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Novel molecular mechanisms of dendritic cell-induced T cell activation

Vanessa E. Woodhead, Timothy J. Stonehouse, Michael H. Binks, Katharina Speidel, David A. Fox[1], Antoni Gaya[2], Deborah Hardie[3], Anthony J. Henniker[4], Vaclav Horejsi[5], Kimitaka Sagawa[6], Keith M. Skubitz[7], Hristo Taskov[8], Robert F. Todd III[9], Andre van Agthoven[10], David R. Katz and **Benjamin M. Chain**

Department of Immunology, Windeyer Institute of Medical Sciences, UCL Medical School, 46 Cleveland Street, London W1P 6DB, UK
[1]Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109, USA
[2]Service d'Immunologie, Hospital Clinic, Barcelona 08036, Spain
3Department of Immunology, University of Birmingham, Birmingham B15 2TT, UK
[4]Institute of Clinical Pathology and Medical Research, Westmead Hospital, Westmead, NSW 2145 Australia
[5]Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic
[6]Department of Transfusion Medicine, Kurume University, Kurume, Fukouka 830, Japan
[7]University of Minnesota, Minnesota Medical School, University Hospital, Minneapolis, MN 55455, USA
[8]National Centre of Infectious and Parasitic Diseases, 1504 Sofia, Bulgaria
[9]Department of Internal Medicine, Division of Haematology/Oncology, University of Michigan Medical Centre, Ann Arbor, MI 48109-0374, USA
[10]Immunotech, 13276 Marseille Cedex 9, France

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Abstract

In this study we have re-examined the molecular mechanisms involved in activation of T cells by dendritic cells (DC). Human peripheral blood DC (PBDC) were derived by 2 h adhesion followed by 7 day culture in a combination of granulocyte macrophage colony stimulating factor and IL-4, and depletion of residual T and B cells. These PBDC were used to induce autologous T cell proliferation in a CD3-dependent response, and antibodies against CD11a/18 and CD86 were used as control inhibitors of accessory function. Antibodies against five of the cell surface molecules that we have recently identified on the surface of DC, CD13, CD87, CD98, CD147 and CD148, and an antibody which recognizes a molecule that has not as yet been identified, all inhibited the CD3-induced T cell proliferation. These findings were observed not only when antibodies were present throughout the culture, but also when they were prepulsed on to the surface of the DC, suggesting the inhibition was mediated via the antigen-presenting cells rather than the T cell. The same set of antibodies also inhibited an allospecific mixed lymphocyte reaction, confirming that the inhibitory effect was not dependent on the use of a CD3 antibody as the stimulating agent. All the antibodies of known specificity inhibited both CD4 and CD8 T cells equally. Unlike CD87, CD98 and CD147 antibodies, which inhibited activation of both CD45RA (naive) T cells and CD45RO (memory) T cells, CD13 and CD148 appeared to be involved in activation of naive cells only. The molecules identified in this study have not previously been demonstrated to play a role as accessory molecules on DC, the cells that are pivotal for immune induction. Therefore they may provide new potential targets for modulation of the immune response at the APC level.

Correspondence to: D. R. Katz

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Introduction

Dendritic cells (DC) are potent, professional antigen-presenting cells (APC) conventionally identified *in vitro* by their ability to activate naive T cells, by high MHC expression and by unusual dendritic processes (1). Analysis of this crucial cell type has always posed a problem to investigators, particularly in man, because of the low frequency of DC (or DC precursors) in the peripheral blood (PB), and because of lack of specific qualitative cell surface markers. Functional characterization of DC is, however, of vital importance, as these cells play a critical role in primary antigen-specific responses *in vivo* and are thus increasingly acknowledged as a putative site for immunological intervention, either as adjuvants to increase responses or as the most effective target for inhibition.

The reasons for the exceptional stimulatory capacity of DC are still incompletely understood. Two signals are required to produce a T cell response: the first is recognition of antigenic peptide by the TCR presented in the context of the MHC. The second, 'co-stimulus' signal is conferred either by cell surface molecules on the APC which interacts with corresponding T cell co-receptors or by soluble mediators released by the APC (2). This signal is antigen non-specific and not restricted by MHC. Co-stimulator molecules that have been implicated include CD54 (ICAM-1), CD102 (ICAM-2) and CD50 (ICAM-3) interacting with CD11a/18 (LFA-1), CD58 (LFA-3) with CD2, B7 (both CD80 and CD86) with CD28, and CD40 with CD154 (CD40 ligand) (2–8). These molecules may also increase adhesion between the APC and the T cell, thus increasing the strength and duration of the interaction (9), and leading to the formation of clusters of T cells around the DC (10–12). Providing both signals are present, interaction between T cells and APC leads to activation, expansion and differentiation of T cells, and hence to an immune response against potential pathogens. For an efficient response it may be necessary for the APC to express more than one co-stimulatory molecule to synergize with the TCR signal (13). Prolonged TCR occupancy (facilitated by adhesion molecules) and functional T cell actin cytoskeleton are both required for sustained signaling (14) leading to T cell activation at low antigen concentration.

The role of adhesion/co-stimulatory molecules in DC-mediated antigen presentation has been investigated via a variety of different approaches that are not dependent upon addition of antigen into an *in vitro* assay. For example, previous studies in our laboratory used an autologous system where T cells pretreated with sodium periodate in an oxidative mitogenesis assay provided evidence for the role of β_2 integrins in the early clustering step (15). In allogeneic mixed lymphocyte reactions (MLR) the extent of inhibition of T cell proliferation has thus been regarded as an index of the importance of a particular molecule (such as B7) in co-stimulation (3,16,17). In other studies of the allogeneic reaction, CD3 mAb have been used to provide an alternative TCR stimulus (18). These studies showed that a stronger TCR-mediated signal interaction requires less co-stimulation for optimal responses than a lesser TCR interaction.

There are several different methods that have been used to separate human PBDC for these *in vitro* functional studies. One possibility is to use elutriated mononuclear cells as a starting point (19). Others have used expression of selected cell-surface markers, such as CD14, to define the precursor cell (20). A wide range of additives have been incorporated into these cultures, ranging from unpurified monocyte conditioned medium to single recombinant cytokines. Recently it has become clear that incubating 2 h adherent cells with IL-4 and granulocyte macrophage colony stimulating factor (GM-CSF) for 7 days (21,22) will yield a potent DC population. We used this model previously, to study the differentiation of DC from monocyte precursors and to monitor phenotypic changes over time. In this study we extend the analysis further, investigating DC–T cell interaction and exploring a role for the novel co-stimulatory molecules that we have identified (22) to see if any of them help to explain the exceptional properties of the DC. For this purpose, an autologous DC–T cell assay was developed, using a low dose of CD3 mAb as a TCR signal, thus ensuring a stringent requirement for co-stimulation. The results demonstrate that five of these molecules found on the DC surface, CD13, CD87, CD98, CD147 and CD148, may play a hitherto unrecognized role in DC-induced T cell activation and that this may be because they provide a co-stimulatory signal during the APC–T cell interaction.

