Published in final edited form as:

Vaccine. 2008 January 17; 26(3): 419–426.

Infection of monocytes or immature dendritic cells (DCs) with an attenuated rabies virus results in DC maturation and a strong activation of the NFκB signaling pathway

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Abstract

To assess the potential role of dendritic cells (DCs) or monocytes in the development of a protective immune response, we infected human immature DCs or monocytes with a live rabies virus (RV) vaccine strain (SPBNGAS-GAS) and a pathogenic RV (DOG4). Both cell types were infected with SPBNGAS-GAS and DOG4 and both RVs were similarly potent in inducing maturation of immature DCs or monocytes. However, in contrast to DOG4, SPBNGAS-GAS induced very high levels of IFN-α1 mRNA in monocytes and DCs. Furthermore, at least 26 other genes related to the NFκB signaling pathway were strongly upregulated in SPBNGAS-GAS-infected DCs, but only somewhat increased in DOG4-infected cells. Thus, the extent of upregulation of NFκB pathway-related genes in DCs infected with the live RV vaccine strain might explain the strong protective activity of SPBNGAS-GAS.

Keywords

rabies virus; dendritic cell maturation; NFκB signaling pathway

1. Introduction

Rabies virus (RV), a member of Rhabdoviridae family, is a fatal human and animal pathogen. A variety of species, including raccoons, skunks, coyotes and bats serve as the primary reservoirs of RV strains in the Americas. Oral immunization of wildlife with live RV vaccines is the most effective method to control and eventually eradicate rabies [1,2]. Over the past decade, more than 15 million baits containing recombinant vaccinia-rabies virus glycoprotein virus (VRG) have been distributed in the US through programs designed to control rabies among free-ranging raccoons, foxes and coyotes [3]. Vaccination using modified-live rabies vaccines, such as ERA, SAD B19, SAG-1 and SAG-2, have resulted in almost complete eradication of vulpine rabies in Western Europe [3].

The precise mechanism by which oral immunization with modified-live RV vaccines confer protective immunity is not known. However, it has been shown that the tonsils, a major lymphoid tissue that contains B and T cells as well as antigen-presenting cells (APCs) including

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dendritic cells (DCs), is a primary site of infection and replication of the RV vaccine strains [4]. This finding, together with the observation that the RV vector is able to infect both immature and mature DCs in vitro [5], suggest that DCs play a pivotal role in the development of a protective immune response following oral immunization with live attenuated RV vaccines. In this context, it has been suggested that targeting live viral antigens to DCs may increase cellular responses against the foreign antigens expressed by the vector. For example, canarypox viruses expressing HIV-1 proteins can infect mature DCs and stimulate strong HIV-1-specific CD4⁺ and CD8⁺ T-cell responses in vitro [6].

DCs, which are the most efficient APCs, are a key element of both innate and adaptive immune responses to viral infection [7]. The detection and capture of viruses by immature DCs trigger stimulus-specific maturation and activation, which are critical for their ability to stimulate T cells, and for the virus-specific adaptive immune responses [8,9].

Many viruses, including influenza virus, **dengue** virus and Newcastle disease virus, induce DCs to express IFN- α/β [10-12], which is thought to play a pivotal role in defense against these infections. Several viruses, such as influenza virus and RV, have developed strategies to inhibit the expression of IFN- α/β [11,13]. For rabies **virus infection**, the RV P protein inhibits the IFN- α - and IFN- γ -induced transcriptional responses, thereby impairing the IFN-induced antiviral state [14,15]. However, different RV strains differ in their ability to inhibit IFN responses. The evasion of host innate immune responses in the central nervous system by the highly pathogenic silver-haired bat RV (SHBRV), but not by the attenuated CVS-B2C is probably related to an effect on type I IFN signaling pathways [16].

Here, we show that both pathogenic and non-pathogenic RVs can infect human monocytes and immature DCs, thereby triggering the generation of mature DCs. However, the induction of innate immune markers, such as IFN- α/β , was significantly more enhanced by infection with the non-pathogenic RV strain, which may account for the protective immune response induced by this strain.

