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Canine Recombinant Adenovirus Vector Induces an Immunogenicity-Related Gene Expression Profile in Skin-Migrated CD11b⁺-Type DCs

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Abstract

Gene expression profiling of the blood cell response induced early after vaccination has previously been demonstrated to predict the immunogenicity of vaccines. In this study, we evaluated whether the analysis of the gene expression profile of skin-migrated dendritic cells (DCs) could be informative for the in vitro prediction of immunogenicity of vaccine, using canine adenovirus serotype 2 (CAV2) as vaccine vector. CAV2 has been shown to induce immunity to transgenes in several species including sheep and is an interesting alternative to human adenovirus-based vectors, based on the safety records of the parental strain in dogs and the lack of pre-existing immunity in non-host species. Skin-migrated DCs were collected from pseudo-afferent lymph in sheep. Both the CD11b⁺-type and CD103⁺-type skin-migrated DCs were transduced by CAV2. An analysis of the global gene response to CAV2 in the two skin DC subsets showed that the gene response in CD11b⁺-type DCs was far higher and broader than in the CD103⁺-type DCs. A newly released integrative analytic tool from Ingeny systems revealed that the CAV2-modulated genes in the CD11b⁺-type DCs clustered in several activated immunogenicity-related functions, such as immune response, immune cell trafficking and inflammation. Thus gene profiling in skin-migrated DC in vitro indicates that the CD11b⁺ DC type is more responsive to CAV2 than the CD103⁺ DC type, and provides valuable information to help in evaluating and possibly improving viral vector vaccine effectiveness.

Introduction

Gene expression profiling of the in vivo innate immune response to vaccines in peripheral blood mononuclear cells (PBMCs) has recently been demonstrated to be predictive of the subsequent magnitude of the antibody and CD8⁺ T cell responses in humans [1,2]. We reasoned that because dendritic cells (DCs) are key actors in bridging the innate and adaptive immune systems, their early gene response might offer an in vitro evaluation tool of vaccine vector immunogenicity. In support of this proposal, a recent study showed that the gene expression profile of bone marrow-derived sheep DCs in response to recombinant myxoma virus vectors included many genes that were previously shown to predict vaccine efficacy [3]. As skin DCs and skin-migrated DCs are main targets of nanoparticle-based vaccines that are delivered via the skin route [4,5], they represent particularly pertinent targets for analyzing the in vitro DC response to vaccines. Skin-migrated DCs can be obtained from human or animal skin explants [6] and from afferent lymph in species that are amenable to lymph duct catheterization (ruminants, pigs) [7,8]. In sheep, skin-migrated DCs collected from pseudo-afferent lymph include two major subsets, the CD26⁺ CD103⁺/CD103⁺ DC subset is specialized in CD8⁺ T cell activation in mixed leukocyte reactions and in soluble antigen cross-presentation [9]. Canine adenovirus serotype 2 (CAV2) is one of the most attractive non-human adenovirus for use as a recombinant vaccine vector [10,11]. CAV2 represents an alternative to the largely documented human adenovirus serotype 5 (Ad5) vector whose clinical application is impaired due to the frequent pre-existing immunity that interferes with the development of the desired immune response [12,13]; furthermore, in the context of regulatory constraints in many countries, non-human adenoviruses are likely to be preferred over Ad5 as vaccine platforms for veterinary species. Wild type CAV2 has been used since over 30 years to vaccinate dogs against the Rubarth Hepatitis and has
proven to be safe in dogs. A non replicative CAV2 (Cav R0) has been generated by deletion of the E1 gene and has been documented to efficiently transduce neurons and to be poorly inflammatory in mice [14]. Several studies document its effectiveness as a recombinant vaccine [13]. For instance, recombinant CAV2 encoding for the G protein of the Seoul virus provided long-term immunity against this Hantavirus in mice [16]. Furthermore recombinant CAV2 encoding for the rabies G protein induced protective immunity to rabies in dogs, cats, pigs and sheep with high levels of neutralizing antibodies and T cell responses [17,18,19,20]. The demonstrated immunogenicity of CAV2 and the availability of skin-migrated DCs in sheep led us to assess the gene response profiling of DCs to vaccine vectors in this model system. We found that CAV2 transduced both the CD11b+ and the CD103+-type DCs in vivo, with a slightly higher efficiency in the later, but only the CD11b+-type DC subset presented a broad pattern of gene responses to CAV2 that is associated to dendritic cell maturation, communication between innate and adaptive immune cells, immune cell trafficking, and immune response. Thus our data suggest that the skin-migrated gene response could be used as an in vivo tool to help in vaccine design and evaluation of effectiveness.

