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Orf virus-encoded interleukin-10 inhibits maturation, antigen presentation and migration of murine dendritic cells

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Orf virus (ORFV) belongs to the genus *Parapoxvirus* and induces cutaneous pustular lesions in sheep, goats and humans. ORFV is unusual in that it has the ability to reinfect its host and this suggests that the generation of immunological memory has been impaired, thus exposing the host to subsequent infection. The discovery that ORFV encodes an IL-10-like virokine raises the question of whether this factor adversely affects the cells that initiate the acquired immune response. We examined the effect of ORFV-IL-10 on immature murine bone marrow-derived dendritic cells (BMDC). Immature BMDC are activated on exposure to antigen and undergo maturation. This process is characterized by increased expression of CD80, CD86 and MHC class II and reduced antigen uptake. We found that the maturation of BMDC is impaired in cells treated with ORFV-IL-10 prior to antigen exposure and this was exemplified by the reduced expression of the cell-surface markers described above. We have also shown that the activation of a haemagglutinin peptide (HAT)-specific T cell hybridoma by dendritic cell-mediated presentation of HAT and heat-inactivated influenza virus AP8/34 was markedly reduced following exposure to ORFV-IL-10. Finally, we examined the effect of ORFV-IL-10 on Langerhans' cell (LC) migration using cultured murine skin explant tissue and showed that this virokine impaired the spontaneous migration of LC from the epidermis and induced changes in LC morphology. Our findings suggest that ORFV-IL-10 has the capacity to impair the initiation of an acquired immune response and hence inhibit the generation of immunological memory necessary for immunity on subsequent exposure.

INTRODUCTION

The initiation of an acquired immune response to a virus requires that antigen be taken up by dendritic cells (DC) in the periphery, processed and transported to the draining lymphoid tissue to be presented to T

cells (reviewed in Palucka & Banchereau, 2002). For this sequence of events to occur, DC must first become activated by signals transmitted through the interaction of viral products with pattern recognition receptors expressed on the surface of these cells. DC form the vital bridge between the innate response and the acquired response. In doing so they become an attractive target for virally mediated immunosuppression.

Viruses evade the immune response of the host by an array of mechanisms including the production of cytokine homologues capable of deflecting, down regulating or aborting cell-mediated immunity (Alcami & Koszinowski, 2000). Interleukin (IL)-10 homologues are produced by Epstein–Barr virus (EBV) (Moore *et al.*, 1990), equine herpesvirus (EHV) (Rode *et al.*, 1993), cytomegalovirus (Kotenko *et al.*, 2000), Yaba-like disease virus (Lee *et al.*, 2001) and orf virus (ORFV) (Fleming *et al.*, 1997). Mammalian IL-10, a prototypic anti-inflammatory cytokine, is capable of both immunosuppressive and immunostimulatory functions (reviewed in Fickenscher *et al.*, 2002). This cytokine is known to inhibit DC function and ultimately the induction of anti-virus immunity. IL-10 prevents the differentiation of DC from monocytes (Buelens *et al.*, 1997) and inhibits the downregulation of receptor-mediated endocytosis and macropinocytosis following exposure to a soluble immunogen (Morel *et al.*, 1997). DC treated with IL-10 prior to antigen exposure are poor at stimulating T cell responses (Morel *et al.*, 1997; Faulkner *et al.*, 2000) and the production of IL-12 is reduced (Huang *et al.*, 2001). Recent work has suggested that IL-10 may intervene at the level of antigen processing within the cell so that antigen is not degraded effectively and MHC class II molecules fail to load with peptide (Fiebiger *et al.*, 2001). This cytokine also impairs DC migration to the local draining lymphoid tissue (Wang *et al.*, 1999) that may occur via the downregulation of chemokine receptors important for chemotaxis to the secondary lymphoid tissue (Takayama *et al.*, 2001). All of these immunosuppressive effects would be advantageous to enhanced survival of an infecting virus.

ORFV is the type species of the genus *Parapoxvirus* and infects sheep, goats and humans. The virus infects sheep via breaks in the skin around the mouth and causes contagious pustular dermatitis or scabby mouth that spontaneously resolves after 4–6 weeks (McKeever *et al.*, 1988). ORFV has the ability to reinfect its host despite an apparently normal host immune response (reviewed in Haig & McInnes, 2002), albeit these lesions are smaller and resolve faster (10–15 days) (Robinson *et al.*, 1981; McKeever *et al.*, 1988; Haig *et al.*, 1996). Antibody and cell-mediated responses to the virus are induced, although recovery is dependent only on the latter (Buddle & Pulford, 1984; Mercer *et al.*, 1994; Czerny *et al.*, 1997; Lloyd *et al.*, 2000). Infection is also associated with a rapid influx of cells characterized as dendritic cells on the basis of morphology, acetylcholinesterase staining and high MHC class II expression (Jenkinson *et al.*, 1991; Lear *et al.*, 1996). Genetic analysis of ORFV has revealed that it encodes a number of potential immunomodulators and these include an IFN resistance factor (Haig *et al.*,

1998; McInnes *et al.*, 1998), a GM-CSF/IL-2 binding protein (Deane *et al.*, 2000), a chemokine binding protein (unpublished) and a homologue of IL-10.