2. Materials and Methods

2.1. Viruses and cells

The non-pathogenic recombinant RV strain SPBNGAS-GAS, which was derived from SPBNGA-S [17] by inserting an extra copy of the glycoprotein gene from SPBNGA-S as previously described [18], was propagated in BSR cells [19]. Briefly, cells grown in DMEM medium supplemented with 10% fetal bovine serum (FBS) were infected at MOI 0.1 and incubated for 1 hr at 37°C. The inoculum was removed and the cells were replenished with OptiPro SFM medium (Invitrogen, Grand Island, NY) supplemented with 4mM glutamine and incubated for 3 days at 34°C. The pathogenic RV strain DOG4, which was isolated from brain tissue of a human rabies victim, was propagated in mouse neuroblastoma cells (NA) as described [20].

Human peripheral blood mononuclear cells were isolated from buffy coats from the blood of healthy adult volunteers obtained from the Blood Donor Center in Thomas Jefferson Hospital by density gradient centrifugation using the Ficoll-Paque Plus lymphocyte isolation solution (GE healthcare, Sweden) according to the manufacture's **instructions**. The work using human blood was approved by the Institutional Review Board of Thomas Jefferson University (IRB control # 05E.49).

2.2. Isolation of monocytes and generation of DCs

Monocytes were purified using a magnetic cell separator (Miltenyi Biotec, Auburn, CA) and human CD14 MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. Cell

sorting of cells stained with FITC-conjugated anti-CD14 (Miltenyi Biotec) indicated positive staining in 98% of the isolated cells (data not shown). Purified monocytes were cultured in RPMI 1640 medium supplemented with 10% FBS in the presence of 800 U/ml granulocyte monocyte-colony stimulating factor (GM-CSF, R&D Systems, Minneapolis, MN) and 1000 U/ml IL-4 (R&D Systems) as described [21], or 800 U/ml GM-CSF and 1000 U/ml IFN- α 2b (PBL Biomedical Laboratories, Piscataway, NJ) as described **for differentiation into DCs** [22]. Cells were incubated at 37°C for 5 to 7 days, and the medium was replenished with cytokines every 2 days. As a positive control, maturation of DCs was induced by addition of 1 μ g/ml lipopolysaccharide (LPS, Sigma, St. Louis, MO).

2.3. Infection and treatment of immature DCs and monocytes

After 5 to 7 days incubation, immature myeloid DCs were infected with live or UV-irradiated SPBNGAS-GAS, or with live DOG4 at MOI 10, or treated with LPS at 1 μ g/ml, and incubated for 48 hr at 37°C. CD14 positive monocytes were similarly infected and treated and incubated for 7 days.

2.4. Fluorescence-activated cell sorting (FACS)

Cells were fixed with 4% paraformaldehyde and stained with FITC-, phycoerythrin (PE)-, or allophycocyanin (APC)-conjugated anti-CD14, anti-CD80, anti-CD83, or anti-CD86 antibodies (Biolegend, San Diego, CA). Isotypic IgG1 was used as a control for non-specific staining. RV G expression on cell surfaces was detected using rabbit anti-RV G polyclonal antibody as primary antibody and Alexa 488-conjugated goat anti-rabbit IgG was used as secondary antibody. Samples were analyzed using the BD FACSCalibur System (BD Bioscience, San Jose, CA) with CellQuest software or the EPICS XL Flow Cytometer (Beckman Coulter, Fullerton, CA). Data were analyzed by Flowjo version 7.2 (Tree Star, San Carlos, CA).

