Pulmonary transcriptomic responses indicate a dual role of inflammation in pneumonia development and viral clearance during 2009 pandemic influenza infection (#17133)

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Craig Roberts / 3 Jun 2017

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Pulmonary transcriptomic responses indicate a dual role of inflammation in pneumonia development and viral clearance during 2009 pandemic influenza infection

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Background: The interaction between influenza virus and the host response to infection clearly plays an important role in determining the outcome of infection. While much is known on the participation of inflammation on the pathogenesis of severe A (H1N1) pandemic 09-influenza virus, its role in the course of non-fatal pneumonia has not been fully addressed.

Methods: A systems biology approach was used to define gene expression profiles, histology and viral dynamics in the lungs of healthy immune-competent mice with pneumonia caused by a human influenza A (H1N1) pdm09 virus, which successfully resolved the infection.

Results: Viral infection activated a marked pro-inflammatory response at the lung level paralleling the emergence of histological changes. Cellular immune response and Cytokine Signaling were the two signaling pathway categories more representative of our analysis. This transcriptome response was associated to viral clearance, and its resolution was accompanied by resolution of histopathology.

Discussion: These findings suggest a dual role of pulmonary inflammation in viral clearance and development of pneumonia during non-fatal infection caused by the 2009 pandemic influenza virus. Understanding the dynamics of the host's transcriptomic and virological changes over the course of the infection caused by A (H1N1) pdm09 virus may help identifying the immune response profiles associated to an effective response against influenza virus.

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Abstract

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Introduction

Influenza is one of the most common respiratory infectious diseases and a worldwide public health concern. The World health Organization (WHO) estimates that influenza viruses infect around 5%–15% of the global population, resulting into 250,000 to 500,000 deaths each year. (Vemula et al., 2016).

At the beginning of 2009, a new influenza virus of the subtype H1N1, [A (H1N1) pmd09], was detected in Mexico. The vast majority of infections caused by this new strain were mild and self-limiting upper respiratory tract illness. However, a small percentage of patients infected by the A H1N1 pm09 virus developed primary viral pneumonia, resulting in respiratory failure, acute respiratory distress, multi-organ failure and death (Health Protection Agency et al., 2009). A large proportion of this severe cases occurred in young adults with accompanying co-morbidities (chronic respiratory disease, cardiovascular disease, hypertension, obesity and diabetes) (Jain et al., 2009).

The host response to the infection clearly plays an important role in determining the outcome of the patients infected by influenza virus. (Almansa, Bermejo-Martín & de Lejarazu Leonardo, 2012). In this regard, severe patients infected by the influenza A (H1N1) pdm09 virus was characterized by the presence of high plasmatic levels of cytokines, chemokines and other immune mediators accompanying the presence of pneumonic infiltrates (Bermejo-Martin et al., 2009), (Hagau et al., 2010), (To et al., 2010). Moreover, we have shown that systemic levels of these mediators were directly associated with viral levels secreted by the respiratory tract from the beginning of the disease (Almansa et al., 2011a). In addition, persistence of viral secretion has been found in the patients with the worst outcomes (Lee et al., 2009), paralleling the presence of impaired expression of a number of genes participating in adaptive immune

64 responses. Depression of adaptive immunity response has been previously put in relationship
65 with poor control of infection and maintenance of inflammation, and secondarily with the
66 generation of damage to the infected tissues with the development of further respiratory failure
67 (Bermejo-Martin et al., 2010).
68 While much is known about the immune alterations and the participation of inflammation on the
69 pathogenesis of severe A (H1N1) pandemic influenza, their role in the course of non-fatal
70 pneumonia has not been enough studied. Aimed to clarify this role, we employed a systems
71 biology approach to study gene expression profiles and its relation to histology and viral
72 dynamics in the lungs of healthy immune-competent mice with pneumonia caused by human
73 influenza A (H1N1) pdm09 virus, which successfully resolved the infection.

Material and methods

Ethics statement

The ethical protocol and the research were reviewed and approved by the Animal and human Experimentation Ethical Committee of the Autonomous University of Barcelona (Internal Register Number 1124M2R) and the Ethical Animal Experimentation Commission of the Catalan Government (Register Number: 5767).

All the animal experiments were done at the Biosafety level 3 (BSL3) facilities of the Centre de Recerca en Sanitat Animal (CReSA, Barcelona, Spain). Animal care was performed according to the standard procedures of the center (Martínez-Orellana et al., 2015). Seven weeks-old C57BL6/JOlaHsd (C57BL6) female mice (Harlan Laboratories, Barcelona, Spain) were housed in groups in experimental isolation cages for one week in acclimation (72 animals in total). Throughout the experiment, all mice were provided with commercial food pellets and tap water *ad libitum.*

A (H1N1) pdm 2009 Catalonian virus and mice infection.

A human pandemic Influenza A virus, A/Catalonia/63/2009 (CAT09) (GenBank accession numbers GQ464405-GQ464411 and GQ168897) was used for animal infection (Busquets et al., 2010). CAT09 was passaged in MDCK two times and the viral stock had a titer of 10^6 PFU/ml. Animals were divided into two groups of 32 mice each; distribution was done as follows: untreated control group (mock group) and pdmH1N1 2009 infected-group (CAT09). To evaluate the pathogenicity mice were infected through intranasal instillation with 50 µL CAT09 at 10^4 PFU/mice as described previously (Itoh et al., 2009) and confirmed by our experimental work. Control non-infected mice were treated with 50 µL phosphate-buffered saline (mock infection) to reproduce CAT09 infection.

Mice monitoring and sampling

During ten days, mice were observed daily to record changes in body weight and clinical signs. Based on our previous experimental work, the day showing the most important histological changes in the lung following infection caused by CAT09 is day 5, which resolve by day 10 (data not shown). In consequence, necropsies of 12 animals per group were performed at days 1, 5 and 10 post infection (dpi). Animals were euthanized with intraperitoneal inoculation of penthobarbital under anesthesia with 5% isofluprane and tissue samples of lung were dissected from dead animals using the standard surgical procedures. Lung samples of six animals per group were used for viral load determination and histological examination. Lung samples were snap frozen on dry ice and stored at –80°C until further processing. Gene expression profiling was performed for whole lungs of the other six animals per group by using microarrays.

Determination of viral load

Viral quantification was determined by plaque assay determining Plaque forming units (PFU) following our laboratory standard operating procedures (Martínez-Orellana et al., 2015). Briefly, supernatants were obtained after weighing, homogenizing and centrifuging lung samples. 0.1 ml of 10-fold supernatant dilutions were incubated with MDCK cells plated in 12-well tissue cultures plates for 1 hour. Then, cells were washed with phosphate buffer saline and plates were overlaid with 1.4% noble agar (Becton Dickinson, France), mixed 1:1 with 0.5 µg/ml of bovine trypsin and Minimum essential medium eagle (MEM) (both of Sigma-Aldrich SA, Madrid, Spain) supplemented with 100 UI/ml penicillin and 100ug/ml streptomycin (Invitrogen ®, Barcelona, Spain). After 4 days of incubation, cells were fixed for 20 min using 10% formalin (Sigma-Aldrich SA, Madrid, Spain) and then overlaid with 1% crystal violet (Anorsa, Barcelona,

120 Spain). Finally, cells were washed with water in order to visualized plaques, which were counted
121 and compared to uninfected cells.

