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Migratory sub-populations of afferent lymphatic dendritic cells differ in their interactions with *Mycobacterium bovis* Bacille Calmette Guerin

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ABSTRACT

Understanding how pathogens or vaccine antigens are targeted to dendritic cell (DC) subsets is important for disease pathogenesis studies and vaccine design. We characterised the sub-populations of migrating bovine DC with functional and phenotypic diversity present in pseudoafferent lymph draining the skin. These skin draining DC exist as a series of maturation dependent subsets with differential capacities for antigen uptake and cytokine expression, and include both Langerhans' cells (LC) and dermal derived cells. Furthermore, *Mycobacterium bovis* Bacille Calmette Guerin, a vaccine which is administered by the intradermal route, was only taken up by a small number of the migrating DC, which were SIRPα$^+$ and expressed the mannose receptor and CD1b. This was evident following *in vitro* infection and also *in vivo* following inoculation of green fluorescent BCG over the lymphatic cannulation site. Only the SIRPα$^+$ DC were able to present antigen to T cells isolated from BCG vaccinated calves. Furthermore, presentation of BCG antigens by DC to T lymphocytes was ineffective compared to mycobacterial proteins. However, mycobacterial antigen 85 was delivered more effectively to DC *via* an adenoviral vector and the magnitude of the subsequent antigen-specific T cell response was significantly increased.

This study further extends our understanding of the biology of migrating DC, identifies potential explanations for the modest success of BCG vaccination and demonstrates that targeted delivery of antigens *via* adenoviruses to DC can improve antigen presentation.

1. Introduction

Dendritic cells (DC) are unique in their capacity to stimulate responses of naive T lymphocytes and are central to the induction of immune responses following infection or vaccination. Understanding the early immune responses induced by DC will provide insights into potential mechanisms for immune manipulation to increase protective immunity or vaccine efficacy.

Studies of DC have identified the presence of subsets with reported differences in phenotype and function. It is proposed that subsets of DC at different sites are involved both in the control of steady state immune homoeostasis or tolerance and/or in activation of immune responses (following infection). However, many of the reported studies of DC rely on the isolation and culture of cells *in vitro* which does not necessarily reflect the *in vivo* biological properties of DC subsets. Cannulation of afferent lymphatic vessels has been reported for ruminants, rats and mice [1–4]; enabling the collection of *ex vivo* DC which migrate in the lymph. These models provide a significant advantage as the *ex vivo* DC have not been subjected to long periods of culture *in vitro*, enzymatic treatment or other separation techniques that affect DC function.

Studies on afferent lymphatic DC (ALDC) in all species examined thus far have defined major sub-populations [5–9] with functional and phenotypic heterogeneity. Interestingly a number of inter-species parallels have been described for ALDC populations with evidence for conserved functional specialisation and common molecular signatures [10]. In cattle, major sub-populations of skin draining ALDC with differential expression of SIRPα (CD172a), CD11a, CD26 and CD13 [6,9,11,12] interact with and stimulate T cells differently, and have differential cytokine secretion profiles [5,6,13,14]. Studies of ALDC draining the intestine of rats and the head mucosae of sheep [15–18] concluded that a major population

Abbreviations: ALDC, afferent lymph dendritic cells; BCG, Bacille Calmette Guerin; MR, mannose receptor; SIRP, signal regulatory protein.

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of SIRPα⁻ ALDC which contained cytokeratin inclusions were involved in the maintenance of peripheral tolerance [19,17]. These observations emphasise the differences and complexity of *in vivo* DC subsets and indicate that differing DC sub-populations may be involved in the control of the immune response. This highlights the requirement for detailed understanding of *in vivo* populations of DC at relevant anatomical sites which is key to the design of targeted approaches to vaccination or immunotherapeutic strategies.

The interaction of ALDC with pathogens has not been extensively documented. In sheep, the efficiency of infection of ALDC with *Salmonella abortusovis* [20,21] was low, and this was suggested as a reason for poor immune responses to salmonella. By contrast sheep ALDC were productively infected by bluetongue virus, and this enhanced the survival of DC [22], this may be a mechanism by which the virus facilitates early dissemination *via* lymphatic vessels to regional lymph nodes. Other studies suggested differential interactions with subsets of bovine ALDC [6] that could be important in polarising immune responses to respiratory viruses.

In this study we have performed a detailed assessment of the subpopulations of ALDC draining the skin in cattle and provided further evidence to suggest that subset specific functionality is an important feature of DC biology. We have also assessed the capacity of ALDC to interact with both a virulent, and an attenuated form of *Mycobacterium bovis*. *M. bovis* is the causative agent of tuberculosis in cattle, a disease increasing rapidly in incidence in cattle herds in developed countries including the UK, New Zealand and the USA. There are no currently licensed vaccines for the control of bovine tuberculosis but numerous experimental and field studies have assessed the efficacy of the human vaccine Bacille Calmette Guerin (BCG), an attenuated form of *M. bovis* [23,24]. These studies demonstrated that BCG has variable efficacy in cattle and the induction of sterile immunity has not been consistently demonstrated.