Methods

Cell culture

PB mononuclear cells (PBMC) were obtained from healthy volunteers. The standard protocol used 60 ml of blood taken between 09:00 a.m. and 11:00 a.m. directly into a heparinized syringe (1.5–2.0 U/ml Monoparin sodium heparin; CP Pharmaceuticals, Wrexham, UK). This was diluted 1:2 in PBS, layered over Histopaque 1077 (Sigma, Poole, UK) and centrifuged for 30 min at 1600 r.p.m. at room temperature. The interface was recovered and washed 3 times in HBSS. PBMC were incubated in six-well tissue culture plates for 2 h at 37°C/5% CO_2 in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% FCS, 50 mM 2-mercaptoethanol (Gibco), 100 U/ml penicillin/streptomycin and 2 mM L-glutamine (Imperial Cancer Research Fund, London, UK). Non-adherent cells were removed, and the adherent cells cultured in fresh medium with 100 ng/ml human recombinant GM-CSF (a gift from Dr S. Devereux, Department of Haematology, UCL Medical School, London, UK and from Schering-Plough, Madison, NJ) and 50 ng/ml human recombinant IL-4 (PeproTech, London, UK and from Schering-Plough).

After 7 days incubation loosely adherent cells were collected and any remaining lymphocytes removed by incubation with CD3 (mouse supernatant mAb UCH T1, IgG1, gift of Professor P. C. L. Beverley) and CD19 (mouse ascites mAb BU12, IgG1, gift of D. Hardie) followed by anti-mouse Ig-coated immunomagnetic beads (Dynal, Merseyside, UK). These cells were washed 3 times in HBSS and used as DC. The DC population always consisted of $<5\%$ CD3 or CD19 positive cells (as judged by staining with the same mAb).

T cells were obtained from the non-adherent population removed on day 1. B cells and monocytes were depleted by incubation with CD19, HLA class II (HLA-DR, L243 supernatant, mAb, gift of Professor P. C. L. Beverley; and HLA-

DQ, Ia3, ascites, IgG2a mouse mAb, gift of Professor R. Winchester) and CD14 (HB246, IgG2b mouse supernatant mAb, gift of Professor P. C. L. Beverley) followed by anti-mouse Ig-coated immunomagnetic beads. This protocol also removed all HLA-DR-expressing (activated) macrophages and resulted in a T cell population that was >80% $CD3^+$, with a mean fluorescence intensity in the region of 100. This phenotype was confirmed using mAb against CD14, HLA-DR and HLA-DQ, and later with mAb against CD13 and CD87 (see below), and all showed <5% positive cells, with negligible mean fluorescence. To separate T cell subsets, these T cells were incubated with CD8 (UCH-T4, IgG2a mouse supernatant mAb, gift of Professor P. C. L. Beverley) when $CD4^+$ T cells were required, with CD4 (Q4120, IgG1 mouse supernatant mAb, gift of Dr Q. Sattentau) when $CD8^+$ T cells were required, with CD45RO (UCH-L1, IgG2a mouse supernatant mAb, gift of Professor P. C. L. Beverley) for $CD45RA^+$ T cells and CD45RA (SN130, IgG1 mouse supernatant mAb, gift of Professor P. C. L. Beverley) for $CD45RO^+$ T cells. $CD4^+$ T cells were found to be 82% pure (as judged by staining with QS4120) and $CD8^+$ T cells were found to be 75% pure (as judged by staining with UCH-T4) (data not shown). $CD45RA^+$ T cells were found to be 95% pure (as judged by staining with SN130) and $CD45RO^+$ T cells were found to be 97% pure (as judged by staining with UCH-L1) (data not shown). T cells were frozen at –80°C in 90% FCS/10% DMSO during the DC culture period prior to assay.

Cell viability was monitored using both Trypan blue exclusion and a MTT assay. For the latter assay, briefly, a 5 mg/ml MTT stock solution was prepared in PBS and stored at 4°C (always for <2 weeks). Then 10 μg MTT (in 20 μl volume/well) was added to each test cell population in a 96-well ELISA plate and the plates incubated at 37°C for 4 h. Then 100 μl of 10% SDS–0.01 M HCl was added and the plates were read on an ELISA reader at an optical density of 570–630 nm. The test populations consisted of both DC and T cells separately, as well as DC but with mAb present during the 4 h period, and both DC and T cells that been pre-pulsed with mAb and then washed 3 times in RPMI to remove any remaining mAb (as outlined below).

mAb

Apart from the mAb listed above, many of the mAb used to characterize the phenotype of DC and T cells were obtained initially from the Sixth Human Leukocyte Differentiation Antigen Workshop, and subsequently from the originators. Those used in this study were against CD11a (CD11a-5E6, IgG1; CD11a-6B7, IgG1); CDw12 (M67, IgG1); CD13 (MCS-2, ascites, IgG1; clone 72, IgG1; and 7H5, ascites, IgG2a); CD18 (AZN-L18, IgG1; AZN-L27, IgG1); CD30 (11D1.H10, IgG2a); CD33 (4D3, ascites, IgG2b); CD40 (MAB89, IgG1); CD86 (BU63, ascites, IgG1); CD87 [clone 109, ascites, IgG2a; clone 3B10, ascites, IgM; IIIF10, purified antibody, IgG1 and IID7, purified antibody, IgG1 (Professor V. Magdolen) (23), HD-uPA-R13.1, purified antibody, IgG1 and HD-uPA-R15.4.1, purified antibody, IgG1 (Professor M. D. Kramer) (24) and VIM-5, ascites, IgG1 (Professor W. Knapp) (25)]; CD98 (2E12, IgG2a; MEM-108, ascites, IgG1; J1-G3B/AHN-18.1, ascites, IgG1; J3-E1B/AHN-18, ascites, IgG1; BK19.9, purified antibody, IgG1; CAF7, ascites, IgG1; IPO-T10, ascites, IgM; BU53, ascites, IgG2a; BU89, ascites, IgG1; 2G12, IgM; and 4F2, IgG2a); CD147 [HIM6 and HI197, both purified ascites, IgG1 (26); H84, ascites, IgG2b]; CD148 (143-41, ascites, IgG1); CD164 (105A5, IgG3) and the unclassified WM78 (purified culture supernatant, IgG1) and CNA42 (IgM). Second label antibody used was FITC-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark).