122 ***Histopathology***

123 Lung samples were collected for macroscopical and histological examination according to our
124 laboratory standard operating procedures (Martínez-Orellana et al., 2015) (Vidaña et al., 2014).
125 The procedures involved lung sample fixation using neutral-buffered 10% formalin for 48 hs,
126 followed by embedment in paraffin wax. Next, sections of 3 μm were stained using
127 haematoxylin and eosin (HE). Cross sections of the lungs of each mouse were analysed
128 separately. Finally, the cross sections were screened for the presence of lesions caused by the to
129 Influenza A Virus infection.

130 ***RNA extraction and microarray processing and analyzing***

131 At designated time points (1, 5 and 10 dpi), C57BL6 mice were euthanized and lung tissue was
132 collected in RNA-later and stored at –80°C until further processing. Total RNA was extracted
133 from lung samples using the Ribopure kit (Ambion, Life technology). RNA integrity and
134 concentration were evaluated as previously described (Almansa et al., 2015). A total amount of
135 100 ng of mRNA was processed as described elsewhere to obtain Cyanine 3-CTP-labeled cRNA.
136 Next cRNA was hybridized with Mouse GE 4x44K v2 Microarray Kit (Agilent p/n G4846A)
137 overnight (17hrs) at 65C on a rotator. Image acquisition was performed using an Agilent
138 Microarray Scanner (Agilent G2565CA) and data were extracted using the Agilent Feature
139 Extraction Software 10.7.1.1 following the Agilent protocol GE1-107_Sep09. Raw data were
140 collected and preprocessed by using the GeneSpring GX 12.0 software (Almansa et al., 2015).
141 This software was employed also to perform the statistical analysis, which involved the use of a
142 moderate T test to identify those genes showing significant differences between their expression

levels fixing a p-value < 0.05 with further application of the Benjamini-Hochberg correction for multiple comparisons. A fold change in gene expression ≥ 2 was used to obtain the list of those genes showing the more important variations in their expression levels between groups along time (1, 5 and 10 dpi). Ingenuity pathway analysis (IPA) (Ingenuity Systems-Quiagen, Redwood City, CA) was employed to determine whether a canonical pathway is enriched with genes of interest by using Fisher's exact test.

Microarray Data Accession Number.

Microarray expression data sets were uploaded at the Array Express microarray data repository and are available publicly under accession number E-MTAB-3866.

Validation of gene expression results from microarrays.

Results of gene expression obtained using microarrays were confirmed by using a next generation PCR technology, droplet digital PCR (ddPCR), using the Bio-Rad QX200 ™ Droplet Digital ™ PCR system. 5ng of total mRNA were retro-transcribed to cDNA and analysed by ddPCR using a Bio-Rad QX200 ™ platform as previously described (Tamayo et al., 2014). Quantification of expression levels of target mRNAs was performed using pre-designed TaqMan® Assay Primer/Probe Sets, (FAM labelled MGB probes, Thermo Fisher/Scientific- Life Technologies, Waltham, MA, USA): IL6 gene; interleukin 6 (Reference: Mm00446190_m1) and IFNB1 gene; interferon beta 1 (Reference: Mm00439552_s1). The droplet reader used at least 10000 droplets to determine the percentage of positive droplets and calculation of copy number of cDNA per ng of initial mRNA. Spearman correlation between ddPRC and microarrays results was performed using SPSS 15.0. (Fig. S1).

Statistical analysis

SPSS 15.00 software was employed for perform statistical comparison of weight loss and viral load between groups at all sampling times (SPSS Inc., Chicago, IL, USA). The statistical test used was the U Mann-Whitney, and the significance level (α) was set at 0.05. All graphs used for represent the variations on weight loss and viral load were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

Results

A (H1N1) pdm09 virus infection induced moderate weight loss during the first five days of infection.

Weight was evaluated each day during the first 10 days following infection with the pandemic CAT09 virus. Even though the percentage of body weight loss in CAT09-infected animals was not dramatic, CAT09-infected mice showed significantly higher body weight loss on the first five days compared to uninfected controls ($p < 0.05$). After 5 dpi, infected mice began to recover their normal weight with no significant differences compared to mock mice (Figure 1a).

Human A (H1N1) pdm09 virus causes a productive infection in the lower respiratory tract of mice.

Virus titers in lung homogenates measured on 1, 5 and 10 dpi are shown in Figure 1b (n= 6 mice per group). The highest value in viral load detected was one day after mice infection (average: 1.08E+05 PFU/g, SD: 143052.661). However, day 5 pi, infected animals were still secreting virus in lungs [1.01E+04 PFU/g (8623.8447)], becoming undetectable at day 10 p.i.

CAT09-infected mice developed pneumonia at day 5 post-infection, fully recovering at day 10 post infection.

Lung tissues from 6 animals per group were histopathologically examined at day 1, 5 and 10 pi. As expected, control animals showed no histopathological lesions (Figure 2). At 1 dpi, three of six infected mice presented histopathological lesions, two of them exhibited necrotizing bronchiolitis and the other one presented bronchointerstitial pneumonia. At day 5 pi, five of six animals presented severe bronchointerstitial pneumonia consisting of moderate to high numbers of lymphoplasmacytic cells and neutrophils infiltrated the bronchiole and surrounding alveoli

(Figure 2). Nevertheless, day 10 pi was characterized by the total resolution of lung lesions in the CAT09-infected animals.

A (H1N1) pdm09 virus induced changes in gene expression levels in the lungs

Gene expression profiles (GEP) at lungs were compared between six infected animals and six mock mice at days 1, 5 and 10 pi. No differences in GEP were found at day 1 pi, but important differences were observed at day 5 pi, paralleling the development of histological pneumonia (Figure 3a and Table S2). In the CAT09-infected mice group, 1264 genes showed a significant variation of their expression levels by day 5 pi compared to the control group (418 up-regulated and 847 down expressed) (Figure 3a and Table S2). Genes showing the most important differences between both groups were interleukin 6 (IL6) (Fold change FC: 86.6), interferon beta 1 (IFNb) (FC: 62.6) and chemokine (C-X-C motif) ligand 10 (IP10), (FC: 43.3). Expression levels of the vast majority of genes normalized by day 10, coinciding with virus clearance and resolution of histological changes (Figure 3a and Table S2). Only 30 out of the 1264 genes kept on showing altered expression levels by day 10 p.i (Figure 3b-c and Table S2). Interestingly, expression levels of IL6 persisted remarkably high by this time point (FC:10.91) along with those of granzyme K (Gzmk) (FC:15.8).

A (H1N1) pdm09 infection turned on the expression of genes involved in the innate response and in the switch to adaptive immunity by day 5 pi.

Since most differences in gene expression were found by day 5 pi, we focused the Ingenuity pathway analysis (IPA) on that day. Notably, Cellular immune response and Cytokine Signaling were the two signaling pathway categories more representative of our analysis (Table S3). The most significant canonical pathways identify by IPA are describe in table 1.