ORFV-IL-10 shows high levels of aa identity to IL-10 of sheep (80 %), cattle (75 %), humans (67 %), mice (64 %) and EBV IL-10 (63 %) and EHV IL-10 (67 %) (Fleming *et al.*, 1997). Studies on the effects of ORFV-IL-10 on the immune response using murine and ovine cells have shown that it inhibits the production of TNF- α and IL-8 from activated macrophages and keratinocytes and IFN- γ and GM-CSF from peripheral blood mononuclear lymphocytes (Haig *et al.*, 2002; Imlach *et al.*, 2002). In addition, ORFV-IL-10 costimulates mast cell and thymocyte proliferation (Fleming *et al.*, 1997; Haig *et al.*, 2002; Imlach *et al.*, 2002). These activities are consistent with the effects of mammalian IL-10. We have used the mouse model to investigate the effects of ORFV-IL-10 on the phenotype and function of DC *in vitro* and on the migration of Langerhans' cells (LC) *in vivo*. We report here that ORFV-IL-10 impairs both the downregulation of antigen uptake following antigen stimulation and the upregulation of MHC class II and the costimulatory molecules CD80 and CD86. It also inhibits the ability of DC to stimulate antigen-specific T cells and impairs the migration of LC from the epidermis. These findings suggest that ORFV may not only suppress the innate immune response but also target the acquired response.

METHODS

Mice. Six- to twelve-week-old BALB/c male and female mice, bred conventionally, were obtained from the Hercus Taieri Resource Unit (HTRU), University of Otago, Dunedin, New Zealand and the Animal Resource Centre (Perth, WA, Australia) and approved for use in these experiments by the Committee on Ethics in the Care and Use of Laboratory Animals at Otago and Sydney Universities.

Cytokines, antibodies and antigen. Commercially produced murine recombinant (m) IL-10 was purchased from R&D Systems. ORFV-IL-10 was purified by affinity chromatography using anti-FLAG resin from the supernatants of 293 EBNA cells transfected with pAPEX expressing the viral IL-10-FLAG (Imlach *et al.*, 2002). Recombinant murine granulocyte/macrophage-colony stimulating factor (GM-CSF) was prepared as described previously (Faulkner *et al.*, 2000).

The following monoclonal phycoerythrin (PE)-conjugated antibodies and their isotype controls were purchased from Pharmingen: 3/23 (rat anti-mouse CD40; IgG2a), 16-10A1 (hamster anti-mouse CD80; IgG), GL1 (rat anti-mouse CD86; IgG2a), 2G9 (rat anti-mouse I-A^{b/d} (MHC class II); IgG2b), 1B1 (rat anti-mouse CD1; IgG2b), HL3 (hamster anti-mouse CD11c; IgG), 3E2 (hamster anti-mouse CD54; IgG). The mAb PE-conjugated AMT-13 antibody (rat anti-mouse CD25; IgG2a) was purchased from Sigma. Undiluted supernatant from the TIB120 hybridoma was used as a source of mAb against I-A^d/I-E^d (MHC

class II) for immunohistochemistry.

PPD-B [purified protein derivative prepared from heat-killed *Mycobacterium bovis* (BCG), strain AN5 (CSL Ltd, Parkville, Victoria Australia)] was used at a final concentration of 40 $\mu\text{g ml}^{-1}$. Fluorescein isothiocyanate-labelled γ D-mannosylated ovalbumin (FITC-ovalbumin) was purchased from Sigma. Heat-inactivated influenza virus A/PR/8/34 was prepared from influenza virus A/Puerto Rico/8/34 (H1N1) (ATCC no. VR-95; GenBank accession no. J02143) grown in the allantoic cavity of 9- to 10-day-old embryonated hens eggs for 48 h at 37 °C. The infected allantoic fluid was collected after an overnight incubation at 4 °C. The virus preparation was quantified by end-point titration in Madin–Darby canine kidney cells (see below) with a TCID₅₀ of 10^{-6.5} corresponding to 1.2×10⁸ virus particles ml⁻¹ and by a standard haemagglutination assay with the last positive titre of 4096. Virus stocks were heat-inactivated at 56 °C for 30 min and used at a dilution of 1:50. Haemagglutinin (HA) peptide, HAT (aa 110–120; S-F-E-R-F-E-I-F-P-K-E-NH₂) was from Auspep, with more than 90 % purity as judged by HPLC. HAT was used from a stock solution of 4000 mg ml⁻¹.