Immunofluorescent flow cytometry

Both DC and T cells were incubated on ice in HBSS with 10% normal rabbit serum (NRS) (Gibco) for 15 min to prevent non-specific binding. Primary mAb was then added to the cells at a titre of 1:500 (unless otherwise shown) without pre-washing and incubated for 45 min on ice. Cells were washed 3 times in HBSS/10% NRS. Fluoresceinated mAb (rabbit anti-mouse IgG) was added (diluted 1:20 in HBSS/10% NRS) and cells incubated for 45 min on ice. Cells were washed 3 times in HBSS/10% NRS, fixed overnight in 3.7% formaldehyde/PBS, and data acquired on a Becton Dickinson FACScan and analysed using WinMDI software. For each sample not less than 5000 events were acquired. The data was examined relative to a negative control sample with no primary mAb where the percentage positive cells was always <5%. The FACScan was calibrated using Quantum 26 microbeads (Flow Cytometry Standards, San Juan, Puerto Rico). DC or T cells were gated via forward and side scatter so as not to include any cell debris for analysis. All analyses were carried out on a minimum of three different individuals.

Proliferation assays

DC (irradiated, 3000 rad) were incubated at 37°C/5% CO_2 with autologous T cells, and with test mAb where noted, for 2 h in flat-bottom 96 well plates (Nunc, Roskilde, Denmark). CD3 (Harlan-Sera, Loughborough, UK) was added at to give a final volume of 200 μl. After the initial titrations, 3×10^4 DC, 10^5 T cells and 0.1 μg/ml CD3 mAb were used in all subsequent experiments. The DC–T cell–CD3 cultures were incubated for 48 h and pulsed with 1 mCi [^{3}H]thymidine (ICN Biomedical, High Wycombe, UK) for 16 h. Cells were harvested and proliferation measured by liquid scintillation counting. All assays were performed in triplicate. Inhibition is expressed as a percentage of thymidine incorporation of triplicates with no test mAb.

For prepulse assays, DC were incubated with test mAb for 1 h at 37°C/5% CO_2, then washed 3 times in RPMI before addition of T cells and CD3 as before. To fix the DC, cells were treated with 0.05% glutaraldehyde for 30 s at room temperature and the fixative quenched with 0.2M lysine (pH 7) for 3 min at room temperature. They were then washed 3 times in RPMI before addition of T cells and CD3 mAb as before.

Allogeneic assays were performed with T cells from a different donor to that of the DC. Then 3×10^4 DC (irradiated) were incubated at 37°C/5% CO_2 with 10^5 allogeneic T cells and mAb of interest, without CD3 mAb, for 6 days before measuring [^{3}H]thymidine incorporation as before.

For studies of T cell activation in the absence of DC, 25 ng/ml phorbol myristate acetate (PMA) and 4 μg/ml ionomycin were added to 2×10^5 T cells/well in a 96-well plate, and dilutions of inhibitory mAb were added to triplicate wells. The

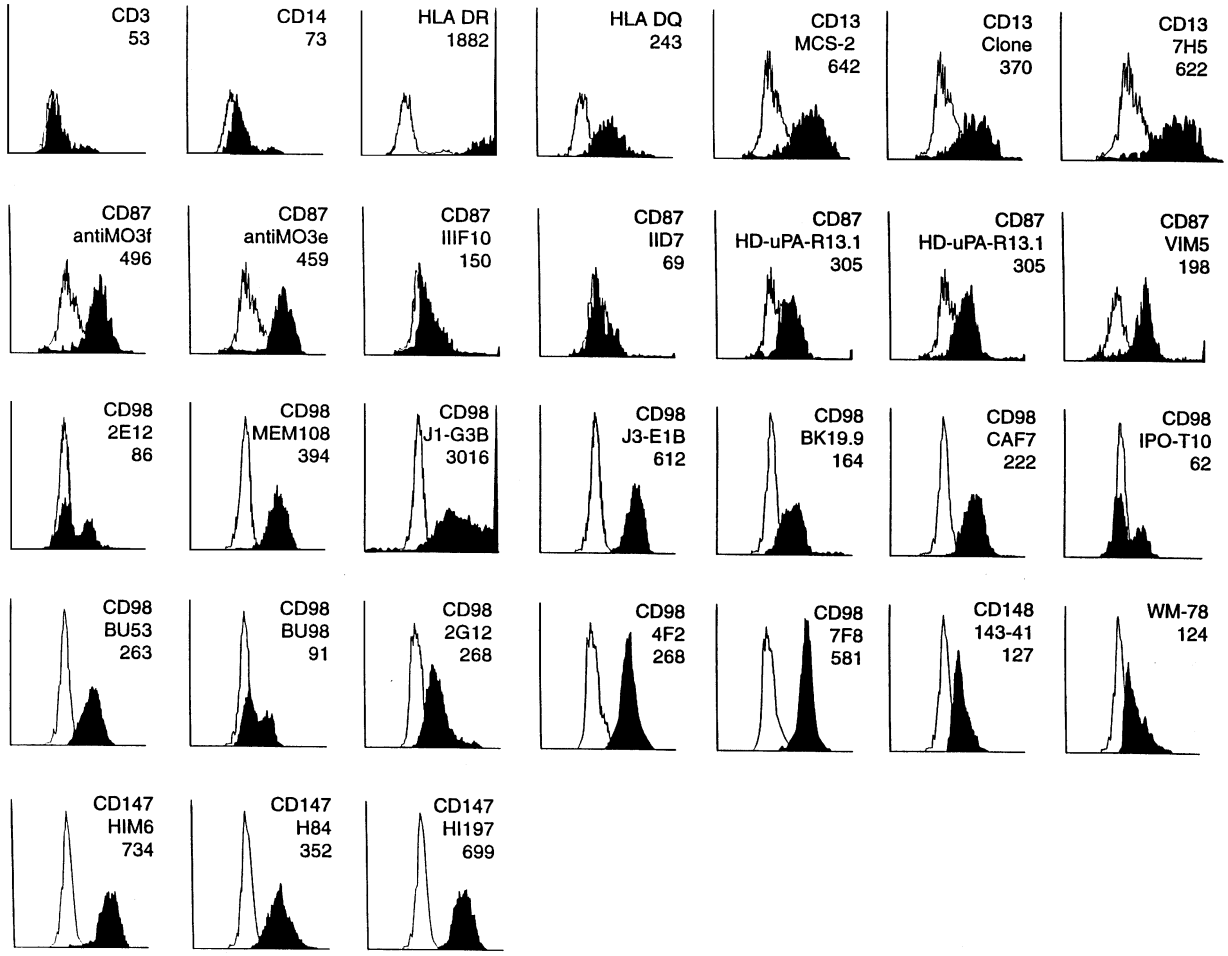

Fig. 1. PBDC were isolated, incubated with a panel of mAb, and examined using the FACScan as outlined in Methods. The data were analysed using WinMDI software. All experiments were performed on at least three individuals and 5000 events recorded. The results of a representative experiment are shown (shaded areas) including not only the names of the molecules against which the mAb used are targeted, but also the particular mAb(s) used in the study. These profiles are relative to the controls (no primary mAb used, unshaded). MFI of the shaded profiles are recorded.

subsequent incubation and assay conditions were the same as those for the DC–T cell–CD3 assays.