215 Most of these pathways were involved in the innate immune response and inflammation: [Role
216 of Hypercytokinemia/ hyperchemokinemia in the Pathogenesis of Influenza (Figure 4), Hepatic
217 Fibrosis/Hepatic Stellate Cell Activation, Agranulocyte Adhesion and Diapedesis, TREM1
218 Signaling, Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-
219 17A and IL-17F, Granulocyte Adhesion and Diapedesis, Altered T Cell and B Cell Signaling in
220 Rheumatoid Arthritis, Differential Regulation of Cytokine Production in Macrophages and T
221 Helper Cells by IL-17A and IL-17F, Role of IL-17F in Allergic Inflammatory Airway Diseases,
222 Graft-versus-Host Disease Signaling, Role of Macrophages, Fibroblasts and Endothelial Cells in
223 Rheumatoid Arthritis, Role of Pattern Recognition Receptors in Recognition of Bacteria and
224 Viruses and Wnt/β-catenin Signaling pathway]. The vast majority of the genes involved in these
225 pathways codify for cytokines (Table S3). H1N1 virus induced also alterations in pathways
226 participating in the switch from innate to adaptive immunity: [Communication between Innate
227 and Adaptive Immune Cells, Crosstalk between Dendritic Cells and Natural Killer Cells, T
228 Helper Cell Differentiation].
229

Discussion

The overarching aim of this work was to study the role of inflammation at pulmonary level during a non-fatal infection caused by the 2009 pandemic influenza virus using the mice model. In this sense, we analyzed the gene expression profiles (GEP) and its relation to histology and viral dynamics in the lungs of healthy immune-competent mice with pneumonia caused by human influenza A (H1N1) pdm09 virus. Previous works like that performed by Pommerenke et al. have evaluated the transcriptomic response in the lungs of mice infected with the mouse-adapted influenza A virus PR8 (H1N1) (Pommerenke et al., 2012). Nonetheless, the transcriptomic response to 2009 pandemic influenza at the pulmonary level has not been sufficiently studied to the present moment.

Our GEP analysis allowed us to identify the presence of marked activation of innate immunity genes by day 5 post infection, paralleling the existence of extensive pneumonic/cellular infiltrates in the lung, and active viral replication. The innate immune response is the first line of defence against invading viruses (Iwasaki & Pillai, 2014). Infection of the respiratory tract induced thus a typical antiviral response response characterised by the activation of pro-inflammatory cytokines and interferon (IFNs) response genes (ISGs). In our analysis, the genes showing higher differences for their expression levels between infected mice and controls were IL6, IFNb, and IP10. These molecules, along with TNF and IL1b (also over-expressed at day 5), are the major cytokines limiting viral replication during influenza infection, recruiting immune cells to the sites of infection and producing inflammation(Nicholls, 2013).

IL6 is a pro-inflammatory cytokine which role in the pathogenesis of the A (H1N1) pdm09 remains unclear. There is a consensus in the literature about the existence of high systemic levels of IL6 in severe patients infected by A (H1N1) pdm09 virus (Bermejo-Martin et al., 2009) (To et

al., 2010) (Zúñiga et al., 2011). This molecule induces pro-inflammatory responses such as leukocyte recruitment into the lung. Excessive production of IL6 has been associated with several pathological manifestations(Ho, Luo & Lai, 2015) (Baillet et al., 2015). However, Paquette et al demonstrated in IL6 deficient mice infected with A (H1N1) pdm09, that no significant differences in survival, weight loss, viral load, or pathology were observed between IL6 deficient and wild-type mice following infection. Based in our results, presence of high expression levels of this cytokine in the lung at day 10 could indicate that this cytokine plays a role in viral clearance and tissue repair after pneumonia. Other mouse models support the idea of a protective role of IL6 in influenza infections (Lauder et al., 2013).

IFNb is a cytokine member of type I interferon family. It induces an antiviral state in infected and neighbouring cells (Ramos & Fernandez-Sesma, 2015). To do so, IFNs induce the transcription of hundreds of ISGs, which leads to numerous changes in the transcriptome of the cell. Interestingly, in our analysis, some OAS genes (OAS1a, OAS1f, OASL1 and OAS2), IFIT genes (IFIT1, IFIT2 and IFIT3), MX1 and CXCL10, all of them IGS genes, showed high expression levels in the infected mice compared with controls.

The activation of a group of genes involved in the "Role of hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza" pathway, evidence the existence of a local "cytokine storm" in the lung, following infection by A (H1N1) pdm09 virus. The virus activated Th1 and chemokine responses mediated by IL1a, IL1b, IL12b,TNF, MCP1 and RANTES. These results are similar to those found at systemic level in patients with primary viral pneumonia (Bermejo-Martin et al., 2009) (Hagau et al., 2010) (To et al., 2010). In our model, the marked inflammatory program observed by day 5 in the lung got deactivated by day 10, paralleling resolution of histological changes and viral replication.

In turn, the activation of genes involved in “Agranulocyte Adhesion and Diapedesis”, “TREM1 Signaling”, “Granulocyte Adhesion and Diapedesis”, “Graft-versus-Host Disease Signaling and “Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses” might confirm the existence of a transcriptomic program aimed to recruit lymphocytes, monocytes and neutrophils to the site of infection. Histological studies at day 5 pi confirmed the presence of extensive pneumonic/cellular infiltrates into the lung. Although the primary role of the innate immune response is limiting viral replication, excessive activation of innate immunity could induce tissue damage (Vidaña et al., 2014) (de Jong et al., 2006). This phenomenon seems to occur also in the context of autoimmunity diseases such as Rheumatoid Arthritis (Catrina et al., 2016). In fact, “Altered T Cell and B Cell Signaling in Rheumatoid Arthritis” and “Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis” are two of the significant pathways identified by IPA in our analysis. In influenza disease, an exaggerated inflammatory response has been cited as the cause of pulmonary oedema, alveolar haemorrhage and acute respiratory distress syndrome, conditions associated with necrosis and tissue destruction (To et al., 2001).

IPA identified also three pathways related to interleukin 17: “Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F, “Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F and “Role of IL-17F in Allergic Inflammatory Airway Diseases”. Th-17 immunity participates in clearing pathogens during host defence reactions but is involved also in tissue inflammation in several autoimmune diseases, allergic diseases, and asthma (Nalbandian, Crispín & Tsokos, 2009) (Cheung, Wong & Lam, 2008). In severe influenza it has been proposed to play a beneficial role (Iwakura et al., 2008) (Bermejo-Martin et al., 2009) (Almansa et al., 2011b).

IPA also identified low expression levels of a group of genes involved in the "Wnt/β-catenin Signaling pathway" at 5 pi. It has been previously described that influenza virus down-regulates the expression of proteins of this pathway like FZD (Shapira et al., 2009). This is consistent with low expression levels of FZD2 and FZD7 genes found in our analysis. The biological repercussion associated to down-modulation of this pathway remains to be elucidated.

Finally, the activation of those cytokine genes involved in the [Communication between Innate and Adaptive Immune Cells, Crosstalk between Dendritic Cells and Natural Killer Cells, T Helper Cell Differentiation] signalling pathways at day 5 pi. could be reflecting the development of the adaptive immune response against the virus.

Conclusions

In conclusion, our findings suggest a dual role of pulmonary inflammation during non-fatal infection caused by the 2009 pandemic influenza virus. On one side, the activation in the lung of a marked innate immunity transcriptomic program was associated to the appearance of pneumonia, but on the other hand, activation of this program paralleled viral clearance (Figure 5). Understanding the dynamics of the host's transcriptomic and virus changes over the course of the infection caused by A (H1N1) pdm09 might helping getting insight of the immune response profiles associated to effective immune response against influenza virus.

