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(54) **Title:** TOLL-LIKE RECEPTOR 3, ITS SIGNALLING ASSOCIATED MOLECULE TRIF AND THEIR USE IN THE PREVENTION AND TREATMENT OF HOST INFLAMMATION RESPONSE INDUCED BY A RNA VIRUS

(57) **Abstract:** The present invention relates to toll-like receptor 3 (TLR3) and its signalling associated molecule TRIF. More specifically, the present invention relates to screening methods for the identification of TLR3 and TRIF antagonists and methods for treating and/or preventing host inflammation response induced by a RNA virus.

TOLL-LIKE RECEPTOR 3, ITS SIGNALLING ASSOCIATED MOLECULE TRIF AND THEIR USE IN THE PREVENTION AND TREATMENT OF HOST INFLAMMATION RESPONSE INDUCED BY A RNA VIRUS

FIELD OF THE INVENTION

The present invention relates to Toll-like receptor 3 (TLR3) and its signalling associated molecule TRIF. More specifically, the present invention relates to screening methods for the identification of TLR3 and TRIF antagonists and methods for treating and/or preventing host inflammation response induced by a RNA virus, such as an Influenza virus type A.

BACKGROUND OF THE INVENTION

Influenza is a highly contagious, acute respiratory disease that affects all age groups and that can promote exacerbations of obstructive airways disorders including asthma and cystic fibrosis. The etiological agent of the disease, the single-stranded RNA influenza viruses, are responsible for an average of 114 000 hospitalizations and 20 000 deaths each year, in the USA alone (1). Influenza viruses are classified into three types (A, B, C) of which influenza A is the most important clinically (2). The major problem in fighting influenza is the high genetic variability of the virus, resulting in the rapid emergence of variants that escape the acquired immunity induced by the available vaccines, or the resistance of the pathogen to antiviral agents (1,3). In that context, it would be valuable to unravel the mechanisms of virus-host cell interactions that are responsible for the "flu" syndrome. Indeed, several studies suggest that the inflammatory response to respiratory viral infections contributes to the pathogenesis of the airway symptoms. In that regard, it is of note that the viral replicative intermediate double-stranded RNA (dsRNA) is critical for the outcome of the infection (reviewed in (4,5)). For instance, synthetic dsRNA and dsRNA isolated from influenza virus infected lungs are each able to induce both the local and systemic cytotoxic effects typical of flu (6-8).

Cells are armed with various latent mechanisms that are able to sense viral components and initiate intracellular signal transduction to respond rapidly to virus infections. Previously, the interferon (IFN)-inducible RNA-dependent protein kinase R (PKR) was considered to be central in the interaction with dsRNA (5). However, cells from PKR-deficient mice still respond to polyinosinic-polycytidylic acid (poly(I:C)), a synthetic dsRNA analog, suggesting the existence of a more critical receptor involved in the sensing and signaling in response to this viral component. Alexopoulou *et al.* demonstrated that dsRNA recognition relies on the Toll-like receptor (TLR)3, a member of the conserved family of host innate immune receptors, essential for detecting pathogen-associated molecular patterns (9). The stimulation of TLR3 by dsRNA transduces signals to activate the transcription factors NF-κB and interferon regulatory factor (IRF)/interferon-sensitive response element (ISRE) *via* myeloid differentiation factor 88 (MyD88)-dependent and/or independent signaling pathways. The last of these involves a distinct adapter molecule, namely the Toll-IL-1 receptor (TIR)-domain containing adaptor inducing interferon (IFN)-*β* (TRIF), also called the TIR-domain containing adaptor molecule (TICAM)-1 (10,11). This molecule elicits an anti-viral response, especially through the production of type I IFN.

In humans, TLR3 mRNA is detected in the lung, placenta, pancreas, liver, heart and brain. It is expressed in dendritic cells (12) and in intestinal epithelial cells (13) but does not seem to be present in monocytes, lymphocytes, polymorphonuclear leukocytes or natural killer cells (14). Interestingly, although TLR3 expression *per se* was not reported, a study by Gern *et al.* showed recently that viral dsRNA activates bronchial epithelial cells (15). Lung epithelial cells are the primary target and the principal host for influenza viruses, causing cytopathic effects to the respiratory tract as well as shedding of infective viral particles. These epithelial cells also play a key role in the initiation of innate and subsequently adaptive immune responses to the virus (16,17).

Surprisingly, very little information is available concerning the expression and localization of TLR3 in pulmonary epithelial cells. Indeed, the role of epithelial TLR3, its regulatory mechanisms and the signaling pathways underlying the response to influenza A virus have not previously been investigated.

Therefore, there is a need for new tools and methods in the prevention and treatment of host inflammatory response triggered by a RNA virus, such as an Influenza virus Type A.

SUMMARY OF THE INVENTION

Therefore, an object of the present invention is to provide tools for preventing and/or treating excessive inflammation response induced by a RNA virus.

More specifically, that aspect is achieved by using a Toll-like-receptor 3 (TLR3) molecule or its signalling-associated molecule Toll-IL-1 receptor (TIR)-domain containing adaptor inducing interferon (IFN)-β (TRIF) in the design of molecules to prevent a host inflammation response induced by a RNA virus or a viral dsRNA.

Another object of the invention relates to a method for screening a TLR3 antagonistic expression, comprising the steps of:

- contacting a functional TLR3 molecule with a candidate agent, under conditions and for a time sufficient to permit interaction between TLR3 molecule and the candidate agent; and
- evaluating the capacity of said candidate agent to interfere with the biological role of said functional TLR3 molecule.

A further object of the invention is to provide a method for preventing and/or treating excessive inflammation response induced by a RNA virus, comprising the step of administering to an animal in need thereof an effective amount of an antagonist of TLR3 and/or an antagonist of TRIF.

Yet, another object of the invention is to provide a method for screening a TRIF antagonistic expression, comprising the steps of:

- contacting a functional TRIF molecule with a candidate agent, under conditions and for a time sufficient to permit interaction between TRIF molecule and the candidate agent; and
- evaluating the capacity of said candidate agent to interfere with the biological role of said functional TRIF molecule.

Other embodiments of the invention are the use of an antagonist of TLR3 and/or of an antagonist of TRIF obtained by the methods as defined above in the preparation of a medicament for preventing and/or treating a host inflammation response induced by a RNA virus.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Comparison of the activation of bronchial epithelial cells induced by dsRNA and influenza A virus.

Monolayers of BEAS-2B cells were stimulated for 24 h with a series of concentrations of poly(I:C) (0.01; 0.1; 1; 10 µg/ml (***1A***)) or with 1 µg/ml poly(I:C) for various times (1, 3, 6 and 24 h; (***1B-1E***)). Supernatant fluids were tested for IL-8 (***1A***, ***1B***), IL-6 (***1C***), RANTES (***1D***) and IFN-α (***1E***) by ELISA. BEAS-2B cells were stimulated for 24h or not (NS) with 1 µg/ml LPS, 1 µg/ml poly(I:C) or 20 ng/ml TNFα and ICAM-1 expression was assayed by FACS analysis (***1F***). Results are expressed as mean fluorescence intensity (MFI). Monolayers of BEAS-2B cells were stimulated for 24 h by increasing concentrations of influenza A virus (0.5; 1; 5 and 10 x 104 PFU/ml (***1G***, ***1I*** and ***1K***)) or with 5 x 104 PFU/ml for various times (6, 12, 24, 48 and 72 h (***1H*** and ***1J***)). Supernatant fluids were tested for IL-8 (***1G***, ***1H***), RANTES (***1I***, ***1J***), and IL-6 (***1K***) by ELISA. ***1L***: BEAS-2B cells were stimulated or not for 24 h with 5 x 10^4 PFU/ml of influenza A virus, of UV-treated Influenza A virus, or of virus after cell pretreatment with 1 µg/ml of amantadine. Supernatant fluids were tested for IL-8 by ELISA. Data are means ± S.D. of triplicate determinations of a representative experiment performed three times. Closed boxes are poly(I:C)- or virus-treated cells and open boxes represent non-treated samples.

Figure 2. Expression and localization of TLR3 in pulmonary epithelial cells.

2A: Representative RT-PCR showing TLR3 expression in human alveolar (A549) and tracheobronchial (BEAS-2B, NT-1 and CFT-2) epithelial cell lines. The human

macrophage cell line U-937, a known source of TLR3, was used as a positive control. ***2B***: Representative FACS analysis of TLR3 expression in two different lung epithelial cell lines. A549 and BEAS-2B were permeabilized ((+)perm., *right panels*) or not ((-) perm., *left panels*) and immunostained with N-15 or H-125 as described in the methods section. The light and dark line histograms represent the fluorescence signal obtained using isotype–control antibody and TLR3-specific antibody, respectively. Fluorescence tracings are representative of three independent experiments.

Figure 3. TLR3 gene regulation by pro-inflammatory stimuli.

BEAS-2B cells were stimulated or not (NS) with 1 µg/ml LPS, 1 µg/ml poly(I:C), 5 x 104 PFU/ml of influenza A virus, 20 ng/ml TNFα, 50 ng/ml IL-1β or 15 nM PMA for 24 h. Total RNA was extracted and TLR3 mRNA was analysed by RT-PCR. ***3A***: A representative result out of three is shown ***3B***: Histogram bars show the value for TLR3 normalized against that for β-actin and are the means ± S.D. of three independent experiments performed in triplicate.

Figure 4. Signal transduction induced by dsRNA and influenza A virus.

4A: Time course of the activation of MAPKs p38, JnK, Erk1/2 and the PI-3K-dependent kinase, Akt. BEAS-2B cells were stimulated or not (NS) with 1 µg/ml poly(I:C) for different time intervals, lysed, and phosphorylation of these enzymes was determined by immunoblotting with specific antibodies (all diluted 1/2000). To confirm equal loading, membranes were reprobed with an anti-ß-actin antibody (diluted 1/15000). Data are representative of three independent experiments. ***4B***, ***4C***: The role of MAPK- and PI-3K/Akt–dependent signaling in the activation of epithelial cells by poly(I:C) and influenza A virus. ***4B***: BEAS-2B cells were pretreated for 1 h with inhibitors of ERK1/2 (PD98059), p38 (SB203580), and PI-3K (LY294002), then stimulated with 1 µg/ml poly(I:C) for 6 h. ***4C***: BEAS-2B cells were pretreated for 1 h with the same inhibitors used at 10 µM, then stimulated with 5 x 10^4 PFU/ml of influenza A virus for 24 h. Supernatant fluids were

tested for IL-8 (*left panels*) and RANTES (*right panels*) by ELISA. Data are means ± S.D. of triplicate determinations of a representative experiment performed three times.

Figure 5. Involvement of TLR3 and TRIF pathway in dsRNA and influenza A virusinduced NF-κB and IRF/ISRE activation in pulmonary epithelial cells.

5A: BEAS-2B cells were transfected with 200 ng of a NF-κB or IRF/ISRE luciferase reporter construct and then stimulated with 1 μg/ml poly(I:C) for 6 h. Cell lysates were prepared and assayed for luciferase activity. Results are expressed as relative luciferase units (RLU) and are means ± S.D. of triplicate determinations of a representative experiment performed 3 times. ***5B***: BEAS-2B cells were stimulated with 1 μg/ml poly(I:C) for 30 and 90 min. Then, cells were immunostained with an anti-NF-κB antibody and examined by confocal microscopy as described in the methods section. ***5C***, ***5D***: BEAS-2B cells were co-transfected with 200 ng of a NF-κB- or IRF/ISRE luciferase reporter construct and with 500 ng of the expression plasmid encoding dominant-negative (DN) forms of MyD88 or TRIF, or with the corresponding control plasmids (pcDNA3 and pCMV). The transfected BEAS-2B cells were then stimulated with 1 μg/ml poly(I:C) for 6 h and cell lysates were prepared and assayed for luciferase activity. ***5E***: BEAS-2B cells were transfected with 200 ng of a NF-κB luciferase reporter plasmid and with 500 ng of the plasmid encoding DN forms of MyD88, TRIF or TLR3 (pZERO-hTLR3), or the respective control plasmids (pcDNA3 and pCMV). The transfected BEAS-2B cells were then stimulated with 5 x 10^4 PFU/ml influenza A virus or 1 μg/ml poly(I:C) for 24 h and cell lysates were prepared and assayed for luciferase activity.

Figure 6. Time-course of dynamic parameters in wild-type mice infected by a lethal influenza virus challenge.

Male C57BL/6 mice were infected intranasally with 300 pfu of influenza A virus and different parameters were analyzed during the course of infection. ***6A***: survival of mice. ***6B***: body weight changes. ***6C***: leukocyte recruitment into the airways (■ all leucocytes,

● polymorphonuclear cells, ▲ mononuclear cells). ***6D*** and ***6E***: RANTES and IL-6 production in BAL fluids. ***6F***: total protein amount in BAL fluids as an index of alveolocapillar permeability. All these results are the means ± S.D. obtained from three distinct animals and are representative of three independent experiments. ***6G***: profile of inflammatory mediators levels at day 3 (□) and day 10 (■) post-viral infection, in BAL fluids. The data were normalized to internal positive controls spotted on the same protein array membrane and are expressed as relative units.