Generation of murine bone marrow dendritic cells. Bone marrow-derived dendritic cells (BMDC) were generated from mouse bone as described previously (Faulkner *et al.*, 2000). Briefly, bone marrow cell suspensions were prepared, RBC-depleted with pre-warmed ammonium chloride, washed twice in 5 % Dulbecco's phosphate-buffered saline (DPBS) and cultured in complete Dulbecco's minimum essential medium (cDMEM; Gibco-BRL) containing 5 % foetal calf serum (FCS) and 20 ng GM-CSF ml⁻¹ at 2×10⁶ cells ml⁻¹ at 37 °C with 10 % CO₂. On day 4, cells were fed by replacing 3 ml of the supernatant with fresh medium. On day 6, non-adherent cells were subcultured in cDMEM containing 10 ng GM-CSF ml⁻¹ prior to use in assays.

Assays for antigen uptake and activation marker expression. BMDC were subcultured with either 10 ng GM-CSF ml⁻¹ alone, or combined with either mIL-10 or ORFV-IL-10. Cells were counted after 24 h and diluted to 1.0×10⁶ cells ml⁻¹. Particulate or soluble antigens were added and incubated overnight at 37 °C with 10 % CO₂. After approximately 24 h incubation, cells were pelleted and resuspended in DPBS+5 % FCS+0.01 % azide and kept on ice until analysed by flow cytometry. All flow cytometry analysis was performed on individual samples of 10 000 cells using a FACSCalibur (Becton Dickinson). All data collected were analysed using CellQuest software.

To determine changes in cell surface marker expression indicative of activation and maturation, variously treated BMDC were incubated with PE-conjugated antibodies for 40 min on ice (antibody dilution: 1:500 for Pharmingen antibodies; 1:200 for Sigma antibody). After washing, the cells were subjected to flow cytometry analysis as described above.

Assay for antigen presenting function. The efficacy of presentation of antigen was assessed using the A5 T cell hybridoma which expresses a T cell receptor specific for the influenza HAT presented in the context of MHC class II I-E^d molecules and containing a triple NF-AT promoter linked to green fluorescent protein (GFP) (Casares *et al.*, 1997; Andersen *et al.*, 2001). BMDC were subcultured overnight with 10 ng GM-CSF ml⁻¹, or with 10 ng GM-CSF ml⁻¹ and mIL-10 or ORFV-IL-10, and then pulsed with antigen for various periods prior to addition of the A5 cells: an overnight pulse for heat-inactivated influenza virus or addition immediately prior to A5 cells (1.0×10⁶ cells ml⁻¹) for HAT peptide. After 24 h incubation, the nonadherent cells were harvested and analysed using flow cytometry.

Preparation of epidermal sheets and enumeration of Langerhans' cells. The dorsal region of each mouse was shaved, depilated and squares of skin were removed and either immediately incubated in 20 mM EDTA/PBS for 2.5 h at 37 °C to facilitate separation of the epidermis from the underlying tissue or cultured for 2 h to stimulate LC migration from the epidermis prior to this treatment. Murine IL-10 (10 ng ml⁻¹) or ORFV-IL-10 (100 ng ml⁻¹) or supernatant from cells transfected with the pAPEX expression vector (without ORFV-IL-10) was included in designated culture media. Epidermal sheets were removed mechanically from the EDTA-treated skin pieces, fixed in acetone for 20 min, washed and incubated in undiluted anti I-A^d hybridoma supernatant overnight at room temperature. The sheets were then washed, incubated with biotinylated goat anti-mouse antibody for 2 h, washed again, incubated with streptavidin-alkaline phosphatase and, finally, the levamisole/new fuchsin/naphthol substrate (Johnston *et al.*, 1996) was added. The sheets were incubated for 5–10 min, dried and mounted. MHC class II-positive cells were counted by light microscopy under 40× magnification.

RESULTS

ORFV-IL-10 impedes BMDC maturation

Immature DC have been shown to take up soluble antigens via macropinocytosis and receptor-mediated endocytosis and phagocytose a range of particles including inert beads, antibody-coated erythrocytes, yeasts, parasites and bacteria (Steinman & Swanson, 1995; Austyn, 1996; Schakel *et al.*, 1998; Henderson *et al.*, 1997; Filgueira *et al.*, 1996; Su *et al.*, 1998). Here we investigated the effect of ORFV-IL-10 on antigen uptake by BMDC that are characterized as immature DC.

First, we determined whether or not ORFV-IL-10 affected soluble antigen uptake by BMDC. BMDC were cultured with ORFV-IL-10 for 24 h and then exposed overnight to soluble FITC-labelled ovalbumin. The proportion of cells taking up antigen and their mean fluorescence were measured by flow cytometry. Fig. 1(a) shows that exposing cells to increasing doses of antigen resulted in higher numbers of fluorescing cells. Significantly, 50 % of cells not grown in the presence of ORFV-IL-10 took up

antigen whereas more than 70 % of cells cultured with ORFV-IL-10 prior to antigen exposure took up FITC-ovalbumin (at the highest concentration). The results obtained by measuring mean fluorescence intensity (MFI), which is indicative of the degree of FITC-ovalbumin uptake of BMDC within the population, followed this pattern (Fig. 1b). BMDC exposed to mIL-10 and ORFV-IL-10 had a higher MFI than those cells without this exposure. There was little difference between the effect of mIL-10 and ORFV-IL-10 on the percentage of the BMDC taking up the antigen but treatment with mIL-10 resulted in a greater degree of FITC-ovalbumin uptake by cells within the population than ORFV-IL-10. Experiments carried out to investigate the effect of ORFV-IL-10 on particulate antigen uptake showed that the viral homologue had little effect on the phagocytosis of either fluorescent beads or recombinant BCG-expressing GFP (data not shown).