Materials and Methods

Recombinant CAV2 Vectors

The replication-deficient CAV2 vectors used in this study were derived from the Manhattan strain that was deleted of the E1 region. The E1-deleted base vector was named Cav-null R0. A E1-deleted vector expressing the Green Fluorescent Protein (GFP) was generated (Cav-GFP R0). The VP7 and NS1 sequences from Bluetongue Virus (BTV) Corsican Strain BTV2 were cloned in E1-deleted vector (Cav-VP7 R0 and Cav-NS1 R0). Cav-GFP, NS1, -VP7 and Cav-null R0 were propagated in DK-E1 cells, purified by double banding on CsCl gradients and titrated by end-point dilution. Infectious titers were expressed as TCID50 ml−1.

Sheep and Ethics Statement

Precaire female sheep (between 1 and 4 years old) were bred in the Institut National de la Recherche Agronomique (INRA) animal experimentation unit in Jouy-en-Josas, France. Pseudo-afferent prescapular skin draining lymph duct cannulations were performed in sheep as previously described [7]. Low-molecular heparin (enoxaparin (Lovenox), Sanofi-Aventis) was injected intradermally in the shoulder skin every 12 h (2,000 IU anti-Xa per injection). Sheep #61 was vaccinated with commercial BTV2 vaccine (Btevap Alsap2, Merial), 7 and 3 weeks before CD103+ T cells isolation from lymph. The cannulated sheep were housed in the INRA animal facilities in Jouy-en-Josas under the authority of a license issued by the Direction des Services Vétérinaires de Versailles (accreditation numbers 78-93, 78-15, and A76-730). The animal experiment protocols were carried out in strict accordance with the recommendations of the « charte nationale portant sur l'éthique en expérimentation animale » established by the Comité national de réflexion éthique sur l’expérimentation animale (CNREEA, Ministère de l’Enseignement Supérieur et de la Recherche et Ministère de l’Agriculture et de la Pêche). They were approved by the Committees on the Ethics of Animal Experiments of the INRA research center in Jouy-en-Josas and AgroParisTech (approval #11019).

Lymph Cells Collection

Lymph was collected twice a day in flasks containing 500 IU heparin + 10000 IU penicillin + 10 mg streptomycin. Total lymph cells were spun down at 700g. Low density lymph (LDL) cells were obtained after centrifugation on a 1.065 density iodixanol gradient (Optiprep, Nycomed Pharma, Denmark) as previously described [21].

Immunolabeling and Flow Cytometry Analyses

Lymph cells were labeled as previously reported [22] using anti ruminant determinant-reacting mAbs, including anti CD1b (Th97A, [IgG2a]), anti CD26 (CC69, [IgG1]), anti CD80 (ILA139, [IgG1]), anti CD86 (ILA190, [IgG1]), anti MHC class 2 (CAT82A, [IgG1]), anti CD40 (ILA156, [IgG1]) and appropriate isotype control (ISC) mAbs. In preliminary experiments of DC activation with Cav-null R0 (Figure S1), DCs were isolated from LDL cell preparations by negative selection using Dynabeads as previously described by us [9]. For experiments with DC subsets, the CD26+CD11b+ (CD103+-type DCs) and CD26−CD11b+ (CD11b+-type DCs) lymph DCs were sorted from LDL frozen cells by flow cytometry at a 99 % purity level (MoFlo XDP Beckman Coulter). A minimum of 80×10⁶ LDL cells from 6 to 8 successive lymph collections of the same animal were needed to obtain 1.5×10⁶ CD26+CD11b+ and CD26−CD11b+ after sorting. In the rest of the study, the CD26+CD11b+ will be designated as CD103+-type DCs and the CD26−CD11b+ DCs will be designated as CD11b+-type DCs. Each subset was cultured for 16 hours in mock conditions (7.5×10⁶ cells) or with Cav-null R0 (7.5×10⁶ cells), 50 TCID₅₀/μl, see below).

Transduction and Activation of Lymph Cells with Recombinant CAV2 Vectors

Lymph cells (LDL cells or selected DC subsets) were spun down at 700 g at 20°C for one hour with 50 TCID₅₀ CAV2 vector per cell to optimize contact of the adenovirus with the target cells [23]. A pilot experiment indicated that 50 TCID₅₀/μl cell was optimal to transduce lymph cells, as used in other comparable studies with adenoviruses [24,25]. Less than 15 % of the analyzed cells were dead after 36 hours culture and culture with Cav-null R0 had no effect on lymph cell viability. Cells were then cultured for the mentioned time periods in complete medium at 37°C.