Figure 7. Altered inflammation and lethality in influenza virus-infected TLR3-/- mice

7A: pulmonary expression and regulation of TLR3 during influenza infection. Mice were either non infected (Ctrl) or infected with 300 pfu of influenza virus by intranasal route and whole lungs were harvested at 2, 3, 4 and 9 days post-infection. Total RNA was extracted and TLR3 mRNA was analysed by RT-PCR; a representative result out of two is shown. β-actin mRNA was assessed as a control for RNA loading. ***7B***: lethality induced by influenza virus in *TLR3-/-* and *MyD88-/-* mice in comparison with wild type mice. Age-matched *TLR3-/-* (●, n=35), *MyD88-/-* (◎, n=8) and wild-type (○, n=65) male mice received intranasally 300 pfu of influenza virus. Wilcoxon test for comparisons of Kaplan-Meier survival curves indicated a significant increase in the survival of *TLR3-/-* mice compared to that of wild-type animals (****, $P< 0.0001$) but not to that of *MyD88-/-* mice. ***7C***: basal respiratory function of wild-type (□) *vs* *TLR3-/-* (■) mice before (day 0) and 3 days post-viral infection. This was measured using a barometric plethysmographic chamber and is expressed as Penh (*cf.* methods section for details), the increase of which is an indicator of deterioration changes in airway mechanics (**, $P< 0.01$). ***7D***- ***7F***: BAL fluids levels of total protein, RANTES and IL-6 in wild-type (□) *vs* *TLR3-/-* (■) mice before (day 0) and 3 days post-viral infection (*, $P< 0.05$; ***, $P< 0.001$; ****, $P< 0.0001$). Histograms are the means ± S.D. obtained from five animals and are representative of at least three independent experiments.

Figure 8. Distinct inflammatory profile in wild-type and TLR3-/- mice infected by a lethal influenza virus challenge.

8A: BAL fluids levels of inflammatory mediators at day 9 post-infection. The data were normalized to internal positive controls spotted on the same protein array membrane and are expressed as relative units. *Inset* : example of a protein array blot probed with BAL collected from wild-type and *TLR3-/-* mice. Relevant spots are highlighted: (1) G-CSF, (2) IL-12p40p70, (3) IFN- γ and (4) RANTES. ***8B***: BAL fluids levels of IFN-γ in wild-type (□) and *TLR3-/-* (■) mice before (day 0) and 4, 7, 9 days post-infection by 300 pfu influenza virus (*, $P< 0.05$). Histograms are the means ± S.D. obtained from five animals and are representative of 3 independent experiments. ***8C***: macroscopic examination of lungs isolated from wild-type and *TLR3-/-* mice at day 9 post-viral infection.

Figure 9. Viral load in the lungs of influenza virus-infected mice.

Viral load in *TLR3-/-* (●) and wild-type (○) mice challenged intranasally by 300 pfu of influenza virus. Results are the means ± S.D. obtained from 4 distinct animals and are expressed as RNA copies normalized to β-actin expression levels, as determined by real-time PCR.

Figure 10. Wild-type and TLR3-deficient mice raise a contrasted leukocyte content in their lungs after infection by influenza A virus.

BAL cells were collected from influenza A virus infected mice at day 9 post-infection. To characterize the recovered leukocyte cell types, polymorphonuclear neutrophils (PMN), macrophages (MΦ), CD4+ T-lymphocytes (CD4) and CD8+-T lymphocytes (CD8) were stained with fluorescently labelled specific antibodies. *Far right and far left dot-plots*: representative BAL cell composition of naive wild-type and *TLR3-/-* mice. *Right and left dot-plots*: representative BAL cell composition of wild type mice and *TLR3-/-* mice at day 9 post-infection by 300 pfu of influenza virus. *Central histograms*: results are the means ± S.D. obtained from 12 wild-type mice and 7 TLR3-/- mice (**, $P< 0.01$, ***: $P< 0.001$, ****: $P< 0.0001$).

DETAILED DESCRIPTION OF THE INVENTION

The present invention has yielded the unexpected discovery that TLR3 is constitutively expressed in distinct human alveolar and bronchial epithelial cells and therefore, the inventors have described its intracellular localization. Moreover, the inventors have shown that TLR3 and its signaling-associated molecule TRIF play a key role in the immune response of respiratory epithelial cells to both dsRNA and influenza A virus.

It is therefore a first embodiment of the invention to use a Toll-like-receptor 3 (TLR3) molecule or its signalling-associated molecule TRIF in the design of molecules, such as an antagonist of TLR3 or TRIF, to prevent an excessive host inflammation response induced by a RNA virus. By the term "inflammation response" is intended, for the purpose of this invention, a localized protective response produced by a host and elicited injury or destruction of tissues infected by a RNA virus which serves to destroy, delete or wall off both the RNA virus and the injured tissue, characterized in the acute form by the classical sequence of pain, heat, redness, swelling an loss of function.

As used herein, the term "antagonist" refers to an agent or compound that reduces or decreases the activity of TLR3 or TRIF. An antagonist may be directly active on a TLR3 or TRIF molecule, or it may be active on one or more constituents in a pathway that leads to reduced or decreased activity of a TLR3 or TRIF molecule.

According to a preferred embodiment of the invention, the RNA virus consists of a single-stranded or a double-stranded RNA virus, and more specifically, it consists of an influenza virus. Although influenza virus of type A represents a preferred embodiment of the invention, other influenza virus including, without limitation, type B and C are included in the invention.

The present invention also provides as another embodiment, a method for screening a TLR3 antagonistic expression, comprising the steps of:

a. contacting a functional TLR3 molecule with a candidate agent, under conditions and for a time sufficient to permit interaction between TLR3 molecule and the candidate agent; and

b. evaluating the capacity of said candidate agent to interfere with the biological role of said functional TLR3 molecule.

Another embodiment is to provide a method for screening a TRIF antagonistic expression, comprising the steps of:

a. contacting a functional TRIF molecule with a candidate agent, under conditions and for a time sufficient to permit interaction between TRIF molecule and the candidate agent; and
b. evaluating the capacity of said candidate agent to interfere with the biological role of said functional TRIF molecule.

As used herein, the term "functional TLR3 or TRIF molecule" refers to a molecule of TLR3 or TRIF which possesses biological role or activity that is identified through a defined functional assay and which is associated with a particular biologic, morphologic, or phenotypic alteration in a cell or cell mechanism.

In others embodiments of the invention, such antagonists of TLR3 and/or TRIF preferably obtained by the methods of the invention are advantageously used in methods and in preparation of a medicament for preventing and/or treating a host inflammation response induced by a RNA virus. By the term "treating" is intended, for the purpose of this invention, that the symptoms of the host inflammation response be ameliorated or eliminated. Whereas, the term "preventing" refers to a process by which symptoms of the host inflammation response are obstructed or delayed.

Consequently, the present invention also provides a method for preventing and/or treating host inflammation response induced by a RNA virus, comprising the step of administering to a mammal in need thereof an effective amount of an antagonist of TLR3 and/or an antagonist of TRIF. As used herein, effective amount is the quantity of a TLR3 and/or an antagonist of TRIF necessary to prevent, to cure, ameliorate, or at least partially arrest, a symptom of host inflammation response induced by a RNA virus in an animal or of a disease state associated therewith. As used herein, the term "animal" refers to any animal susceptible to be infected by a RNA virus, such as an Influenza of

type A. Specifically, such an animal may be, but not limited to, pig, poultry, horse and human. More specifically, the animal consists of a human.

Compounds useful as potential TLR3 and/or TRIF antagonists, including chemical or biological molecules such as simple or complex organic molecules, metal-containing compounds, carbohydrates, peptides, proteins, peptidomimetics, glycoproteins, lipoproteins, nucleic acids, antibodies, and the like. Libraries containing large numbers of natural and synthetic compounds also can be obtained and screened from commercial sources. Combinatorial libraries of molecules can be prepared using well known combinatorial chemistry methods (Gordon et al., J. Med. Chem. 37: 1233-1251 (1994); Gordon et al., J. Med. Chem. 37: 1385-1401 (1994); Gordon et al., Acc. Chem. Res. 29:144-154 (1996); Wilson and Czarnik, eds., Combinatorial Chemistry: Synthesis and Application, John Wiley & Sons, New York (1997)).

For use as a TLR3 and/or TRIF antagonist, the compound can be formulated with a pharmaceutically acceptable carrier to produce a pharmaceutical composition or a medicament, which can be administered to the individual, which can be a human or any other animal susceptible to be afflicted by a RNA virus such as influenza. A pharmaceutically acceptable carrier can be, for example, water, sodium phosphate buffer, phosphate buffered saline, normal saline or Ringer's solution or other physiologically buffered saline, or other solvent or vehicle such as a glycol, glycerol, an oil such as olive oil or an injectable organic ester. A pharmaceutically acceptable carrier can also contain physiologically acceptable compounds that act, for example, to stabilize or increase the absorption of the TLR3 and/or TRIF antagonists. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier depends, for example, on the route of administration of the medicament and age of the individual.

The present invention will be more readily understood by referring to the following examples. These examples are illustrative of the wide range of applicability of the present invention and are not intended to limit its scope. Modifications and variations can be made therein without departing from the spirit and scope of the invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred methods and materials are described.

EXAMPLES

EXAMPLE 1: Involvement of Toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and Influenza virus

Influenza A is a highly contagious single-stranded RNA virus, which infects both the upper and lower respiratory tracts of humans. The host innate immune Toll-like receptor (TLR)3 was previously shown in cells of myeloid origin to recognize the viral replicative intermediate double-stranded RNA (dsRNA). Thus, dsRNA may be critical for the outcome of the infection. Here, the inventors first compared pulmonary epithelial cells activation triggered by either influenza A virus or dsRNA. They established that TLR3 is constitutively expressed in human alveolar and bronchial epithelial cells and they describe its intracellular localization. Expression of TLR3 was positively regulated by influenza A virus and by dsRNA but not by other inflammatory mediators including bacterial lipopolysaccharide, the cytokines tumor necrosis factor-á and IL-1β, and the protein-kinase C activator PMA. The inventors also demonstrate that TLR3 contributes directly to the immune response of respiratory epithelial cells to influenza A virus and dsRNA and propose a molecular mechanism by which these stimuli induce epithelial cell activation. This model involves mitogen-activated protein kinases, PI-3K/Akt signaling and the TLR3-associated adapter molecule TRIF, but not MyD88-dependent activation of the transcription factors NF-κB or interferon regulatory factor/interferon-sensitive response element pathways. Ultimately, this signal transduction elicits an epithelial response that includes the secretion of the cytokines IL-8, IL-6, RANTES and interferon-β and the up-regulation of the major adhesion molecule ICAM-1.

Materials and Methods

Reagents and antibodies. RPMI 1640, F-12K nutrient mixture (Kaighn's modification), antibiotics, glutamine, Hank's Balanced Salt Solution and Trysin-EDTA were from GIBCO Life Technology, Ltd (Paisley, UK). Fetal calf serum (FCS) was from Hyclone (Logan, UT). Leupeptin, aprotinin, SBTI, PMSF, benzamidine, paraformaldehyde (PFA), polyinosinic-polycytidylic (poly(I:C)) acid, forskolin and phorbol 12-myristate 13-acetate (PMA) were from Sigma Chemical Corp (Saint Louis, MO). The p38- (SB203580) and ERK1/2 (PD98059) inhibitors were obtained from Calbiochem (La Jolla, CA) and Cell Signaling technology (Beverly, MA), respectively. The PI-3K inhibitor, LY294002, was obtained from Cell Signaling technology. Horseradish peroxidase-conjugated secondary antibody was from Pierce (Rockford, IL). Anti-TLR3 antibodies used included the goat polyclonal antibody N-15, directed against a N-terminus region of human TLR3 (Santa Cruz Biotechnology, Santa Cruz, CA) and the rabbit polyclonal antibody H-125 specifically raised against a peptide close to the transmembrane domain (aminoacids 653- 777) of TLR3 (Santa Cruz Biotechnology). The anti-human ß-actin antibody was from Sigma, and the anti-NF-κB-p65, anti-phospho-Erk1/2 and anti-phospho-Jnk antibodies were from Santa Cruz. The anti-phospho-p38 was purchased from Cell Signaling.Fluorescein isothiocyanate (FITC)-labeled anti-goat and anti-rabbit antibodies were obtained from Dako and Rockland (Gilbertsville, PA). Anti-human-ICAM-1 antibody was from R&D systems (Minneapolis, MN).

Cell and culture conditions. The human promonocytic cell line U-937, the human alveolar epithelial cell line A549 and the human bronchial epithelial cell line BEAS-2B were obtained from the American Type Cell Collection (Rockville, IL). The human tracheal epithelial cell lines CFT-2 and NT-1 were a kind gift from Dr. A. Paul (INSERM U402, Paris, France). CFT-2 was derived from primary tracheal epithelia homozygous for the common cystic fibrosis mutation .F508, and NT-1 was derived from normal primary tracheal epithelial cells (18). Cells were cultured as described previously (19).

Virus preparation and inactivation. Influenza A/Scotland/20/74(H3N2) virus was grown on Madin-Darby canin kidney (MDCK) cells in the presence of 2 µg/ml TPCK-treated trypsin. The supernatant was harvested on day 3 and clarified by centrifugation at 680 x *g* for 15 min. Viral stocks were stored in aliquots at -80°C. Virus titers were determined by a standard plaque assay using MDCK cells. A noninfected cell culture was used for preparation of the control inoculum. UV light-inactivated virus was prepared by exposing stock virus solution (0.5 ml/6-cm petri dish) to a 15 W UV light at a distance of 20 cm for 15 min.

RT-PCR. Total RNA was extracted using an RNeasy kit (Qiagen, Courtaboeuf, France). RT was performed using 0.5 µg of total RNA extracted as previously described (19). PCR was performed using specific primers (Proligo, Evry, France) for human TLR3 (sense: 5' AAA TTG GGC AAG AAC TCA CAG G 3'; antisense: 5' GTG TTT CCA GAG CCG TGC TAA 3'). As an internal control, we used primers for human ß-actin (sense 5' AAG GAG AAG CTG TGC TAC GTC GC 3'; antisense 5' AGA CAG CAC TGT GTT GGC GTA CA 3'). Amplifications were performed in a Peltier thermal cycler apparatus (MJ Research, Watertown, MA) using the Qbiotaq polymerase (Qbiogene, Illkirch, France). To detect TLR3, the thermocycling protocol was: 95°C for 3 min, 36 cycles of denaturation at 95°C for 45 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min. To detect ßactin, only 30 cycles were used and the annealing temperature was 62°C. Amplification products were resolved on 1.5% agarose gel containing ethidium bromide. Band intensities on gels were recorded after amplification with an Ultra-Lum system (Ultra-Lum, Claremont, CA). Samples for each point were serially diluted to verify that PCR was performed in the linear phase of the amplification reaction.

