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Research paper

Characterisation of local immune responses induced by a novel nano-particle based carrier-adjuvant in sheep

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ABSTRACT

Most adjuvants require danger signals to promote immune responses against vaccine antigens. Our previous studies have characterised a powerful nano-particulate antigen delivery system, which by itself does not induce inflammation, and which further appears to induce substantial immune responses in mice and sheep without the requirement for added stimulators of toll like receptors or other pathogen recognition receptors. In the present study we dissect the nature of the early induction phase of the immune response stimulated by such a vaccine comprising 40 nm polystyrene nano-particles conjugated to the antigen. We analyse the kinetics of export from an individual draining lymph node from the sheep, of antibodies and cytokines as well as antigen responsive CD4 and CD8 T cells. Our results indicate that simple inert nano-bead based antigen delivery into the draining area of the lymph node is highly efficient at priming combined humoral and T cell antigen specific immunity without the need for added 'danger signals'. Furthermore this nano-bead adjuvant is a potent agent capable of promoting cross-priming for CD8 T cell induction in sheep. Interestingly, using nano-beads, similarly to what has been observed with natural pathogen based lymph node stimulation, a phase of CD4 T cell priming and export preceded CD8 T cell induction, suggesting the engagement of natural priming processes and kinetics.

1. Introduction

The induction of immune responses require the presence of "danger signals" (Matzinger, 1994) which are often provided by the adjuvants as many modern vaccines lack these danger signals. In many cases these "dangers signals" include pathogen associated molecular patterns (PAMPS) and damage associated molecular patterns (DAMPS), which are recognised by pattern recognition receptors (PRR) (Tang et al., 2012). Thus most substances with adjuvant properties contain added potent PAMPS including CpG motives or monophosphoryl lipid A, which activate toll-like receptors (TLRs) (van Duin et al., 2006). Another group of adjuvants was identified as working via TLR-independent mechanisms including Alum (with activity mediated by acid uric induction and stimulation of the NLRP3 inflammasome complex) and MF59 (acting by inducing rapid cell recruitment at the site of infection) (De Gregorio et al., 2009). It is therefore not surprising new effective ways of enhancing immune responses directed against cancer and infectious diseases are most often based on the activation of the immune system through one of these known routes.

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One alternative way of promoting immune responses is to target the antigen to specialised antigen presenting cells such as dendritic cells (DCs) or DC precursors, as these cells are known to play a major role in presenting antigen to naïve lymphocytes (Albert et al., 1998; den Haan et al., 2000; Schnorrer et al., 2006). Particulate vaccines of various sizes and formulations have been extensively investigated for effective antigen delivery to DCs, with subsequent CD8 T cell activation *in vivo* (Gamvrellis et al., 2004). For example, >500 nm particles can target and activate DCs and macrophages *in vivo* and *in vitro* (Falo et al., 1995; Kovacsovics-Bankowski et al., 1993). Particles such as VLPs (Moron et al., 2002; Peng et al., 1998; Rudolf et al., 2001) and 50–90 nm ISCOMs (Beacock-Sharp et al., 2003; Robson et al., 2003), which also contain added danger signals are also adjuvants that target DCs, promoting Th1 and/or Th2 immune responses.

The observation of efficient uptake and presentation of VLP associated-antigen by DCs has promoted the development of synthetic particles made of inert materials such as PLGA, polystyrene or gold, with antigen enclosed or bound onto the particle surface. We have previously demonstrated the immunogenicity of proteins and peptides covalently coupled to inert 40–50 nm polystyrene particles that localise preferentially to DC in draining lymph nodes (LN). The optimal bead size for induction of cellular immune responses in mice was defined within the viral size range (30–50 nm) (Fifis et al., 2004a,b; Mottram et al., 2007). We have also shown that antigen-bound nano-beads are effective in a large animal, since sheep immunised with OVA-conjugated nano-beads developed antigen-specific T cell and antibody responses comparable to responses induced by other adjuvants (Scheerlinck et al., 2006). In addition, nano-beads conjugated to FMDV peptides also induced potent immune responses (Greenwood et al., 2008). By the inert nature these particles do represent a novel mechanism of immune induction, with potentially unusual patterns of induction. This has prompted investigation of the kinetics if the immune response in the draining LN following immunisation with such antigen conjugated nano-beads.