RNA Isolation and Hybridization

Total mRNA from LDL cells (called “reference”) and flow cytometry-purified CD103+- and CD11b+-type DCs mock-cultured or cultured with Cav-null R0 for 16 hours were extracted using the Qiagen RNeasy Micro Kit and checked for quality on an Agilent 2100 Bioanalyzer with RNA 6000 Nano Chip kit (Agilent). Four independent sheep were used for the microarray data generation, corresponding to a total of 16 RNA samples (mock CD103+-type DCs, Cav-null R0+CD103+-type DCs, mock CD11b+-type DCs, Cav-null R0+CD11b+-type DCs). Total RNAs (200 ng) were amplified by linear PCR and the amplification products were labeled with Cy5 using Bioprine Array CGH Genomic Labelling System Kit (Invitrogen, Carlsbad, CA). RNA from the LDL reference preparation was similarly amplified, labeled by Cy5 and used as a reference probe for the hybridization. Each Cy5 labeled cDNA was co-hybridized with the Cy5 reference probe onto ovine 15K 60-mers microarrays (15208 probes, Reference: G4813A, AMADID: 19921, Agilent) following the manufacturer’s protocol. Raw data were extracted from scanned microarray images (.tiff) using Feature Extraction Software v9.5 (Agilent) and normalized using the Quantile method.
adapted to bicolour microarrays. All the protocols used can be obtained by contacting the microarray and sequencing platform of the IGBMC (see also the web site http://www-microarrays.ustrasbg.fr/). The microarray data have been assigned the Gene Expression Omnibus number GSE42502 (www.ncbi.nlm.nih.gov/geo/info/), and they are publicly available.

**Microarray Analysis**

The probe signals corresponding to genes significantly induced by Cav-null R0 vs mock conditions was analyzed by a linear model taking into account treatment of the samples (Cav-null R0 vs mock) and the origin (animal) of the samples using a LIMMA analysis. The p-values obtained have been corrected for multiple testing using the Benjamini and Hochberg procedure. By convention, genes were considered dys-regulated by Cav-null R0 treatment when their corresponding Agilent probe signal showed a > 2 or < 0.5 fold modulation compared to the mock condition, with an adjusted p-value < 0.05. The ovine microarray has been partially annotated by the SIGENAE group (INRA - SIGENAE [http://www.sigenae.org/]). Regarding the differentially expressed genes revealed by non-annotated probes, the corresponding EST were blasted against bovine sequences (RefSeq Bos Taurus) in order to identify the putative bovine orthologous gene (>200 nt with >92 % identity). The visual representation of the different experimental condition effects (Cav-null R0 treatment, cell subsets, animal) was given by the representation of the different experimental condition effects (Cav-null R0 treatment, cell subsets, animal) was given by the

**RT-PCR**

RNA (400 ng) was reverse transcribed using random primers and the Multiscribe reverse transcriptase (Applied Biosystem). Quantitative real-time PCR was carried out using 100 ng cDNA with 300 nM primers in a final reaction volume of 25 µl of 1×SYBR Green PCR Master Mix (Applied Biosystem). The primers used to amplify the ovine IL12 (Forward GAAATTCCTTGAGGAGTGAAAG, reverse GCTGCTCCTGGTATGACCTTC, Reverse GTCGCTCCTGGTATGACCTTC, TNC92: (Forward CAAGGAGGTTTTTACC, reverse GCCACCCATGTCAAGT- TCTG), IL2: (Forward GTGGAATGGTCTGCTGGA, reverse TGTTCACTTGGTTTGCTCTGG), IL16: (Forward GAGCA- CAGCATATAAGATGG, reverse TGAAACAGTCAAGAC- CATGA), MX1: (Forward TGGCGATGCTGACGAGAT, reverse CCAAGATCAGGCTTTTGCAAG), ETS2: (Forward TGCAAGAAGGCTGTAGATG, reverse CGGCGTGTGCTCCTTTTG), TNFRSF13: (Forward GGAAGCTGAAGATTCACGAGAT, reverse GCTGCTCCTGGTAAACCT), STAT1: (Forward ACCECGGAAATGGTCAGCTTCTCT, reverse CAGCTGCAAGTCCTGGAAGAACG), Caspase1: (Forward GCGAAATCTGCAAGACCATA, reverse TCCAGTCTGCTGTTTCCG, reverse TCCATTCTCCCTGTGTATAC), IL-8: (Forward TCCAACTGGCTCTGTAATTG, reverse GCCAGGCTAGCCTGAAAG), EGF: (Forward AGGCTGGCTGAAATGATC, reverse GGCGCCGCTAGCCTGAAAG), IL2: (Forward GTGAAGTCATTGCTGCTGGA, reverse GCCAGGGTTCTTACC, reverse GCCCACCCATGTCAAGT- TCTG), IL10: (Forward TCCAACTGGCTCTGTAATTG, reverse GCCAGGCTAGCCTGAAAG), CCL5: (Forward GTGGAATGGTCTGCTGGA, reverse GCCAGGCTAGCCTGAAAG), C2CR1: (Forward CCAGATGGTCTGCTGGAAGATGC, reverse GCGGATGCTGACGAGAT), ETS2: (Forward TGCAAGAAGGCTGTAGATG, reverse CGGCGTGTGCTCCTTTTG), TNF

**Antigen Presentation to Immune Autologous CD8+ T cells**

CD103+ and CD11b+ -type DCs were sorted as described above from sheep #61 affrent skin lymph. CD8+ T cells, that recirculate in skin-lymph, were selected from affrent lymph on the same sheep, using the CAT80C and 7C2 anti sheep CD8 monoclonal, and goat anti mouse IgG Miltenyi magnetic beads, 3 weeks after the recall injection of commercial BTV8 vaccine. The CD103+ and CD11b+ -type DCs (2×10⁵) were pulsed overnight with 50 PFU/cell Cav-NS1 R0 and Cav-VP7 R0 op vi Cav-null R0 (control) or left alone. Autologous BTV-immune CD8+ T cells (2×10⁵) were added to the wells and the co-culture was pursued for 72 hours. Supernatants were harvested for ruminant IFNγ detection by ELISA as described above.
Statistics
Statistical significance of transduction and functional tests was estimated with paired Student’s t-test. The statistics of the microarray analyses are described in the corresponding section.