Immunoblotting. Epithelial cell extracts were prepared and solubilized as previously described (19). Aliquots (15 µg of protein) were run on 10% acrylamide SDS-PAGE and the proteins were then electrotransferred to a nitrocellulose membrane (Optitran BA-S 85, Schleicher and Schuell, Dassel, Germany) and probed with specific antibodies, as specified in the figure legends. Bound antibodies were detected using ECL+ immunoblotting detection system (Amersham), according to the manufacturer's

instructions. Molecular masses were estimated from calibration standards included in each gel. *Flow cytometry analysis.* Epithelial and monocytic cells were dispensed (1 x 10^6 cells/ml) into conical-bottomed 96-well plastic plates (Nunc A/S, Roskilde, Denmark) and were centrifuged at 100 *g* at 4°C for 10 min. Cells were washed with HBSS/0.5% BSA supplemented with 1 mM Ca2+ and Mg2+ and a saturating concentration of anti-TLR3 antibodies (5 µg/ml), anti-ICAM-1 antibody (1 µg/ml), or non-immune IgG as controls, was then added and the samples incubated for 30 min at 4°C. Cells were washed and incubated for 30 min at 4°C with the corresponding secondary FITC-conjugated antibody (5 µg/ml). For intracellular staining, cells were fixed and permeabilized by incubation for 90 min on ice with a solution of PBS/3.2% PFA /0.2% Tween-20; they were then incubated with an anti-TLR3 antibody (5 µg/ml) and FITC-conjugated secondary antibodies (5 µg/ml). FACScan flow cytometer (Becton Dickinson Immunocytometry System, Mountain View, CA) was used for cytometric analysis.

NF- κB immunostaining. BEAS-2B epithelial cells were cultured on 22-mm glass cell culture coverslips (CML France, Nemours, France). Cells were washed three times with PBS then fixed for 15 min in PBS/3.2% PFA. After washing under gentle shaking, cells were permeabilized for 5 min with 0.1% triton X-100 and washed, as before, prior to incubation with an anti-NF-κB-p65 antibody (0.4 µg/ml) and a specific secondary antibody (5 µg/ml). In control experiments, cells were incubated with non-immune IgG as control isotypes. Finally, the cells were washed extensively with PBS and the coverslips were mounted in fluorescence mounting medium. Fluorescence microscopy was performed with a 63x/1.4 oil objective lens on a confocal microscope (model LSM 510; Carl Zeiss France, Le Pecq, France), using laser excitation at 488 nm.

Epithelial cell transfection and reporter gene studies. BEAS-2B cells were seeded at 5 x 104 on 24-well plates (Costar, New York, NY) 96 h before transfection using FuGENE 6 tranfection reagent (Roche Molecular Diagnostics, Meylan, France), according to the manufacturer's instructions. Each sample contained 200 ng of a NF-κB-luciferase- (kindly provided by Dr. A. Israel, Pasteur Institute, Paris, France), an ISRE-luciferase- or a CREluciferase-reporter plasmid (BD Biosciences Clontech, CA) and 500

ng of vector expressing a dominant-negative form of either MyD88 (Myd88-DN; a kind gift from Dr. M. Muzio, Mario Negri Institute, Milan, Italy) or TRIF (TRIF-DN). The TLR3 construct from which the TIR domain is deleted (pZERO-hTLR3) and encoding a non-functional TLR3 molecule was purchased from InvivoGen (San Diego, CA). The empty plasmids, pcDNA3 (Invitrogen, Carlsbad, CA) and pCMV (BD Biosciences Clontech), were used as controls as appropriate. After 24 hours, cells were left untreated or stimulated for 6 or 24 h at 37°C with influenza A virus or 1 µg/ml poly(I:C). Luciferase activity was measured in the cell lysates as previously described (19), using an EGNG Berthold luminometer. Results are expressed as relative luciferase units.

Cytokine measurements. Human IL-8, IL-6, RANTES and IFN-β concentrations in cell culture supernatants were determined using DuoSet ELISA kits obtained from R&D systems (Minneapolis, MN).

Statistical Analysis. Each point corresponds to the mean ± S.D. of the indicated number of experiments. The statistical significance of differences between groups was tested using the unpaired Student's *t* test with a threshold of $p < 0.05$.

Results

Comparison of the activation of bronchial respiratory cells by dsRNA and influenza A virus.

DsRNA is known to accumulate within infected cells and it has the required physical and biological properties to induce antiviral responses and pathological inflammatory processes (4,5). Thus, it appears important to examine whether influenza A virus–induced activation of pulmonary epithelial cells shares any characteristics with that stimulated by a synthetic dsRNA such as poly(I:C). Figure 1 reports a comparison of the two inducers' effects in the human bronchial epithelial cell line BEAS-2B. Poly(I:C) triggered a strong secretion of IL-8, in a concentration- (Figure 1A) and time-dependent manner (Figures 1B). The secretion started within 3 h and IL-8 accumulated in the

culture medium up to 24 h (Figure 1B). The inventors also observed a time-dependent accumulation of IL-6 (Figure 1C). RANTES was also induced by poly(I:C) but approximately 3 h after the start of production of the other cytokines (Figure 1D). This suggests an autocrine/paracrine activation by another mediator; for example IFN-β might feed-back on RANTES production. In that regard, IFN-β production peaked 6 h post-stimulation but was not detected at 24 h, suggesting a reprocessing of this cytokine by BEAS-2B cells (Figure 1E). To exclude any stimulatory effect associated with contamination of poly(I:C) by bacterial endotoxin, experiments were also performed using poly(I:C) supplemented with 20 µg/ml polymyxin B, a well-characterized LPS inhibitor. Under these experimental conditions, IL-8 secretion by BEAS-2B cells was not modified (not shown).

When BEAS-2B cells were infected with influenza A virus, IL-8 secretion was clearly detected 24 h later, and was dose dependent (Figure 1G and H). RANTES and IL-6 secretion were also dose dependent up to a maximum at 5×10^4 PFU (multiplicity of infection (MOI) 0.25) (Figures 1I and 1K). Cytokine secretion was delayed by 20 h with respect to that by poly(I:C)-stimulated BEAS-2B cells. This delay may be consistent with the time required to 12 generate dsRNA within the infected cell, during the replication of the virus (Figure 1H, 1J).

Epithelial cells in the lung express ICAM-1, which is involved in the recruitment and the local accumulation of inflammatory cells through the binding to leukocyte-associated antigen (LFA)-1 (20). Previous studies have shown that pro-inflammatory mediators including LPS and TNF-α induce ICAM-1 expression on pulmonary epithelial cells (21). These findings were confirmed by flow cytometric analysis and extended them to dsRNA and influenza A virus by demonstrating a potent up-regulation of ICAM-1 expression, which was as strong as that obtained with TNF-α (Figure 1F).

Previous studies have shown that production of inflammatory mediators in response to a viral infection can occur in the presence or absence of multiplication of the pathogen (22). The inventors tested whether viral contact with the plasma membrane and/or viral penetration of the host cell were sufficient to trigger the inflammatory response. Bronchial epithelial cells were infected with UV-treated, and therefore non-

replicative, virus. Also, BEAS-2B cells were treated with an intact virus in the presence of 1 µg/ml amantadine, an influenza-specific inhibitor blocking the early release of the viral genome into the cytoplasm but not the endocytosis of the viral particle into the cell (1). Neither UV-treated virus nor virus in presence of amantadine induced IL-8 release (Figure 1L). Thus, components generated during viral replication are required for the inflammatory response. Similarly, there was no RANTES or IL-6 secretion in the absence of viral replication (data not shown). Hence, the inflammatory response to influenza A virus infection is replication-dependent and is not mediated solely by the initial virus-host cell interaction or by any artifact possibly present in the infectious inoculum.

Expression and localization of TLR3 in pulmonary epithelial cells.

Since the foregoing results suggested that dsRNA is required for the immunostimulatory activity of influenza A virus, the inventors examined in pulmonary epithelial cells the expression of TLR3, a recently described dsRNA sensor (9). RT-PCR was first used to test for the presence of TLR3 mRNA in unstimulated human respiratory epithelial cells. As shown in Figure 2A, TLR3 mRNA was detected in PMA-differentiated U-937 cells and in both human alveolar (A549) and tracheo-bronchial (BEAS-2B, NT-1 and CFT-2) epithelial cells lines. RT-PCR analysis of β-actin mRNA confirmed the quality of all RNA preparations. Then two antibodies, N-15 and H-125 were used to test for TLR3 protein in human pulmonary epithelial cells. The specificity of these antibodies was confirmed by westernblotting with a recombinant TLR3 glycosylated peptide, consisting of amino-acids 21 to 711. Protein expression level of TLR3 was analyzed by flow cytometry in A549 and BEAS-2B cells (Figure 2B). No TLR3 signal was detected at the cell surface of these respiratory cells (*left panels*) but abundant intracellular TLR3 was revealed using a mildfixation and permeabilization protocol (*middle and right panels*).

Epithelial TLR3 expression is up-regulated by poly(I:C) and influenza A virus.

Next, the inventors examined whether different stimuli regulate the epithelial TLR3 mRNA. BEAS-2B cells were exposed to an optimal concentration of poly(I:C)

(1µg/ml), LPS (1µg/ml), TNFα (50 ng/ml), IL-1β (20 ng/ml) or the potent protein kinase C activator PMA (15 nM) as well as to influenza A virus (5 x 10^4 PFU) for 24 h. Under these conditions, the different cell activators were fairly equipotent, as assessed by the measurement of IL-8 secretion. Expression of TLR3 mRNA was normalized to that of β-actin and is reported as histogram bars in Figure 3B. This semi-quantitative densitometric measurement clearly shows that only influenza A virus infection and cell stimulation by the viral RNA mimetic component specifically upregulated TLR3 mRNA; the other treatments had no significant effect (Figure 3A and 3B).

IL-8 but not RANTES secretion triggered by dsRNA or influenza A virus shares a common signaling pathway.

Virus infection of susceptible cells activates multiple signaling pathways, including dynamic protein phosphorylations, that orchestrate the induction of genes contributing to the innate immune response. The kinases involved include p38, extracellular signal-regulated kinase (ERK) 1/2 and Jun aminoterminal kinase (JNK). Little is known concerning the involvement of PI-3K in virus induced- and/or TLR signaling. PI-3K catalyses the production of PI(3,4,5)P3 which allows the recruitment of signaling proteins, including the serinethreonine kinase Akt (23). Therefore, we examined whether dsRNA activates p38, JNK, ERK1/2 and Akt in bronchial epithelial cells. Treatment with poly(I:C) strongly up-regulated phosphorylation of these signaling components. The maximum level of phosphorylation was between 15 to 60 min and declined thereafter, except for p38 phosphorylation which increased until 360 min post-stimulation (Figure 4A). To specifically delineate the role of these kinases in the production of epithelial cytokines, BEAS-2B cells were pretreated with specific inhibitors: PD98059 for ERK1/2, SB203580 for p38 and LY294002 for PI-3K (Figure 4B). None of these inhibitors caused cytotoxic effects on BEAS-2B cells at the concentrations used in these experiments. However, all inhibitors significantly reduced the dsRNA-induced production of IL-8 (Figure 4B) and IL-6 (not illustrated). By contrast, only the PI-3K/Akt pathway inhibitor reduced RANTES production.

When pulmonary epithelial cells were infected by influenza A virus in the presence of 10 µM of the above inhibitors, IL-8 secretion was similar to that induced by

synthetic dsRNA under the same experimental conditions: approximately ~45% inhibition in the presence of PD98059, and a secretion almost abolished in the presence of SB203580 or LY294002. By contrast, RANTES release triggered by either stimulus does not exhibit a similar inhibitory pattern. Remarkably, while the p38 inhibitor did not affect dsRNA-induced RANTES secretion, it strongly inhibited RANTES secretion following influenza A virus infection (Figure 4C).

The TLR3/TRIF pathway is essential for dsRNA and influenza A virus-induced NF-kB and IRF/ISRE activation in pulmonary epithelial cells.

Activation of transcription factors is pivotal to many signal transduction pathways. For instance, NF-κB can be activated in response to many different stress conditions including infection, inflammation, and tissue repair. IRFs consist of a growing family of related transcription proteins initially identified as regulators of the IFN-α/β gene promoters, and the ISRE of various IFN-stimulated genes. Activators of the cyclic AMP response element (CRE) contribute to diverse physiological processes, including the control of cellular metabolism and cell survival. The inventors assessed the involvement of these regulatory signaling elements in the innate immune response induced by dsRNA and influenza A virus. In that purpose, BEAS-2B cells were transfected with a set of vectors each of which contains a different *cis*-acting enhancer element (NF-κB, ISRE or CRE) upstream from a luciferase reporter gene. As shown in Figure 5A, NF-κB and IRF/ISRE were strongly activated upon dsRNA challenge in bronchial epithelial cells. By contrast, CRE was not activated under the same experimental conditions, even though forskolin (10 μM), a potent activator of the cAMP signaling pathway, confirmed the CRE vector was functional (data not shown). NF-κB activation was also confirmed by immunofluorescence staining as its translocation from the cytoplasm to the nucleus was clearly visible within 90 min of epithelial cell activation by poly(I:C) (Figure 5B).

The inventors further investigated whether MyD88 and/or TRIF were involved in the NF-κB and IRF/ISRE signaling pathways activated by dsRNA and influenza A virus, using luciferase reporter plasmids and either dominant negative (DN)- or control vectors. dsRNA-mediated activation of both NF-κB and IRF/ISRE in BEAS-2B cells transfected with 500 ng of the expression vector encoding DN-TRIF were approximately ~70 % and

approximately ~50 % lower, respectively, than in control plasmid-transfected cells (Figures 5C and 5D). Influenza A virus also failed to activate NF-κB in cells transfected with the vector encoding DN-TRIF (Figure 5E, *left panel*). By contrast, transfection of BEAS-2B cells with a plasmid encoding DN-MyD88 did not alter dsRNA- or influenza A virus-mediated NF-κB and/or IRF/ISRE activation (Figures 5C-E, *left panel*). As a positive control for DN-MyD88 efficiency, the inventors verified that IL-1β-induced NF-κB activation, known to require MyD88, was inhibited by prior transfection with 500 ng of DN-MyD88 vector. Conversely, IL-1-β-induced NF-κB activation was not affected by the DN-TRIF plasmid.