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References:

Almansa R., Anton A., Ramirez P., Martin-Loeches I., Banner D., Pumarola T., Xu L., Blanco J., Ran L., Lopez-Campos G., Martin-Sanchez F., Socias L., Loza A., Andaluz D., Maravi E., Gordón M., Gallegos MC., Fernandez V., León C., Merino P., Marcos MA., Gandía F., Bobillo F., Resino S., Eiros JM., Castro C., Mateo P., Gonzalez-Rivera M., Rello J., de Lejarazu RO., Kelvin DJ., Bermejo-Martin JF. 2011a. Direct association between pharyngeal viral secretion and host cytokine response in severe pandemic influenza. *BMC infectious diseases* 11:232. DOI: 10.1186/1471-2334-11-232.

Almansa R., Bermejo-Martín JF., de Lejarazu Leonardo RO. 2012. Immunopathogenesis of 2009 pandemic influenza. *Enfermedades Infecciosas Y Microbiología Clínica* 30 Suppl 4:18–24. DOI: 10.1016/S0213-005X(12)70100-3.

Almansa R., Heredia-Rodríguez M., Gomez-Sanchez E., Andaluz-Ojeda D., Iglesias V., Rico L., Ortega A., Gomez-Pesquera E., Liu P., Aragón M., Eiros JM., Jiménez-Sousa MÁ., Resino S., Gómez-Herreras I., Bermejo-Martín JF., Tamayo E. 2015. Transcriptomic correlates of organ failure extent in sepsis. *The Journal of Infection* 70:445–456. DOI: 10.1016/j.jinf.2014.12.010.

Almansa R., Socias L., Ramirez P., Martin-Loeches I., Vallés J., Loza A., Rello J., Kelvin DJ., León C., Blanco J., Andaluz D., Micheloud D., Maraví E., Ortiz de Lejarazu R., Bermejo-Martin JF. 2011b. Imbalanced pro- and anti-Th17 responses (IL-17/granulocyte colony-stimulating factor) predict fatal outcome in 2009 pandemic influenza. *Critical Care (London, England)* 15:448. DOI: 10.1186/cc10426.

Baillet A., Gossec L., Paternotte S., Etcheto A., Combe B., Meyer O., Mariette X., Gottenberg J-E., Dougados M. 2015. Evaluation of Serum Interleukin-6 Level as a Surrogate Marker of Synovial Inflammation and as a Factor of Structural Progression in Early Rheumatoid Arthritis: Results From a French National Multicenter Cohort. *Arthritis Care & Research* 67:905–912. DOI: 10.1002/acr.22513.

Bermejo-Martin JF., Martin-Loeches I., Rello J., Antón A., Almansa R., Xu L., Lopez-Campos G., Pumarola T., Ran L., Ramirez P., Banner D., Ng DC., Socias L., Loza A., Andaluz D., Maravi E., Gómez-Sánchez MJ., Gordón M., Gallegos MC., Fernandez V., Aldunate S., León C., Merino P., Blanco J., Martin-Sanchez F., Rico L., Varillas D., Iglesias V., Marcos MÁ., Gandía F., Bobillo F., Nogueira B., Rojo S., Resino S., Castro C., Ortiz de Lejarazu R., Kelvin D. 2010. Host adaptive immunity deficiency in severe pandemic influenza. *Critical Care (London, England)* 14:R167. DOI: 10.1186/cc9259.

Bermejo-Martin JF., Ortiz de Lejarazu R., Pumarola T., Rello J., Almansa R., Ramírez P., Martin-Loeches I., Varillas D., Gallegos MC., Serón C., Micheloud D., Gomez JM., Tenorio-Abreu A., Ramos MJ., Molina ML., Huidobro S., Sanchez E., Gordón M., Fernández V., Del

Castillo A., Marcos MA., Villanueva B., López CJ., Rodríguez-Domínguez M., Galan J-C., Cantón R., Lietor A., Rojo S., Eiros JM., Hinojosa C., Gonzalez I., Torner N., Banner D., Leon A., Cuesta P., Rowe T., Kelvin DJ. 2009. Th1 and Th17 hypercytokinemia as early host response signature in severe pandemic influenza. *Critical Care (London, England)* 13:R201. DOI: 10.1186/cc8208.

Busquets N., Segalés J., Córdoba L., Mussá T., Crisci E., Martín-Valls GE., Simon-Grifé M., Pérez-Simó M., Pérez-Maíllo M., Núñez JI., Abad FX., Fraile L., Pina S., Majó N., Bensaid A., Domingo M., Montoya M. 2010. Experimental infection with H1N1 European swine influenza virus protects pigs from an infection with the 2009 pandemic H1N1 human influenza virus. *Veterinary Research* 41:74. DOI: 10.1051/vetres/2010046.

Catrina AI., Joshua V., Klareskog L., Malmström V. 2016. Mechanisms involved in triggering rheumatoid arthritis. *Immunological Reviews* 269:162–174. DOI: 10.1111/imr.12379.

Cheung PFY., Wong CK., Lam CWK. 2008. Molecular mechanisms of cytokine and chemokine release from eosinophils activated by IL-17A, IL-17F, and IL-23: implication for Th17 lymphocytes-mediated allergic inflammation. *Journal of Immunology (Baltimore, Md.: 1950)* 180:5625–5635.

Hagau N., Slavcovici A., Gonganau DN., Oltean S., Dirzu DS., Brezoszki ES., Maxim M., Ciuce C., Mlesnite M., Gavrus RL., Laslo C., Hagau R., Petrescu M., Studnicska DM. 2010. Clinical aspects and cytokine response in severe H1N1 influenza A virus infection. *Critical Care (London, England)* 14:R203. DOI: 10.1186/cc9324.

Health Protection Agency, Health Protection Scotland, National Public Health Service for Wales, HPA Northern Ireland Swine influenza investigation teams 2009. Epidemiology of new influenza A (H1N1) virus infection, United Kingdom, April-June 2009. *Euro Surveillance: Bulletin Européen Sur Les Maladies Transmissibles = European Communicable Disease Bulletin* 14.

Ho L-J., Luo S-F., Lai J-H. 2015. Biological effects of interleukin-6: Clinical applications in autoimmune diseases and cancers. *Biochemical Pharmacology* 97:16–26. DOI: 10.1016/j.bcp.2015.06.009.

Itoh Y., Shinya K., Kiso M., Watanabe T., Sakoda Y., Hatta M., Muramoto Y., Tamura D., Sakai-Tagawa Y., Noda T., Sakabe S., Imai M., Hatta Y., Watanabe S., Li C., Yamada S., Fujii K., Murakami S., Imai H., Kakugawa S., Ito M., Takano R., Iwatsuki-Horimoto K., Shimojima M., Horimoto T., Goto H., Takahashi K., Makino A., Ishigaki H., Nakayama M., Okamatsu M., Takahashi K., Warshauer D., Shult PA., Saito R., Suzuki H., Furuta Y., Yamashita M., Mitamura K., Nakano K., Nakamura M., Brockman-Schneider R., Mitamura H., Yamazaki M., Sugaya N., Suresh M., Ozawa M., Neumann G., Gern J., Kida H., Ogasawara K., Kawaoka Y. 2009. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. *Nature* 460:1021–1025. DOI: 10.1038/nature08260.