Results

DC and T cell phenotype

The phenotype of the PBDC is illustrated in Fig. 1. As has been reported previously, PBDC were negative for CD3 and CD19. A small percentage (14%) were weakly positive for CD14, which is consistent with other studies where it is only the most 'activated' DC populations that have no $CD14^+$ cells present at all. These cells were >95% positive (range 94–100%, over five individuals) with a high mean fluorescence intensity (MFI) (range 4537–5789) for HLA-DR and >80% positive (range 76–88%) with a lower range of MFI (143–490) for HLA-DQ. PBDC also expressed CD1a (70–95%, MFI 989–1887), CD18 (95–100%, MFI 2356–5187), and CD86 (67–96%, MFI 249–604). These findings are in accordance with the established PBDC phenotype. In addition, PBDC were also noted to express CD13, CD87, CD98, CD147 and CD148, and to bind one mAb (WM78) which recognizes a molecule that has not yet been assigned CD nomenclature. T cells, known to be positive for CD3 and negative for CD14, HLA-DR, HLA-DQ, CD13 and CD87, showed variable levels of detectable CD98 depending on which mAb was used. For example, one mAb, J1-G3B/AHN-18.1, bound to 100% of cells, with an MFI in a representative experiment of 370, whereas although the J3-E1B/AHN-18 mAb bound to >90% of the same T cells the MFI was only 24 (compared to 612 on PBDC in the same experiment). The other three CD98 mAb that showed binding on a proportion of T cells (MEM108, CAF7 and BU53) all showed mean fluorescence of <20, i.e. 10-fold less than that seen on PBDC. CD147 was expressed on a high percentage of T cells (75–95%), but the MFI (e.g. maximum 35 in a representative experiment) was much lower than that seen on PBDC. The levels of CD148 expression were also much lower on T cells (e.g. MFI of 8, compared to 127 on PBDC) and only 10–15% of the cells expressed this molecule.

DC-induced autologous T cell co-stimulation assay

The first part of the study aimed to establish a reliable autologous assay of DC co-stimulatory function. The approach selected was based upon previous related similar studies (23–25). The experiments were performed using soluble CD3, rather than CD3 mAb coated to the culture well, in order that the TCR signal be delivered to the T cell in the context of a direct physical interaction with the PBDC, which could be monitored visually using the formation of clusters around the DC. Thus in this assay the CD3 mAb provides the first signal and the second signal comes from the DC. By morphology, the clustering was striking, whereas in the absence of CD3 mAb only small clusters were observed and in the absence of PBDC no clusters were observed (data not shown).

To confirm the co-stimulation dependence of the assay further, the different components were titrated into the assay, and the outcome monitored using tritiated [^{3}H]incorporation. Figure 2(a) shows that titration of PBDC results in significant proliferation with as few as 10^4 PBDC, and that the proliferative response correlated with the numbers of PBDC present. PB monocytes at concentrations of 10^4 and 3×10^4 did not induce a response (data not shown). Similar experiments with different T cell numbers (Fig. 2b) and CD3 concentrations (Fig. 2c) showed that 0.1 μg/ml CD3 and 10^5 T cells could be used, and that these levels gave sub-maximal responses, thus allowing for both positive and negative effects to be observed.

In order to confirm that the T cell activation in this assay was dependent on delivery of classical adhesive–co-stimulatory signals from the DC, the effects of mAb directed at CD11a and CD18 were used. The results of a representative experiment are shown in Fig. 3. In all cases >80% inhibition of proliferation by these mAb was observed and the results were statistically significant (Welch two-tailed *t*-test $P < 0.05$). In similar experiments CD80 gave ~50% inhibition, CD86 mAb gave between 32 and42% inhibition, and when the two mAb were added together mean inhibition was 60%, with similar statistical significance. A panel of isotype-matched mAb (against CDw12,IgG1; CD30, IgG2a; CD33, IgG2b; CD164, IgG3; and an unknown molecule recognized by the mAb CNA42, IgM), all of which bind to DC, were not inhibitory. Therefore these inhibitory and non-inhibitory mAb were used as standard controls for subsequent assays.

Based upon these experiments, a series of test mAb were studied in the same assay system. All were chosen because they were expressed on PBDC (Fig. 1), but had not been implicated in DC function previously. Figure 4 shows that mAb against CD13 (7H5), CD98 (J3-E1B/AHN-18), CD147 (H84) and CD148 (143-41) are all inhibitory; and that, furthermore, the level of inhibition is dependent upon the concentration of mAb used. The CD30 mAb (11D1-H10) had no effect. All three CD13 mAb gave similar inhibition (>40% in each of three experiments). Two of the three CD147 mAb tested inhibited the response (HIM6 and H84) but the third mAb (HI197) (data not shown) did not. The CD148 mAb (143-41) also gave a consistent pattern of inhibition and likewise WM-78, a mAb to a molecule not yet assigned a CD number, showed percentage inhibition in a similar range (data not shown).

In these experiments, all mAb to CD87 inhibited the response, but there was some variability in the degree of inhibition depending upon which mAb was used. Figure 5(a) shows a representative experiment. These differences in percentage inhibition were reproducible, and statistically significant compared to the test assays ($P < 0.05$). For example, HD-uPA-R15.4.1 was consistently the most potent inhibitor (percentage inhibition range 72–91% in three experiments) and this was seen even at a dilution of 1:1000 (data not shown). The mAb IIIF10 (range 34–67%) and IID7 (range 41–