Results
The CAV2 Vector Transduces both CD103\(^+\) and CD11b\(^+\) type DCs

We evaluated whether CAV2 can transduce the skin-migrated sheep DC subsets collected from afferent lymph. A pilot experiment showed that 50 TCID\(_{50}\) of Cav-GFP R\(^0\)/cell was optimal to induce transduction of low density lymph (LDL) cells that are enriched in DCs (data not shown). As shown in Fig. 1A in LDL cells, the GFP transgene encoded by CAV2 was found mainly expressed in CD11b\(^+\) cells that correspond to DCs in sheep afferent lymph [7] and CD11b\(^+\) cells appeared little transduced (Fig. 1A). From 6 independent infections using LDL cell preparations from 2 sheep, we found that 3.8 ± 0.8 % of the skin-migrated DCs were transduced by Cav-GFP R\(^0\) (Fig. 1B). Cav-GFP R\(^0\) transduced a slightly higher percentage of cells in the CD103\(^+\) type DCs subset than in the CD11b\(^+\) type DCs that represent the major part of the skin-migrated DCs (> 85%) [7] (Fig. 1B, 6.2 ± 2.3 versus 3.6 ± 0.9, p<0.05). Despite a higher receptivity of CD103\(^+\) type DCs to CAV2, both FACS-sorted subsets when transduced with Cav R\(^0\) expressing Bluetongue NS1 and VP7 antigens, were capable of activating autologous immune CD8\(^+\) T cells, with the CD11b\(^+\) type DCs being slightly more efficient (Fig. S1). Thus a small but significant fraction of skin-migrated DCs are transduced by CAV2 vector and can directly stimulate autologous CD8\(^+\) T cells.

The CAV2 Vector Triggers a Broad Gene Response in Skin-migrated DCs, Especially in the CD11b\(^+\) -type DC Subset

We next analyzed the global gene response of CD11b\(^+\) and CD103\(^+\) -type DCs to Cav-null R\(^0\) (50 TCID\(_{50}\)/cell) was established from 4 different sheep after 16 h culture. We used the ovine Agilent microarrays that include 15000 ovine long 60-mers. They are the only commercially available gene arrays for sheep that partially cover the sheep genome that is not yet annotated [28]. The gene expression analysis of the CD11b\(^+\) and CD103\(^+\) -type DCs response to Cav-null R\(^0\) was informative. In order to visualize the different experimental conditions effects, the normalized probe signals of each mock and CAV2-activated DC subset were subjected to principal component analysis. As shown Fig. 2A, a strong “animal effect” is observed, that is expected from studies with primary cells from outbred animals. Despite this strong animal effect, Fig. 2B shows that the samples grouped according to the CAV2 treatment (Y-axis, dim 4) and to the DC subset type (x-axis, dim 3). The lists of the genes dys-regulated by Cav-null R\(^0\) were established for the CD11b\(^+\) and CD103\(^+\) -type DCs using the LIMMA R package and the p-values have been adjusted for multiple testing using the Benjamini-Hochberg procedure. The up- and down-modulated genes were selected when they presented a > 2-fold and < 0.5-fold modulated expression. In the CD11b\(^+\) -type DCs, 222 up-regulated genes (Table S1) and 29 down-modulated genes were identified (Table S2). In the CD103\(^+\) -type DCs, only 21 up-regulated genes were found (Table S1). The heat map representation of the expression levels of the 222 up-regulated genes and 29 down-modulated genes (Fig. 3) shows that all the up-regulated genes in the CD103\(^+\) -type DCs are also up-regulated in the CD11b\(^+\) -type DCs, and usually at a higher extent in the latter. Thus overall, our result show that CD11b\(^+\) -type DCs are more transcriptionnaly activated by Cav-null R\(^0\) than are the CD103\(^+\) -type DCs, and that no gene is specifically modulated in the CD103\(^+\) -type DCs.

The transcriptomic results provided by the LIMMA analysis were confirmed and completed by qRT-PCR analysis using 3 to 4 different sheep. As shown in Table S1 and Fig. 4 for the CD11b\(^+\) -type DCs, MX1, TNFA, ETS2, TNFSF13B, CCL5, STAT1, CXC1L10, EIF2AK2 (PKR) gene expressions were found up-regulated by the LIMMA selection and were confirmed by qRT-PCR (Fig. 4). For the CD103\(^+\) -type DCs, only MX1 of this qRT-PCR analysis was selected by the LIMMA filter. However, the other gene mRNAs were also up-regulated but at a clearly lower extent than in CD11b\(^+\) -type DCs, and often between 1.5 and 2 folds (Table S1).