Iwakura Y., Nakae S., Saijo S., Ishigame H. 2008. The roles of IL-17A in inflammatory immune responses and host defense against pathogens. *Immunological Reviews* 226:57–79. DOI: 10.1111/j.1600-065X.2008.00699.x.

Iwasaki A., Pillai PS. 2014. Innate immunity to influenza virus infection. *Nature Reviews. Immunology* 14:315–328. DOI: 10.1038/nri3665.

Jain S., Kamimoto L., Bramley AM., Schmitz AM., Benoit SR., Louie J., Sugerman DE., Druckenmiller JK., Ritger KA., Chugh R., Jasuja S., Deutscher M., Chen S., Walker JD., Duchin JS., Lett S., Soliva S., Wells EV., Swerdlow D., Uyeki TM., Fiore AE., Olsen SJ., Fry AM., Bridges CB., Finelli L., 2009 Pandemic Influenza A (H1N1) Virus Hospitalizations Investigation

Team 2009. Hospitalized patients with 2009 H1N1 influenza in the United States, April-June 2009. *The New England Journal of Medicine* 361:1935–1944. DOI: 10.1056/NEJMoa0906695.
de Jong MD., Simmons CP., Thanh TT., Hien VM., Smith GJD., Chau TNB., Hoang DM., Van Vinh Chau N., Khanh TH., Dong VC., Qui PT., Van Cam B., Ha DQ., Guan Y., Peiris JSM., Chinh NT., Hien TT., Farrar J. 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nature Medicine* 12:1203–1207. DOI: 10.1038/nm1477.
Lauder SN., Jones E., Smart K., Bloom A., Williams AS., Hindley JP., Ondondo B., Taylor PR., Clement M., Fielding C., Godkin AJ., Jones SA., Gallimore AM. 2013. Interleukin-6 limits influenza-induced inflammation and protects against fatal lung pathology. *European Journal of Immunology* 43:2613–2625. DOI: 10.1002/eji.201243018.
Lee N., Chan PKS., Hui DSC., Rainer TH., Wong E., Choi K-W., Lui GCY., Wong BCK., Wong RYK., Lam W-Y., Chu IMT., Lai RWM., Cockram CS., Sung JJY. 2009. Viral loads and duration of viral shedding in adult patients hospitalized with influenza. *The Journal of Infectious Diseases* 200:492–500. DOI: 10.1086/600383.
Martínez-Orellana P., Martorell J., Vidaña B., Majó N., Martínez J., Falcón A., Rodríguez-Frandsen A., Casas I., Pozo F., García-Migura L., García-Barreno B., Melero JA., Fraile L., Nieto A., Montoya M. 2015. Clinical response to pandemic h1n1 influenza virus from a fatal and mild case in ferrets. *Virology Journal* 12. DOI: 10.1186/s12985-015-0272-x.
Nalbandian A., Crispín JC., Tsokos GC. 2009. Interleukin-17 and systemic lupus erythematosus: current concepts. *Clinical and Experimental Immunology* 157:209–215. DOI: 10.1111/j.1365-2249.2009.03944.x.
Nicholls JM. 2013. The battle between influenza and the innate immune response in the human respiratory tract. *Infection & Chemotherapy* 45:11–21. DOI: 10.3947/ic.2013.45.1.11.
Pommerenke C., Wilk E., Srivastava B., Schulze A., Novoselova N., Geffers R., Schughart K. 2012. Global transcriptome analysis in influenza-infected mouse lungs reveals the kinetics of innate and adaptive host immune responses. *PloS One* 7:e41169. DOI: 10.1371/journal.pone.0041169.
Ramos I., Fernandez-Sesma A. 2015. Modulating the Innate Immune Response to Influenza A Virus: Potential Therapeutic Use of Anti-Inflammatory Drugs. *Frontiers in Immunology* 6:361. DOI: 10.3389/fimmu.2015.00361.
Shapira SD., Gat-Viks I., Shum BOV., Dricot A., de Grace MM., Wu L., Gupta PB., Hao T., Silver SJ., Root DE., Hill DE., Regev A., Hacohen N. 2009. A Physical and Regulatory Map of Host-Influenza Interactions Reveals Pathways in H1N1 Infection. *Cell* 139:1255–1267. DOI: 10.1016/j.cell.2009.12.018.
Tamayo E., Almansa R., Carrasco E., Ávila-Alonso A., Rodríguez-Fernández A., Wain J., Heredia M., Gomez-Sanchez E., Soria S., Rico L., Iglesias V., Martínez-Martínez A., Andaluz-Ojeda D., Herreras JIG., Eiros JM., Bermejo-Martin JF. 2014. Quantification of IgM molecular response by droplet digital PCR as a potential tool for the early diagnosis of sepsis. *Critical Care (London, England)* 18:433. DOI: 10.1186/cc13910.
To KF., Chan PK., Chan KF., Lee WK., Lam WY., Wong KF., Tang NL., Tsang DN., Sung RY., Buckley TA., Tam JS., Cheng AF. 2001. Pathology of fatal human infection associated with avian influenza A H5N1 virus. *Journal of Medical Virology* 63:242–246.
To KKW., Hung IFN., Li IWS., Lee K-L., Koo C-K., Yan W-W., Liu R., Ho K-Y., Chu K-H., Watt C-L., Luk W-K., Lai K-Y., Chow F-L., Mok T., Buckley T., Chan JFW., Wong SSY., Zheng B., Chen H., Lau CCY., Tse H., Cheng VCC., Chan K-H., Yuen K-Y. 2010. Delayed

clearance of viral load and marked cytokine activation in severe cases of pandemic H1N1 2009 influenza virus infection. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America* 50:850–859. DOI: 10.1086/650581.

Vemula SV., Zhao J., Liu J., Wang X., Biswas S., Hewlett I. 2016. Current Approaches for Diagnosis of Influenza Virus Infections in Humans. *Viruses* 8:96. DOI: 10.3390/v8040096.

Vidaña B., Martínez J., Martínez-Orellana P., García Migura L., Montoya M., Martorell J., Majó N. 2014. Heterogeneous pathological outcomes after experimental pH1N1 influenza infection in ferrets correlate with viral replication and host immune responses in the lung. *Veterinary Research* 45. DOI: 10.1186/s13567-014-0085-8.

Zúñiga J., Torres M., Romo J., Torres D., Jiménez L., Ramírez G., Cruz A., Espinosa E., Herrera T., Buendía I., Ramírez-Venegas A., González Y., Bobadilla K., Hernández F., García J., Quiñones-Falconi F., Sada E., Manjarrez ME., Cabello C., Kawa S., Zlotnik A., Pardo A., Selman M. 2011. Inflammatory profiles in severe pneumonia associated with the pandemic influenza A/H1N1 virus isolated in Mexico City. *Autoimmunity* 44:562–570. DOI: 10.3109/08916934.2011.592885.

Figure legends:

Figure 1: Changes in body weight and lung viral load induced by A (H1N1) pdm09 virus.

A) Average weight curve for C57BL6 mice infected through intranasal instillation with 50 µL CAT09 at 10^4 PFU A/Catalonia/63/2009 (H1N1pdm) and mock.