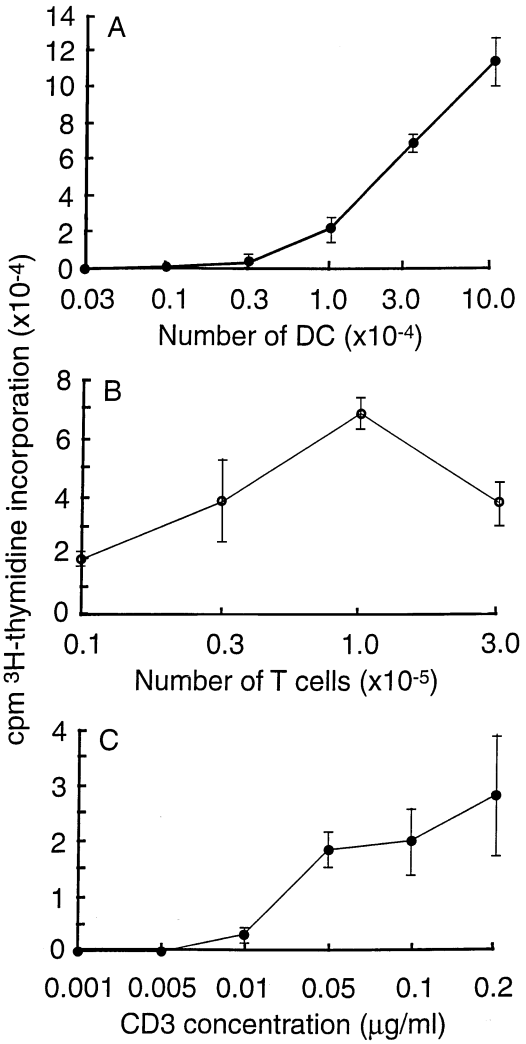

Fig. 2. (A) PBDC (irradiated) titration (horizontal axis) in an assay using 10^5 autologous T cells and 0.1 μg/ml CD3, incubated for 48 h and pulsed with [^{3}H]thymidine for 16 h. PBDC at 3×10^4 was taken as the optimum concentration for subsequent experiments. (B) T cell titration (horizontal axis) in an assay using 3×10^4 PBDC (irradiated) and 0.1 μg/ml CD3, incubated for 48 h and pulsed with [^{3}H]thymidine for 16 h . T cells at 10^5 was taken as the optimum concentration for subsequent experiments. (C) CD3 titration (horizontal axis) in an assay using 3×10^4 PBDC and 10^5 autologous T cells incubated for 48 h, and pulsed with [^{3}H]thymidine for 16 h. All experiments were performed on at least three individuals. Results are shown as mean ± SD of triplicate wells and data is from a representative experiment. PBDC and CD3 alone gave 356 c.p.m., T cells and CD3 alone gave 272 c.p.m., and PBDC and T cells with no CD3 gave 1854 c.p.m.

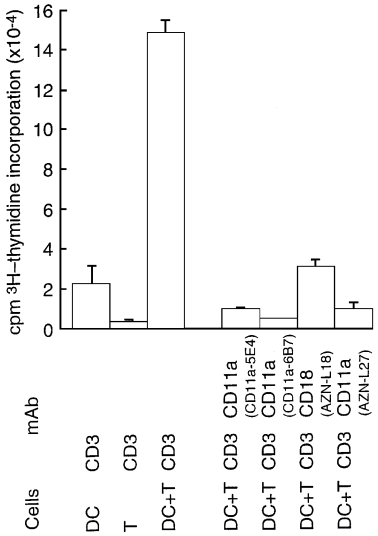

Fig. 3. Inhibition of T cell proliferation by CD11a and CD18 mAb. In total, 3×10^4 irradiated PBDC, 10^5 autologous T cells and 0.1 μg/ml CD3 were incubated for 48 h and pulsed with [^{3}H]thymidine for 16 h. The mAb against CD11a (CD11a-5E4, 2.8 μg/ml; CD11a-6B7 and AZN-L27, 0.3 μg/ml) and CD18 (AZN-L18, 0.3 μg/ml) were added at 1:500 dilution as indicated. Control mAb (data not shown) were against CDw12, CD30, CD33 and CD164. Results are shown as mean ± SD of triplicate wells and data is from a representative experiment.

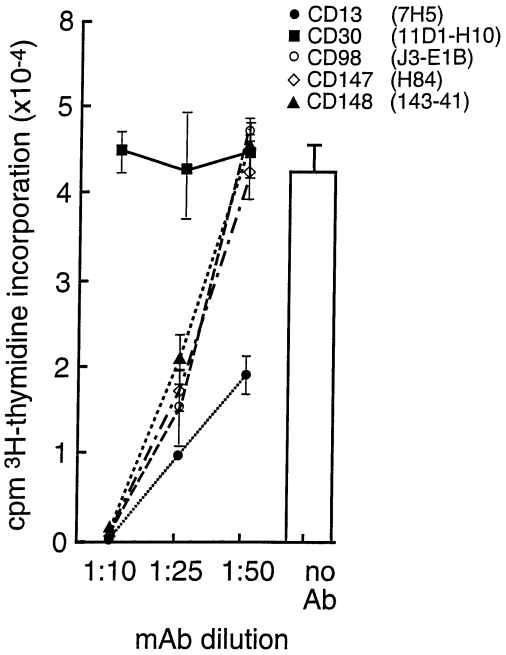

Fig. 4. In total, 3×10^4 irradiated PBDC, 10^5 autologous T cells and 0.1 μg/ml CD3 were incubated for 48 h, and pulsed with [^{3}H]thymidine for 16 h. The mAb against CD13 (7H5, 0.8 μg/ml), CD30 (11D1-H10, 0.1 μg/ml), CD98 (J3-E1B/AHN-18, 3.2 μg/ml), CD147 (H84, 0.8 μg/ml) and CD148 (143-41, 1.1 μg/ml) were added to the assay at three different dilutions. Results are shown as mean ± SD of triplicate wells and data is from a representative experiment.

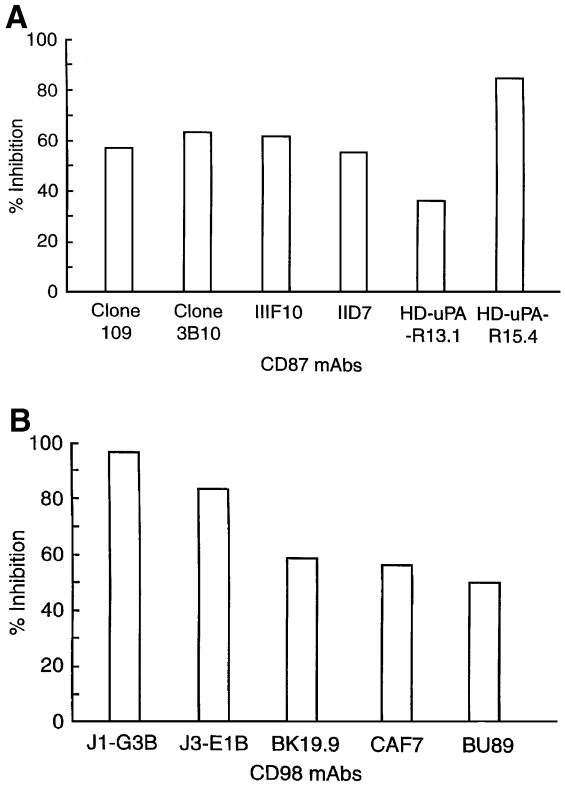

Fig. 5. (A) In total, 3×10^4 irradiated PBDC, 10^5 autologous T cells and 0.1 μg/ml CD3 were incubated for 48 h, and pulsed with [^{3}H]thymidine for 16 h. A panel of mAb against CD87 (0.1–3.0 μg/ml) were added to the assay at dilutions ranging from 1:100 – 1:1000. Results from a representative experiment are shown at 1:100 dilution, expressed as percentage inhibition of a standard assay. (B) In total, 3×10^4 irradiated PBDC, 10^5 autologous T cells and 0.1 μg/ml CD3 were incubated for 48 h and pulsed with [^{3}H]thymidine for 16 h. A panel of mAb against CD98 (0.1–3.0 μg/ml) were added to the assay at dilutions ranging from 1:100 to 1:1000. Results from a representative experiment are shown at 1:100 dilution, expressed as percentage inhibition of a standard assay.

