Immunité Anti-Grippale A(H1N1)2009 au cours des formes sévères/fatales de l'infection

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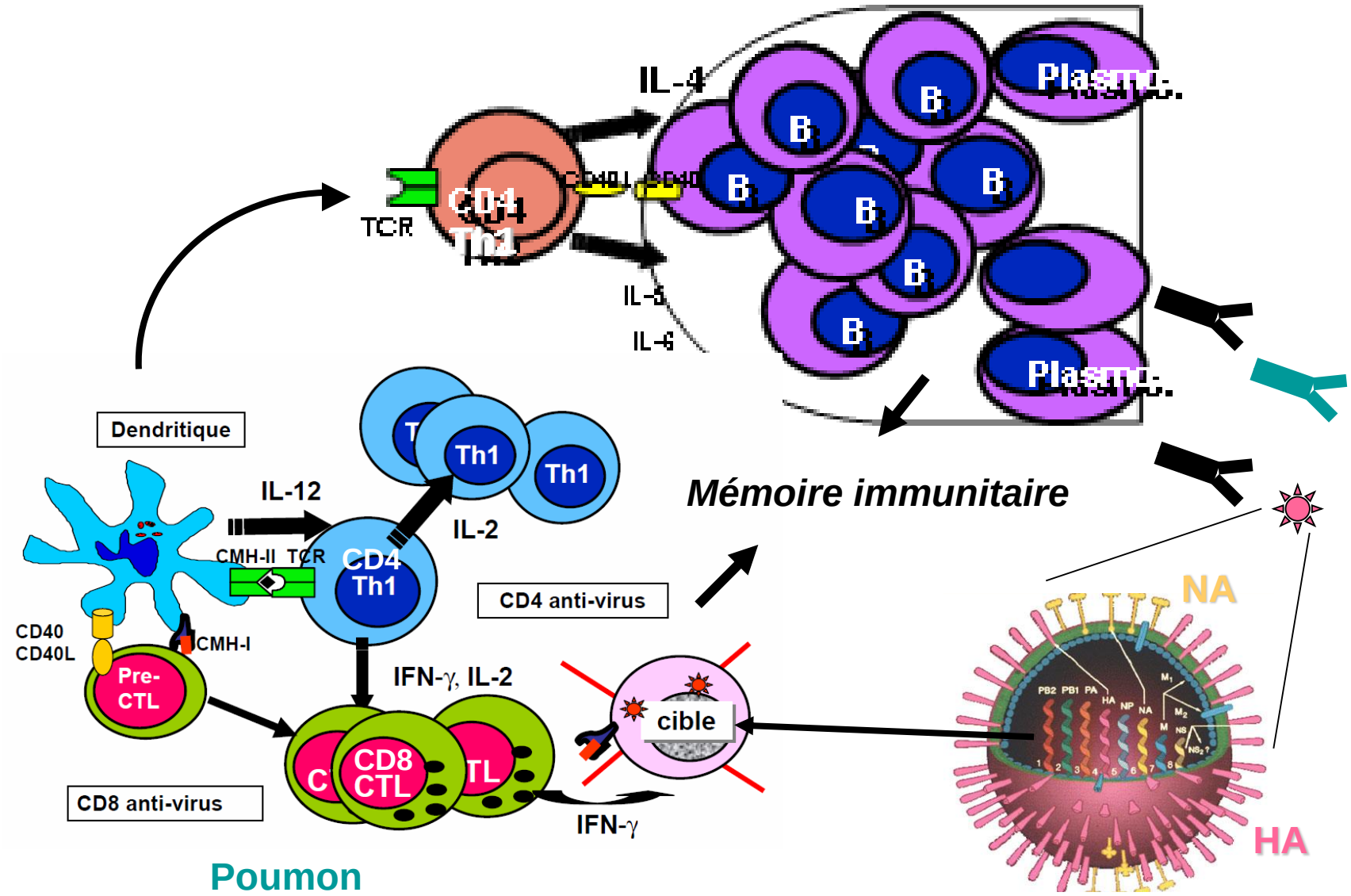
Both influenza-specific T cell and B cell responses participate to viral control



Humoral and cellular responses to influenza differ in target and capacities of viral clearance

Humoral responses :

- Neuraminidase, **Hemagglutinine** :
  - principal target of neutralizing antibodies, annual mutations++
  - poor cross reactivity

⇨ Protective responses for vaccines
Prevent infection

Cellular responses :

- Directed against **most influenza proteins** : NP, PA, M, HA, NS…
- Broad **cross-reactive** capacities

McMichael et al, NEJM 1983; 309:13

- CD8 cells control secondary infection in the absence of B cells and antibodies

Epstein et al, J Immunol 1998;160:322

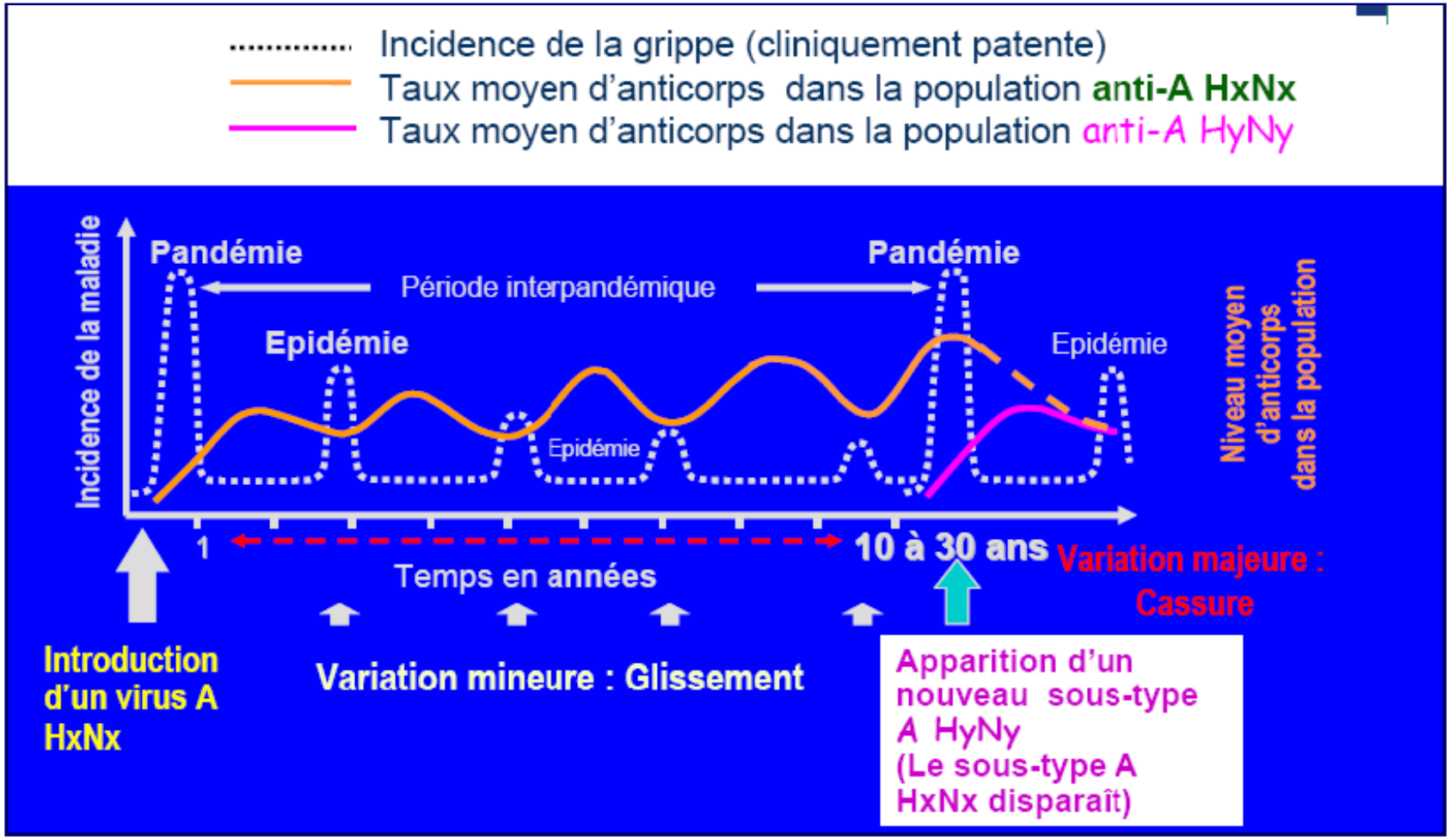
⇨ **Limit morbidity**

Influenza-specific immune responses depend on previous antigen encounter

HUMORAL RESPONSES	Low affinity antibodies Low cross reactivity Stimulation of Naïve B cell pool	Low to moderate affinity antibodies Cross-reactive antibodies Memory B cells Naïve B cell pool stimulation	High affinity antibodies High Cross-reactivity Memory B cells
CELLULAR RESPONSES	Low affinity TCR Recognition of conserved T cell epitopes Stimulation of Naive T cell pool	Low to moderate affinity TCR TCR directed against conserved epitopes Effector/memory cross-reactive T cells Stimulation of Naive T cell pool	High affinity TCR High Cross-reactivity TEM And TCM

Combadière B et al, Pathol Biol 2010;58:e-79

Influenza epidemics and pandemics are linked to immune memory responses to the virus



CMIT- Fédération française d'infectiologie 2009

T cell epitopes from previously circulating H1N1 strains are conserved in the A(H1N1)2009 virus

Table 2. Distribution of epitopes among the influenza proteins

	B-cell		T-cell, CD8+		T-cell, CD4+		Overall		
Protein	Total	Cons.	Total	Cons.	Total	Cons.	Total	Cons.	Cons. (%)
HA	5	1	4	1	34	3	43	5	12
NA	1	0	2	1	3	0	6	1	17
M1	4	1	17	13	28	14	49	28	57
M2	4	1	1	0	3	0	8	1	13
NS1	1	0	2	1	2	1	5	2	40
NS2	0	0	1	1	1	0	2	1	50
NP	9	4	19	15	43	21	71	40	56
PA	0	0	7	4	1	1	8	5	63
PB1	2	1	23	17	21	16	46	34	74
PB1-F2	0	0	0	0	0	0	0	NA	NA
PB2	0	0	2	1	3	1	5	2	40

The total number of epitopes in the H1N1 seasonal flu strains from 1988–2008 (Total) as well as the number of epitopes conserved in swine-origin H1N1 influenza virus (S-OIV) (Cons.) are listed.