B) Viral load in lung homogenates collected at days 1, 5 and 10 pi. (n=6 for all groups). Infection of Madin-Darby Canine Kidney cells was employed to measure viral titers. The U Mann-Whitney test was used to compare weight loss and viral load between groups at all sampling time. The significance level (α) was set at 0.05. Asterisks indicate significant differences between groups.

Figure 2: Histopathology of mice belonging to control and CAT09 groups at day 1, 5 and 10 pi.

At day 1 pi, infected groups presented histopathological lesions that went from a mild necrotizing bronchiolitis to an interstitial pneumonia lesion. At day 5 pi, infected animals presented severe bronchointerstitial pneumonia consisting of moderate to high numbers of

485 lymphoplasmacytic cells and neutrophils infiltrated the bronchiole and surrounding alveoli. At
486 day 10 pi, no histopathological lesions were observed in any group. Hematoxilin/Eosin stain.
487
488 ***Figure 3: Pulmonary gene expression profiles at day 1, 5 and 10 post infection.***
489 A) Volcano plots for the representation of the number of genes with significant variation
490 of their expression levels between CAT09 and mock groups, at different time points
491 (1, 5 and 10 dpi). The level of significance was fixed in $p < 0.05$, with Benjamini-
492 Hochberg multiple testing corrections and Fold change ≥ 2.
493 B) Venn diagram showing those genes whose expression levels differed from controls
494 either at day 5 and day 10, and those which differed only at one time point.
495 C) Heatmap of the common signature across different time points. The colour is
496 proportional to their fold change (FC) compared to mock group, with the scale
497 ranging from -4.2 FC (blue) to 4.2 FC (red).
498
499 ***Table 1: Top 20 Canonical signaling pathways altered by A (H1N1)pdm 09 virus.***
500 This table summarized the most significant canonical pathways identify by "Ingenuity pathway
501 analysis". The IPA system implements Fisher's exact test to determine whether a canonical
502 pathway is enriched with genes of interest (the level of significance was fixed in $p < 0.05$). The
503 ratio show the number of genes whose expression levels were different between CAT09 and
504 mock groups, of the total of genes that have been described previously in each pathway.
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506 ***Figure 4: Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza***
507 ***signaling pathway.***

"Ingenuity pathway analysis" identified this route as the most altered pathway of the analysis. Red: genes up-regulated in the infected group compared with non-infected mice.

Figure 5: Model of uncomplicated A (H1N1) pdm09 viral infection:

The virus induced the activation of a marked pro-inflammatory program at the lung level paralleling the emergence of histological changes. This program was associated to viral clearance, and its resolution was accompanied by resolution of pneumonia.

Supplemental information:

Fig. S1: Droplet digital PCR validation of microarray data: Expression values obtained from the microarrays for IFNB1 and IL6 genes showed a significant positive correlation, confirmed by using digital droplet PCR.

Table S2: List of genes differentially expressed between infected mice and controls. FC: fold change.

Table S3: Genes involved in the top 20 canonical signalling pathways altered by A (H1N1) pdm 09 virus at day 5 post infection.

FC: fold change.

Figure 1

Changes in body weight and lung viral load induced by A (H1N1) pdm09 virus.

A) Average weight curve for C57BL6 mice infected through intranasal instillation with 50 µL CAT09 at10^4PFU A/Catalonia/63/2009 (H1N1pdm) and mock.

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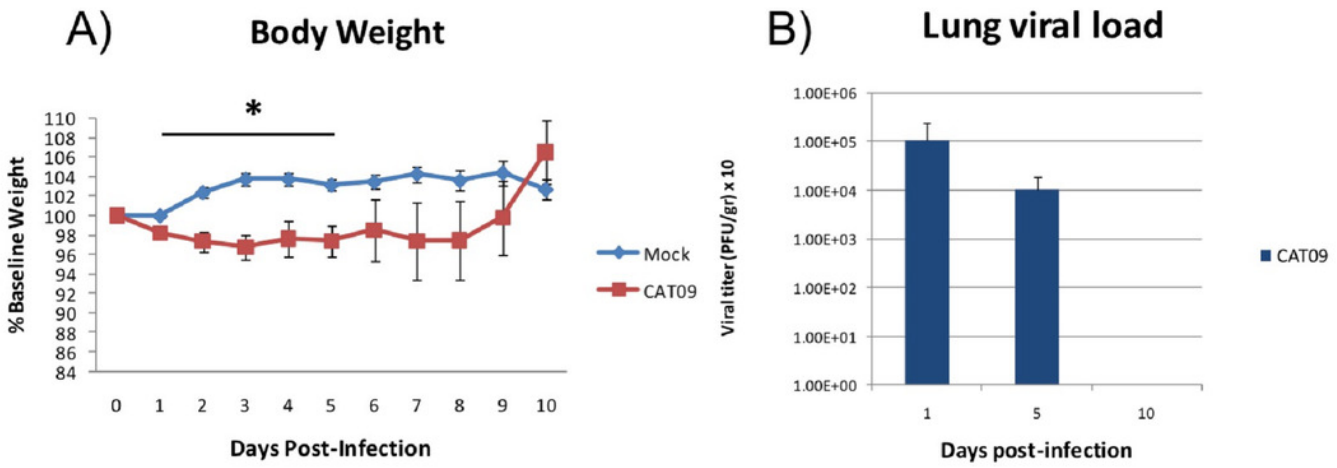

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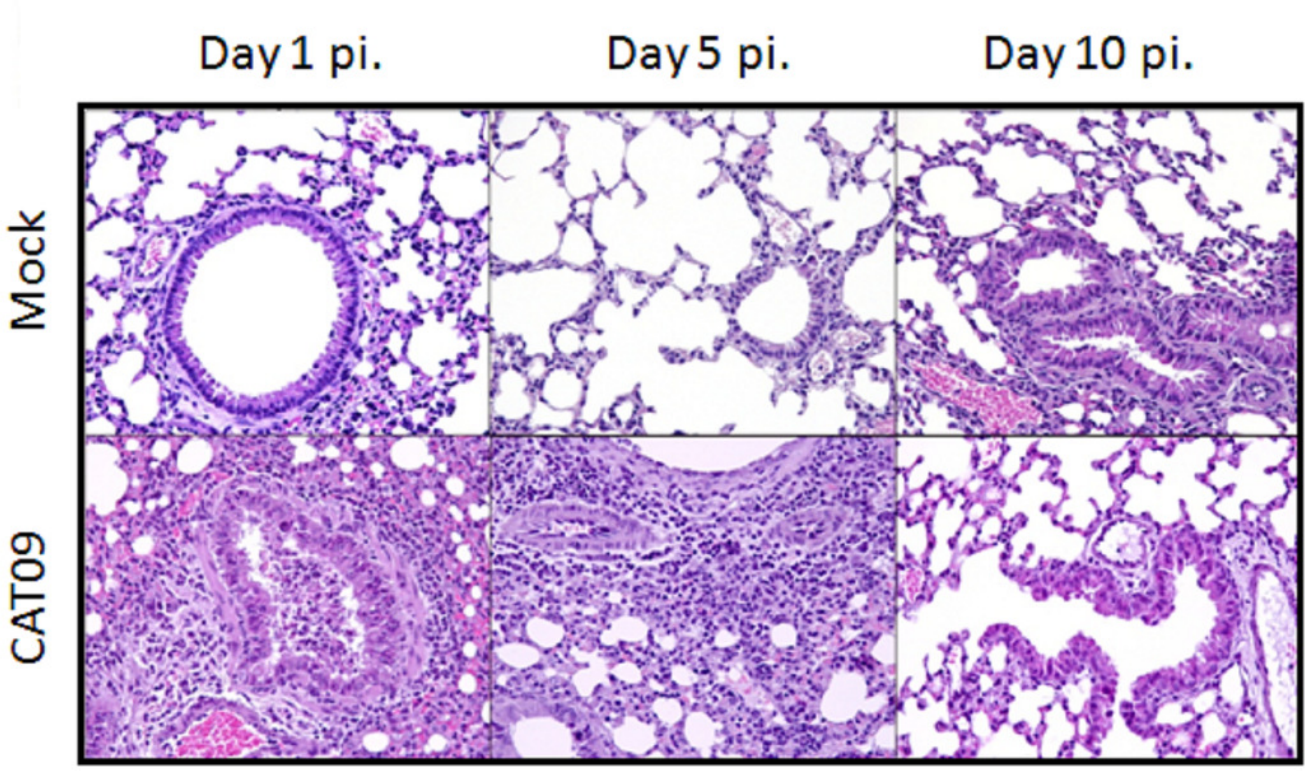