As our goal was to evaluate the immunity-related functions induced in DCs by Cav-null R\(^0\), we extended our qRT-PCR...
Figure 2. Principal component analysis of microarray data from the CD103<sup>+</sup> and CD11b<sup>+</sup>-type DCs activated by the CAV2 vector. The expression value for each gene in mock and CAV2-activated CD103<sup>+</sup> and CD11b<sup>+</sup>-type DCs from 4 sheep (A: sheep #58; B: sheep #74; C: sheep #66; D: sheep #61) were used for the analysis. The expression values for the 4 first components (Dim 1, 2, 3, 4) of the principal component analysis were plotted. (A) The Dim 1 and dim 2 axes may be interpreted as the "animal" component representing 18.58 % and 16.28 % of the microarray data variance; CD11b<sup>+</sup> and CD103<sup>+</sup> stands for CD11b<sup>+</sup> and CD103<sup>+</sup>-type DCs; Mock stands for DCs cultured alone, CAV stands for Cav-null R0 treated DCs. (B) the Dim 3 may be interpreted as the "subset" component (12.71 % of the array data variance) and dim 4 represents the "CAV2 treatment" component (10.21 % of the array data variance).

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The in vitro Gene Responses of Skin-migrated DC Subsets to the CAV2 Vector is Associated to Immune Response Activation

The CAV2-modulated genes in CD103<sup>+</sup> and CD11b<sup>+</sup>-type DCs completed with the genes confirmed by qRT-PCR (i.e. first list + IL8, IFRF1, CCR7, IL12B, IRF7) were submitted to the Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com/) to determine the statistical over-representation of functional gene clusters and the functional annotations of downstream effects of the gene expression data (see material and methods). The analysis of dysregulated gene list in Cav-null R<sup>0</sup> stimulated CD11b<sup>+</sup>-type DCs provided z-scores that predicted the activation of several functions based on the knowledge data base of IPA (Table 1). As found in previous transcriptomic analyses with human adenovirus [29], the function “Proliferation of cells” is predicted to be activated in the CD11b<sup>+</sup>-type DCs. Many molecules in the “Proliferation of cells” list are also found in the “Cell death” function list, although this latter function is not activated (z-score = 1.67). Four functional clusters related to immunity are predicted to be activated in the CD11b<sup>+</sup>-type DCs, and correspond to “activation of immune responses” (z-score = 2.4), “inflammation” (2.16) “cell movement of myeloid cells” (2.06) and “activation of mononuclear leukocytes” (2.07). Conversely none of these functions were predicted to be activated in the CD103<sup>+</sup>-type DCs, despite the fact that several immune genes are stimulated in this subset such as CCL5, IFIH1, IL27, ISG15, and MX1. In this subset, only the “cell proliferation and cell death” functions were significantly predicted to be activated (z-score 2.34 and 1.98).

Canonical pathways induced by Cav-R<sup>0</sup> in the CD11b<sup>+</sup>-type DCs were also revealed by IPA (Table 2). The top ones were the “communication between innate and adaptive immune cells” (p-value = 10<sup>−7.72</sup>) and the “activation of IRF by cytosolic pattern recognition receptors” (p-value = 10<sup>−7.05</sup>) and “interferon signaling” (p-value = 10<sup>−5.0</sup>) (Table 2). None of these pathways were found significantly activated in the CD103<sup>+</sup>-type DCs. Finally the most meaningful biological network revealed by IPA in CD11b<sup>+</sup>-type DCs corresponded to the “Antimicrobial, Inflammatory and Infection” network (23 up-regulated genes over the 59 members of the corresponding IPA network) that was centered on IFN related pathways (Fig. 5).

Altogether, our results show that the in vitro gene response of skin-migrated CD11b<sup>+</sup>-type DCs to Cav-null R<sup>0</sup> is broad and clusters into activated functions that are related to the immunogenicity.

Discussion

In this study, the global gene response profile of skin-migrated DCs revealed that CAV2 stimulation in vitro induces an immunogenicity-related gene expression profile primarily in the CD11b<sup>+</sup>-type DCs, whereas both CD103<sup>+</sup> and CD11b<sup>+</sup>-type DCs are transduced by the vector.