2.5. Reverse transcription and quantitative real-time PCR

Total cellular RNA was extracted from 10⁶ cells using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription was performed with ReactionReady First Strand cDNA Synthesis Kit (SuperArray, Frederick, MD) according to the manufacturer's instructions. For cDNA synthesis, quantitative real-time PCR was performed with 1 μ g RNA in 20 μ l reaction buffer using RT² Real-Time SYBR Green Master Mixes (SuperArray) and primer sets for IFNA1, NFKB1, STAT1, CASP8, FASLG, TNFSF10, IL6, TLR7, or ACTB (SuperArray, www.superarray.com). All samples were run in triplicate in a 96-well reaction plate. Data were analyzed using the ABI Prism 7000 sequence detection system and version 2.1 Software (Applied Biosystems, Foster City, CA). The threshold cycle (Ct) is the cycle number at which the change in fluorescence exceeds a fixed threshold during a real-time PCR run. Gene expression was measured by a comparative Ct method [23] and normalized using the β -actin gene. Relative expression levels of IFN- α 1 RNA in infected or treated cells were calculated as normalized Ct values divided by the normalized Ct values obtained from control cells. One-way ANOVA was used for statistical analysis.

2.6. PCR array analysis

Expression of genes related to NF κ B-mediated signal transduction was analyzed using the RT² Profiler PCR Array (SuperArray) according to the manufacturer's instructions. Values obtained with infected DCs were expressed as fold increase compared to values obtained with uninfected DCs.

3. Results

3.1. Infection of immature DCs with non-pathogenic and pathogenic RVs

Immunofluorescence microscopy revealed RV G-specific surface staining in SPBNGAS-GAS-infected cells but not in LPS-treated or untreated **immature** DCs (data not shown). Virus titers in the tissue culture supernatant of infected **immature** DCs increased from 10^4 FFU at day 1 p.i. to 10^5 at day 4 p.i., indicating productive RV infection in the DCs. FACS analysis of RV G expression showed higher FITC staining intensity in SPBNGAS-GAS-infected immature DCs than in DOG4-infected immature DCs (Fig. 1A), suggesting that SPBNGAS-GAS-infected **immature** DCs expressed more RV G than did DOG4-infected cells. The difference in G protein expression levels is unlikely due to different affinities of the polyclonal anti RV G antibody to these G proteins. We have shown that the polyclonal anti RV G antibody recognizes similarly distinct RV G proteins that have only 84 % amino acid homology [24]. FACS analysis also showed that 96.3% of immature DCs were infected with SPBNGAS-GAS, whereas only 59.3% cells were infected with DOG4 (Fig. 1B), suggesting a higher susceptibility of immature DCs for infection with non-pathogenic RV.

3.2. Induction of DC maturation after RV infection of immature DCs

The ability of RVs to trigger maturation of DCs was tested in immature DCs infected with live SPBNGAS-GAS, live DOG4, or UV-inactivated SPBNGAS-GAS. LPS-treated immature DCs served as a positive control to monitor DC maturation. RV-infected and LPS-treated cells showed morphological characteristics typical of mature DC, whereas untreated cells retained the morphology of immature DCs (Fig. 2). FACS analysis revealed expression of the maturation marker molecule CD83 in DCs infected with live SPBNGAS-GAS but not with its UV-inactivated counterpart (Fig. 3A), indicating that RV replication is essential for RV-induced maturation of DCs. Expression levels of the co-stimulatory molecules CD80, CD86 and of the maturation marker CD83 upon infection with live DOG4 were similar to those after infection with live SPBNGAS-GAS (Fig. 3B), indicating that both pathogenic and non-pathogenic RVs can similarly trigger DC maturation.

3.3. Induction of monocyte differentiation into DCs after RV infection

To examine whether RV infection can directly trigger the differentiation of monocytes into DCs, monocytes were infected with SPBNGAS-GAS or DOG4, or treated with IL-4 or IFN- α 2b, both of which can trigger monocyte differentiation into DCs [22,25,26]. FACS analysis indicated that both SPBNGAS-GAS and DOG4 can infect CD14-positive monocytes (left panel in Fig. 4A), and that infection resulted in expression levels of CD80, CD83, and CD 86 molecules similar to that detected as in IFN- α -treated cells (Fig. 4A). CD14 expression was lost in 49.6% of the SPBNGAS-GAS-infected cells and in 64.0% of the DOG4-infected cells, compared to 98.7% of IL-4-treated and 82.1% of IFN- α -treated monocytes (Fig. 4B). These results indicate that RV-infection of monocytes can induce the differentiation of these cells into mature DCs without the addition of IL-4.