Figure 3

Pulmonary gene expression profiles at day 1, 5 and 10 post infection.

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B) Venn diagram showing those genes whose expression levels differed from controls either at day 5 and day 10, and those which differed only at one time point.

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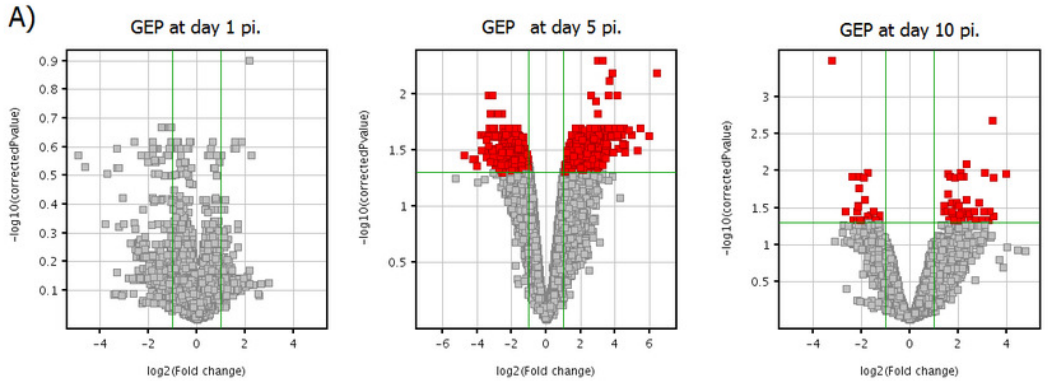
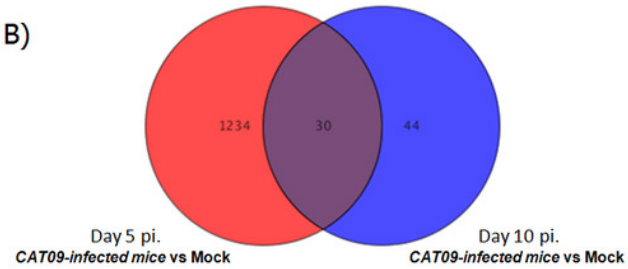

A)
GEP at day 1 pi.
GEP at day 5 pi.
GEP at day 10 pi.
-log10(correctedPvalue)
log2(Fold change)


B)
1234
30
44
Day 5 pi.
CAT09-infected mice vs Mock
Day 10 pi.
CAT09-infected mice vs Mock

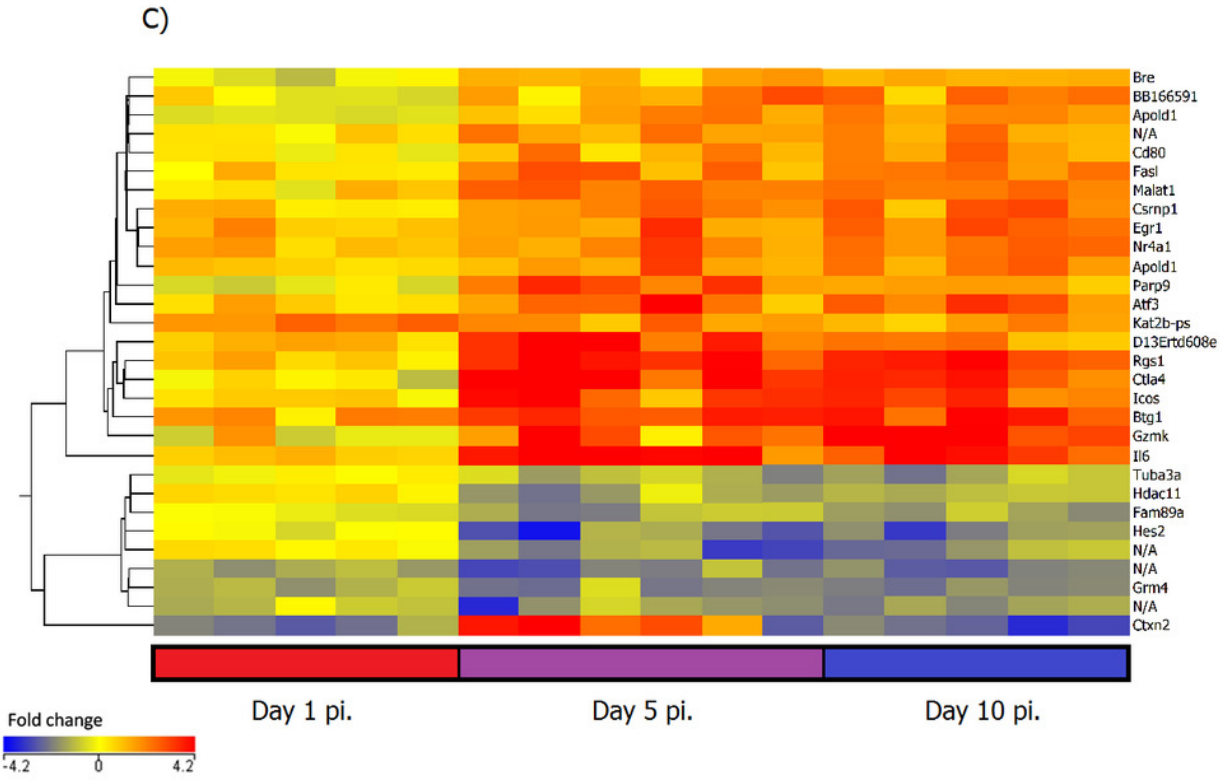

C)
Bre
BB166591
Apold1
N/A
Cd80
Fasl
Malat1
Csrnp1
Egr1
Nr4a1
Apold1
Parp9
Atf3
Kat2b-ps
D13Ertd608e
Rgs1
Ctla4
Icos
Btg1
Gzmk
Il6
Tuba3a
Hdac11
Fam89a
Hes2
N/A
N/A
Grm4
N/A
Ctxn2
Day 1 pi.
Day 5 pi.
Day 10 pi.
Fold change
-4.2
0
4.2

Table 1(on next page)

Top 20 Canonical signaling pathways altered by A (H1N1)pdm 09 virus.