Skin-migrated DCs were significantly transduced by CAV2 in vitro, contrasting with the refractory state of human monocyte derived-DCs (Mo-DCs) to CAV2 transduction [24], when used at similar multiplicity of infection. Mo-DCs and skin-migrated DCs are distinct DC types: the later originate from common DC precursors in the bone marrow [30], whereas the former are derived from monocytes cultured with GM-CSF and IL-4 in vitro.
Indeed several groups have reported that skin-derived DCs, and especially skin-migrated DCs, are more permissive to Ad5 or Ad35 transduction than Mo-DCs [6,31]. Furthermore, skin-migrated DCs from cattle were previously found to be highly susceptible to Ad5 transduction [25]. Difference of species (human/sheep) may also explain the discrepancy of the results for CAV2 transduction in sheep skin-migrated and human Mo-DCs, that may implicate differences in viral receptors and/or intracellular trafficking. For instance Ad35 shows species-specific interactions with target cells, due to the selective expression of its receptor CD46 in primate cells [32]. Nevertheless, the percent of sheep skin lymph DCs transduced by CAV2 remained relatively low in the two CD103⁺ and CD11b⁻-type DCs (6.2 and 3.6 % respectively). Of note, the increase in co-stimulatory molecules expression could be detected in the overall DC population cultured with CAV2, much beyond the limited fraction of transduced cells. Thus it is likely that CAV2 activates DCs via sensing mechanisms that do not require complete transduction by the vector. Indeed UV-
| Function annotation                  | CD11b⁺-type DCs                                                                 | CD103⁺-type DCs                                                                 |
|-------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| Molecules                           | Z-score | P-value | #   | Molecules                           | Z-score | P-value | #   |
| Proliferation of cells             |         |         |     |                                     |         |         |     |
| ANP32A,ANPEP,ATF3,AURKA,AURKB,BIRC5,BUB1,  | 2.488   | 4.71E-11 | 82  | BUB1,CCL5,CSK2,IL27,  | 1.97    | 8.4E-03 | 9  |
| CASP1,CAT,CC4,CC5,CCD3,CCR7,CD14,CD80,  |         |         |     | ISG15,NCAPG,PDZK1,PI3M2,TTK    |         |         |     |
| CDCA7,COR1,CORB,CFR,CFRA,CKS1B,CSK2,CSK3,CTSL2,  |         |         |     |                                     |         |         |     |
| CXCL10,CXR3,CYP2D2,DAWX,DDX,CEN1,EFA2A,K2,EF2,EFL,ETS2,FGF10,FL,T1,GGC,GC,CL,HMGFW,  |         |         |     |                                     |         |         |     |
| HSPA1A/HSPA1B,IFITM1,LT2B,IL27,IL6,IL18R1,ISG15,JAG1,KCNJ2,KIF2C,  |         |         |     |                                     |         |         |     |
| KLF4,AT,MARC851,1,MBP,MT2A,MYCBP,MYLK,NA-MPT,  |         |         |     |                                     |         |         |     |
| NCAPG,NK2,PARP1,PARP10,PDZK1,PELI1,  |         |         |     |                                     |         |         |     |
| PIM2,PLAUR,PLK2,PLM1,PRDM1,PRDX1,  |         |         |     |                                     |         |         |     |
| SDC4,SERTAD1,SLAMF7,SMAD1,STAT1,TACC3,  |         |         |     |                                     |         |         |     |
| TNF,TNFAIP8,TNFSF13B,TOM1L1,TTK,  |         |         |     |                                     |         |         |     |
| TXNIP,ZC3HAV1                      |         |         |     |                                     |         |         |     |
| Immune responses                   |         |         |     |                                     |         |         |     |
| ALOX5AP,AMICA1⁺,ANPEP,ATF3,CASP1,CASP5,CC4,CC5,  | 2.454   | 5.33E-07 | 50  |                                     | 2.163   | 3.10E-07 | 27  |
| CCR7,CD14,CD200,CD80,CFB,CSF1,CTSL1,CXCL10,CXCR3,  |         |         |     |                                     |         |         |     |
| EDN1,EFA2A,K2,FL,T1,GM2A,GNLY,FH44,IFH1,IL2B,IL27,IL6,  |         |         |     |                                     |         |         |     |
| IL18R1,IRF3,IRF7,ISG15,KLF4,LA,MBP,MT2A,MT1,MYLK,PELI1,  |         |         |     |                                     |         |         |     |
| PLAUR,PLM1,PRDM1,SLC2A4,STAT1,STAT2,TNF,TNFSF13B,TRIM5,  |         |         |     |                                     |         |         |     |
| TXNIP,ZC3HAV1                      |         |         |     |                                     |         |         |     |
| Inflammation                       |         |         |     |                                     | 2.081   | 4.00E-05 | 20  |
| ALOX5AP,ATF3,CASP1,CASPS,CC4,CC5,  | 2.081   | 4.00E-05 | 20  |                                     | 2.071   | 1.26E-04 | 18  |
| CCL4,CC5,CC7,CD14,CD80,CFB,CSF1,SLC2A4,STAT1,STAT2,  |         |         |     |                                     |         |         |     |
| CXCL10,CXR3,EDN1,FL,T1,IL1B,IL27,IL6,  |         |         |     |                                     |         |         |     |
| IL18R1,MT2A,PLAUR,SLC2A4,STAT1,TNF,TNFSF13B  |         |         |     |                                     |         |         |     |
| Cell death                         |         |         |     |                                     | 1.67    | 1.14E-15 | 98  |
| ADA,ANP32A,ANPEP,ATF3,AXIN1,ATXN3,AURKA,BIRC5,BUB1,  | 1.67    | 1.14E-15 | 98  | BUB1,CCL5,CSK2,IL27,  | 1.742   | 3.68E-03 | 11  |
| CASP1,CASP5,CC4,CC5,CCD3,CCR7,  |         |         |     | ISG15,NCAPG,PDZK1,PI3M2,TTK    |         |         |     |
| CD14,CD200,CD80,CD90,CD91,CDK2,CDK1,CDK20,CL,CTR,CKAP2,CSF1,CTSL2,  |         |         |     |                                     |         |         |     |
| CXCL10,CXR3,EDN1,FGL1,FSK1,FSL1,ET52,  |         |         |     |                                     |         |         |     |
| FANC,D2,FAP,FGF10,FL1,GGC,GNL,GNLY,GSR,HMGFW,  |         |         |     |                                     |         |         |     |
| HSPA1A/HSPA1B,IFH1,IL2B,IL27,IL6,  |         |         |     |                                     |         |         |     |
| IL18R1,IRF3,IRF7,ISG15,KLF4,LA,MBP,MT2A,MT1,MYLK,PELI1,  |         |         |     |                                     |         |         |     |
| PLAUR,PLM1,PRDM1,SLC2A4,STAT1,STAT2,TNF,TNFSF13B,TRIM5,  |         |         |     |                                     |         |         |     |
| TXNIP,ZC3HAV1                      |         |         |     |                                     |         |         |     |