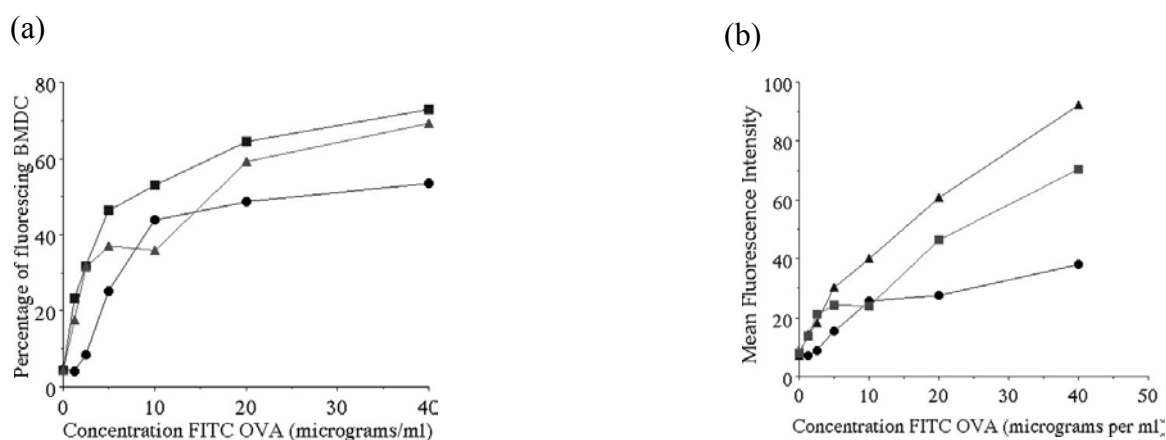


Fig. 1. Effect of ORFV-IL-10 on phagocytosis by BMDC. BMDC at a concentration of 0.5×10^6 cells ml^{-1} were subcultured overnight in the presence of 10 ng GM-CSF ml^{-1} with 100 ng ORFV-IL-10 ml^{-1} (■) or 20 ng mIL-10 ml^{-1} (▲) or without IL-10 (●). The cells were then exposed overnight to mannosylated FITC-ovalbumin (OVA). The cells were analysed by flow cytometry. The percentage of cells that have taken up antigen is shown (a) as well as the MFI (b). Three experiments were performed in each case and the results of one representative experiment are shown.

ORFV-IL-10 reduces phenotypic marker expression of antigen-stimulated BMDC

We first determined the optimal concentration of ORFV-IL-10 that inhibited the maturation of BMDC upon exposure to antigen. BMDC were cultured with ORFV-IL-10 over a titration range of 6.25–200 ng ml^{-1} . Fig. 2(a) shows that BMDC grown in the presence of ORFV-IL-10 for 24 h and then exposed to the antigen PPD showed an inverse relationship between levels of MHC class II expression and the amount of ORFV-IL-10 used to pre-treat cells. The maximum inhibition of MHC class II expression by ORFV-IL-10 was seen at 100 and 200 ng ml^{-1} in several experiments. In comparison, the maximum inhibition of MHC class II expression by mIL-10 over a number of titration experiments was seen at a substantially lower concentration, in the order of 10 and 20 ng ml^{-1} of mIL-10 (data not shown).