This table summarized the most significant canonical pathways identify by “Ingenuity pathway analysis”. The IPA system implements Fisher's exact test to determine whether a canonical pathway is enriched with genes of interest (the level of significance was fixed in $p < 0.05$). The ratio show the number of genes whose expression levels were different between CAT09 and mock groups, of the total of genes that have been described previously in each pathway.

1 ***Top 20 Canonical signaling pathways altered by A (H1N1)pdm 09 virus***

Ingenuity Canonical Pathways	*p value*	Ratio	Top Functions & Diseases
Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza	<0.001	0.244	Cell-To-Cell Signaling and Interaction; Cellular Movement; Hematological System Development and Function
Hepatic Fibrosis / Hepatic Stellate Cell Activation	<0.001	0.122	Organismal Injury and Abnormalities; Cardiovascular System Development and Function; Organismal Development
Communication between Innate and Adaptive Immune Cells	<0.001	0.165	Cell-To-Cell Signaling and Interaction; Cellular Growth and Proliferation; Hematological System Development and Function
Wnt/β-catenin Signaling	<0.001	0.124	Gene Expression; Cellular Development; Tissue Development
Agranulocyte Adhesion and Diapedesis	<0.001	0.116	Cell-To-Cell Signaling and Interaction; Tissue Development; Hematological System Development and Function
TREM1 Signaling	<0.001	0.173	Cell-To-Cell Signaling and Interaction; Hematological System Development and Function; Immune Cell Trafficking
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	<0.001	0.304	Cell-To-Cell Signaling and Interaction; Hematological System Development and Function; Immune Cell Trafficking
Granulocyte Adhesion and Diapedesis	<0.001	0.113	Cell-To-Cell Signaling and Interaction; Hematological System Development and Function; Immune Cell Trafficking
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	<0.001	0.148	Hematological System Development and Function; Tissue Morphology; Cellular Development
Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	<0.001	0.333	Cell-To-Cell Signaling and Interaction; Hematological System Development and Function; Immune Cell Trafficking
Role of IL-17F in Allergic Inflammatory Airway Diseases	<0.001	0.205	Connective Tissue Disorders; Immunological Disease; Inflammatory Disease
Crosstalk between Dendritic Cells and Natural Killer Cells	<0.001	0.146	Cell-To-Cell Signaling and Interaction; Cellular Growth and Proliferation; Hematological System Development and Function
HMGB1 Signaling	<0.001	0.125	Cell-To-Cell Signaling and Interaction; Cellular Movement; Hematological System Development and Function
Graft-versus-Host Disease Signaling	<0.001	0.188	Cellular Immune Response; Disease-Specific Pathways
T Helper Cell Differentiation	<0.001	0.155	Cell-mediated Immune Response; Cellular Development; Cellular Function and Maintenance
Atherosclerosis Signaling	<0.001	0.122	Cell-To-Cell Signaling and Interaction; Cellular Movement; Hematological System Development and Function
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	<0.001	0.087	Cell Death and Survival; Cellular Development; Cellular Growth and Proliferation
Colorectal Cancer Metastasis Signaling	<0.001	0.093	Cell Death and Survival; Cell Cycle; Cellular Development
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	0.001	0.091	Hematological System Development and Function; Tissue Morphology; Cellular Development
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	0.001	0.110	Antimicrobial Response; Inflammatory Response; Infectious Disease

Figure 4

Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza signaling pathway.

"Ingenuity pathway analysis" identified this route as the most altered pathway of the analysis. Red: genes up-regulated in the infected group compared with non-infected mice.

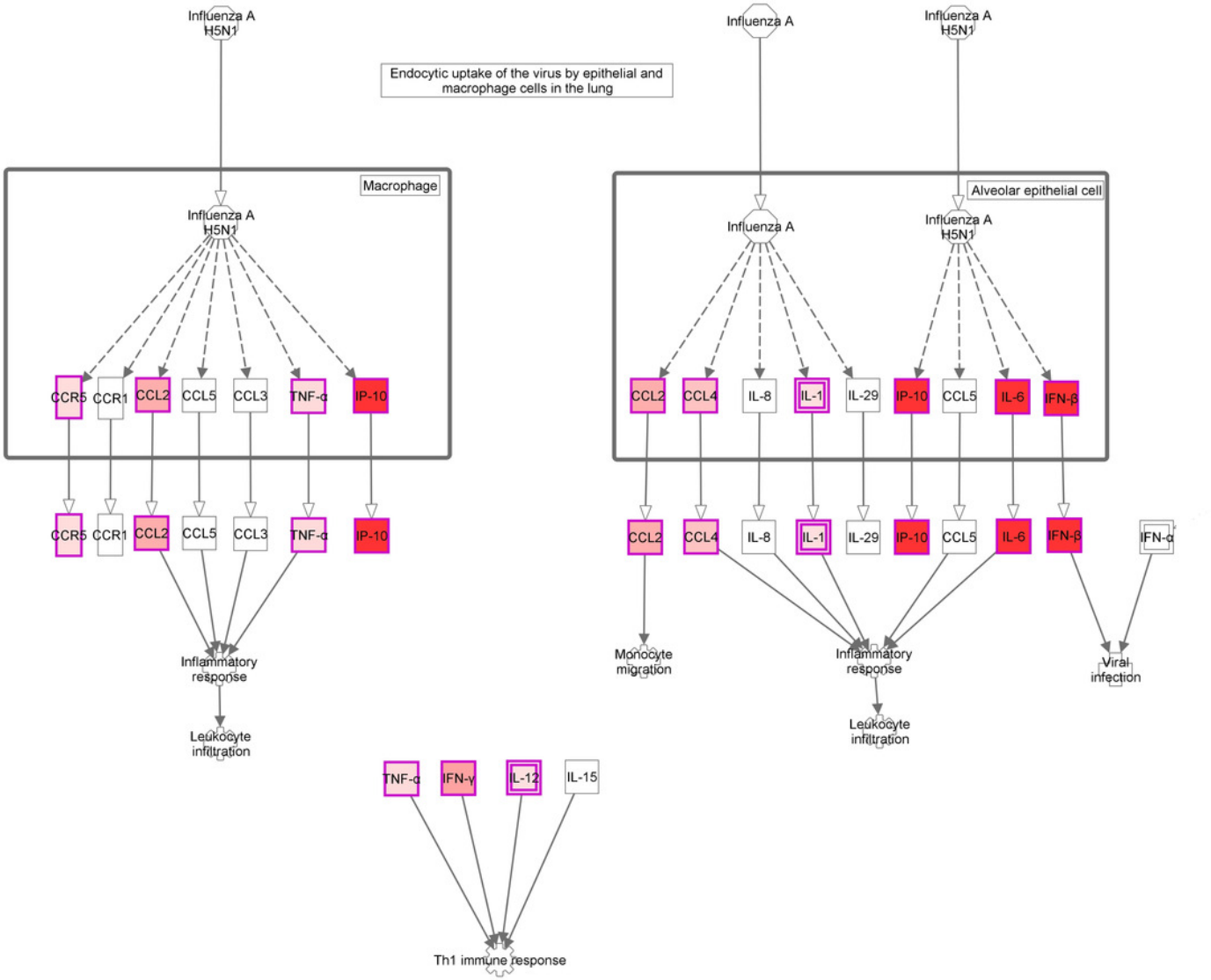


Figure 5

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