Therefore, the conservation of a large fraction of T-cell epitopes suggests that the severity of an S-OIV infection, as far as it is determined by susceptibility of the virus to immune attack, would not differ much from that of seasonal flu.

Greenbaum et al. PNAS 2009

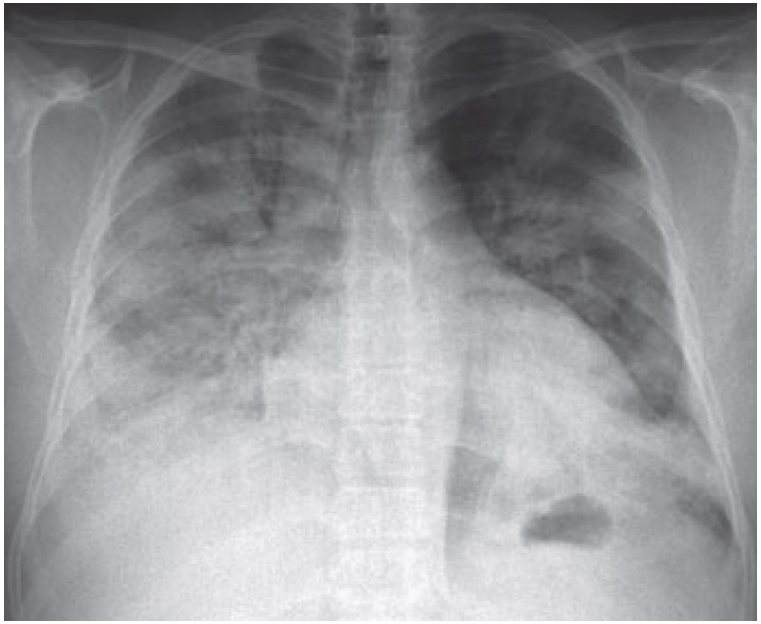
Influenza A(H1N1)2009 variant induces severe pneumonia with ARDS

- Pandemic influenza A (H1N1) 2009 virus
- **Severe respiratory disease** : 28.7 case/million people
- ARDS 50% in ICU

ANZIC investigators, NEJM 2009;361

- 65-75% of ICU patients undergo mechanical ventilation

Rello, Crit Care 2009;13:R148



Perez-Padilla et al, NEJM 2009;361:680

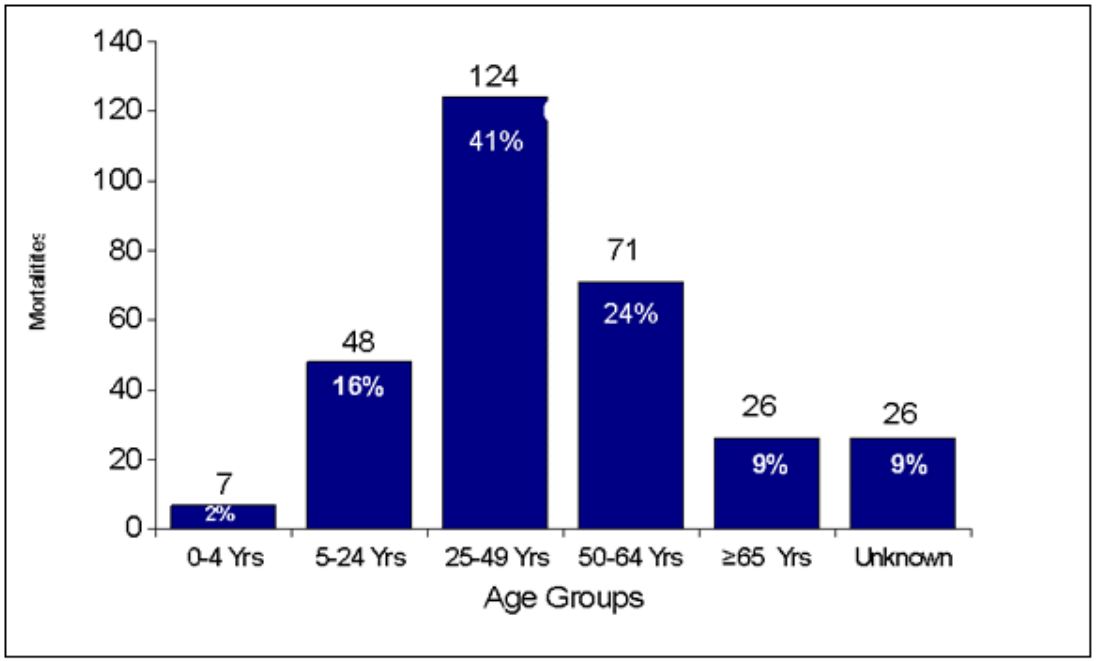
Risk factor	Cases (n = 32)
Obesity	10
BMI > 40 / BMI 30/40	4 / 6
Asthma	5
COPD	4
Pregancy	2
Cardiac failure	1
Hypertension	1
Diabetes	1
HIV	1
Neur-muscular disease	1
Hemopathy	1
None	15

Influenza A(H1N1)2009 variant: higher mortality rates in young adults

- **High mortality in young patients** : unusual during seasonal influenza epidemics
- 30% of severe cases **without** comorbidities

ANZIC investigators, NEJM 2009;361

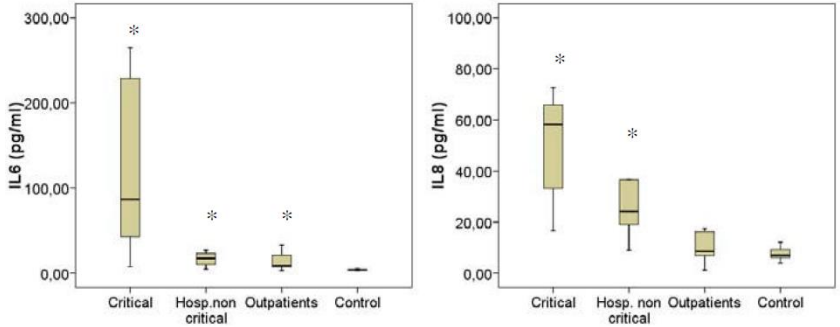
Graph C: Novel H1N1 U.S. Deaths, By Age Group



http://www.cdc.gov/H1N1FLU/surveillanceqa.htm (winter 2009)

Immunopathology of severe pulmonary influenza infection (H1N1)2009 : cytokines

- **Pro-inflammatory cytokine environment:**



n=10 pts in each group

Bermejo-Martin et al, Critical Care 2009;13,R201

Tramontana et *al, Emerg Infect Dis. 2010;16:1068*

Yu et al, PlosOne 2011;6:28680

  - In more severe forms of H1N1v pneumonia: IL-6, IL-8, MCP-1, sTNFR-1

Lee et al, Options for the Control of Influenza VII, 2010, O-828

- **IL-6 plasma levels associated with severity**

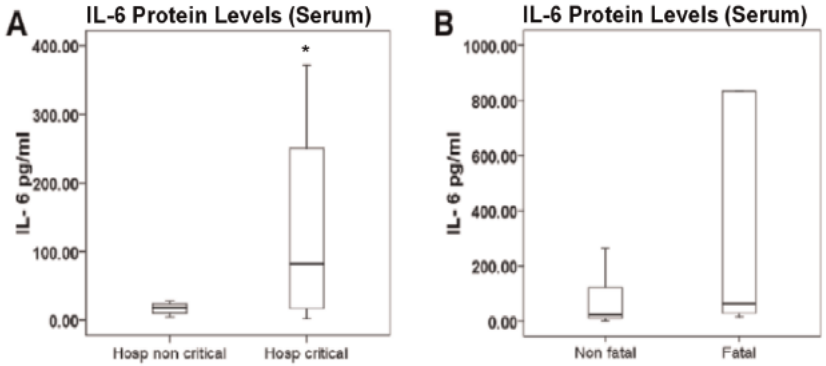
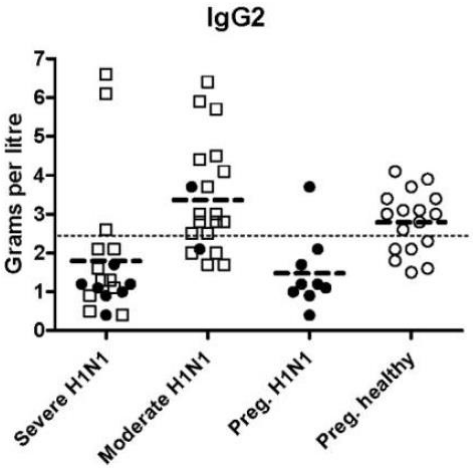


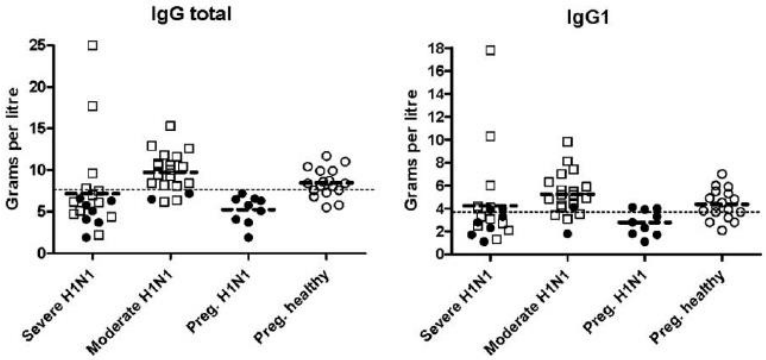
Figure 1. IL-6 levels associated strongly with disease severity in patients hospitalized with H1N1pdm infection. Serum IL-6 levels in hospitalized patients with laboratory-confirmed H1N1pdm infection. Comparison between different groups: between patients requiring critical (n = 35) or non-critical (n = 10) care (A) and between patients who survived (n = 35) or died (n = 10) (B). Mann-Whitney U test was applied to assess statistical significance of differences between groups; p-values <0.05 are indicated by an asterisk (*).
doi:10.1371/journal.pone.0038214.g001