*the expression of the underlined molecules is down-modulated.

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inactivated Ad5 activates DCs to the same extent as non UV-irradiated Ad5 for the production of inflammatory cytokines and NF-kB activation [33]. Sensors such as the heparin-sensitive adenovirus receptor [34], TLR9 [13] and the helicase DDX41 [35] that are involved in Ad5 sensing need to be tested for their implication in CAV2 signaling in DCs.

The direct transduction of skin-migrated DCs by CAV2 observed in our in vitro experiments may participate in CAV2 immunogenicity but may not be the key mechanism of CAV2 vector immunogenicity in vivo. Indeed in the case of Ad5 in mice, DCs were required for induction of immunity to transgene but their direct transduction seemed not to be required for inducing immunity [36]. In addition Hangalapura et al documented that Ad5 essentially transduced non DC cells when given intradermally [37]. The preferential transduction of non DC cells may possibly due to their expression of the Coxsachie and Adenovirus Receptor (CAR), the Ad5 primary receptor, whereas DCs do not express CAR and use a not yet defined fiber shaft receptor [34]. In the case of CAV2 that also utilizes CAR to transduce epithelial and neuronal cells [38], the transduction of non DC cells in the skin may play an important role in its immunogenicity, leading to cross presentation by the DCs in the lymph node [36]. Notably the general activation of the skin-migrated DCs by CAV2, that we report here, may allow the proper priming of DCs for triggering efficient antigen presentation both after their direct transduction and after capture of non DC transduced cell fragments (cross presentation).

The distinct capacities of the two skin-migrated DC subsets to induce gene responses to CAV2 cannot be explained by difference in transduction efficiency because CD103+ -type DCs are better transduced by CAV2 than are the CD11b+ -type DCs (Fig. 1). We can not exclude that technical artifacts such as differential response/activation to sorting conditions or differential timing for optimal response explain the differences of the DC subset response to CAV. However despite limited global gene activation in response to CAV2, the CD103+ -type DCs still presented their intrinsic properties, with higher IL-12p40 mRNA basal levels and further increase upon CAV2 stimulation (Fig. 4). In addition, several genes were induced under our CAV2 activating conditions in the CD103+ -type DCs, at the same or higher extent than in the CD11b+ -type DCs (IL12p40, PDZK1, TACC3, CEBPB, MBP, IL8, IRF7, IRF1, Table S1 ) suggesting that the CD103+ -type DCs kept its functionality and capacity for being activated. Indeed the CD103+ -type DCs were efficient at activating antigen-specific CD8+ T cells upon CAV2 stimulation (Fig. S1). In addition, the viability of both DC subsets cultured with CAV2 is similar (>90% as measured by 7-Amino-Actinomycin D exclusion, data not shown) and both subsets show similar z-scores for the cell death function in the 2 subsets (Table 1). The higher and broader gene response to CAV2 stimulation encountered in the CD11b+ -type DCs might be explained by the different expression of viral sensors in the two subsets. Indeed murine CD8+ and CD11b+ DCs [39] and sheep lymph DC subsets [40] express different sets of TLR receptors. In addition, murine CD8+ DCs largely lack the receptors required to sense certain viruses in the cytoplasm, such as retinoic acid-inducible gene I [41,42]. However we could not detect consistent differences between the two lymph DC subsets in the mRNA expression of known adenovirus sensors, (our microarray data, not shown). Other yet unknown adenovirus sensors may be involved, and proteomic studies are also needed to detect differences of sensor expression.