59%) were also inhibitory at 1:100 dilution, despite almost no detectable binding to the PBDC surface by FACS analysis. One CD87 mAb (VIM5) did not inhibit the response (data not shown). These differences were not due to variability in mAb concentration, which ranged from 0.025 to 1.5 μg/ml.

Figure 5(b) shows a similar experiment with CD98 mAb. Again the results were all statistically significant compared to controls. The mAb J1-G3B/AHN-18.1 (1.5 μg/ml), J3-E1B/AHN18 (3.2 μg/ml), BK19.9 and CAF7 (0.8 μg/ml) were consistently inhibitory. For example, addition of J1-G3B/AHN-18.1 resulted in a percentage inhibition range from 97 to 99% inhibition in three experiments when used at 1:500 dilution; the other inhibitory mAb gave 50–80% inhibition at this dilution. Four CD98 mAb [2E12 (0.8 μg/ml), MEM108 (0.025 μg/ml), IPO-T10 (0.8 μg/ml) and BU53 (0.4 μg/ml)] were not inhibitory.

Neither Trypan blue exclusion nor the MTT assay demonstrated changes in cell viability in the populations which contained mAb compared to controls with no mAb (data not shown).

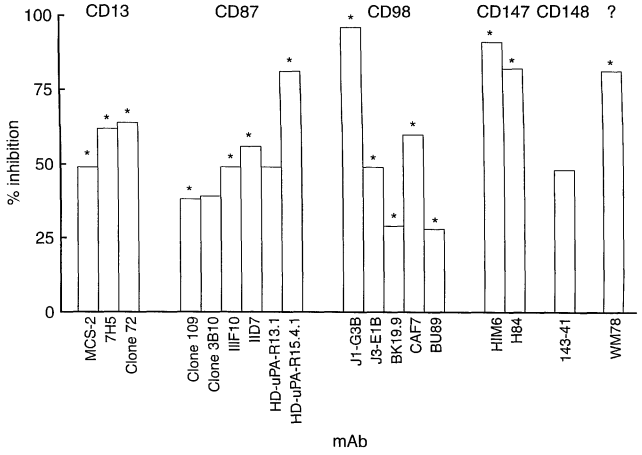

Fig. 6. PBDC were prepulsed with a panel of mAb for 1 h at 37°C/5% CO_2 before being washed 3 times in RPMI. T cells and CD3 were added as before (as outlined in Methods). Results from a representative experiment are shown, expressed as percentage inhibition. Control mAb against CD11a and CD18 were inhibitory, and against CDw12, CD30 and CD164 were not (data not shown). Asterisks indicate statistically significant inhibition compared to cells prepulsed with non-inhibitory isotype-matched control mAb ($P < 0.5$)

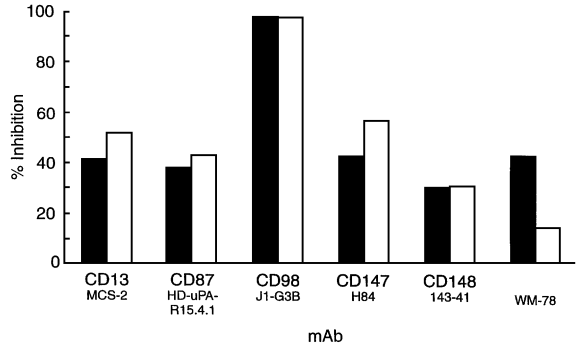

Fig. 7. PBDC CD3-induced proliferation of autologous $CD4^+$ T cells (black columns) and $CD8^+$ T cells (white columns) was examined. The data is from a representative experiment and is expressed as percentage inhibition, in an assay where DC and CD3 alone gave 381 c.p.m., $CD4^+$ T cells and CD3 alone gave 13 c.p.m. [^{3}H]Thymidine incorporation, $CD8^+$ T cells and CD3 alone gave 1710 c.p.m., DC and $CD4^+$ T cells and CD3 gave 110,908 c.p.m., and DC and $CD8^+$ T cells and CD3 gave 54,463 c.p.m.

Effect of prepulse mAb on autologous DC-induced T cell proliferation

In order to ascertain whether the effects observed by these mAb were mediated via binding to the PBDC, PBDC were pulsed with mAb of interest for 1 h at 37°C, before being washed 3 times in RPMI to remove any unbound mAb. Aliquots both of pulsed PBDC and of control T cells were examined by Trypan blue exclusion and in the MTT assay, and there was no difference in cell viability indicating that the effects seen were not due to cytotoxicity. T cells and CD3 were added into the assay as outlined previously. The results are shown in Fig. 6, and resemble those of the previous assay closely, again with the CD87 mAb HD-uPA-R15.4.1 and the CD98 mAb J1-G3B/AHN18.1 being the most striking inhibitors for each respective specificity. Inhibition ranged from 62 to 73% over three experiments for HD-uPA-R15.4.1 (pulsing concentration 1:100) and from 97 to 99% over three experiments for J1-G3B/AHN18.1 (pulsing concentration 1:500). Where the PBDC were fixed after the prepulsing step, the CD98 (J1-G3B/AHN18.1) inhibition remained >90%. For CD13 (MCS-2) the percentage inhibition was 68%, for CD147 (HI84) 98% and for CD148 (143-41) 96%. These results were all statistically significant ($P < 0.05$).

DC induced allogeneic responses

To confirm that the inhibition documented above (Figs 4–6) was not a characteristic of CD3-mediated T cell activation only, we tested the mAb further in a 6 day MLR. Inhibition was observed with CD13 (MCS-2) (20%), CD87 (Clone 109) (49%), CD98 (J1-G3B/AHN18.1) (46%), CD147 (H84) (77%), CD148 (143-41) (19%), WM-78 (24%) and CD86 (50%).

Effect of mAb on DC-independent T cell activation

In view of the possibility that many of these cell surface molecules are expressed on T cells as well as on DC, and may therefore have an effect on T–T interactions as well as on DC–T cell interaction, the same panel of mAb were also tested at the same concentrations, but using PMA and ionomycin instead of DC and CD3 as the activating signal. In two consecutive assays neither CD13 nor CD87 had an inhibitory effect. In contrast, the results for CD98 mAb were similar to those seen in the other activation assays, i.e. maximum (>90%) inhibition was seen with both J1-G3B/AHN18.1 and J3-E1B/AHN18. Four other CD98 mAb tested (BK19.9, BU53, BU89 and 4F2) showed significant inhibition but only between 20 and 30%. Likewise, CD147 (H84) and CD148 (143-41) mAb showed >90 and 50% inhibition respectively.