Paquette PlosOne 2012;7:e38214

Immunopathology of severe pulmonary influenza infection (H1N1)2009 (3)

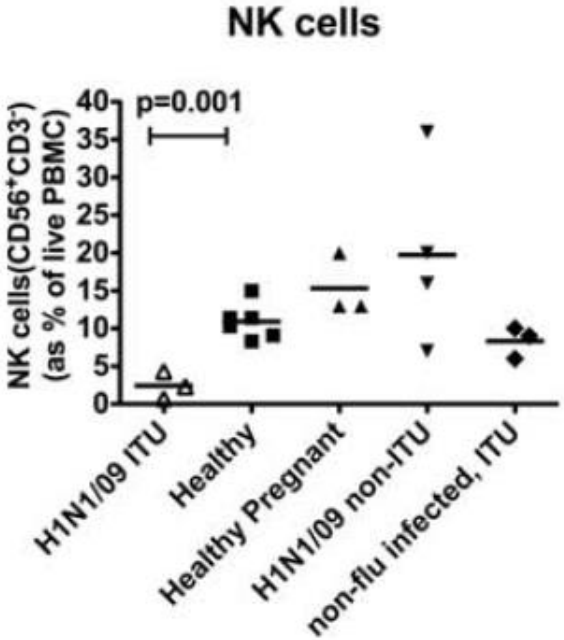
- **Lack of serum IgG2:**





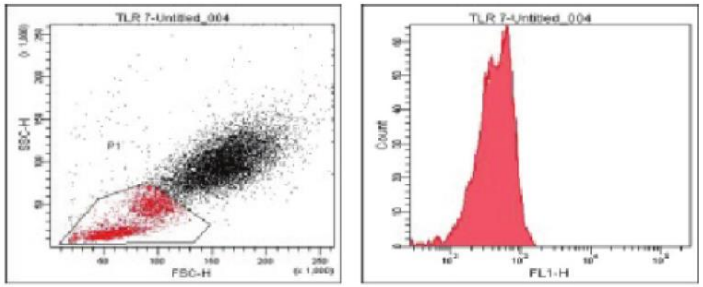
Gordon et al, Clin Infect Dis 2010; 50:672

- **Lack of blood NK cells:**



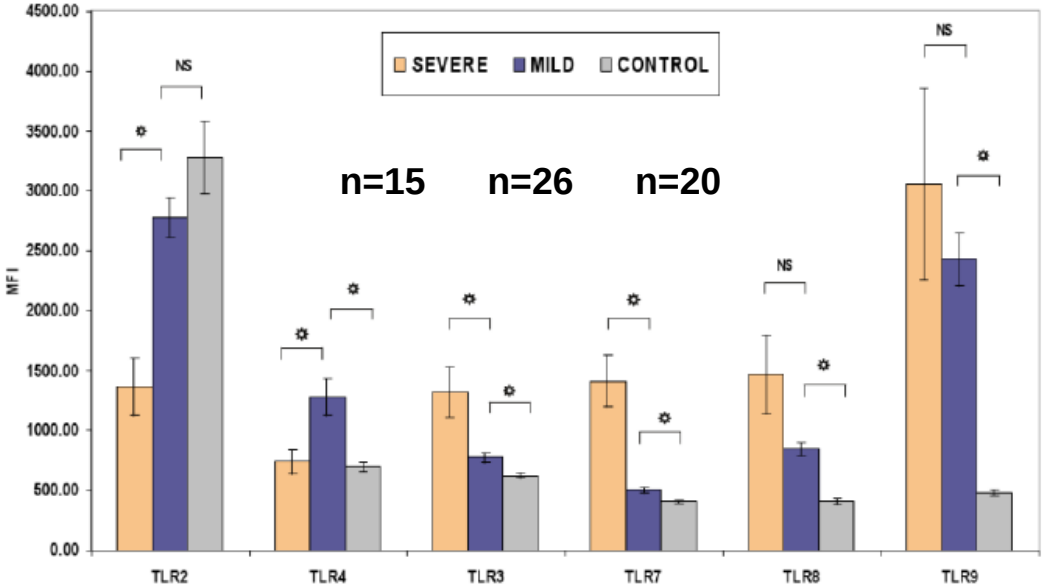
Denney et al, Plos One 2010;5:e10675

TLR receptors sensing viral infections are overexpressed in PBMC during severe A(H1N1)2009 infection

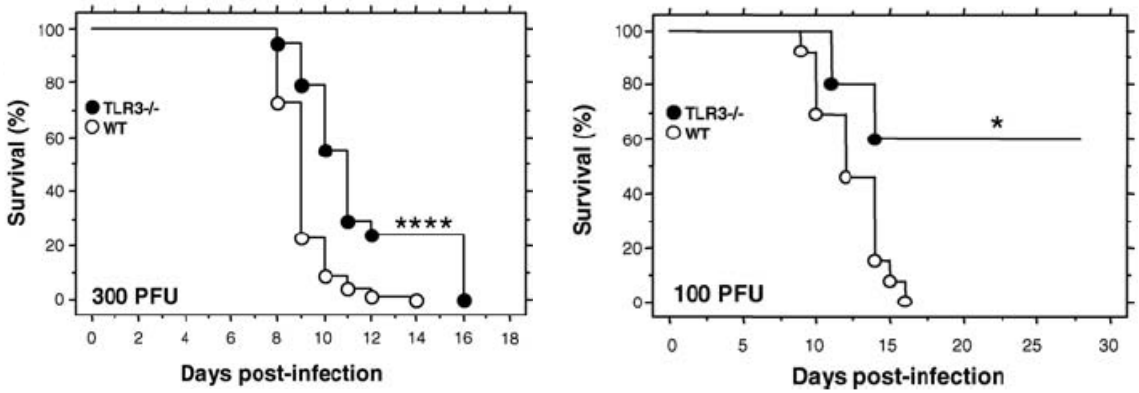


TLR 7

For intracellular staining, 100 µl of the whole blood was fully lysed with BD FACS lysing solution (Becton Dickinson), washed twice with Perm-wash buffer (BD bioscience) and processed for staining with the following anti-human antibodies: PE- TLR 3, FITC- TLR 7, FITC- TLR8 and PE-TLR 9 respectively.



Arankalle et al, Plos One 2010;5:e13099



Le Goffic, Plos Pathog 2006;2:e53

(B) Lethality induced by IAV in *TLR3*$^{-/-}$ and *MyD88*$^{-/-}$ mice in comparison with wild-type mice. Age-matched *TLR3*$^{-/-}$, *MyD88*$^{-/-}$, and wild-type male mice received intranasally 300, 100, or 30 pfu of IAV. 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 (****$p < 0.0001$, *$p < 0.05$) but not to that of *MyD88*$^{-/-}$ mice.

Projet FluBAL

Caractérisation immuno-virologique *in situ* des formes pulmonaires graves de la grippe A H1N1v pandémique chez des patients ventilés en réanimation

Promoteur : Institut de Microbiologie et Maladies infectieuses (IMMI)

(dans le cadre des projets grippe A H1N1v)

Investigateur principal :
Dr Amélie Guihot
Dr Antoine Parrot

Maladies infectieuses

Inserm
Institut national de la santé et de la recherche médicale

Guihot et al, Am J Respir Crit Care Med. 2014 Mar 19. [Epub ahead of print]

FluBAL

FluBAL patients: mechanical ventilation for severe A(H1N1)v pneumonia in ICU

Baseline characteristics:

n=	**35**
Age (years): *median (range)*	34 (14-64)
Sex ratio: *M/F*	17/18 (1)
Predisposing factors: *n (%)*	
Obesity (BMI>30)	9/35 (25)
Pregnancy	5/35 (14)
Cardio-pulmonary disease	4/35 (11)
Immunosuppressive condition	8/35 (22)

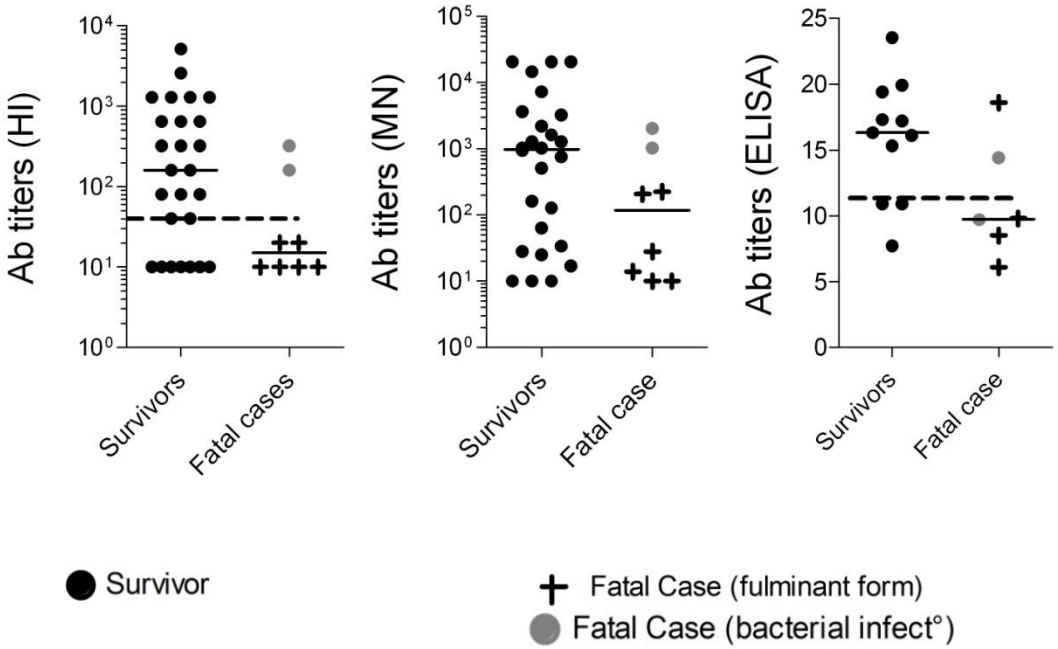
At 1st sample in ICU:

Days from first flu symptoms: *median (SD)*	9 (4,9)
Days from antiviral therapy: *median (SD)*	3 (2.5)
ARDS: *n (%)*	28/35 (80)
ECMO: *n (%)*	24/35 (68)

Lack of influenza A(H1N1)2009-specific antibody response in fulminant forms of H1N1 infection

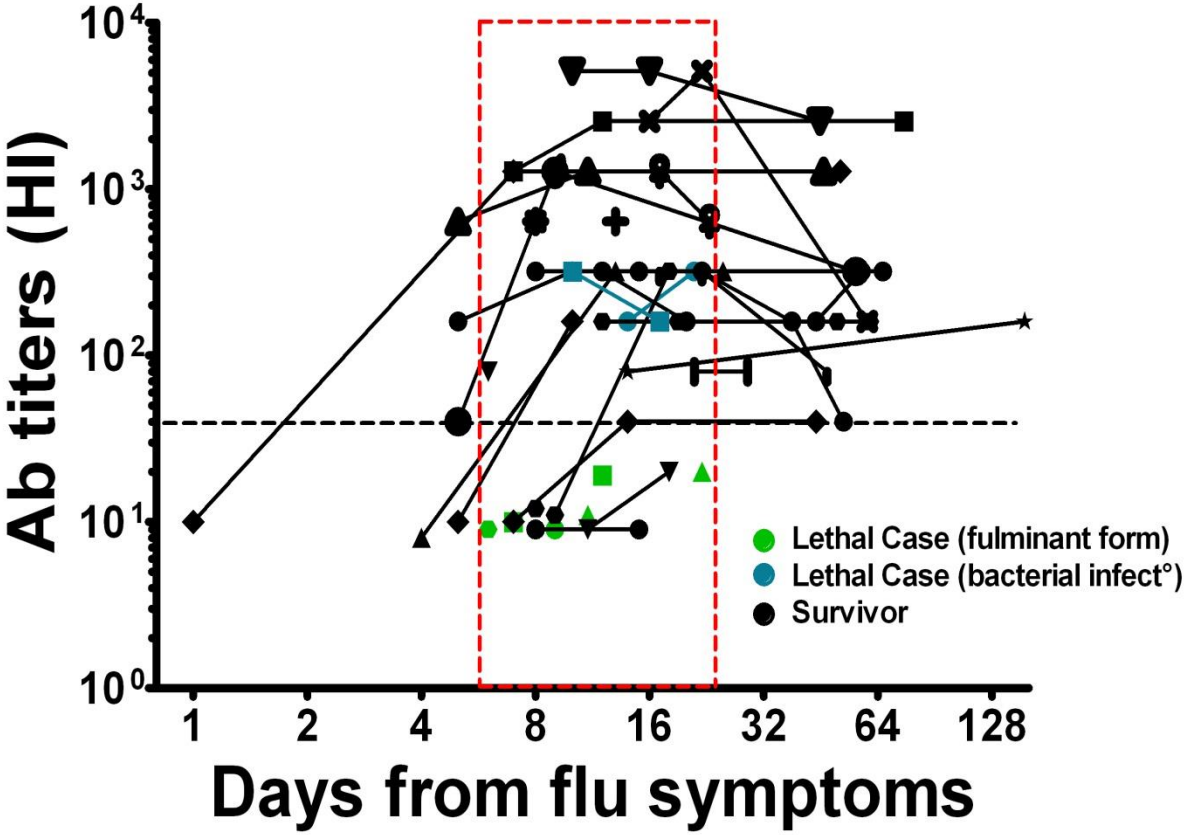
Sex/ Age	Comorbidities	Days from flu symptoms	Cause of death
M/40	Steroids + myc. mofetil	9	MOF
M/35	Obesity	7	MOF
M/63	Waldenstrom	14	PAVM
M/23	-	10	MOF
M/54	-	12	MOF
M/34	-	6	MOF
M/42	-	10	PAVM
F/35	CVID (γ=3g)	22	MOF

CVID, Common variable immunodeficiency; MOF, Multiorgan failure; PAVM, pneumopathie acquise sous ventilation mécanique



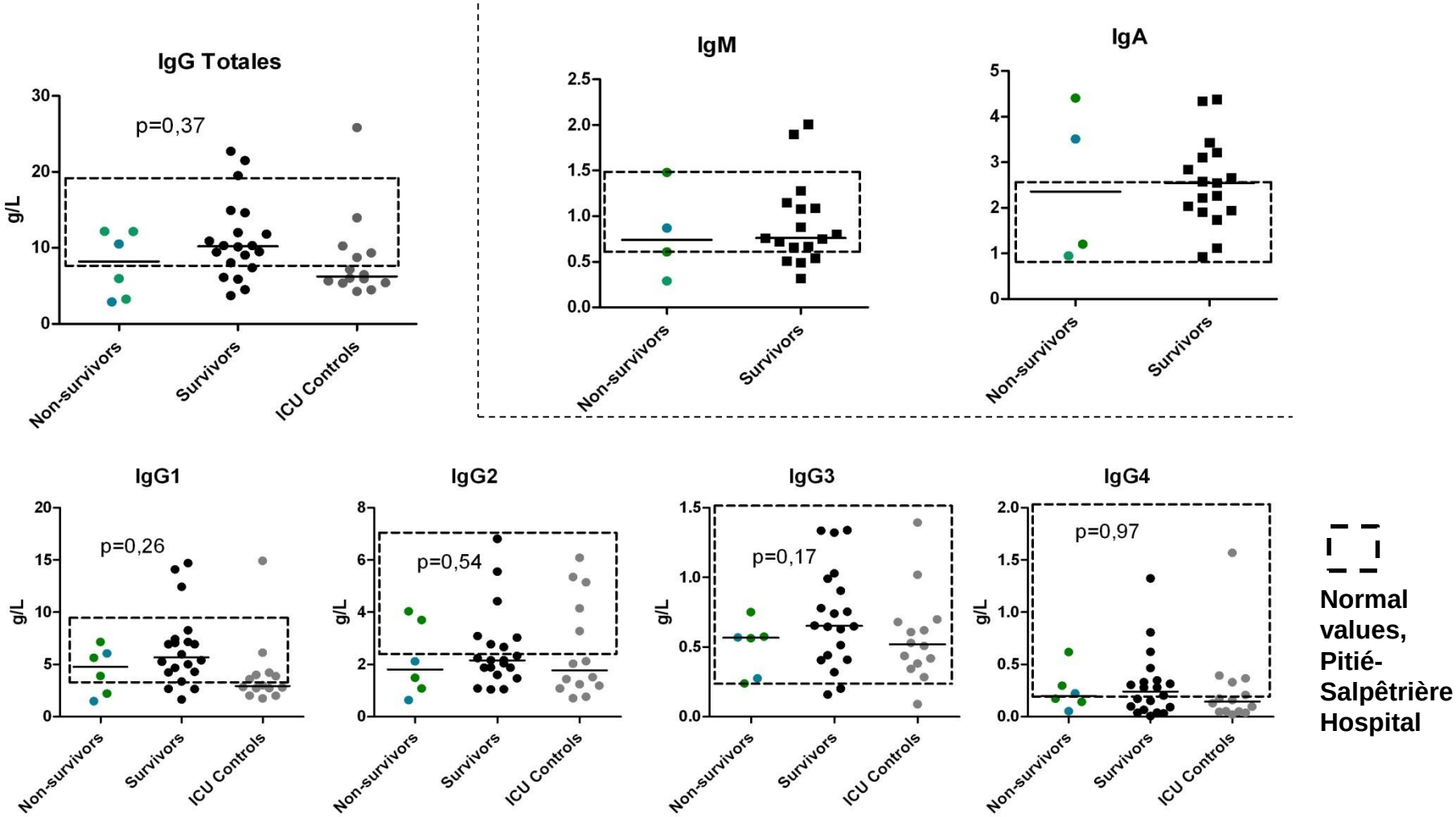
Inhibition Hemagglutination and Microneutralisation assays using influenza A/California/04/09 H1N1 strains in n= 8 fatal cases, n=22 H1N1v surviving patients and n=12 ICU controls at first sample in ICU (median = 9 days from first flu symptoms). IHA positive threshold: 1:40 Ab titer ---- ; *p<0.05 (fulminant forms versus survivors), ***p<0.0005

Lack of influenza A(H1N1)2009-specific antibody response in fulminant forms of H1N1 infection : kinetic