3.4. Induction of IFN- α 1 mRNA expression in DCs and monocytes after RV infection

Since IFN- α induces monocyte differentiation into DCs, we asked whether this cytokine mediates the differentiation of monocytes into DCs observed after RV infection. Compared to IFN- α 1 mRNA levels detected by real-time PCR in uninfected monocytes, levels after SPBNGAS-GAS or DOG4 infection were 132 times ($p < 0.001$) and 8 times ($p < 0.001$) higher, respectively (Fig. 5A). Similar results were obtained with immature DCs in which IFN- α 1 mRNA levels were 221 ($p < 0.001$) and 32-fold ($p < 0.001$) higher after infection with SPBNGAS-GAS and DOG4, respectively (Fig. 5B).

Thus, both SPBNGAS-GAS and DOG4, induced upregulation of IFN- α 1 mRNA expression, infection of monocytes or immature DCs, however, the amount of IFN- α 1 mRNA was significantly higher in SPBNGAS-GAS- than in DOG-4-infected cells ($p < 0.001$).

3.5. RV-induced expression of factors involved in the NF κ B pathway

Since NF κ B is an important factor in the induction of IFN- α , responsive gene, we asked whether RV infection triggers the expression of other NF κ B-related factors. Of 84 key genes related to NF κ B-mediated signal transduction, 26 were upregulated in SPBNGAS-GAS-infected DCs as determined using the human NF κ B signaling pathway RT² Profiler PCR assay (Table 1). The highest increases in expression were seen in the STAT1 and EDARADD genes, which are involved in the activation of the NF κ B pathway, the FASLG, and TNFSF10 genes that play a role in the positive regulation of I κ B kinase and the NF κ B cascade, the NF κ B response genes BF, CCL2, IFN- α 1, IFN- β 1, and IL-6, and in TLR7, another gene involved in the NF κ B pathway (see www.superarray.com for functional grouping of NF κ B pathway-related genes). Real time RT-PCR to confirm and compare the upregulation of some of the NF κ B pathway-related genes in SPBNGAS-GAS-infected and DOG4-infected DCs revealed significantly higher expression levels of STAT 1, FASLG, TNFSF10, and TLR7, in the SPBNGAS-GAS-infected DCs (Fig. 6).

4. Discussion

DCs are widely considered the most efficient APCs in initiating protective immune responses against viral infections [7-9]. These cells also play a role during oral immunization with live attenuated RV vaccines, where they may function not only as APCs, but also as primary target cells for RV infection to allow production of RV antigens at levels sufficient to trigger a protective immune response. In this context, it should be noted that oral immunization with inactivated RV or purified RV G was either ineffective in inducing immunity or protection was only conferred when extremely high concentrations of these antigens were administered [27].