Having demonstrated that TRIF-dependent signaling is involved in dsRNA- and influenza A virus-induced respiratory epithelial cell stimulation, we next verified the upstream role of TLR3 *per se* in sensing and cell activation by these viral entities. BEAS-2B cells were transfected with a plasmid (pZERO-hTLR3) encoding TLR3 from which the TIR domain had been deleted, to compete with the endogenous functional TLR3. Figure 5E (*right panel*) shows that pZERO-hTLR3 abolished the cell responses to both dsRNA and influenza A virus. Overall, these data demonstrate a major role for the interaction between TLR3 and influenza A virus-derived dsRNA in the innate immune response of infected pulmonary epithelial cells.

Discussion

Influenza A virus causes pulmonary inflammation and exacerbates chronic lung diseases, due to an infiltration of inflammatory cells and an increased airway hyperresponsiveness. Bronchial epithelial cells play an important role in the pathogenesis of this viral infection (24). However, while many of the molecular events in influenza A virus replication have been described, the underlying mechanisms by which virus-epithelium interaction triggers the inflammation process have yet to be fully characterized. The discovery of TLR3 as a key receptor for dsRNA led the inventors to investigate the contribution of this receptor to the activation of pulmonary epithelial cells by dsRNA and influenza A virus. The inventors show: (*i*) that TLR3 is constitutively expressed in respiratory epithelial cells in an intracellular compartment; (*ii*) that TLR3

expression is upregulated either by influenza A virus or by dsRNA, but not by other major inflammatory mediators; (*iii*) that TLR3 plays a central role in the immune response of bronchial epithelial cells triggered by these stimuli; and *(iv)* that influenza A virus and dsRNA induce epithelial cell activation through MAPK, PI-3K/Akt signaling and TRIF- but not MyD88-dependent activation of the transcription components NF-κB and IRF/ISRE.

It is unclear how much true dsRNA, *i.e.* full duplexes between positive and negative sense RNA, is present in infected cells upon replication of influenza A and other singlestranded RNA viruses. Nevertheless, RT-PCR experiments and binding of anti-helical dsRNA antibodies to viroplasm from whole cell extracts suggest that true dsRNA accumulates within virus-infected cells (5). Also, previous studies reported that as little as one molecule *per* cell can have profound effects on cellular physiology (5). Thus, dsRNA is likely the most immunostimulatory entity of influenza A virus, eliciting epithelial antiviral and inflammatory responses, as suggested by the results of the experiments using amantadine- and UV-treated virus. Moreover, the inventors found that dsRNA can substitute for the virus in terms of secretion of the cytokines IL-8, IL-6, RANTES and INF-β that may promote leukocyte infiltration. Of note is that the kinetic of cytokine release differs between the two stimuli, and this interval may be consistent with the time required for the virus replication process to generate dsRNA (25).

The present findings demonstrate that human lung epithelial cells express TLR3 only in an intracellular compartment. The same is true for immature human dendritic cells subsets (26). The life-cycle of a virus includes entry into the target cell by endocytosis and transport of the viral genome either to the cytoplasm or to the nucleus where it is transcribed and amplified (27). Thus, the intracellular localization of TLR3 is rather consistent with its sensing role of viral replicative elements. Nonetheless, part of our epithelial cell activation experiments were performed using exogenously added poly(I:C), which is reminiscent of viral dsRNA leaking from dying cells, that may act directly on neighboring cells (28). Consequently, for exogenous dsRNA to encounter TLR3, it must penetrate the cell. In that regard, internalization was found to be essential for poly(I:C) to induce interferon activity or cell toxicity in murine LM cells (29). Hence, exogenous dsRNA is presumably internalized in pulmonary epithelium after cell surface

recognition that may involve proper binding structures, including scavenger receptors as it has been demonstrated in macrophages (4).

Previous studies of the regulation of TLR expression report diverse findings depending on the stimulus and the cell type or tissue considered (30). In addition, Heinz *et al.* reported a species-specific regulation of the TLR3 gene (31). We therefore examined whether various inflammatory mediators regulate the expression of TLR3 in human respiratory epithelial cells. Of the mediators we tested, only influenza A virus and dsRNA up-regulated TLR3 expression, suggesting that the signaling pathways controlling the induction of this gene are restricted. An attenuated strain of the measles virus also stimulates the expression of TLR3 through a type I IFN-, and an ISRE-dependent regulatory mechanism (32).

The present findings also demonstrate that to some extent, dsRNA and influenza A virus use different signaling mechanisms to induce inflammatory epithelial responses: dsRNA triggers IL-8 secretion *via* at least the signal-transducing molecules ERK, p38 and PI-3K/Akt whereas RANTES release appears to be mainly dependent on a PI-3K/Akt activation. These results are in agreement with recent work showing p38 dependent-production of IL-8 but not of RANTES, in lung epithelial cells following dsRNA treatment (15). By contrast, the inventors found that in influenza A-infected epithelial cells, RANTES production required both PI-3K/Akt and p38 MAP-kinase activation. These results implicate PI-3K/Akt and p38 signaling pathways rather than ERK ½ signaling pathways in the epithelial immune response to dsRNA and influenza A virus infection. The effect of the p38 inhibitor on the virus- but not the synthetic dsRNA-induced RANTES secretion suggests, unsurprisingly, that the molecular interactions governing influenza A infection are more complex than those induced by dsRNA alone. Presumably, in addition to dsRNA, other viral entities such as virion-associated proteins, may contribute to virus-induced signal transduction (33). Generally, IL-8 gene regulation is considered to be highly dependent on NF-κB activation whereas RANTES expression required the combination of both NF-κB and IRF (34,35). Thus, our study can be used to suggest a model of how dsRNA activates human respiratory epithelial cells. After either a receptor-mediated transfer of exogenous dsRNA across the plasma membrane, or a viral replication-dependent accumulation within the cell, dsRNA encounters intracellular

TLR3, triggering a downstream signaling. This process involves at least the TLR3 adaptor TRIF but not MyD88. The kinases p38, JNK, ERK1/2 MAPK and PI3-K/Akt are important mediators of this cell activation process, activating the transcription components NF-κB and/or IRF/ISRE to regulate selectively the expression of various inflammatory mediators, including IL-8 and RANTES.

Interestingly, a recent work claimed that TLR3 signaling pathways do not appear to influence significantly the generation of effective host responses in murine models of infection to four different viruses (lymphocytic choriomeningitis virus, vesicular stomatitis virus, murine cytomegalovirus and reovirus (36)). However, the present study clearly indicates that human pulmonary mucosa does interact with viral products and elicits inflammatory and/or anti-viral immune responses through TLR3 signalling. These contradictory results may be explained either by the virus itself and/or the species of the TLR3 considered. In that regard, it is of note that different TLR3 expression patterns have been reported in mice and humans, a phenomenon also observed for other TLR family members. Differences between species include the presence of TLR transcripts in different cell types and dissimilar regulation of transcription following cellular activation (30). Therefore, it cannot be excluded that differences in cell types and the patterns of basal or induced expression of TLR3 influence the immune responses in the two species. Besides, TLR7 and TLR8 were recently characterized as new sensors for viral nucleic acids with single-stranded RNA (ssRNA) being their ligands and innate antiviral responses following influenza A virus infection of mouse dendritic cells have been demonstrated to be TLR7-dependent (37-39). In fact, genetic complementation experiments with human TLR7 and TLR8 suggest that TLR8, not TLR7, is responsible for the recognition of ssRNA in humans (37). Moreover, TLR7 and TLR8 are not expressed in human bronchial epithelial cells (40) and the inventors' experiments using a vector encoding a dominant negative form of MyD88 rule out a role for those receptors in the activation of influenza A-infected human epithelial cells. Indeed, it is established that MyD88 is essential for the signalling downstream from TLR7 and TLR8 (37-39, 41).

Influenza A virus infections impose a considerable socio-economic burden upon society, despite annual vaccination campaigns. Therefore, the present example on the detailed function of TLR3 in human respiratory cells helps to elucidate the pathogenesis

of influenza A infection and thereby contribute to the design of new molecules to prevent the excessive host inflammatory response produced by this virus.

Non-standard abbreviations used: DN, dominant-negative; dsRNA, double-stranded RNA; ERK, extracellular signal-regulated kinase; IRF, interferon regulatory factor; ISRE, interferon-sensitive response element; MyD, myeloid differenciation; NS, nonstimulated; TLR, Toll-like receptor; poly(I:C), polyinosinic-polycytidylic acid; PKR, RNA-dependent protein kinase R; TIR, Toll-IL-1 receptor; TRIF, TIR-domain containing adaptor inducing interferon-β.

EXAMPLE 2: THE TOLL-LIKE RECEPTOR (TLR)-3 CONTRIBUTES TO INFLUENZA A VIRUS-INDUCED ACUTE PNEUMONIA.

Influenza A virus is the etiological agent of highly contagious acute respiratory disease that causes epidemics and considerable mortality annually. In Example I, the inventors demonstrated using an *in vitro* approach, that the pattern recognition Toll-like receptor (TLR) 3 plays a key role in the immune response of lung epithelial cells to influenza virus. This example was designed to evaluate the *in vivo* role of TLR3 during the course of influenza infection. The time-course of several dynamic parameters was compared, including animal survival, respiratory suffering, viral clearance, leukocyte recruitment into the airspaces and secretion of critical inflammatory mediators, in wild-type and *TLR3-/-* mice infected by a lethal viral challenge. The inventors first found that the pulmonary expression of TLR3 is constitutive and markedly up-regulated following influenza infection in control mice. Notably, inflammatory mediators, including RANTES, IL-6 and IL-12p40p70 and the number of CD8+ T lymphocytes were significantly reduced in the bronchoalveolar spaces of infected *TLR3-/-* animals, in comparison with wild-type mice. Furthermore, in spite of persistent viral production in the lungs, the lower inflammatory response in infected TLR3-deficient animals was concomitant to an improved survival. The present findings demonstrate that TLR3 contributes to the debilitating effects of a detrimental host inflammatory response triggered by influenza virus infection.

Materials and Methods

Virus preparation and quantification. Influenza A/Scotland/20/74 (H3N2) virus was generously provided by N. Escriou and S. Van Der Werf (Unité de Génétique Moléculaire des Virus Respiratoires, Institut Pasteur, Paris, France). The virus was prepared as previously described (53). Viral RNA was isolated from the viral stock with a RNeasy mini kit (QIAGEN, Hilden, Germany), and quantified with a Nanodrop ND-1000 spectrophotometer (Rockland, DE). cDNA derived from the viral RNA sample was used

as a standard for a quantitative reverse transcription-PCR (qRT-PCR). qRT-PCR was then performed using specific primers (sense : 5' AAG ACC AAT CCT GTC ACC TCT GA 3' and antisense : 5' CAA AGC GTC TAC GCT GCA GTC C 3'; Proligo, Evry, France) that complement 20 temporally and spatially divergent influenza A matrix protein gene sequences, as previously described (54).

Mouse strains. Males C57BL/6 mice were purchased from the Centre d'Elevage R. Janvier (Le Genest Saint-Isle, France) and were used at about 8 weeks of age. MyD88- and TLR3-deficient mice were generated as described earlier (9, 55). MyD88-/- mice were obtained from Dr. S. Akira (Osaka University, Japan). Each type of mice was backcrossed at least eight times with C57BL/6 to ensure similar genetic backgrounds. Mice strains were bred in an animal facility in pathogen free conditions. Mice were fed with normal mouse chow and water *ad libitum* and were reared and housed under standard conditions with air filtration. For experiments of infection by influenza A virus, mice were housed in cages inside stainless steel isolation cabinets that were ventilated under negative pressure with HEPA-filtered air. Mice were treated in accordance with Pasteur Institute guidelines in compliance with the European animal welfare regulation.

Animal fluids collection. Mice were anaesthetized by a mixture of ketamine-xylazine (1 and 0.2 mg *per* mouse, respectively) and infected intranasally with 50 µl of PBS containing 300 pfu influenza A virus. Mice were observed daily for signs of morbidity. Alternatively, mice were euthanized at different time points by intraperitoneal injection of 300 $mg.kg^{-1}$ sodium pentobarbital and 1 ml of heparinized blood was collected by vena cava. After centrifugation at 300 *g*, the resulting plasma was stored. Also, airways were washed twice with 1ml saline, and the bronchoalveolar lavage (BAL) was collected to further determine cell differential counts and percentages using a Coulter counter (Coulter-Electronics, Margency, France) as well as a Diff-Quik staining (Baxter-Dale, Dudingen, Germany) of cytospin slides. Aliquots of BAL fluids were stored for total protein and cytokine measurement. For flow cytometry analysis, cells were eventually counted and stabilized with Cyto-Chex (Sireck, Omaha, NE). Under these conditions, cells could be stored 1 week at 4°C before labelling for flow cytometry analysis.

Monoclonal antibodies (mAb) for flow cytometry analysis. MAb reactive to CD11b (Mac-1, M1/70, rat IgG2a), Ly-6G-Gr1 (clone RB6-8C5, rat IgG2b), CD8 (clone 53-6.7, rat IgG2a), CD4 (clone RM4-5, rat IgG2a), were purchased from BD Pharmingen (San Diego, CA) as conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or cy-Chrome. PE-conjugated F4/80 (clone C1 :A3-4, rat IgG-2b) was purchased from Caltag laboratories, Burlingame, CA). Before flow cytometry analysis, cells were washed in PBS containing 5% FCS and stained for 30 minutes at 4°C with the conjugated Abs. Cells were further washed twice and analyzed on a FACScan flow cytometer (BD).