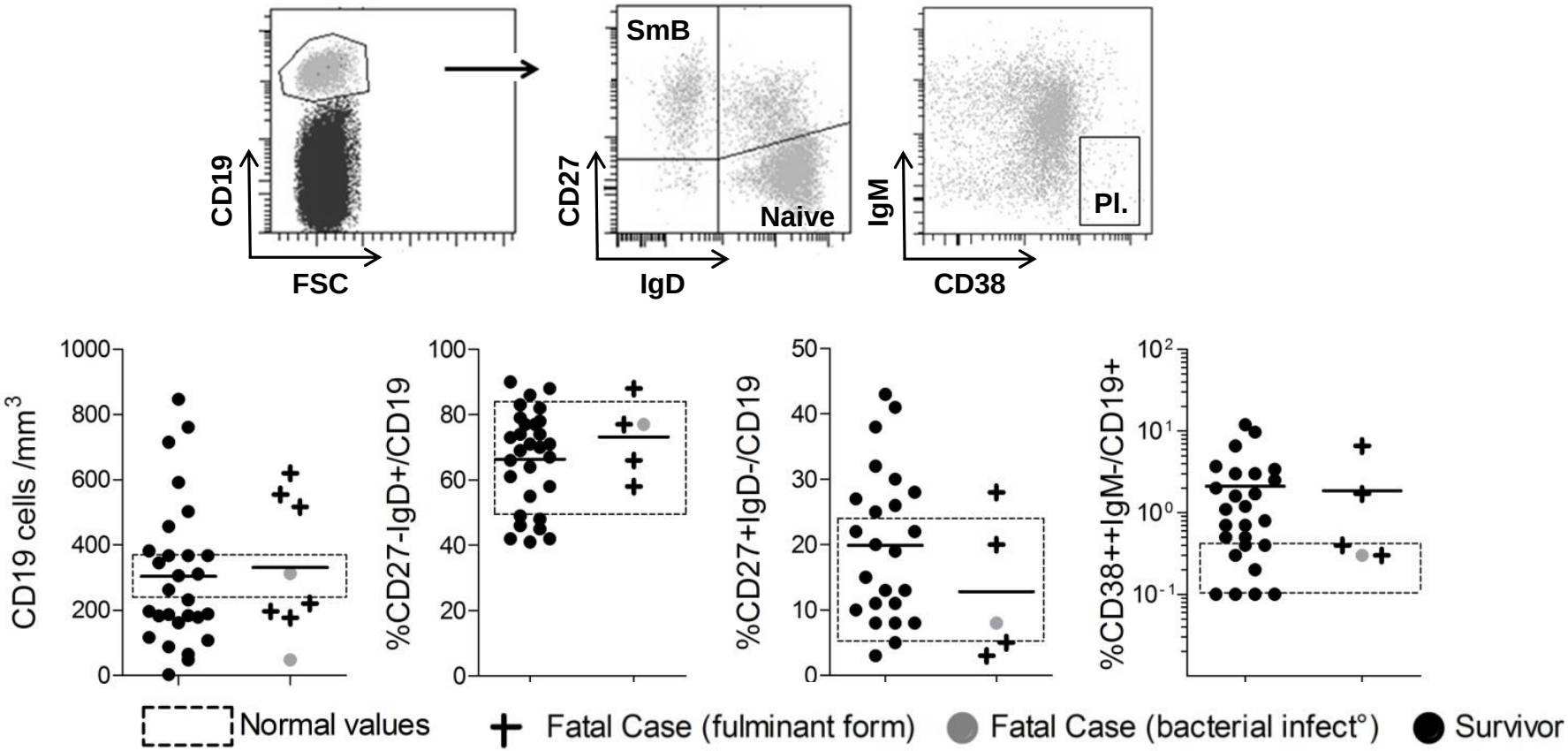
Inhibition hemagglutination assay using influenza A/California/04/09 H1N1 strains in n= 6 fatal cases, n=22 H1N1v surviving patients and n=12 ICU controls at first sample in ICU (median = 9 days from first flu symptoms). HIA positive threshold: 1:40 Ab titer ----

Non-survivors of (H1N1)2009 infection do not present serum IgG/IgA immunoglobulin deficiency



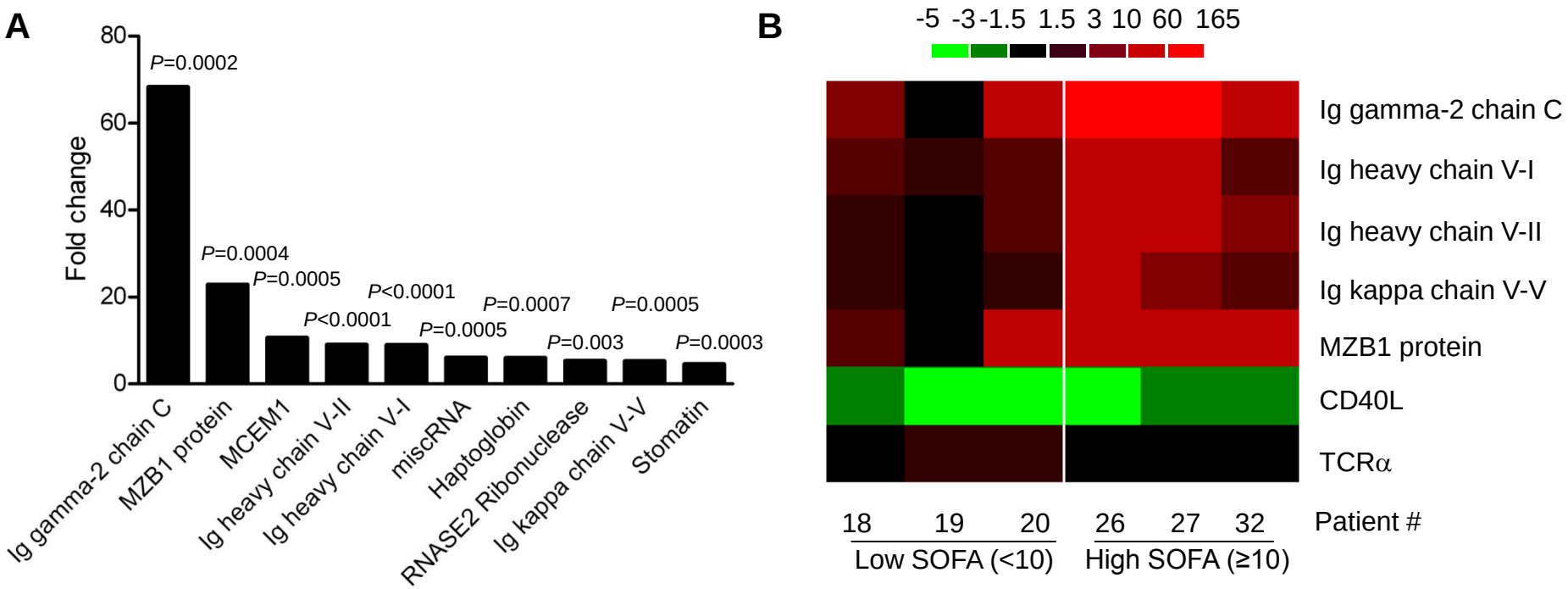
Total Serum IgG/A/M and IgG subclasses in n= 6 fatal cases, n=27 H1N1v surviving patients at first sample in ICU (median = 9 days from first flu symptoms).

B cell immunophenotyping do not differ during lethal cases of influenza infection



Fresh blood cells were stained with the following monoclonal antibodies: CD3-FITC, CD19-PE-Cy5 (Beckman Coulter), on a Beckman Coulter Epics XL flow cytometer for lymphocyte phenotyping and counting, and CD19-APC Cy7, CD27-APC, IgM-PC5 (BD Biosciences Pharmingen), IgD-FITC (Dako) on a BD FACSCanto flow cytometer for the phenotyping of B cells. Normal values for each lymphocyte population were defined as mean +/- 2 standard deviation values in n=15 healthy donors.

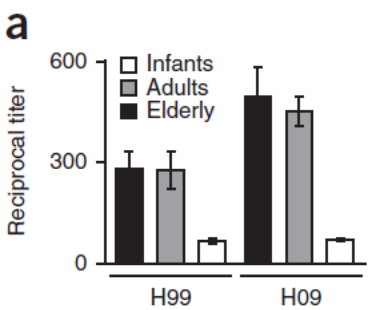
Peripheral upregulation of immunoglobulin genes in very severe influenza infection



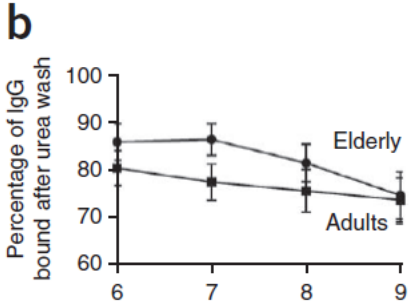
The genome-wide gene expression profiles were evaluated on blood sample (PAXgene tube, PreAnalytix®) for n=3 patients with a low SOFA score (SOFA 5-6, patients #19, 20, 21) and n=3 patients with a high SOFA score (SOFA 10-16, patients #27, 28, 33) infected by H1N1, and compared to n=3 healthy blood donors. expression chips (Illumina ® HT-12 Expression BeadChip V4). A false-discovery rate (FDR) threshold of 0.05 as statistical cutoff was chosen.