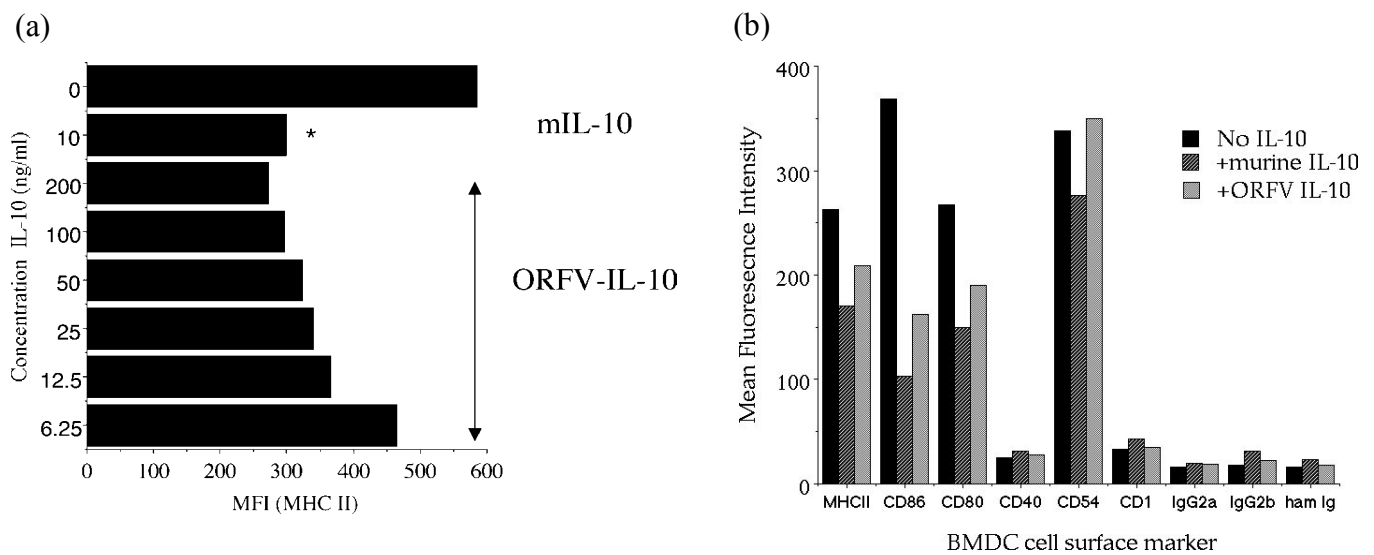


Fig. 2. Effect of ORFV-IL-10 on BMDC phenotypic marker expression in response to PPD-B. (a) Titration of ORFV-IL-10. BMDC at a concentration of 1.0×10^6 cells ml^{-1} were subcultured overnight in the presence of 10 ng GM-CSF ml^{-1} with various concentrations of ORFV-IL-10 or 10 ng mIL-10 ml^{-1} (*) or without IL-10 (top bar). The cells were then exposed to PPD-B at a final concentration of 40 $\mu\text{g ml}^{-1}$ for 24 h. BMDC were stained with PE-labelled anti-MHC class II antibody and analysed by flow cytometry. (b) BMDC at a concentration of 0.9×10^6 cells ml^{-1} were incubated overnight in 10 ng GM-CSF ml^{-1} with 100 ng ORFV-IL-10 ml^{-1} or 20 ng mIL-10 ml^{-1} or without IL-10. The cells were then exposed to PPD-B at a final concentration of 40 $\mu\text{g ml}^{-1}$ for 24 h. The cells were labelled with PE-conjugated antibodies [raised against MHCII, CD86, CD80, CD40, CD54 and CD1; serum isotype controls were IgG2a, IgG2b and ham Ig (hamster Ig)] and analysed by flow cytometry. Three experiments were performed in each case and the results of one representative experiment are shown.

We then examined the effects of ORFV-IL-10 on a range of phenotypic markers expressed by BMDC. BMDC were cultured with viral IL-10 for 24 h prior to exposure to PPD-B. The expression of the markers MHC class II, CD80 and CD86 was lower when BMDC were treated with ORFV-IL-10 and PPD-B compared with those treated with PPD-B alone (Fig. 2b). There was little effect on CD54 expression when cells were pretreated with ORFV-IL-10 but mIL-10 inhibited upregulation of this marker. These results are consistent with those of Faulkner *et al.* (2000) for mIL-10. It was apparent in this assay that mIL-10 was having a greater effect on MHC class II, CD80, CD86 and CD54 expression than ORFV-IL-10. Other markers that we examined included the costimulatory marker CD40 and a nonpolymorphic MHC class I molecule associated with the presentation of lipid moieties, CD1. These showed little increase in expression when BMDC were exposed to PPD-B without exogenous cytokine so it was not possible to determine whether ORFV-IL-10 affected their expression.

ORFV-IL-10 inhibits antigen presentation by BMDC

The effect of ORFV-IL-10 on antigen presentation was examined using the A5 T cell hybridoma assay system (Faulkner *et al.*, 2001). These cells are transfected with a gene encoding GFP under the control of an NF-AT (IL-2) promoter. The T cell receptor is specific for the influenza virus HA peptide, HAT,

presented on MHC class II. The interaction of the HAT peptide complexed to MHC class II molecules on BMDC with the T cell receptor on A5 cells results in the activation of the IL-2 promoter and expression of GFP.

Preliminary experiments were conducted to optimize the T cell hybridoma assay. The variables that we examined were time of exposure of BMDC with antigen, dose of antigen, and the ratio of BMDC to A5 cells. The time of exposure of BMDC to antigen was dependent on the form of the antigen used. The antigens that were used were the HAT peptide and heat-inactivated influenza virus. The HAT peptide requires minimal processing for loading onto MHC class II molecules whereas production of peptides from crude heat-inactivated influenza virus within BMDC requires a longer period. A further variable that was critical in this assay was the ratio of A5 cells to BMDC. The optimal ratio of A5 cells to BMDC used in all assays was 2:1 and therefore the maximum level of responding A5 cells in a mixed population was approximately 66 %.