We show herein that subpopulations of afferent lymph DC interact variably with *Mycobacteria* and that presentation of antigens from BCG to T lymphocytes is ineffective compared to the presentation of mycobacterial proteins or peptides. However, effective stimulation of antigen-specific T cells was observed when antigens were delivered to DC *via* adenoviral vectors suggesting that adenoviral vaccines are more likely to induce effective immunity than BCG against *M. bovis* in cattle.

2. Materials and methods

2.1. Pseudoafferent lymphatic cannulation

Conventionally reared Friesian Holstein calves (*Bos taurus*) from the Institute for Animal Health (IAH) herd were used for these studies. Cannulations were performed essentially as previously described [4]. Lymph was collected into sterile plastic bottles containing heparin (10 U/ml), penicillin and streptomycin. Bottles were replaced every 8–12 h. The lymph collected was either used fresh (for uptake studies) or was centrifuged ($300 \times g$, 8 min), resuspended in FCS/10% DMSO and the cells stored in liquid nitrogen prior to use. For uptake studies the mononuclear cells were isolated from the afferent lymph by density gradient centrifugation over Histopaque (1083, Sigma). Afferent lymph from 10 different animals was collected and analysed. The animal experiments were approved by the IAH ethics committee according to national UK guidelines.

2.2. Monoclonal antibodies and flow cytometric analysis of cell surface molecule expression

The mouse anti-bovine mAb used in this study have been described in detail previously [6,8,11,25,26]. These were CC98 and IL-A53 (both anti-DEC205), IL-A99 (anti-CD11a), CC81 (anti-CD13), CC21 (anti-CD21), CC14 (anti-CD1b) and CC149 (anti-SIRPα). Expression of MR was detected with mAb 3.29B1 (anti-human MR; a gift from Dr. A. Lanzavecchia). Control mAb were isotype and concentration matched anti-avian mAb [27]. The expression of these molecules was detected following staining with mAb by the use of fluorescently labelled isotype specific reagents (Cambridge Biosciences). Where biotinylated mAb were used these were detected using fluorescently labelled streptavidin. The cells were analysed using a FACSCalibur (Becton Dickinson) and staining was assessed using FCS Express (DeNovo Software). Afferent lymph DC were distinguished from other cells on the basis of their high FSc and high intensity expression of DEC205 [9].

2.3. Acetylcholinesterase staining

Cytospin preparations of purified DC subpopulations were fixed in formal calcium (1% $CaCl_2$ in 4% formaldehyde) for 30 s, soaked in 0.22 M sucrose and incubated for 70 min at 37 °C in staining buffer (500 μg/ml acetylthiocholine iodide, 3 mM copper sulphate, 165 μg/ml potassium ferricyanide, 7.5% (w/v) sucrose in sodium maleate buffer pH 7.2). Following washing in dH_2O the slides were incubated in 10 mM ammonium sulphide for 5 min and counterstained with Mayer's Haematoxylin.

2.4. Culture of afferent lymph cells with GMCSF and TNFα

To assess the effects of culture on subsets of ALDC, afferent lymph cells were cultured for 24 h in either RPMI or IMDM (Invitrogen, Paisley, UK) supplemented with 10% FCS, 5×10^{-5} M 2-Me and 50 μg/ml gentamicin (Sigma, UK). Preliminary studies indicated that addition of rbo GMCSF (0.2 U/ml; [28]) and rbo TNFα (4 U/ml; units based on half maximal activity in WEHI bioassay [29]) enhanced cell viability without substantially affecting the expression of SIRPα or DEC205 by the ALDC (*data not shown*). Cell viability was poor in RPMI without the addition of GMCSF + TNFα (<20%) but was significantly enhanced by the presence of cytokines. Cell viability in IMDM was consistently above 90% and all subsequent investigations (including infection studies with *gfp*-BCG) were carried out in IMDM. Cells were analysed at time 0 (directly *ex vivo*) or cultured at 5×10^5 per well in 96 U well plates for 24 h prior to analysis by multicolour flow cytometry.

2.5. Recombinant adenovirus 5 vectors

Replication-deficient human recombinant adenovirus 5 (rhuAdV5) vectors expressing mycobacterial antigen 85A (Ag85A; AdV5-85A) were produced by the Jenner Institute Viral Vector Core Facility University of Oxford, UK.

2.6. Mycobacterial antigens

Purified protein derivative from *M. bovis* (PPD-B) was obtained from the tuberculin production unit at Veterinary Laboratories Agency (VLA), Weybridge, UK. Recombinant antigen 85A (Ag85A) was obtained from Lionex GmbH, Germany.

2.7. Infection of afferent lymph cells with mycobacteria

Bacillus Calmette Guerin (BCG) strain Pasteur labelled with a green fluorescent protein marker (provided by Veterinary Laboratories Agency, Weybridge, UK) was grown at 37 °C in Middlebrook 7H9 medium supplemented with OADC (Difco, Paisley, UK) [30] and 20 μg/ml kanamycin. Aliquots of mid-log phase cultures were stored at −80 °C and bacterial counts (CFU/ml) assessed 3–4 weeks following plating on Middlebrook 7H10 agar for BCG.