Immune complexes lung deposition during severe influenza A(H1N1)2009 pneumonia

Healthy individuals

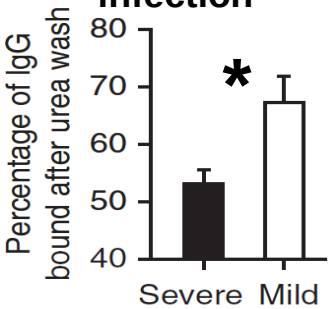


Abs cross reacting with influenza A(H1N1)2009 strain



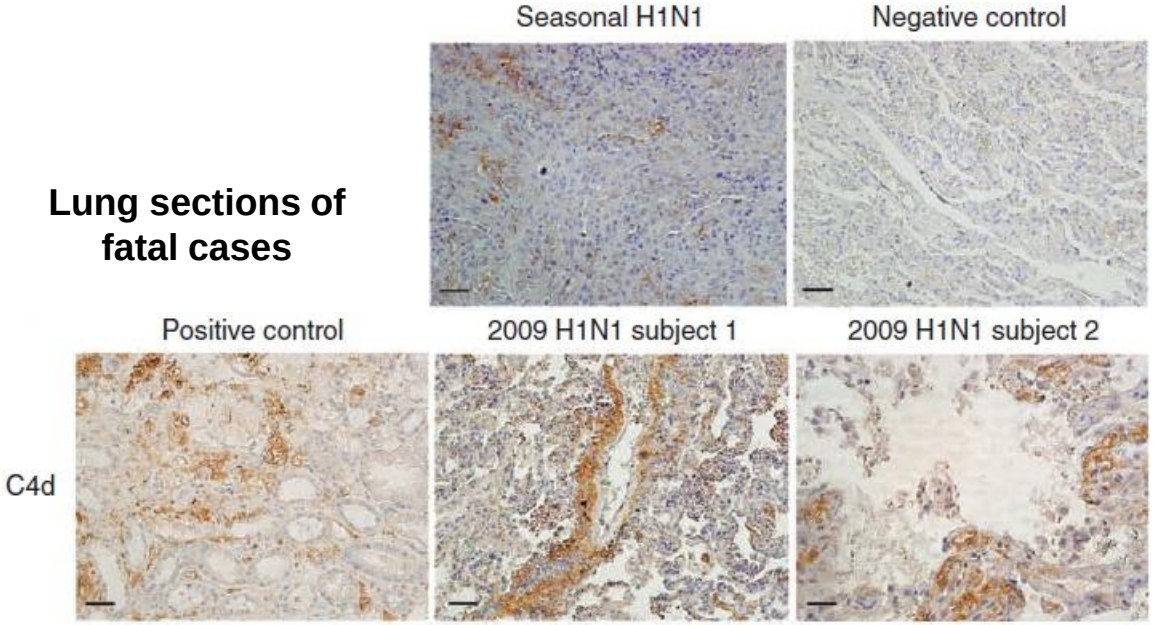
Low avidity cross reacting Abs

Patients with (H1N1)2009 infection



Low avidity of A(H1N1)2009 Abs

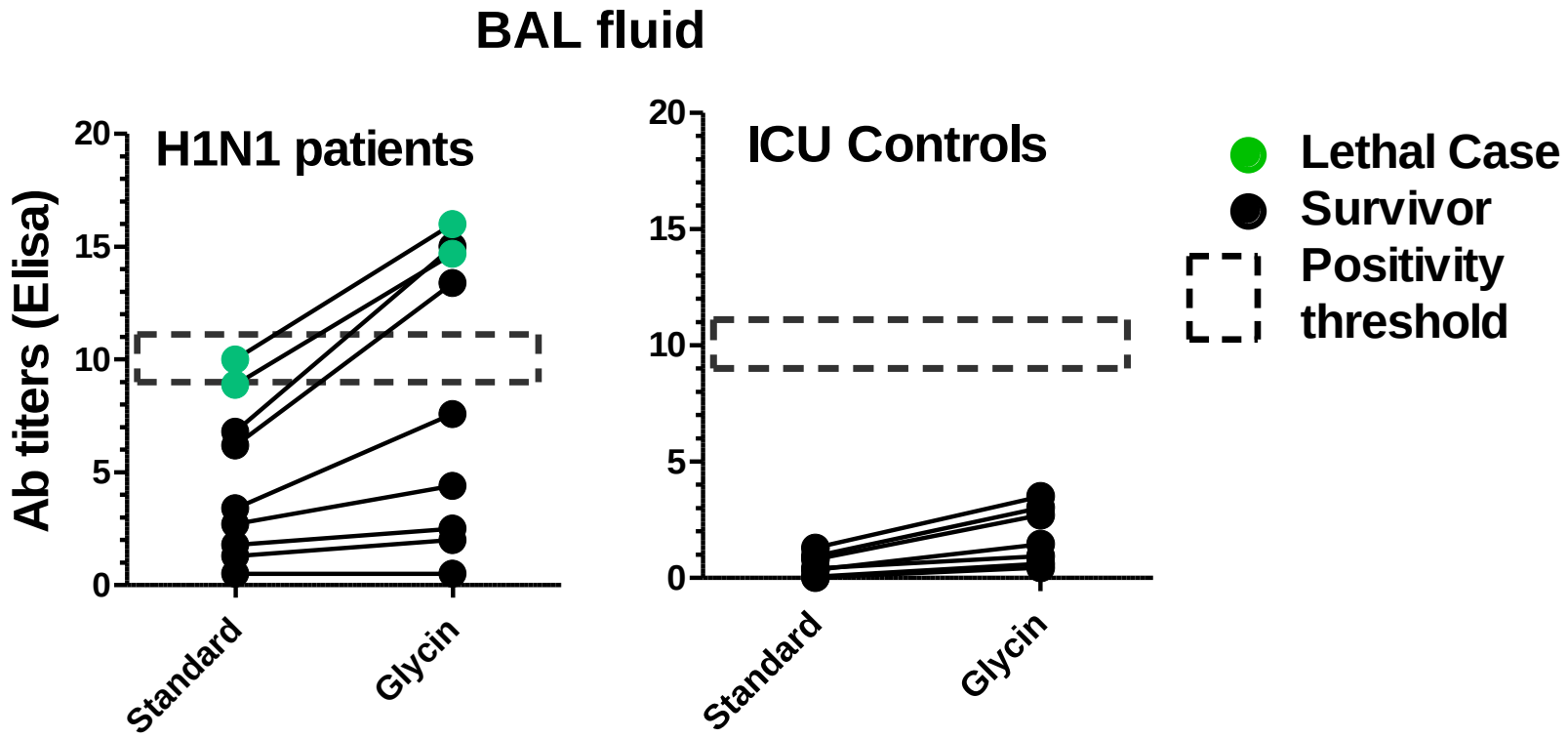
Lung sections of fatal cases



- C4d marker of complement activation mediated by immune complexes
- Same aspects in lung section from fatal cases of 1957 H2N2 infection

Monsalvo et al, Nat Med 2011;17:195

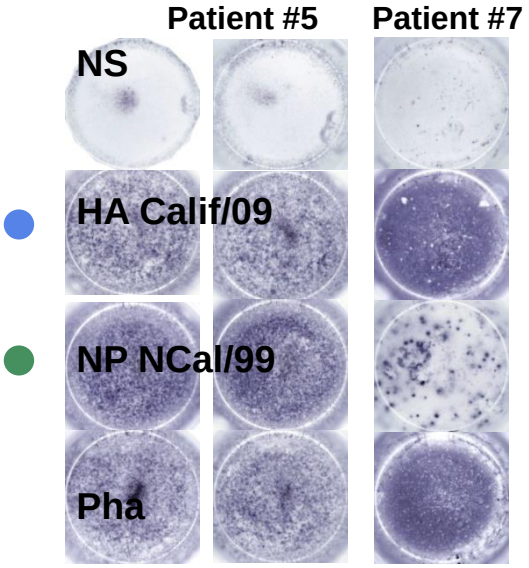
Indirect evidence of H1N1-specific immune complexes in lung during fatal infection

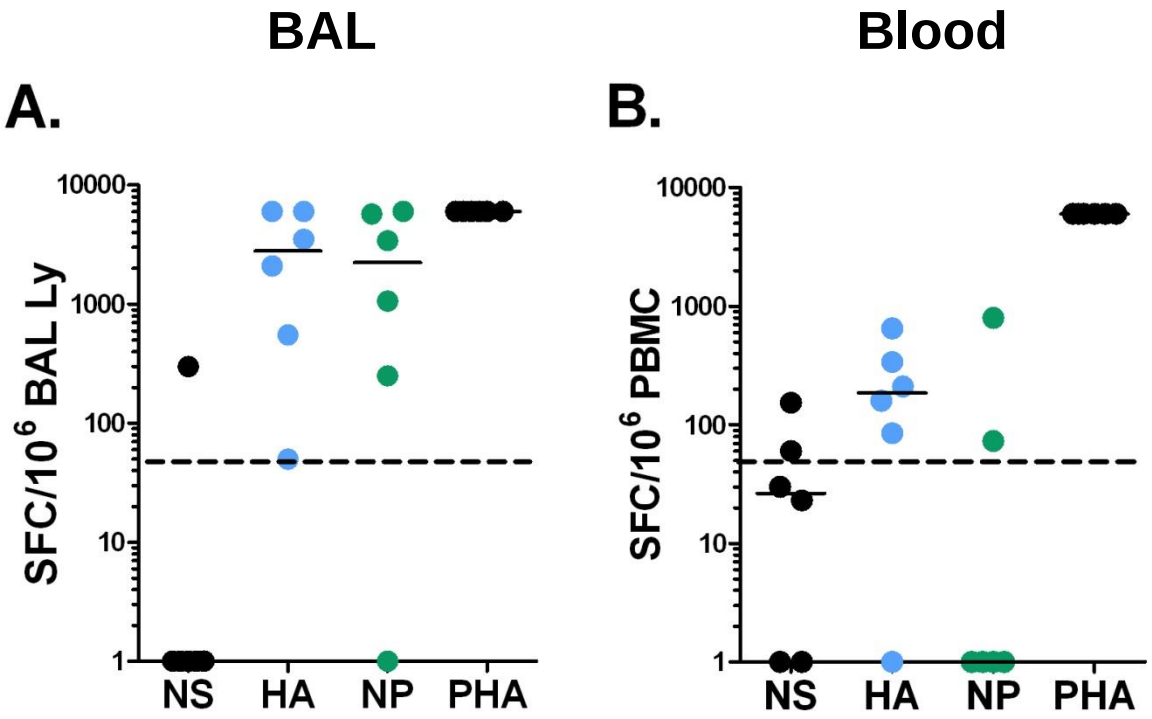


Glycin: After immune complex dissociation in Glycin Buffer

ELISA H1N1&H3N2 assay. Ab titers are expressed as following: <9 Virotech Unity (VU): negative; 9-11 VU: indeterminate; >11 VU: positive. For the dissociation of antibody-antigen immune complex, 70µL of patient serum was treated with 70µl of dissociation buffer (1.5M glycine [pH:2.8]) for a 1:2 dilution and the immune complex was dissociated 1H at 37°C . The reaction was stopped by the adding of 70µl of neutralization buffer (1.5M Tris-HCL [pH:9.7]) to achieve an end dilution of 1:3.