Our data suggest that the in vitro transcriptomic signature of DCs in response to vaccines may bring information on the possible downstream immunogenicity. This approach brings complementary information to classical functional in vitro assays. Indeed, while a direct antigen presentation assay by CAV2-transduced CD103+ and CD11b+ -type DCs showed a slightly higher efficiency of the later DC type for stimulating antigen-specific CD8+ T cells, the transcriptomic analysis provided a broad information on the global DC subset activation by the vector, that may be even more pertinent if indirect antigen presentation is concerned. Indeed the transcriptomic signature unveiled that CAV2-stimulated CD11b+ -type DCs for B and T cell activation and immune cell trafficking. The signature that we obtained here would be strengthened and completed by using RNAseq strategy as the limited array available for sheep studies probably bias the results. Finally subsequent studies need to be conducted to establish gene expression signatures in DCs in vitro that predict the magnitude of the immune response using formal training and validation test processes. These transcriptomic signatures may vary with vectors and vaccine formulations. DC gene response signatures to different vaccine vectors and formulas may importantly help in vaccine improvement.

Altogether, this work indicates that CAV2 activates the CD11c+ -type and not the CD103+ -type DCs to express a transcriptomic signature related to stimulation of immune response. Thus the analysis of the DC gene responses in vitro stands as an interesting and convenient assay to evaluate and possibly improve vaccine effectiveness. The in vitro innate gene response of DCs combined to other parameters such as the strength and duration of transgene expression, structural analyses of predictive antigenicity of the transgene-encoded protein, and classical immunological functional
assays could altogether allow prediction of downstream immunogenicity and offer new avenues to reasoned vaccine developments, while reducing the number of animal tests.

Supporting Information

Figure S1  CD103⁺-like and CD11b⁺-type DCs induce antigen-specific CD8⁺ T cell responses upon transduction with Cav-NS1 R⁰ and Cav-VP7 R⁰. CD103⁺ and CD11b⁺-type DCs (2×10⁴) were cytometry-sorted from sheep #61 and pulsed overnight with Cav-NS1 R⁰ and Cav-VP7 R⁰ (BTV label), with Cav-null R⁰ (null label) or left alone (- label). Autologous BTV-immune CD8⁺ T cells (2×10⁵) were added to the wells (2-4 replicates) and the co-culture was pursued for 72 hours. Supernatants were harvested for IFNγ detection by ELISA. Statistical significance (paired Student t-test) is indicated by ** for p < 0.005 and * for p < 0.05.

Figure S2  CAV2 triggers skin-migrated DC activation. (A) lymph DCs from 2 different sheep (◆ #70 and ▲ #66) were isolated by negative selection and they were cultured alone or with 50 TCID₅₀/cell Cav-null R⁰ for 3, 16 and 24 hours. Cells were lysed for RNA extraction and real time RT-PCR. The ratio of the GAPDH-normalized cytokine mRNA signals in Cav-null R⁰ -stimulated versus non stimulated cultures was calculated. (B) After a 36 hour infection with 50 TCID₅₀/cell Cav-null R⁰, LDL cells from sheep #81 (black bar) and sheep #70 (grey bar) were co-labeled for detection of the CD1b (FL-1) together with the CD80, CD86, CD40 and MHC class 2 molecules (FL-2). The ratio of the

Figure 5. Antimicrobial gene network induced by CAV2 vector in CD11b⁺-type DCs. An antimicrobial gene network centered on IFN was generated by the Ingenuity Pathways Analysis on the selected genes dys-regulated by CAV2 vector in CD11b⁺-type DCs (Table S1, p < 0.05 and fold > 2, or p < 0.05 and folds confirmed by qRT-PCR). Molecule types are represented by symbols: diamonds (enzymes), triangles (kinase), square (cytokine), double circle (complex), oval (transcription regulator), circle (others).

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mean fluorescence intensity (MFI) corresponding to CD80, CD86, CD40, and MHC class 2 expression on the gated CD1b+ cells from cultures with Cav-null R8 versus from mock cultures is reported. Isotype control staining of the control and Cav-null R8-activated cell cultures were identical (not shown). (C) Cytometry profile of CD1b+ cells (infected CD1b+ cells, thick black line; mock cultured CD1b+ cells, grey filling). The increase in CD80/CD86 expression concerns the whole CD1b+ population. (TH)

Table S1 Up-regulated genes induced by CAV2 vector in skin-migrated DC subsets.

Table S2 Down-modulated genes by CAV2 vector in skin-migrated DC subsets.

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Author Contributions

Conceived and designed the experiments: IS-C SZ BK. Performed the experiments: VC CU CB-C M. Bourge M. Bonnae. Analyzed the data: VC LJ IS-C. Wrote the paper: IS-C.

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