RANTES, IL-6 and IFN-γ ELISA. Murine RANTES, IL-6 and IFN-γ concentrations in BAL were determined using DuoSet ELISA kits obtained from R&D Systems (Minneapolis, MN).

Inflammatory protein array. A commercial antibody-based protein array designed to detect 32 inflammatory mediators was used according to the manufacturer's instructions (RayBio® Mouse Cytokine Array II, RayBiotech, Atlanta, GA). Membrane arrays were hybridised with BAL fluids comparing different types of mice and different time points and were always processed simultaneously. Array images were recorded after amplification with an Ultra-Lum system (Ultra-Lum, Claremont, CA) and all scanned images accurately reproduced spots seen on films.

RT-PCR. RNA was extracted from the lung tissue using the FastRNA® GREEN Kit and FastPrep® instrument (Bio 101, Qbiogene, Inc., CA) according to the protocol from the manufacturer. RNA was reverse transcribed using 0.5 µg of total RNA. PCR was performed using specific primers (Proligo, Evry, France) for mouse TLR3 (sense: 5' GCT CAT TCT CCC TTG CTC AC 3'; antisense: 5' CCC GAA AAC ATC CTT CTC AA 3'). As an internal control, we used primers for mouse β-actin (sense GGA CTC CTA TGT GGG TGA CGA GG 3'; antisense 5' GGG AGA GCA TAG CCC TCG TAG AT 3'). Amplifications were performed in a Peltier thermal cycler apparatus (MJ Research, Watertown, MA) using the Qbiotaq polymerase (Qbiogene, Illkirch, France). To detect

mTLR3, the thermocycling protocol was: 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min. To detect β-actin, only 30 cycles were used and the annealing temperature was 62°C. Amplification products were resolved on 1.5% agarose gel containing ethidium bromide. Band intensities on gels were recorded after amplification with an Ultra-Lum system. Samples for each point were serially diluted to verify that PCR was performed in the linear phase of the amplification reaction.

Assessment of the basal respiratory function. Unrestrained conscious mice, infected or not by influenza A virus, were placed in a whole body plethysmographic chamber (Buxco Electronics, Sharon, CT) to analyze the respiratory waveforms. The system measures both the magnitude and the slope of the chamber pressure. After a 10 min stabilization period, the basal respiratory capacity of each individual mouse was estimated by recording the enhanced pause pressure expressed as "Penh", calculated as: Penh = [*Te* (expiratory time)/*Tr* (relaxation time)] - 1 x [*Pef* (peak expiratory flow)/*Pif* (peak inspiratory flow)]. The values of Penh expressed per minute were averaged from three determinations recorded every 20 s. A Penh increase is an indicator of deterioration in airway mechanics.

Statistical analysis. Statistical significance between the individual groups was analyzed using the unpaired Student's *t* test with a threshold of $p < 0.05$. Survival of mice was compared using Kaplan-Meier analysis and log-rank test.

Results

Kinetics of pathologic features associated to influenza virus infection

The pathogenesis and immune response associated to the influenza pneumonia is assumed to be very complex (56-58). To eventually evaluate the role of TLR3 in influenza A virus sensing, we used a murine experimental model to first characterize in infected wild-type animals the time-course of distinct dynamic parameters, including animal mortality and weight, leukocyte recruitment into the airspaces, increase of

alveolocapillar permeability, and secretion of critical mediators. Fig. 6A shows that all C57Bl/6J mice inoculated intranasally with influenza A virus at a dose of 300 pfu per mouse succumbed within 12 days. Signs of piloerection and anorexia associated to a loss of weight (≈32%) appeared after 4 days of infection (Fig. 6B). Animals were euthanized at different intervals post-infection and BAL were collected to assess cellular infiltration and mediators content in the airspaces. Fig. 6C (square symbol) shows a biphasic leukocyte recruitment constituted mainly of polymorphonuclear cells (circle symbol) by day 3 to day 8 post-infection and of mononuclear cells (triangle symbol) afterwards. The kinetic of influenza virus-induced secretion of a major cytokine (IL-6) and a CC chemokine (RANTES) was investigated. While RANTES peaked at day 3 and decreased significantly thereafter (Fig. 6D), IL-6 secretion increased steadily until day 4 and then stabilized (Fig. 6E). As an index of transudation from the vascular compartment into the lungs, the amount of total protein was determined in the BAL (Fig. 6F). Interestingly, the protein concentration curve parallels the one of total leukocyte content (Fig. 6C, square symbol) confirming a connection between increased microvascular permeability and cellular infiltration during acute lung injury (20). BAL fluids were further analyzed by an inflammatory protein array to examine at day 3 and day 10 post-infection, whether other major components, not measured in the initial assays, were affected during influenza pneumonia (Fig. 6G). A total of 32 mediators were measured that included 16 cytokines, 11 chemokines, 3 growth factors, 1 metalloproteinase inhibitor, 1 soluble cytokine receptor. This array analysis indicates selective major increases of G-CSF, IL-6, MCP-1, MCP-5, MIP-2α, RANTES, soluble TNFα receptor and TIMP-1. Expression of other mediators was also observed although to a less extent and was time-related. Some display a restricted early expression (including IL-9, MIP-1α and SCF) while others were induced only later on (G-CSF, vEGF). Also, a number of mediators were not or barely detected at the two analysed time points , i.e. 6Ckine, GM-CSF, IL-2, 3, 4, 5, 17, interferon-γ, leptin and TNF-α. Next, we checked that the lethal pneumonia induced by influenza A virus was not associated to a bacteria superinfection, as it has been reported by others (60). We showed that bacteria counts in blood or BAL of influenza A virus-treated mice at day 3, 6, 8 or 10 post-infection was negligible (constantly below 250 cfu/ml). In addition, intramuscular

administration of the mice with a large spectrum antibiotic (Clamoxyl®, 25 mg/kg) before and during the viral infection did not reduce or delay the survival pattern.

Reduced inflammation and lethality in influenza virus infected TLR3-/- mice

The inventors previously demonstrated that of all the mediators we tested, i.e. bacterial LPS, the cytokines TNFα and IL-1β, the protein kinase C activator PMA, influenza A virus and the synthetic double-stranded RNA poly(I:C), only the two latter stimuli up-regulated TLR3 expression in human pulmonary epithelial cells, suggesting that the signaling pathways controlling the induction of this gene are restricted (53). Given the distinct complexity between in vitro and in vivo systems, there was a need to determine the regulation and the function of TLR3 in relation to the pathogenesis of influenza in an experimental animal model. The inventors first showed that the expression of this receptor in the lungs of infected mice is constitutive and markedly up-regulated following influenza virus administration, peaking at day 3 and slightly decreasing at day 9 post-infection (Fig. 7A). Remarkably, the inventors showed that TLR3-/- mice had a more prolonged survival after a challenge of 300 pfu influenza A virus, than wild-type animals as determined by a Kaplan-Meier test; i.e. ≈26% and 0% survival, respectively at day 12 post-infection ($P<0.0001$, Fig. 2B left panel). Ultimately, all TLR3-/- mice died 3 to 5 days later (not shown). Interestingly, mice deficient for the adapter protein MyD88, which is involved in the signaling of all TLR molecules but TLR3, were as sensitive as wild-type mice to influenza infection (0% survival for both groups, Fig. 7B right panel). Then, to monitor the role of TLR3 in lung dysfunction induced by the viral infection, the respiratory distress index Penh and total protein amount as well as the secretion of inflammatory cytokines and chemokines were evaluated in the BAL fluids of TLR3-/- and wild-type animals at day 3 post-infection (this time point was chosen as it corresponds to the peak of the viral load in the lungs of both animal groups, cf. Fig.9). Fig. 7C shows that the Penh index was significantly diminished in TLR3-deficient mice compared to control mice, i.e 0.95 ± 0.12 vs. 1.74 ± 0.2, respectively, ($n=5$, $P=0.005$). Likewise, total protein, RANTES and IL-6 amount were significantly lower in TLR3-/- than in wild-type mice (Fig. 7D-F). BAL fluids were further analyzed by an inflammatory protein array to examine at day 9, whether the expression

of additional mediators was directly regulated by TLR3. Fig. 8A not only confirms a reduced IL-6 and RANTES production in the lungs of TLR3-deficient mice but also reveals a similar inhibition concerning the inflammatory mediators IL-12p40p70, MCP-1, sTNFR1, TIMP1, TNFα and TPO. On the contrary, the expression of distinct components is increased in TLR3-/- vs wild-type lungs, including not only INF-γ but also G-CSF and to a less extent IL-9 and IL-10. As an additional evidence, the inventors showed by ELISA that the amount of IFN-γ at day 4 to day 7 post-viral infection was similar in both mice groups while a greater release of this cytokine was measured in TLR3-/- mice (approximately 14.5 times that of wild-type animals) at day 9 when mice were beginning to succumb to lethal influenza (Fig. 8B). Furthermore, Fig. 8C presents a typical gross morphological view of perfused lungs isolated from wild-type and TLR3-/- mice at 9 days post-infection by 300 pfu of *influenza virus*. Wild-type animals appear severely injured as manifested by an almost black hemorrhaged lung surface whereas those obtained from TLR3-deficient mice only produced faintly and diffuse red lungs, suggesting that the lesions induced by influenza virus are reduced in absence of TLR3.

Paradoxical increased lung viral load in TLR3-deficient mice

The previous results indicate that a potent inflammatory reaction occurs in the lungs of wild-type mice after influenza infection and that this process is critically reduced or altered in *TLR3-/-* animals. Inflammatory signalling pathways during viral infection have been interpreted in some cases as a protective response of the host whereas in other cases the virus can utilize these pathways to enhance its replication (61). Thus, the inventors investigated whether TLR3-dependent host response might regulate the replication of influenza A virus. Fig. 9 shows that virus replication is major in both wild-type and TLR3-deficient mice with very similar titers when examined on days 1 to 4 post-viral infections. Paradoxically, while control animals had significantly reduced the virus load of their lungs at day 9 post-infection, the virus persisted in *TLR3-/-* mice with an elevated amount (approximately 280 times that of wild-type animals).

Wild-type and TLR3-deficient mice raise a contrasted leukocyte content in their lungs after infection by influenza A virus

Some of the foregoing findings suggested that a TLR3-mediated host immune response plays a harmful role in the pathogenesis of influenza A virus infection. Of note is that several studies revealed a functional redundancy and synergy of different immune cells in the antiviral response to influenza virus (56, 58, 62). In that context, to have an insight into which cell type can possibly participate to the TLR3-regulated detrimental immune response, leukocytes were harvested from the BAL of wild-type and *TLR3-/-* mice 9 days post infection and the percentage and phenotype of the cells were characterized by three-color flow cytometry using the following markers : Gr1 and CD11b (for the polymorphonuclear neutrophils), F4/80 (for the macrophages), CD4 (for the CD4+ T-lymphocytes), CD8 (for the CD8+ cytotoxic T-lymphocytes). Infection of wild-type mice with influenza virus resulted in a significant increase in the number of leukocytes (*cf.* Fig. 6C), and the accumulated cells in the BAL were composed mostly of T-lymphocytes (≈55%), neutrophils (≈23%), macrophages (≈11%; Fig. 10 *left* and *central panels*), those numbers have to be compared to a leukocyte population in naïve mice constituted by ≈90% macrophages (Fig. 10 *left panels* and (24)). Among the T-lymphocytes, the CD8+ T cells were the predominant cell population in the lungs of infected control animals (≈38%). In striking contrast, whereas the absolute number of leukocytes collected in the BALs was similar to that observed in wild-type animals (≈1 x 10^6 cells), the percentage of CD8+, but not CD4+, T-lymphocytes in TLR3-deficient mice was significantly lesser (about a third that of wild-type animals; P=1 x 10^{-6}, Fig. 5 *right* and *central panels*). Likewise, a significant reduction of the percentage of macrophages in *TLR3-/-* mice was observed in comparison with control animals (P=0.004) whereas the percentage of neutrophils was 1.5 times higher in TLR3-deficient mice (P=0.0009; Fig. 5 *right* and *central panels*).

Discussion

The inventors understanding of how the immune system recognizes pathogens and initiates an appropriate response has increased exponentially in recent years due to

the discovery of the TLR family. TLRs play a central role in the detection of pathogen-associated molecular patterns and in the initiation of an effective innate and adaptive immune response to fight against pathogenic microorganisms (49, 50). In agreement with this paradigm, there is accumulating *in vivo* evidence using TLR-deficient mice that support a role for these receptors in antibacterial, antifungal and antiviral defense. Moreover, several clinical reports confirm the contribution of TLRs to the pathophysiology of infectious diseases and polymorphisms in TLR genes are associated with predisposition to severe infections (64).

In view of these major informations, at the start of this investigation to evaluate the *in vivo* role of TLR3 during the course of influenza infection, the inventors speculated that TLR3 would act as a protective component and conversely, its absence in *TLR3-/-* mice would render the animals more susceptible to influenza infection. In contrast, this study reveals that activation of TLR3 can be a double-edged sword for the host as it shows that an unchecked immune response may be hazardous to the host and may cause severe outcome such as death. Thus, the inventors found (*i*) that the pulmonary expression of TLR3 is constitutive and markedly up-regulated following influenza infection, (*ii*) increased levels of inflammatory mediators in the bronchoalveolar spaces, including RANTES, IL-6 and IL-12p40p70 in wild-type mice, that were seriously reduced in *TLR3-/-* animals, (*iii*) a smaller number of the predominant leukocyte population in the airspaces, *i.e.* the CD8+ T cells, in infected *TLR3-/-* mice in comparison with wild-type animals, and (*iv*) a paradoxical higher resistance of TLR3-deficient mice to a lethal challenge of influenza A virus. Based on these findings and in view of previous works describing the major role of CD8+ T lymphocytes and cytokines in the pathogenesis of influenza infection, the inventors discuss below the fact that the enhanced resistance observed in influenza virus-infected *TLR3-/-* might be due to a lower TLR3-mediated release of inflammatory mediators and T-cells infiltration in the lung airspaces.