Stronger effector and effector-memory influenza A(H1N1)v-specific T cell responses in BAL than blood

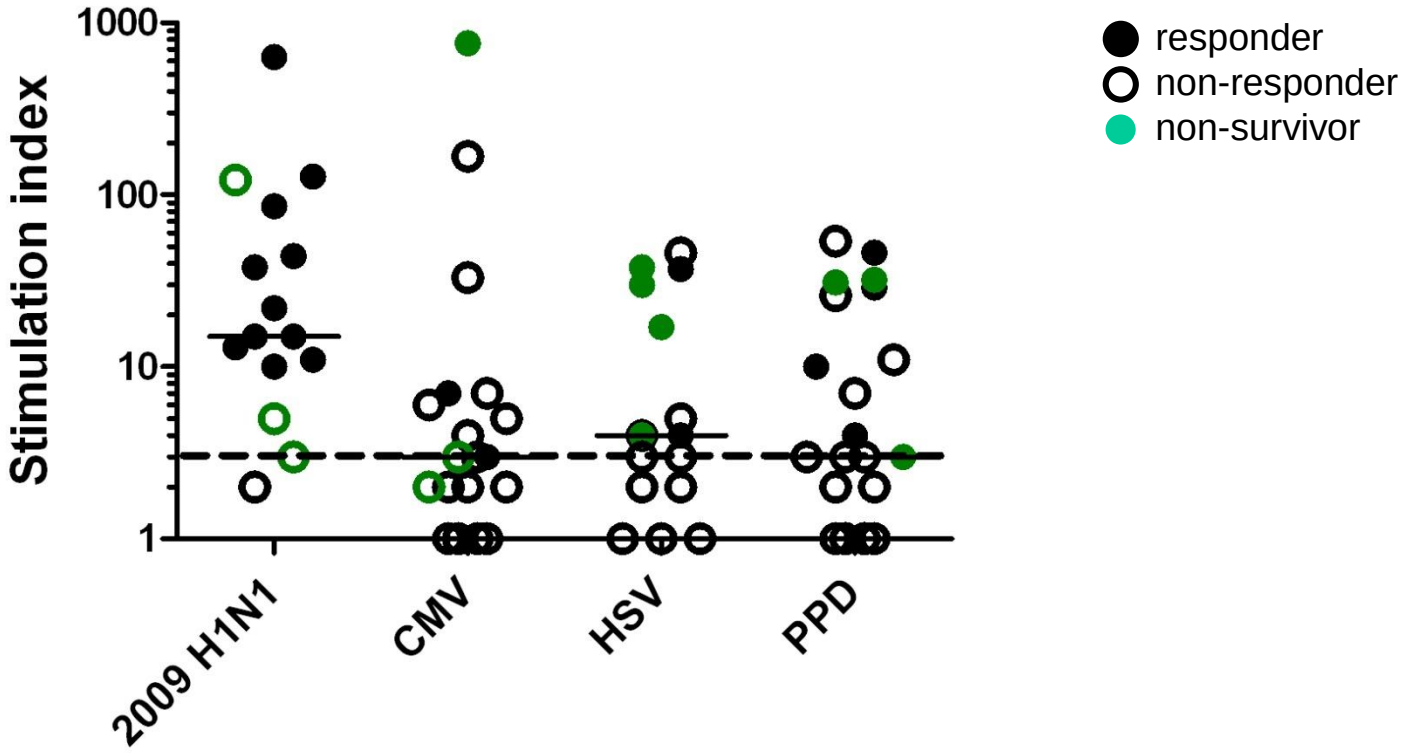




→ + n=2 fatal cases : background values >>

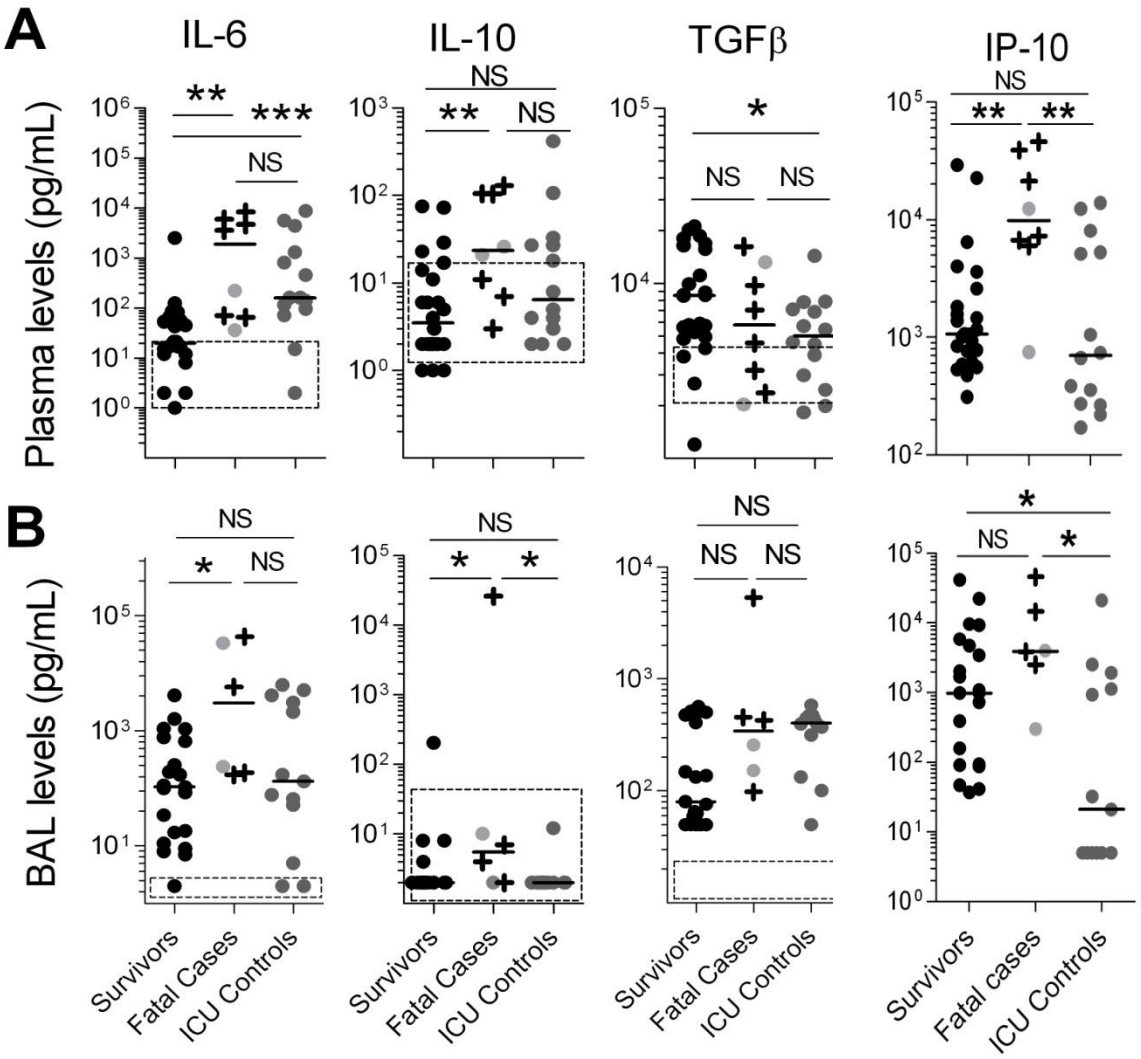
ELISpot IFNγ assay with (A) total BAL cells and (B) PBMC in n=6 surviving H1N1 patients. Positive threshold: 50 SFC/106 BAL ly. Stimulation: 18 or 20-mer peptides covering H1N1/California/2009 hemagglutinin (HA) and H1N1/NewCal/1999 nucleoprotein (NP) sharing 98% sequence homology with the A/California/04/2009 nucleoprotein. PHA : Phytohemagglutinin. NS: non-stimulated

Lack of memory T cell responses to influenza in lethal forms of (H1N1)2009 infection



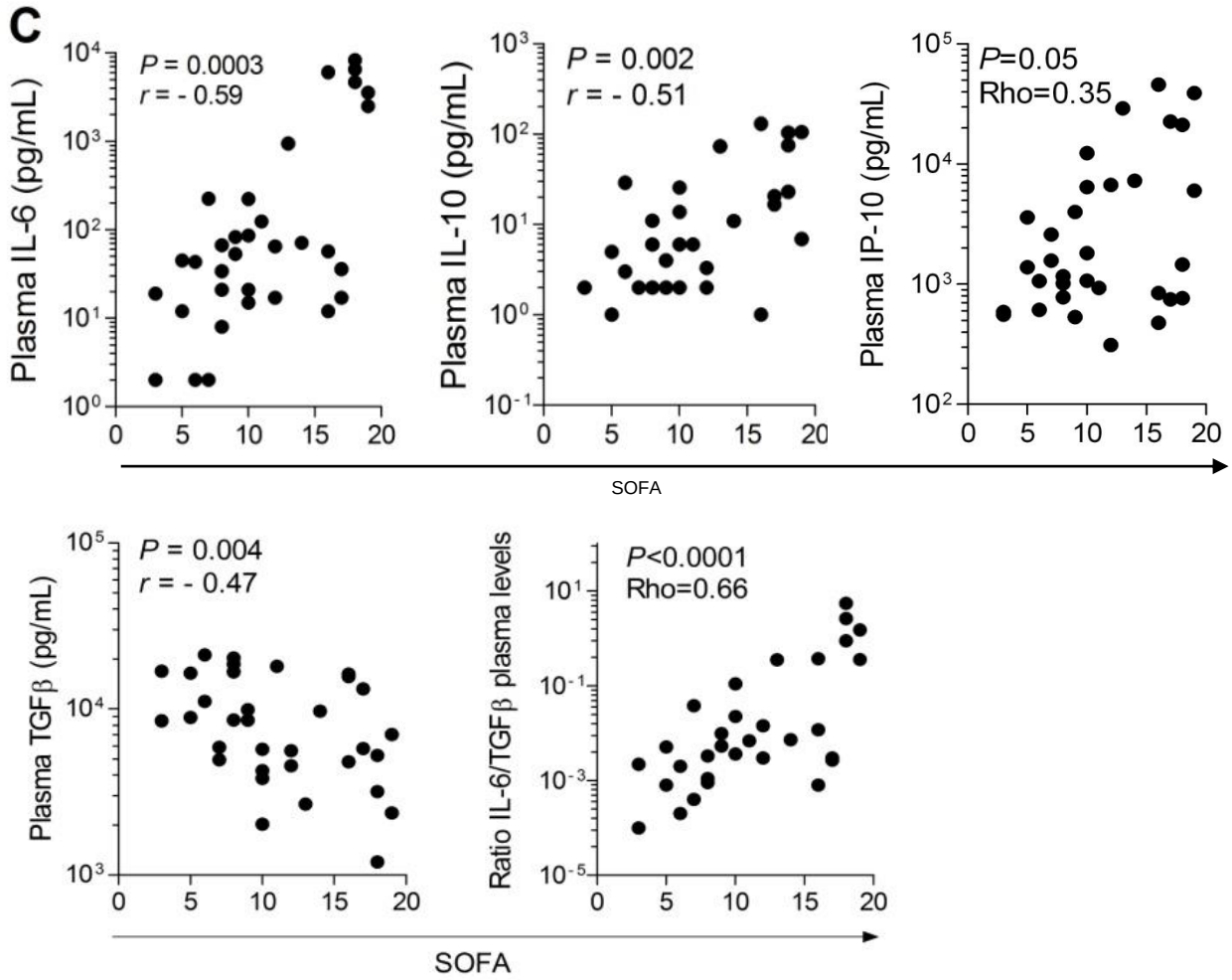
Lymphoproliferative assay. Positive threshold: >3 SI and >3,000 cpm. Stimulation: 2009 H1N1 : Recombinant hemagglutinin H1N1/Calif/09 (HA) or Panenza H1N1 vaccine; CMV crude extract antigen (HSV); purified tuberculin (PPD) in n= 26 H1N1 patients in ICU; n=15 H1N1 patients during ICU stay