First, we examined the effect of ORFV-IL-10 on antigen presentation using the HAT epitope. BMDC that were treated with ORFV-IL-10 for 24 h were mixed with A5 cells and exposed to HAT peptide simultaneously. Fig. 3(a) shows that ORFV-IL-10 reduced the antigen-presenting ability of BMDC as efficiently as mIL-10. The maximal effect of ORFV-IL-10 in this assay was seen at a HAT peptide concentration of between 10 and 30 $\mu\text{g HAT ml}^{-1}$.

The effect of ORFV-IL-10 on antigen presentation was then assessed using heat-inactivated influenza virus (HI APR8/34). In this case, the viral antigen was taken up by the BMDC, processed through the MHC class II pathway and the HAT epitope displayed on the surface of the cell in conjunction with MHC class II. This assay potentially allowed us to examine the affect of ORFV-IL-10 on antigen processing and presentation via the MHC class II pathway. BMDC were treated with ORFV-IL-10 for 24 h and then exposed to inactivated virus particles overnight before addition to A5 cells. The results show that ORFV-IL-10 reduces the ability of BMDC to present antigen derived from HI APR8/34 in a similar manner but to a lesser degree than was seen with the HAT peptide (Fig. 3b).

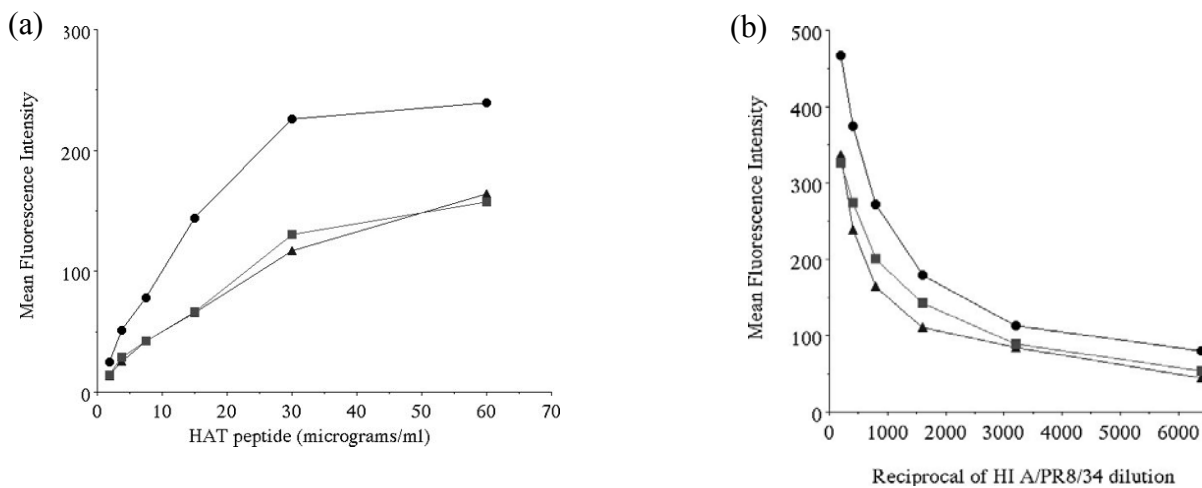


Fig. 3. Effect of ORFV-IL-10 on antigen presentation. (a) HAT antigen. BMDC at a concentration of 2.0×10^6 cells ml^{-1} were subcultured overnight in the presence of 10 ng GM-CSF with 100 ng ORFV-IL-10 ml^{-1} (■) or 20 ng mIL-10 ml^{-1} (▲) or without IL-10 (●). BMDC were then harvested and diluted to 0.3×10^6 cells ml^{-1} in cDMEM without GM-CSF. HAT antigen was added at the concentrations shown followed by A5 cells at 0.6×10^6 cells ml^{-1} . After overnight incubation, cells were analysed by flow cytometry. (b) Heat-inactivated influenza virus (APR8/34). BMDC at a concentration of 2.0×10^6 cells ml^{-1} were subcultured overnight in 10 ng GM-CSF ml^{-1} with 100 ng ORFV-IL-10 ml^{-1} (■) or 20 ng mIL-10 ml^{-1} (▲) or without IL-10 (●). The cells were then diluted to 0.5×10^6 cells ml^{-1} in cDMEM without GM-CSF and exposed to influenza virus at the concentrations shown. After overnight incubation, A5 cells were added to the BMDC at a concentration of 1.0×10^6 cells ml^{-1} . After overnight incubation, the cells were analysed by flow cytometry. Three experiments were performed in each case and the results of one representative experiment are shown.