$CD4^+$ T cell and $CD8^+$ T cell subsets

In order to assess whether mAb preferentially inhibited $CD4^+$ T cells or $CD8^+$ T cells, responder T cell populations were enriched for these phenotypes before the assay. Figure 7 shows percentage inhibition of proliferation using either CD4 or CD8 T cell subsets. All mAb tested inhibited both $CD4^+$ and $CD8^+$ T cells. The mAb WM-78 inhibited $CD4^+$ T cell proliferation by 43–48%, but $CD8^+$ T cells by only 10–15% in two consecutive experiments ($P < 0.0003$); otherwise there were no significant differences between the inhibition of the $CD4^+$ and $CD8^+$ T cells respectively.

$CD45RA^+$ and $CD45RO^+$ T cell subsets

Similar experiments were performed using fractionated $CD45RA^+$ T cells or $CD45RO^+$ T cells (Fig. 8). CD87 (HD-uPA-R15.4.1), CD98 (J1-G3B), CD147 (H84) and mAb WM-78 inhibited both CD45RA and CD45RO T cell proliferation equally. CD13 (MCS-2) and CD148 (143-41) both inhibited CD45RA T cell proliferation to a greater extent than CD45RO T cells. Thus, for example, CD13 (MCS-2) inhibited CD45RA T cells (42–49%, $P < 0.01$), but had a much smaller effect on CD45RO T cells (<10%). Similarly CD148 (143-41) inhibited CD45RA T cell proliferation by 53–56% ($P < 0.003$), whereas with CD45RO T cells the same mAb inhibited by 35–40%.

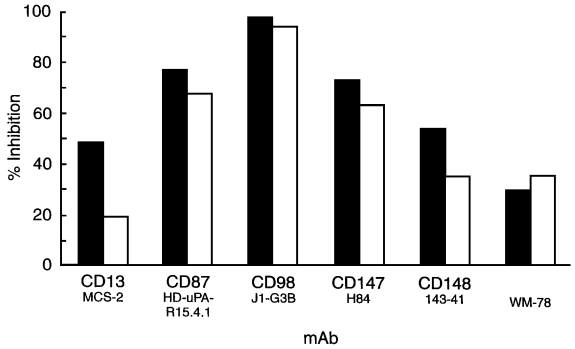

Fig. 8. PBDC CD3-induced proliferation of autologous $CD45RA^+$ T cells (black columns) and $CD45RO^+$ T cells (white columns) was examined. The data is from a representative experiment and is expressed as percentage inhibition, in an assay where DC and CD3 alone gave 584 c.p.m. [^{3}H]Thymidine incorporation, $CD45RA^+$ T cells and CD3 alone gave 71 c.p.m., $CD45RO^+$ T cells and CD3 alone gave 386 c.p.m., DC and $CD45RA^+$ T cells and CD3 gave 41011 c.p.m., and DC and $CD45RO^+$ T cells and CD3 gave 18,780 c.p.m.

Discussion

In this study we have explored the molecular mechanisms that are involved during activation of T cells by PBDC further, using an autologous culture system comprising PBDC, T cells and CD3 mAb. The system showed many of the characteristics of physiological DC presentation: the T cell response was proportional to DC numbers and could be blocked by mAb to known accessory molecules, such as CD18, CD54 and CD86. In addition, the use of soluble CD3, rather than antibody coated to plastic, allowed the formation of DC–T cell clusters reminiscent of those seen in antigen-specific responses *in vivo*. To confirm further that the effects were not an artifact of the CD3-dependent model, the same mAb were also tested in a classic MLR and a parallel inhibitory effect demonstrated.

Based upon these findings we analysed the functional consequences of adding a panel of mAb to a number of cell-surface molecules that we have shown recently are present on PBDC (22). As a result, we identified an accessory role for five CD antigens (and for a potential sixth molecule, recognized by a mAb whose target has yet to be defined) in T cell activation by PBDC. The combination of the CD3 assay ('over-riding' the antigen-specific component and thus focussing on potential co-stimulatory components), the expression of these molecules on the surface of PBDC, the observation that very similar results are seen when the mAb were pre-pulsed on to the PBDC and confirmation of the data in separate parallel work using U937 (promonocyte) cells as accessory cells (27) all highlight the potential role of these molecules in PBDC–T cell interaction and raise the possibility that this role is at the level of the PBDC.

One example of this was CD13, which is highly expressed on PBDC, but not on the T cells. mAb to CD13 inhibited both standard and prepulse autologous assays, had no effect on T cell activation by PMA and ionomycin, and blocked activation of CD45RA (naive) T cell proliferation rather than CD45RO (memory) T cell proliferation. CD13, or aminopeptidase N, is a zinc-dependent metalloprotease, which may remove N-terminal residues from active peptides or convert peptides from inactive forms to active forms (28) and thus modulate signalling. CD13 has been implicated in antigen presentation in murine studies previously (29) in that CD13 was coordinately regulated with MHC class II expression, but no functional analysis was documented. In this study CD13 might be required to generate soluble or cell surface active peptides that are important for initiating signal transduction into the DC, or alternatively which are directly involved in the PBDC interaction with naive T cells.

Likewise, CD87 showed the same inhibitory pattern as CD13. CD87 is the urokinase plasminogen activator receptor (uPAR), that is implicated in leukocyte migration and adhesion. The molecule has been co-isolated with the c-*fyn* tyrosine kinase and forms a functional complex with β_2 integrins, thus facilitating adhesion (30–33). CD87 mediates mechanical force transfer across the cell surface and is coupled to the cytoskeleton (34). Disruption of the cytoskeleton can lead to an increase in CD87 expression (35,36). In functional studies when CD87 binds to uPA this is associated with conversion of plasminogen to plasmin, which facilitates matrix degradation and hence increases metastatic invasion (37). This is supported by the observation that over-expression of CD87 is associated with increased tumour growth and metastasis, particularly in breast cancer (38–40). Thus, *in vivo* CD87 may play a role in migration of DC through tissues, mediated via the same mechanism as that used by the neoplastic cell. In *in vitro* experiments, however, it is more likely that an integrin–cytosteletal association required for an effective DC–T cell interaction is the target site for the mAb.

