T cell anergy by signalling through the γ_c chain of the IL-2 receptor. *Science* **266,** 1039.
25. Huot A.E., Moore A.L., Roberts J.D. & Hacker M.P. (1993) Nitric oxide modulates lymphocyte proliferation but not secretion of IL-2. *Immunol Invest* **22,** 319.