It is established that influenza virus replicates in epithelial cells and leukocytes resulting in the production of chemokines and cytokines that favours the recruitment of mononuclear cell population to the site of infection. There is growing evidence that the mediators that have a central role in the resolution of influenza infection are the same

that can be the cause of many clinical signs related to this pathology (65-67). The inventors confirm in the present study that influenza infection leads to the synthesis of major inflammatory cytokines and chemokines, including IL-6, G-CSF, IL-12p40p70, MCPs, MIPs and RANTES. Remarkably, IL-6 exhibits multifunctional immune activities but its release has been correlated with the symptom pathogenesis during acute influenza; the role of IL-6 in these symptoms being largely explained by its pro-inflammatory activity (66). IL-12 administration was also found to have an adverse effect on the course of influenza infection (67). Other studies have confirmed the involvement of cytokines in influenza pathogenesis, but the effect of blocking individual cytokines can be somewhat partial. On plausible explanation is the substantial redundancy between cytokines (56, 58). Notably, this study clearly establishes that TLR3 plays a major role in the inflammatory cytokine response to influenza virus, the lack of TLR3 resulting in a significant decrease of cytokine synthesis, including that of IL-6, IL-12p40p70 and RANTES. Ongoing loss-of-function *in vitro* studies with human pulmonary epithelial cells also demonstrate an essential function for TLR3 in the production of inflammatory cytokines, including IL-6, after influenza virus challenge. On the contrary, the expression of other mediators is increased in *TLR3-/- vs* wild-type lungs. The mechanism by which TLR3 differentially regulates the expression of cytokines and chemokines will likely be complex to unravel due to the pleiotropic and multiple effects of these mediators on diverse cell types. Moreover, these molecules induce or inhibit the production of other cytokines or mediators from their targets in a complex array of positive and negative feedback loops. Regardless, consistent with the role of TLR3 in viral-induced inflammation, one of us recently established that infection of *TLR3-/-* mice with the west Nile virus induces a lower secretion of cytokines, including IL-6 and TNF-α, compared with wild-type mice, that eventually prevents neuronal injury (52). Similarly, in a study examining the role of another TLR in the host response to herpes simplex virus, Kurt-Jones *et al.* demonstrated an attenuated cytokine response paralleled to a reduction of symptoms of encephalitis in *TLR2-/-* compared to control animals (68).

Interestingly, cytokines play critical roles in shaping subsequent adaptive T cell responses. Although the recruitment of these cells is essential for protective responses,

it is becoming increasingly evident that some T lymphocytes are also associated with the development of influenza-related immunopathological sequelae (69, 70). The inventors confirmed that CD8+ T cells are prominent in the airways of influenza virus-challenged wild-type mice, representing ≈38% of the leukocytes. The drastically decrease of CD8+ T lymphocytes infiltration (down to ≈13%) concomitantly to the prolonged survival of the TLR3-deficient mice to influenza virus infection suggest that a disregulated antiviral, TLR3-dependent, CD8+ T cell response may lead to sustained lung injury. This belief is rather well-supported by previous studies that have reported that mice given antilymphocyte serum (71) or the immunosuppressive drug cyclophosphamide had less pulmonary pathology when infected with influenza virus than untreated mice (72, 73). Moreover, numerous studies reported that athymic nude mice exhibited an increased survival time compared to immunocompetent mice given influenza challenge (74-76). Other authors suggested that CD8+ T lymphocytes exacerbate the influenza pathology and cause mortality at high viral infection (77). Clinical observations also lend support to the model that CD8+ T lymphocytes may contribute to the pathologic manifestations of influenza virus infection (78). Of note is that the detrimental role of the cell-mediated immune system in influenza infection is analogous to the infections with the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV). Indeed, immunodepressed mice infected with this virus do not die as do immunocompetent mice, in spite of persistent viral production (79). This latter feature is especially meaningful in regard to the inability of TLR3-deficient mice as well as athymic mice (74-76) to clear the influenza virus whereas they live longer than control animals.

Overall, it emerges that the etiology and pathogenesis of a very complex disease such as influenza cannot be fully explained by a single cell type such as CD8+ T cells or by the action of the virus alone. Rather, the underlying mechanisms of this pathology likely involve a combination of both as well as the imbalance between the beneficial and harmful effects of mediators released by immune cells. Moreover, a clear conclusion from this work on influenza and from previous experimental viral infection models is that a reduction of TLR-mediated inflammatory infiltrate reduces clinical manifestations (49, 50, 80). Nonetheless, though potentially valuable, there would be no overall benefit from

treating infuenza infection solely with TLR3 regulators as we found that the lack of TLR3 resulted concomitantly in highly increased pulmonary influenza virus load. Hence, while vaccination should remain the basis for influenza prophylaxis (81), this study propose that inhibitors of viral replication combined to modulators of TLR3 are useful in the management of influenza virus infections, particularly in at-risk groups during periods when there is a mismatch between the epidemic strain and the vaccine strain.

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CLAIMS

1. Use of TLR3 or its signalling-associated molecule Toll-IL-1 receptor (TIR)-domain containing adaptor inducing interferon (IFN)-β (TRIF) in the design of molecules to prevent a host inflammation response induced by a RNA virus.

2. Use according to claim 1, wherein said molecule is an antagonist of TLR3 or TRIF.

3. Use according to claim 1 or 2, wherein said RNA virus is a single-stranded virus or a double-stranded virus.

4. Use according to claim 3, wherein said RNA virus consists of Influenza.

5. Use according to claim 4, wherein said Influenza is of type A.

6. Method for screening a TLR3 antagonistic expression, comprising the steps of:
    a. contacting a functional TLR3 molecule with a candidate agent, under conditions and for a time sufficient to permit interaction between TLR3 molecule and the candidate agent; and
    b. evaluating the capacity of said candidate agent to interfere with the biological role of said functional TLR3 molecule.

7. Method for screening a TRIF antagonistic expression, comprising the steps of:
    a. contacting a functional TRIF molecule with a candidate agent, under conditions and for a time sufficient to permit interaction between TRIF molecule and the candidate agent; and
    b. evaluating the capacity of said candidate agent to interfere with the biological role of said functional TRIF molecule.

8. Method for preventing and/or treating a host inflammation response induced by a RNA virus, comprising the step of administering to a animal in need thereof an effective amount of an antagonist of TLR3 and/or an antagonist of TRIF.

9. Method according to claim 7, wherein said RNA virus is a single-stranded virus or a double-stranded virus.

10. Method according to claim 8, wherein said RNA virus consists of Influenza.

11. Method according to claim 9, wherein said Influenza is of type A

12. Method according to any one of claims 8 to 11, wherein said antagonist of TLR3 is obtained by the method as defined in claim 6.

13. Method according to any one of claims 8 to 11, wherein said antagonist of TRIF is obtained by the method as defined in claim 7.

14. Use of an antagonist of TLR3 obtained by the method as defined in claim 6 in the preparation of a medicament for preventing and/or treating a host inflammation response induced by a RNA virus.

15. Use of an antagonist of TRIF obtained by the method as defined in claim 7 in the preparation of a medicament for preventing and/or treating a host inflammation response induced by a RNA virus.

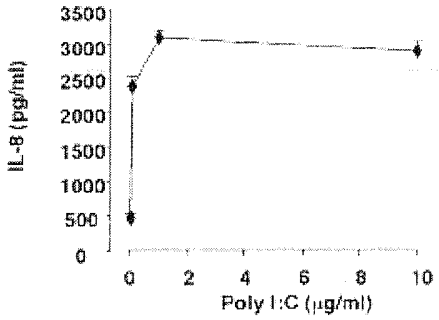
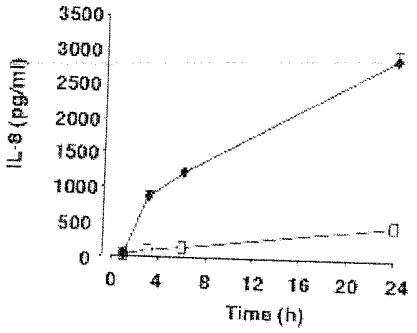


Figure 1A



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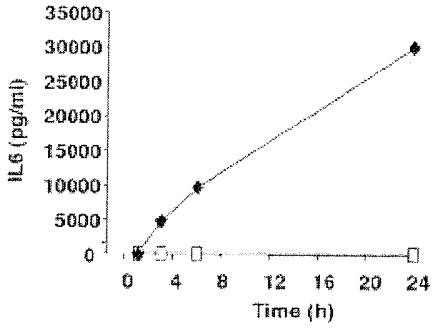

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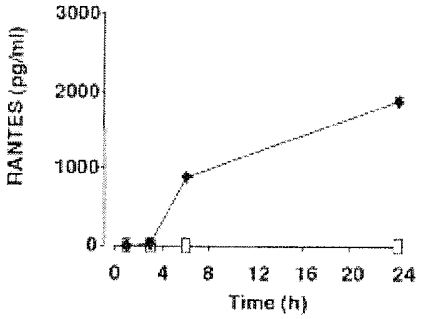

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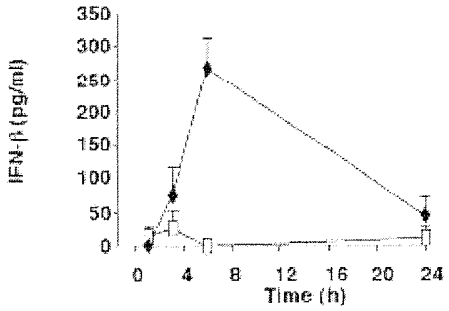

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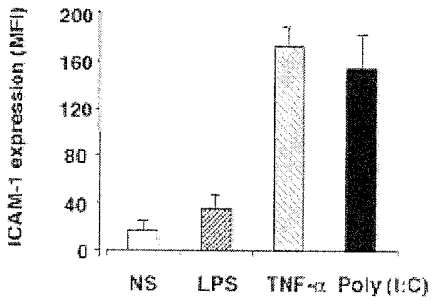

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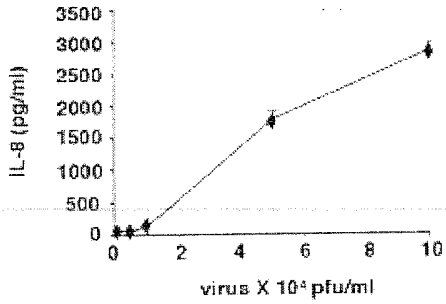
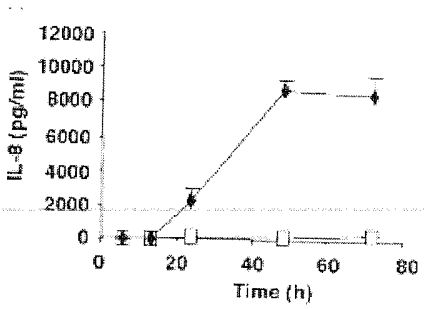


Figure 1G



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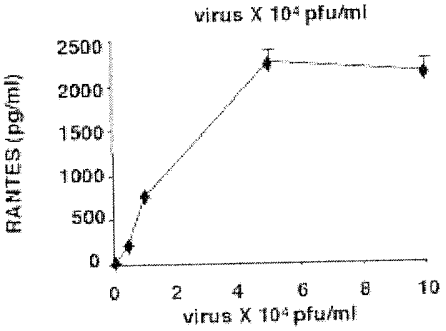
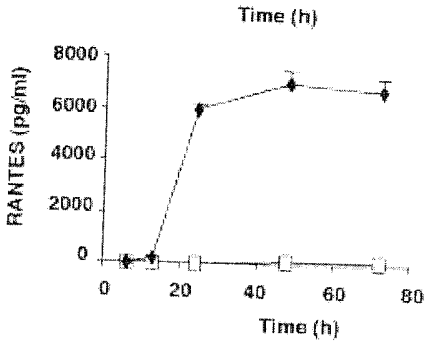


Figure 1I



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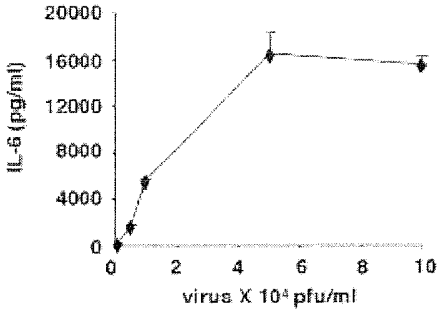

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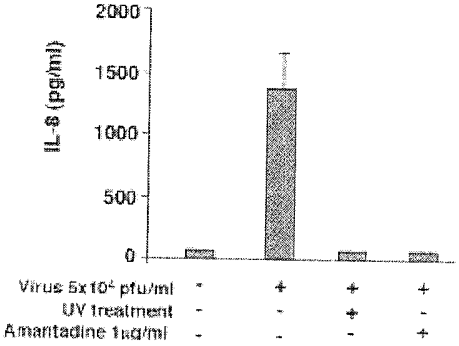