Mouse epidermal sheets cultured with ORFV-IL-10 and mIL-10 retain LC

When explanted skin is cultured, LC migrate out of the epidermis within hours (Kimber *et al.*, 2000). The cytokines IL-1 β and TNF- α released from keratinocytes and IL-1 β produced by LC play a major role in this process (Cumberbatch & Kimber, 1992; Roake *et al.*, 1995). More recent studies have shown that fewer LC are retained in skin explants from IL-10 knock-out mice than in wild-type mice (Wang *et al.*, 1999). We used this model to investigate any comparable effect mediated by ORFV-IL-10. Culturing skin explant tissue in the presence of 10 ng mIL-10 ml^{-1} resulted in higher numbers of epidermal cells staining for MHC class II than was seen in tissue cultured in the absence of cytokine ($P=0.005$; unpaired two-tailed Student's *t*-test) (Fig. 4). When mIL-10 was substituted with 100 ng ml^{-1} ORFV-IL-10, a similar retention of LC was recorded, i.e. there was no significant difference in the numbers of LC in these epidermal sheets compared with those cultured with mIL-10 ($P=0.5545$). By contrast, there was a significant reduction in the numbers of LC in epidermal sheets cultured without exogenous cytokine compared to uncultured sheets ($P=0.0378$). An additional control was included in these experiments in which skin was cultured in medium containing supernatant from the 293 cells transfected with the pAPEX expression vector only. There was no evidence that LC migration was impaired in these samples indicating that the effect was specific to ORFV-IL-10. In addition we noted that changes in LC

morphology, including the loss of a markedly stellate appearance that characterizes the majority of these cells in cultured epidermal sheets, were less apparent in ORFV-IL-10-treated sheets but the changes were by no means uniform.

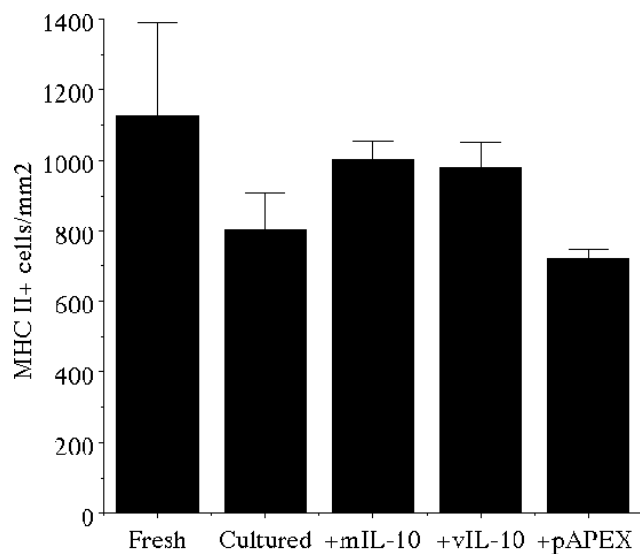


Fig. 4. Effects of murine IL-10 and ORFV-IL-10 on LC migration from cultured skin explants. Murine skin explants were either cultured in the presence of 10 ng mIL-10 ml⁻¹ or 100 ng ORFV-IL-10 ml⁻¹ (vIL-10), or pAPEX vector-transfected 293 cell supernatant (pAPEX) or in the absence of exogenous cytokine. Skin explants were cultured for 2 h and LC were identified by immunohistochemical staining for MHC class II. Uncultured explants were included as a control. Five sheets were analysed for each group and numbers of LC in 10 random fields on each sheet were counted. The data shown are the mean+1SD.

DISCUSSION

Reinfection of the host by ORFV may suggest that the generation of immunological memory has been inadequate, thus exposing the host to subsequent infection. The discovery that ORFV encodes IL-10 raises the question of whether this factor adversely affects the cells that initiate the acquired immune response leading to the production of memory. LC are the sentinels of the skin immune system and initiate specific acquired immune responses against organisms that infect the skin. It is likely that these cells, rather than blood DC, are involved in initiating an acquired immune response against ORFV, as LC are at the site of the primary infection. However, blood DC may also have a role. For practical reasons we did not use tissue-derived LC to examine the effects of ORFV-IL-10 in cell culture, although it was possible to show that ORFV-IL-10 impaired the migration of LC in skin explant tissue. LC are difficult to isolate from skin and undergo maturation in culture (Teunissen *et al.*, 1990) whereas BMDC are readily grown in cell culture, resemble DC morphologically and display almost all of the characteristics of DC (Inaba *et al.*, 1992; Scheicher *et al.*, 1992; Faulkner *et al.*, 2000). BMDC have the ability to take up, process and present antigen, the ability to interact with and stimulate T cell responses (Inaba *et al.*, 1992; Faulkner *et al.*, 2000) and the ability to migrate and function as DC when injected *in vivo* (Inaba *et al.*, 1993).