In this study, we tested the ability of RV to infect immature human DCs and thereby trigger their activation and maturation. Both the non-pathogenic SPBNGAS-GAS and the pathogenic DOG4 strains infected immature DCs, although susceptibility to the non-pathogenic RV was higher and infection resulted in higher RV G expression than that after infection with the pathogenic RV. This finding is in agreement with previous observations indicating that the expression of viral proteins, in particular the RV G in neuroblastoma cells, correlates inversely with the pathogenicity of a RV [28]. However, regardless of the amount of G expressed, both live SPBNGAS-GAS and DOG4, but not UV-irradiated SPBNGAS-GAS, showed a similar ability to stimulate the maturation of DCs. Moreover, both viruses were able to infect CD14-positive monocytes and induce their maturation into mature DCs. The maturation of RV-infected monocytes into DCs occurred without the addition of IL-4, leading our research for other factors that might mediate DC maturation. In that context, many viruses are known to induce IFN- α/β in DCs [11,12]. In turn, IFN- α/β can mediate monocyte differentiation into DCs [22,25,26]. Our study revealed induction of very high levels of IFN- α 1 mRNA in monocytes and DCs infected with the non-pathogenic SPBNGAS-GAS, but only relatively low IFN- α 1 RNA expression after infection with the pathogenic DOG4 resulted, suggesting either that IFN- α/β is extremely potent even at low levels or that other factors contribute to the maturation process. In this regard, studies with negative-strand RNA viruses showed that the interferon induction pathway, but not released interferon, participates in the maturation of dendritic cells [10]. Our PCR array analysis revealed upregulated expression of at least 26 genes related to the NF κ B signaling pathway in SPBNGAS-GAS-infected DCs and expression of several of these genes was also increased in DOG4-infected cells as determined by real time PCR analysis. However, like the induction of IFN- α/β , the expression levels of these genes

were significantly higher in SPBNGAS-GAS-infected than in DOG4-infected DCs. Thus, although both the non-pathogenic and the pathogenic RV can induce activation of genes related to the NF κ B signaling pathway, infection with non-pathogenic SPBNGAS-GAS appears to cause a much stronger activation of this pathway than infection with the pathogenic DOG4. A robust immune response triggered by the strong activation of NF κ B pathway-related genes might serve to confine replication of SPBNGAS-GAS to the primary site of infection and eventually clear the infection, in contrast, to the weaker activation of NF κ B pathway-related genes and consequently weaker immune response induced by pathogenic RVs, which might allow the spread of the virus to the CNS. Indeed, the significantly higher expression of NF κ B pathway-related genes including IFN- α/β in DCs infected with a non-pathogenic RV versus a pathogenic RV may account for the protective activity conferred by live RV vaccines.

Acknowledgement

This study was supported by NIH grants AI45097 and AI060686.

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Figure 1.

FACS analysis of RV G expression in immature DCs. Immature myeloid DCs were infected with SPBNGAS-GAS or DOG4 at MOI 10, or treated with LPS. After incubation for 48 hr at 37°C, cells were stained with rabbit anti-RV G polyclonal antibody, followed by Alexa 488-conjugated goat anti-rabbit IgG. Samples were analyzed using an EPICS XL flow cytometer and Flowjo version 7.2 software. Data are representative of five independent experiments performed with cells from different donors. (A) Histograms showing the intensity of RV G staining in SPBNGAS-GAS- and DOG4-infected DCs. (B) Dot plots of uninfected, LPS-treated, SPBNGAS-GAS- and DOG4-infected DCs. The percentages of RV G-positive cells are given in the right corner of each plot.

Figure 2.

Morphological transformation of DCs. Cells were infected with SPBNGAS-GAS or treated with LPS. After incubation for 48 hr at 37°C, the cells were observed under microscope. The SPBNGAS-GAS-infected cells showed **morphological** characteristics typical of mature DCs, similarly as LPS-treated DCs, whereas the untreated cells retained the morphology of immature DCs.

Figure 3.

Infection of immature myeloid DCs. Cells were infected with live DOG4, live SPBNGAS-GAS, UV-irradiated SPBNGAS-GAS at MOI 10, or treated with LPS. After incubation for 48 hr at 37°C, expression of CD80, CD83, and CD86 on the surface of RV-infected or LPS-treated cells was analyzed using a BD FACSCalibur System with CellQuest software and Flowjo software (see Materials and Methods). (A) Histogram of CD83 expression in DCs infected with live or UV-inactivated SPBNGAS-GAS, or treated with LPS or left untreated. Data are representative of two independent experiments performed with cells from different donors. (B) Histograms of CD80, CD83, and CD86 expression in untreated, LPS-treated, SPBNGAS-GAS-infected and DOG4-infected DCs. Data are representative of five independent experiments with cells from different donors.