Our study (Fig. 1) demonstrated differences in expression between different CD87 epitopes, as reflected in binding by different CD87 mAb. Of seven CD87 mAb analysed, five were positive on these PBDC. The two mAb that did not bind to PBDC recognize epitopes in the D1 and D2 domains, and a different pattern of expression with these two mAb has been reported (41). No CD87 mAb stained T cells, consistent with previous findings, where CD87 was only seen on activated T cells (42). All the CD87 mAb which were positive on PBDC recognize glycosylated forms of CD87, while the other two mAb recognize components of the amino acid backbone, which is not exposed at the cell surface. However, these two mAb nonetheless have an inhibitory effect in the functional assay suggesting that CD87 may undergoes dynamic changes during the course of the assay. This could be due to synthesis of new CD87 in a non-glycosylated form, to deglycosylation on the surface or to change in conformation, rendering the protein core accessible to mAb binding and hence inhibition.

CD98 is a type II transmembrane glycoprotein, recently shown to co-precipitate with the β_1 integrins (43). These studies postulated that up-regulation of integrin function requires clustering of CD98 molecules. Some CD98 mAb also induce HIV gp160-mediated cell fusion to produce multinucleated giant cells (44,45) and the molecule has been implicated in regulation of cell survival—apoptosis (46). A role in calcium flux into cells has been proposed, since CD98 ligation causes an influx of extracellular calcium in neuronal cells (47) and galectin-3 (which is a possible CD98 ligand) also induces calcium flux in CD98-expressing T cells (48). As galectin-3 is synthesized and released by accessory

cells, it may also induce CD98-mediated autocrine effects on monocytes and macrophages themselves (49).

The pattern of CD98 expression on PBDC and T cells shown here suggests that the molecule may exist in more than one form, possibly due to differential glycosylation or to the existence of different isoforms on different cell types. Thus, on PBDC, most mAb bound with high intensity, but 2E12, IPO-T10 and BU89 bound at lower levels and exhibited a biphasic distribution (Fig. 1). This was reflected in the pattern of binding to T cells as well as to PBDC: three mAb showed strong binding (MEM108, J1-G3B/AHN-18.1 and J3-E1B/AHN-18), two showed weak binding (CAF-7 and BU53) and the remaining mAb did not bind. A similar pattern was seen in the functional assays: mAb, J1-G3B/AHN-18.1, J3-E1B/AHN-18, BK19.9 and CAF7 all gave consistent inhibition, whereas 2E12, MEM108, IPO-T10, BU53 and BU89 did not. Thus different CD98 epitopes may have different functional roles in antigen presentation, and this could be due to their relative ability to cluster and induce a signalling cascade in the APC. Alternatively, inhibitory mAb may cause more efficient clustering of CD98 molecules and then sterically hinder the integrin interaction with ligands on the T cell.

These studies are consistent with our findings with CD98 mAb in a U937 model system (27) and with functional studies of CD98 that showed that the molecule acts at the accessory cell level, both in primary T cell responses and in a xenogeneic system (51). The inhibition (unlike that seen with CD13 and CD87 mAb) in the PMA–ionomycin-induced T cell activation is not inconsistent with this view. This may be due to an effect on cell survival (46) or reflect the dynamic T–T cell interaction that contributes to the formation of the APC–T cell complex in addition to the APC–T cell component (52). It is noteworthy that prefixation of bound CD98 before adding T cells and CD3 was also inhibitory. Furthermore, the previous studies of the role of CD98 in accessory cell assays used PBMC depleted by treatment with leucine methyl ester to suggest that the effect was at the level of the monocyte: since the PBDC used here were derived from the same cell fraction, this implies no PBDC would have been present in the assays. Therefore both these findings favour a role for CD98 at the DC level.

CD147, neurothelin, was described originally on endothelial cells at the blood–brain barrier (53) and may play a role in nutrient transport. The distribution of CD147 is regulated by cell–cell interactions and co-localizes with F-actin, exhibiting a polarized distribution on the cell surface (54). Neurothelin knockout mice showed little or no difference in blood–brain barrier function, although paradoxically lymphocytes from the knockout mice produced a greater proliferative response in an MLR, than those of wild-type littermates (55). In our analysis CD147 was expressed on both PBDC and T cells, and CD147 mAb inhibited both the standard and pre-pulsing assays. As for CD98, the effect of CD147 on accessory cell-independent PMA–ionomycin T cell responses indicates a more general role in cell–cell interaction, but one which is reflected in its role on PBDC function and, again, this is supported by the results of fixation experiments.

CD148 is a newly defined receptor tyrosine phosphatase recently described as a marker for other forms of activated monocytes (56). The mAb inhibited all assays except where CD45RO T cells were used, where no consistent pattern of inhibition was found. CD148 may therefore play an important role at the level of PBDC presentation to naive, rather than memory T cells. By analogy with previous findings with CD148, the effects may be to decrease calcium mobilization into the PBDC, but it is also possible that in the presence of CD148 mAb there is unopposed tyrosine phosphorylation and hence activation of inhibitory molecules that are otherwise not readily identified (57). This latter hypothesis would be consistent with the observation that CD148 is also inhibitory in PBDC-independent assays.

Finally, the other mAb with functional effects was WM-78, which has not yet been cloned and allocated CD nomenclature, but was present on PBDC, and showed some expression on T cells. This mAb also inhibited all assays and interestingly was the only one tested that showed selective inhibition of CD4 T cell proliferation rather than of CD8 T cells.

In conclusion, therefore, our study has identified six further molecules which appear to play a role in the antigen presentation event. In every case the mAb which recognized these molecules were inhibitory when pre-pulsed on the APC, suggesting that their predominant effect is mediated via these cells rather than via T cells. Only one mAb showed selective inhibition of CD4 versus CD8 T cells and two mAb preferentially inhibited CD45RA versus CD45RO cells. The latter finding is consistent with current dogma suggesting that naive T cells have a more stringent requirement for accessory interaction than memory T cells (13,58). The precise mechanism whereby each molecule exerts its effect remains to be elucidated in future studies. For example, we do not as yet know whether they act in parallel or in series to known co-stimulatory pathways such as CD28–CD80–CD86. However, irrespective of the specific molecular mechanisms, the study does highlight an added level of complexity to the APC–T cell interaction, but also one which identifies several new potential targets for immunomodulation of DC in the future. Furthermore, it is becoming increasingly clear that DC do indeed serve as the 'sentinels' responding to infection or cell injury in the local micro-environment (59), and that this may be mediated by up-regulation of the molecules on their surface that act as co-stimulators in conjunction with the antigen-specific TCR-mediated events and control migration to lymph nodes; and that the same molecules may select not only for antigen presentation *per se* but also for determining outcome, as reflected by 'tolerance' versus 'responsiveness'. The findings reported here suggest that these six potential novel co-stimulatory molecules all need to be considered as components in this critical stage of response induction.

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Abbreviations

APC	antigen-presenting cell
DC	dendritic cell
MC	mononuclear cells
MLR	mixed lymphocyte reaction

NRS	normal rat serum
PB	peripheral blood
PMA	phorbol myristate acetate

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