In this study, we have shown that treating immature BMDC with ORFV-IL-10 in culture prior to antigen exposure affects antigen uptake and inhibits maturation and antigen presentation. The effects of ORFV-IL-10 on antigen uptake were analogous to those where BMDC were treated with mIL-10 prior to antigen

exposure (Faulkner *et al.*, 2000), showing that in the presence of ORFV-IL-10 the transition from an immature to a mature cell on exposure to antigen is impaired. The reason for the difference in soluble and particulate antigen uptake by immature BMDC is unclear. It is likely that these differences could be explained by the very different mechanisms of antigen uptake for soluble and particulate antigen and that these processes are affected in different ways by IL-10. The inhibitory effect of ORFV-IL-10 on DC maturation is also reflected in a failure of antigen to induce upregulation of MHC class II and the costimulatory molecules CD80 and CD86 as effectively as in untreated cells. This is also consistent with the inhibitory effects of mIL-10 reported by others (Henderson *et al.*, 1997; Demangel *et al.*, 1999; Faulkner *et al.*, 2000). In these experiments, we observed a difference between the effect of ORFV-IL-10 on phenotypic marker expression and that of mIL-10. This difference may not be surprising since ORFV-IL-10 is subtly different to mIL-10 in molecular structure within those regions thought to contact the IL-10 receptor (Imlach *et al.*, 2002). We were unable to detect ORFV-IL-10 mediated upregulation in the expression of the costimulatory marker CD40 or the adhesion molecule CD54, which is important in DC:T cell interactions.

An important test of the immunosuppressive effect of ORFV-IL-10 was to examine its effect on DC antigen-presenting function. We used a HAT-specific T cell hybridoma to assess antigen presentation. Hybridomas, like memory T cells, are relatively easily activated because they do not require high levels of costimulation. Our investigations have shown that T cell activation by DC-mediated presentation of HAT and heat-inactivated AP8/34 influenza virus, is markedly reduced following exposure to ORFV-IL-10. Presentation of HAT peptide, which requires little processing, was impaired, indicating that either or both reduced antigen loading on MHC class II and reduced expression of MHC class II on the cell surface may be involved in the inhibitory activity of ORFV-IL-10.

Immunosuppression can clearly occur at a number of levels with respect to DC function. Assays using BMDC showed unequivocally that ORFV-IL-10 has a suppressive effect on the maturation and the antigen-presenting ability of these cells. In order to examine the effect of ORFV-IL-10 on DC migration, we used mouse skin explant tissue. We have shown that ORFV-IL-10 impairs the spontaneous migration of LC from the epidermis that normally occurs following a period of culture. The implications of this, with respect to differential signalling involved in morphological changes and migration, have yet to be investigated.

Many poxvirus genomes have been completely sequenced including the type species for each Chordopoxvirus genus (University of Victoria poxvirus database; <http://athena.bioc.uvic.ca/pbr/POCs/pocs.html>). There is little evidence to suggest that these viruses produce factors that specifically target the acquired immune response, in particular DC. However, many

of the immune modulators that suppress inflammation and the innate responses, such as soluble receptor homologues of TNF- α and IFN- γ , will also disrupt cytokine signalling pathways involved in the development of acquired immunity. Genetic analysis of ORFV (Mercer *et al.*, 1995; Fleming *et al.*, 1997; Deane *et al.*, 2000; Büttner & Rziha, 2002) suggests that this virus has evolved a unique repertoire of factors that specifically disrupt Th1 immunity. Surprisingly, ORFV does not appear to encode soluble receptor-like factors specifically directed against the inflammatory cytokines such as IL-1 β , IL-18, TNF- α or IFN- γ . In addition to IL-10, ORFV produces a factor (GIF) that specifically binds IL-2 and GM-CSF. This is relevant to DC because IL-2 is produced by these cells and enhances antigen presentation (Granucci *et al.*, 2002) and GM-CSF recruits DC to peripheral tissues (Kaplan *et al.*, 1992). These findings strongly suggest that ORFV has evolved mechanisms to delay the clearance of the virus by a type 1 response and this sets the immune evasion strategies of ORFV apart from those of other poxviruses. It is possible that such a strategy is only successful where virus replication is localized and restricted to specific tissues, such as the skin epithelium in the case of ORFV. The short range effects of the viral immunomodulators produced in this instance are unlikely to compromise the integrity of the host's immune system and expose the host to other pathogens that could occur in more generalized poxvirus infections.

We have shown that ORFV-IL-10 has the capability to suppress the migration of LC and inhibit DC function in addition to suppressing inflammation and the innate responses. Our findings suggest that ORFV-IL-10 has the capacity to impair the initiation of an acquired immune response and hence inhibit the generation of immunological memory necessary for immunity on subsequent exposure. ORFV-IL-10 may be capable of inhibiting the activation of memory cells recruited to infected skin following reinfection, as well as having the potential to inhibit the activation of naïve T cells that would otherwise be brought about by activated LC trafficking to the draining lymphoid tissue. This could be another means of delaying the destruction of virally infected cells and preventing complete immunity on re-exposure to the virus. The implications of this are that on repeated cycles of infection the virus can replicate, shed and be transmitted to other hosts before elimination by the acquired immune response. We are currently investigating this possibility using an IL-10 knock-out strain of ORFV.

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