Figure 1L

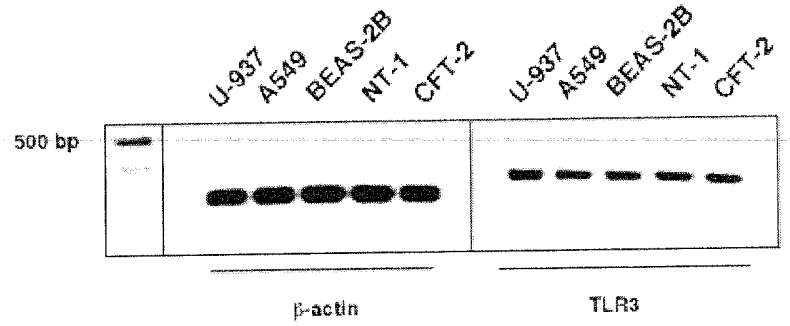
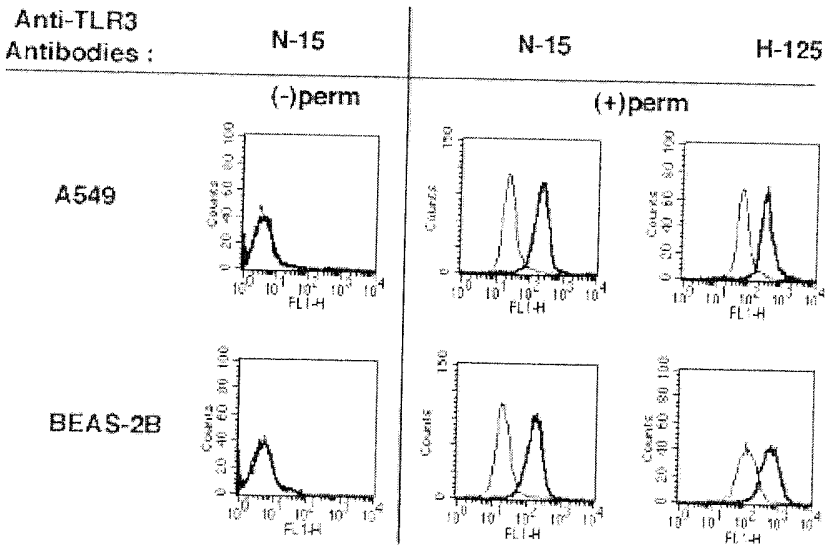


Figure 2A



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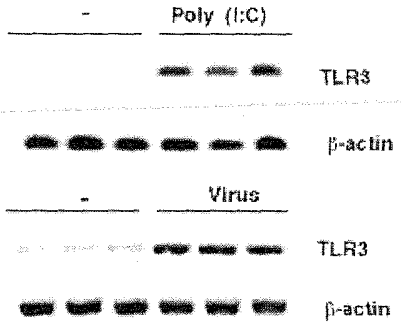

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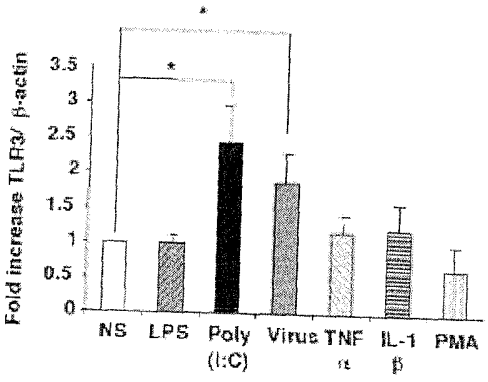

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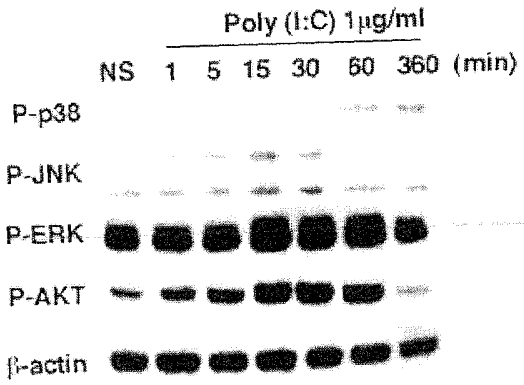

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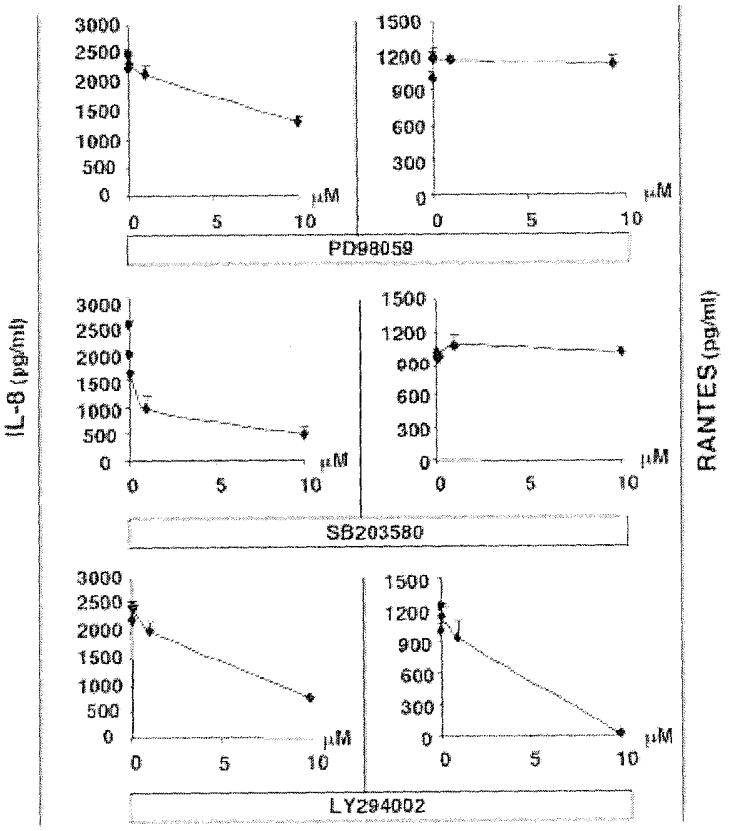

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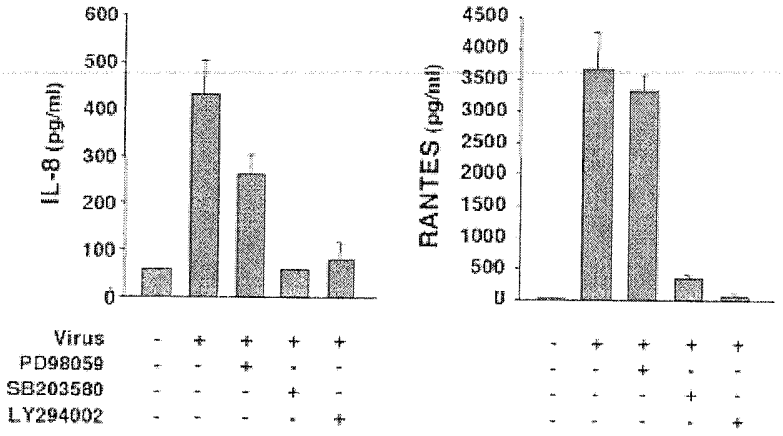
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SB203580 - - - + -
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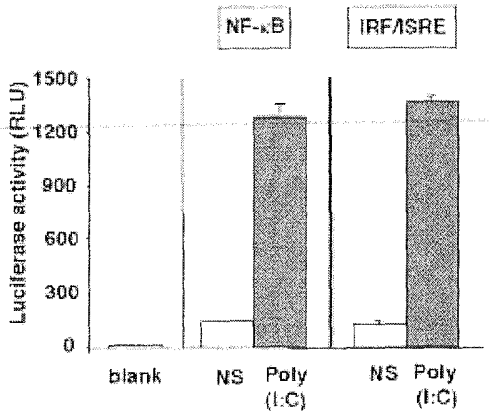
Figure 4C



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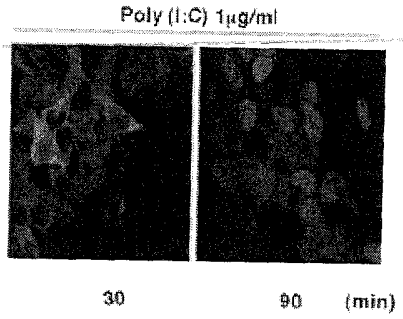

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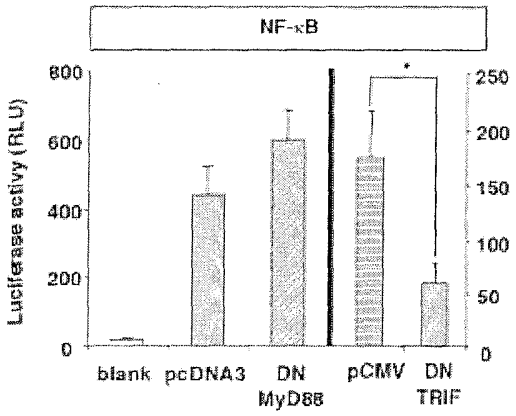

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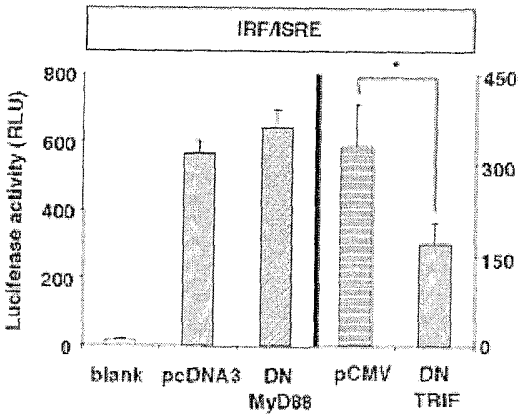

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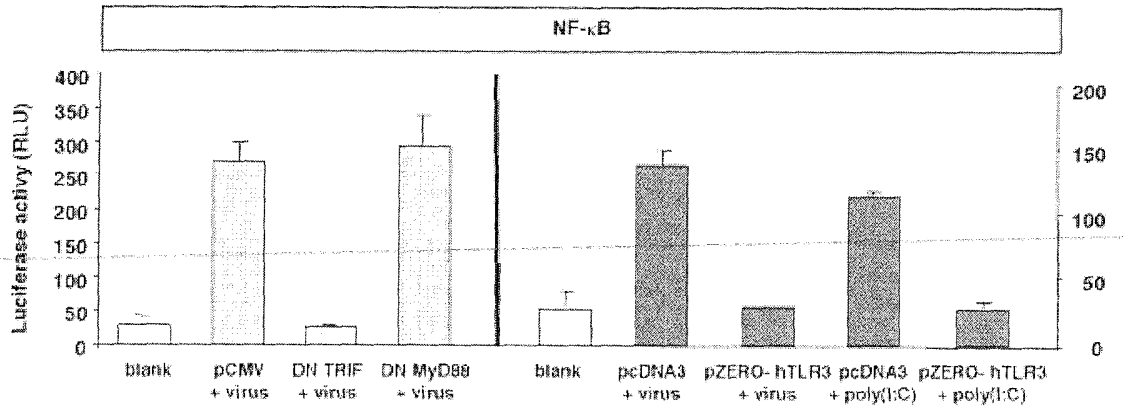

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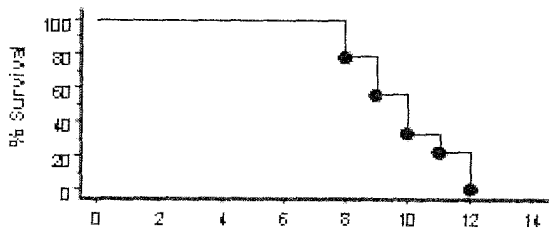

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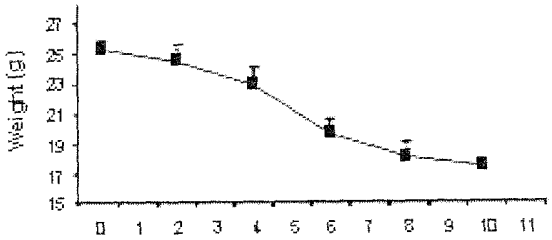

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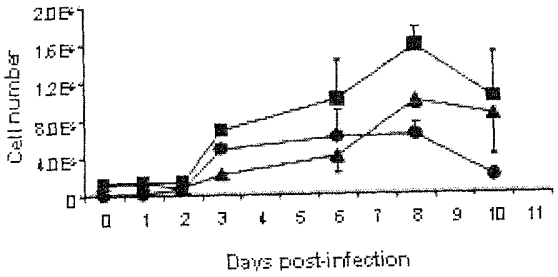

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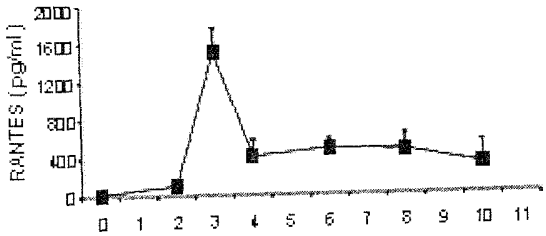

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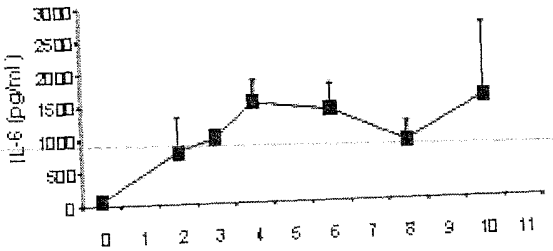

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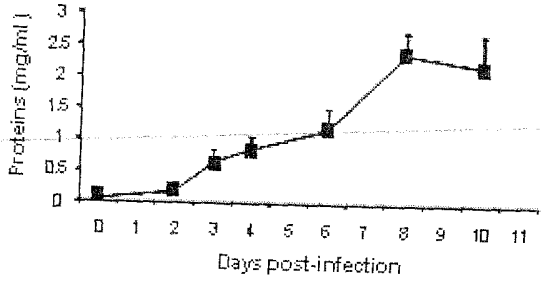