Figure 4.

Differentiation of monocytes into mature DCs after RV infection. CD14-positive monocytes were infected with SPBNGAS-GAS or DOG4, or treated with IL-4 or IFN- α 2b (Materials and Methods). After incubation for 7 days in RPMI 1640 medium supplemented with 10% FBS and 800 U/ml GM-CSF, cells were analyzed using FACS. (A) Histograms showing the expression of RV G, CD80, CD83, and CD86 in monocytes infected with SPBNGAS-GAS or DOG4, or treated with IFN- α 2b. (B) Dot plots showing the expression of CD14 in monocytes treated with IL-4 or IFN- α 2b, or infected with SPBNGAS-GAS or DOG4, or left untreated.

Figure 5.

Induction of IFN- α 1 mRNA expression in DCs and monocytes after RV infection. Monocytes (A) or immature DCs (B) were infected with SPBNGAS-GAS or DOG4, and after 7 or 2 days, **respectively**, RNA was isolated and analyzed for expression of IFN- α 1 mRNA by real time RT-PCR. Data were analyzed using ABI Prism 7000 sequence detection system and version 2.1 software. Gene expression was measured by the $\Delta\Delta C(t)$ method using β -actin mRNA as a reference. Data were normalized to the percentage of RV G-positive cells and represent the fold increase of IFN- α 1 mRNA expression in SPBNGAS-GAS- or DOG4-infected cells as compared to that in uninfected cells. Error bars represent standard deviation of triplicate samples. Stars represent significant difference between DOG4-infected cells, uninfected cells, and SPBNGAS-GAS-infected cells ($p < 0.001$). Data are representative of two independent experiments with cells from different donors.

Figure 6.

Comparison of the expression levels of selected NFκB signaling pathway-related genes in DCs infected with SPBNGAS-GAS or DOG4. Immature DCs were infected with SPBNGAS-GAS or DOG4, and after 2 days, RNA was isolated and examined by real time PCR to measure of mRNAs expression levels for NFκB1, STAT1, caspase 8 (CASP8), Fas ligand (FASLG), member 10 of the tumor necrosis factor superfamily (TNFSF10), interleukin 6 (IL6), and toll-like receptor 7 (TLR7).. Gene expression was measured by the $\Delta\Delta C(t)$ method using β -actin mRNA as a reference. Data represent the fold increase of the different mRNA expression levels as compared to the respective levels measured in uninfected cells. Error bars represent the standard deviations of triplicate samples. Stars represent significant difference ($p < 0.001$).

Table 1

Upregulation of NFκB pathway-related genes after infection with SPBNGAS-GAS*

Functional Gene Groupings	Gene Symbol**	Fold Increase***
Activation of NFκB Pathway	EDARADD	12.58
	IL1B	3.81
	IRAK2	4.30
	MYD88	7.50
	NFKB1	4.26
	STAT1	18.40
Positive Regulation of IκB Kinase and the NFκB Cascade	TLR3	6.23
	CASP1	3.96
	CASP8	4.95
	CFLAR	3.45
	FASLG	5.93
	IKBKE	3.20
	REL	3.84
	TICAM2	3.46
	TNFSF10	170.86
	TRADD	3.20
NFκB Responsive Genes	BF	13.74
	CCL2	42.57
	IFNA1	796.02
	IFNB1	280.79
	IL6	32.48
	LTA	3.37
Other Factors Involved in the NFκB Pathway	IL1A	5.74
	NFKB2	4.07
	TLR7	42.08
	TNFAIP3	4.32

* Gene expression was determined by RT² profiler PCR array analysis.** Gene symbols are explained in the product specification sheet for RT² profiler PCR array (www.superarray.com).

*** Data represent the fold increase of mRNA expression in SPBNGAS-GAS- infected cells as compared to mRNA expression in uninfected cells.