2. Methods

2.1. Sheep

Medium-wool Merino ewes, aged 6–24 months, were brought from pasture into pens to acclimatise prior to experimental procedures. The animals were placed in metabolic cages following surgery, and were fed Lucerne chaff, with *ad libitum* access to water. Upon completion of experimental work, the animals were sacrificed by lethal injection with sodium pentobarbitone (Lethabarb, Boehringer Ingelheim). The University of Melbourne Animal Experimentation Ethics Committee approved all experimental procedures in these studies.

2.2. Surgery

Efferent lymph cannulation provides extensive information about the local immune responses, since the cells and lymph are collected before they can recirculate. Separate LNs in the same animal can be injected with different preparations for comparison. Sheep were immunised with the nano-beads and the cells draining from the LN were analysed by collecting the cellular fractions flowing from the site draining the cannulated LN. Prefemoral nodes were surgically cannulated as previously described (Windon et al., 2000b). Heparinised polyvinyl tubing (CBAS-coated, Carmeda AB, Stockholm, Sweden) of size 0.4 mm i.d. × 0.8 mm o.d. or 0.58 mm i.d. × 0.96 o.d. was used to cannulate the efferent duct.

2.3. Preparation of antigen and adjuvants

Ovalbumin was conjugated to the nano-beads as described previously (Fifis et al., 2004a). Briefly, carboxylated polystyrene microspheres, size 48–53 nm (polysciences), adjusted to 2% solids were mixed 1:1 (vol:vol) with 2 mg/mL of Ovalbumin (OVA, Grade III, Sigma), in 0.05 M MES ([2-*N*-morpholino] ethane sulfonic acid) buffer pH 6.0 for 15 min at room temperature. 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide was added at 4 mg/mL, with a pH ~ 6.5, and rocked for 2 h. One hundred millimolars glycine was added for 30–60 min before overnight dialysis against cold PBS against membranes of 300 kD pore size (Spectrum Laboratories Inc., Australia). Efficiency of conjugation of selected batches of nano-beads-OVA was determined by iodination of OVA (Fifis et al., 2004a). In addition, the potential induction of systemic immunity was monitored regularly by parallel interferon gamma (IFN-γ) ELISPOT assays in C57BL/6 mice (Fifis et al., 2004a). Conjugated particles were used within a day of conjugation and sonicated for 5–10 min before use.

2.4. Injection treatments

The treatment doses were 160 μL of nano-beads-OVA preparation (based on most inefficient conjugation of the nano-beads-OVA batches tested, this represents at least 160 μg of conjugated OVA) or 160 μg soluble OVA. Preparations were diluted to 0.5 mL for intradermal (i.d.) injections at day 0. Sheep were injected i.d. approximately 3 cm above and anterior to the precrual fold (i.e. fold of flank), an area known to drain the cannulated prefemoral LN (Windon et al., 2000b). The vaccination regime is outlined in Table 1.

2.5. Efferent lymph collection, cell counts and preparation of lymph plasma

Lymph collections and determination of cell outputs ($\times 10^6\ h^{-1}$) were performed as described previously (Windon et al., 2000b). Briefly, lymph fluid was collected into sterile collection bottles containing 5000IU heparin (DBL, Melbourne, Australia). After each collection, lymph volume was recorded and total lymphocytes were counted using a Coulter Counter (Industrial D, Coulter Electronics, UK). Lymph fluid from each collection was centrifuged ($600 \times g$, 5 min) and the lymph plasma stored in 1.5 mL aliquots at −20 °C until analysed.