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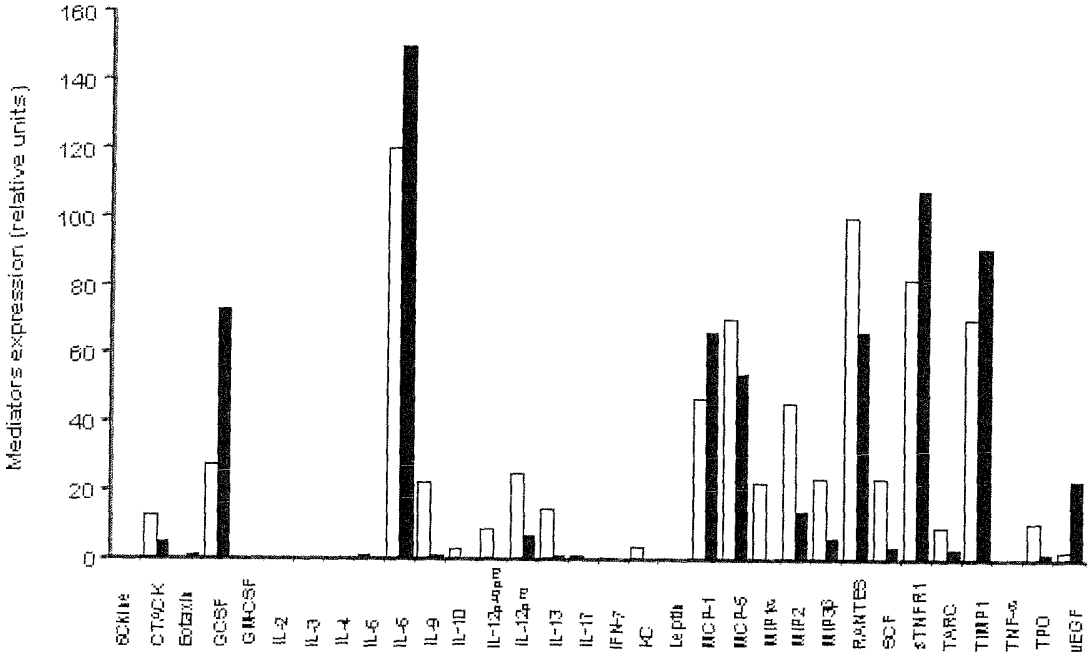

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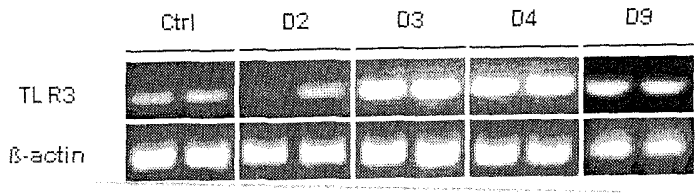

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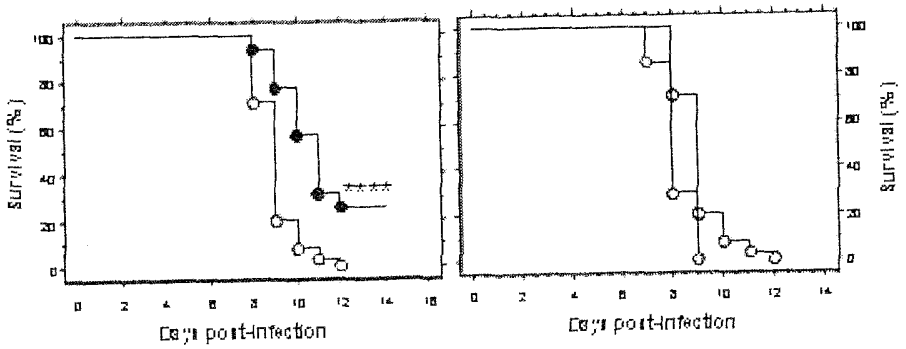

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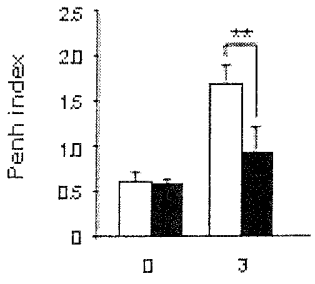

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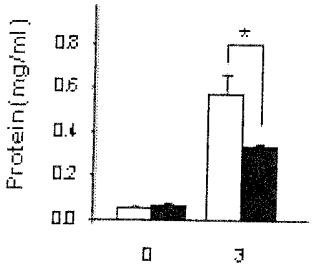

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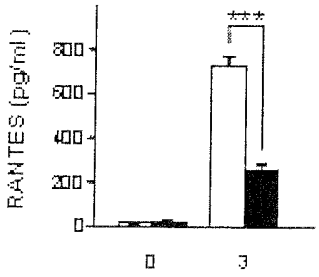

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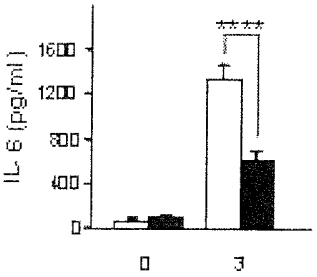

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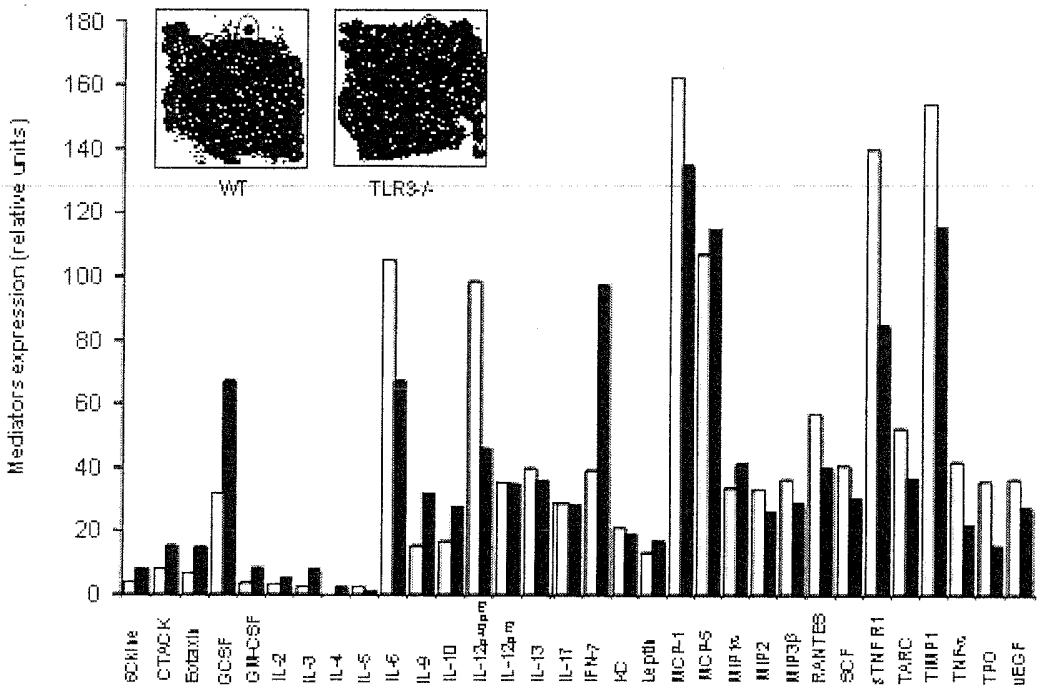

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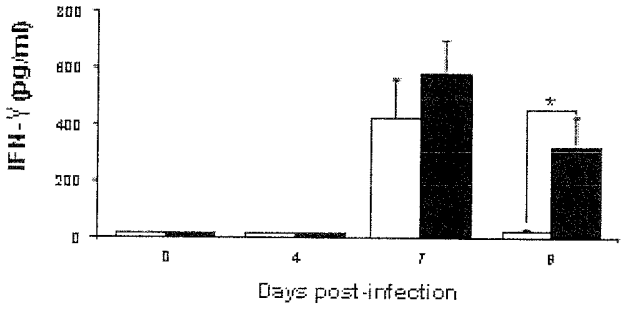

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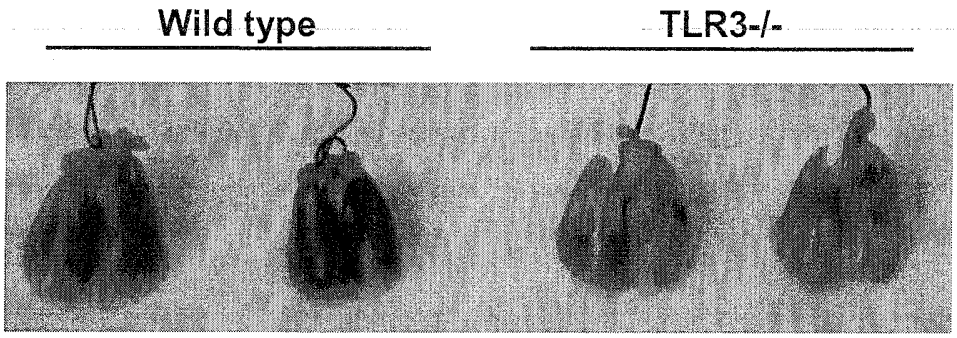

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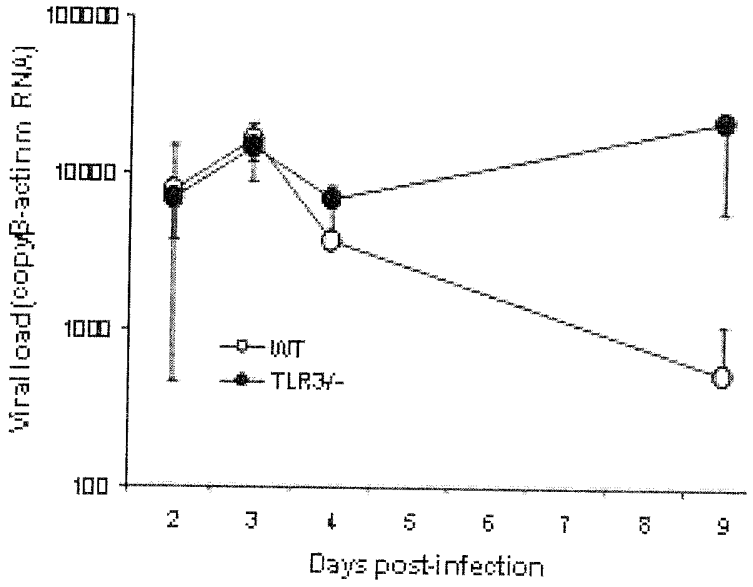

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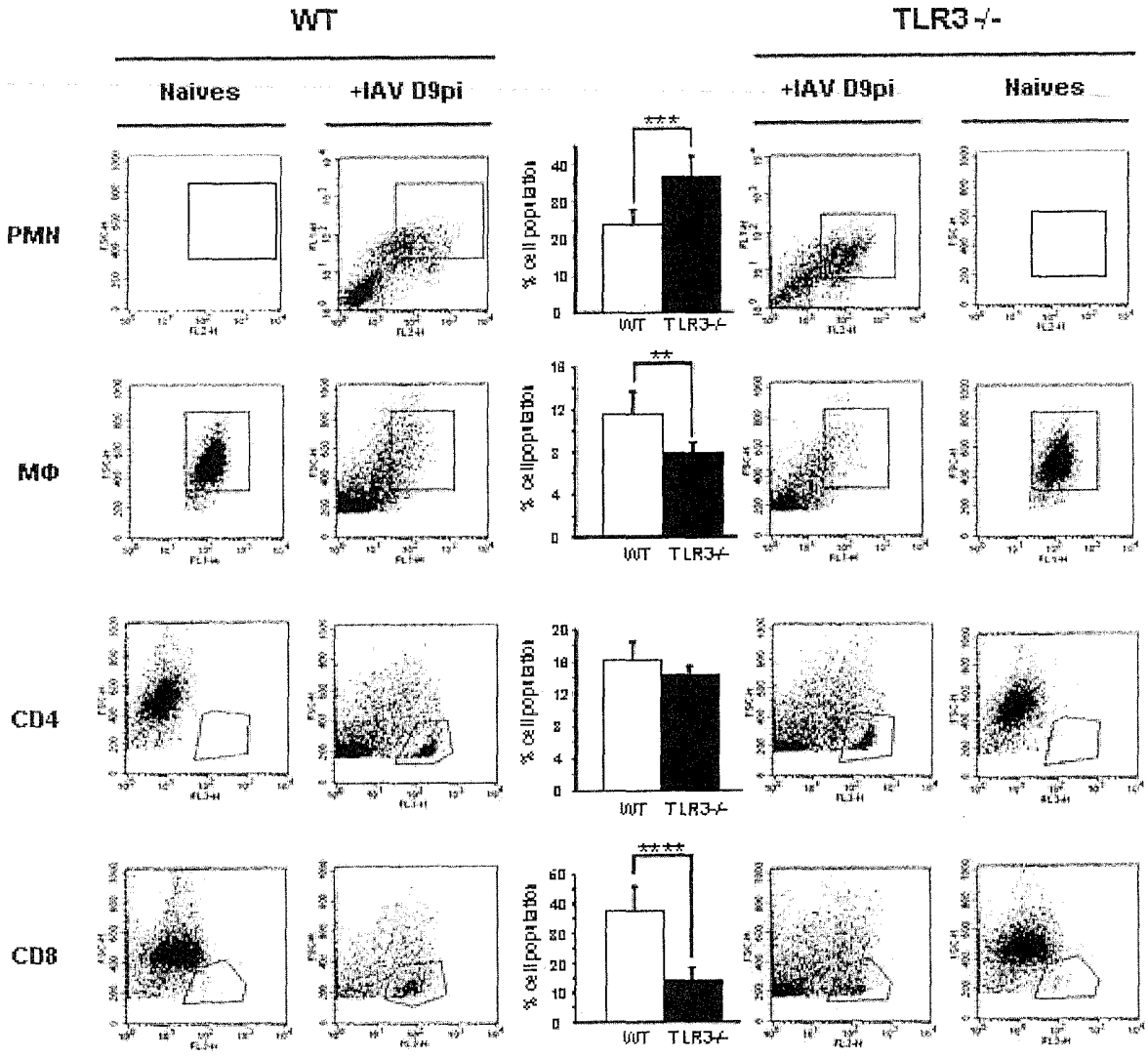
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TLR3-/-
Naives
+IAV D9pi
+IAV D9pi
Naives
PMN
MΦ
CD4
CD8
% cell population
WT
TLR3-/-
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Figure 10