Systemic and pulmonary cytokine profile of lethal cases of influenza A(H1N1)2009 infection



Cytokine/chemokine levels in plasma in Multiplex in n=8 non-survivors and n=25 survivors + 12 ICU Controls at first sample in ICU. **Mann Whitney test *p<0.05, **p<0.005, ***p<0.0005**

Relationship between cytokine/chemokine levels and severity during influenza A(H1N1)2009 infection



Cytokine/chemokine levels in plasma in Multiplex in n=8 non-survivors and n=25 survivors at first sample in ICU. Spearman correlation test

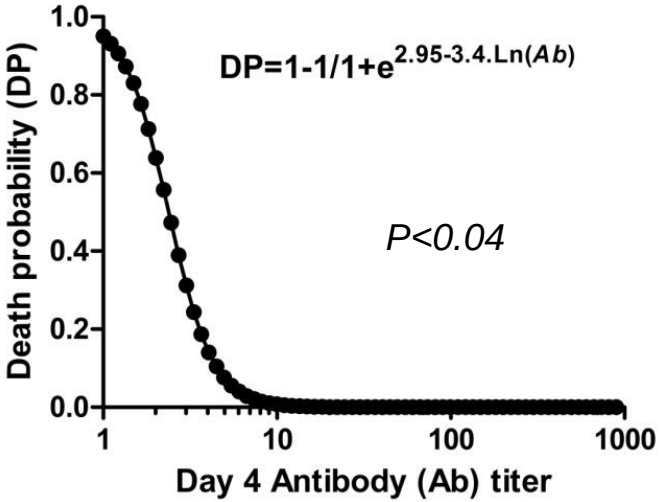
Low titers of seric antibodies inhibiting haemagglutination predict fulminant fatal influenza A(H1N1) 2009 infection

Table. Univariate and multivariate analyses of prognostic factors of death from fulminant influenza infection.

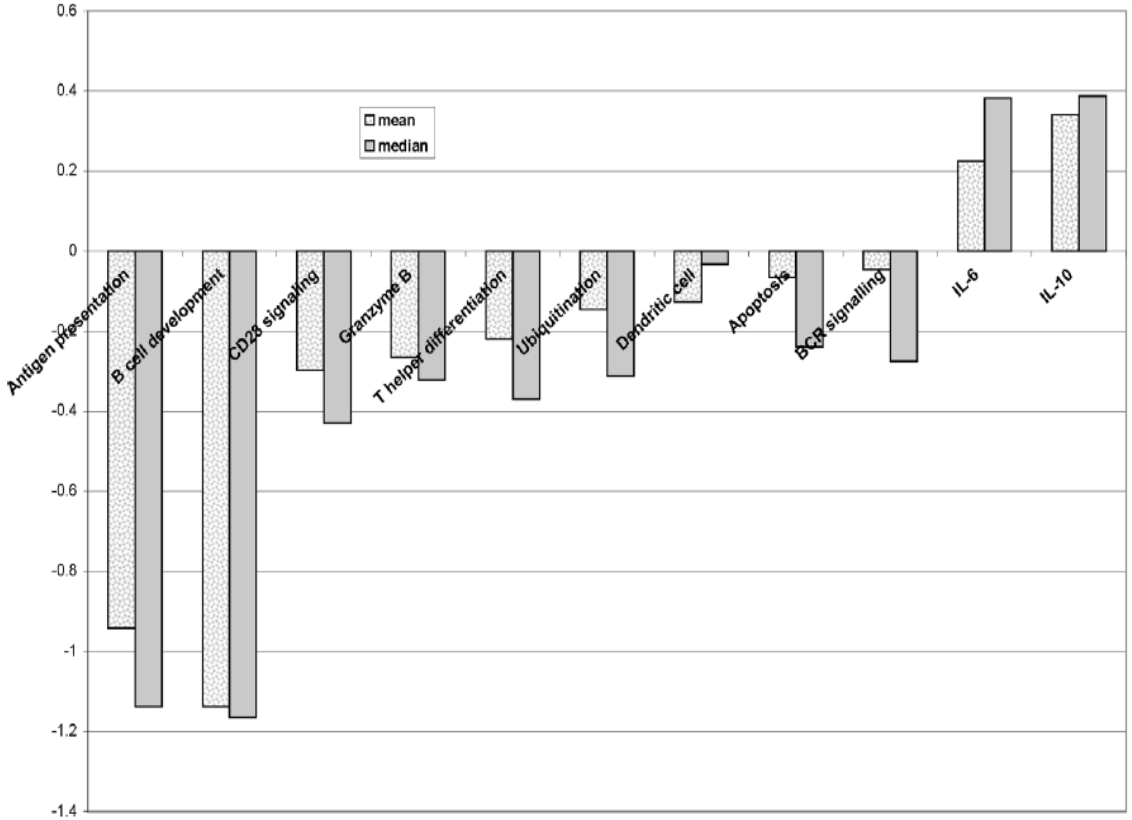
	Univariate analysis					Multivariate analysis			
Prognostic Factor	**Parameter Estimate**	**Odds ratio**	**95% CI of odds ratio**	**p**	**AIC criterion****	**Parameter Estimate**	**Odds ratio**	**95% CI of odds ratio**	**p**
# of clinical risk factors	-1.01	0.36	0.06-2.09	0.26					
Bacteria in BAL (Y/N)	-0.12	0.89	0.13-5.85	0.90					
Viral load (Y/N)	1.79	6.00	0.65-55.6	0.11					
H1N1 HA Mutation 222 (D/G)*	-1.44	0.056	0.004-0.81	**0.034**	34.1				
Plasma IL6/100 units	0.044	1.04	1.00-1.09	0.056					
Plasma IL10	0.028	1.03	1.001-1.06	**0.044**	26.1	0.03 (1.06)	1.03	0.99-1.07	**0.19**
TGFb in plasma/1000 units	-0.094	0.91	0.76-1.09	0.31					
HA proliferation/unit	-0.24	0.78	0.53-1.17	0.23					
Neutralizing Ac/100 units	-0.33	0.72	0.43-1.19	0.19					
IHA J4 (/twofold increase)	-2.28	0.10	0.07-0.93	**0.040**	22.2	-2.36 (-5.25)	0.09	0.01-1.28	**0.07**

BAL, Broncho-alveolar lavage; HA, Hemagglutinin; IHA, inhibition hemagglutination assay; AIC, Akaike information criterion
***N=16. **Rescaled to account for missing data.**

Regression model for survival based on the antibody titer at day 4, estimated for a given individual. The log-titer was treated as the prognostic covariate in a standard logistic regression model predicting the probability of influenza-related death. Therefore univariate and multivariate analyses aimed at characterizing global and specific prognostic factors relied upon logistic regression models predicting the probability of influenza-related death.



Impairment of Antigen Presentation/B cell development during most severe forms of H1N1 infection ?



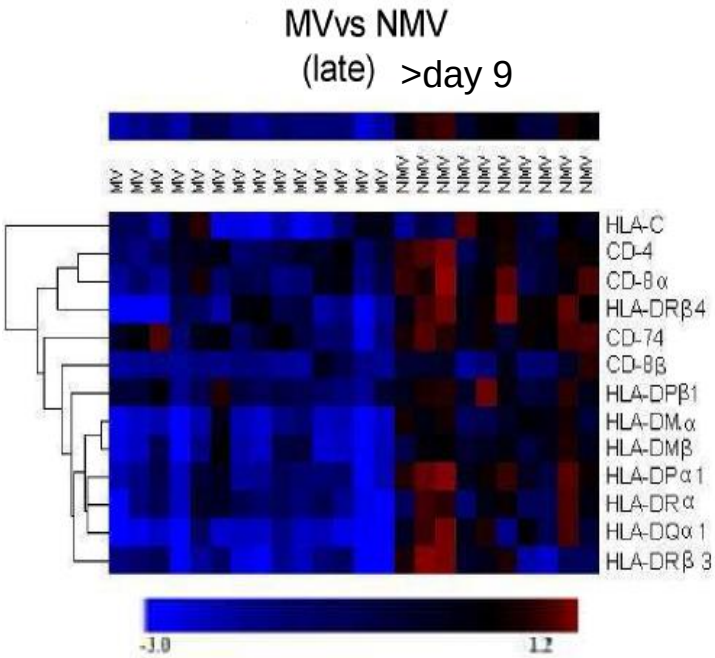
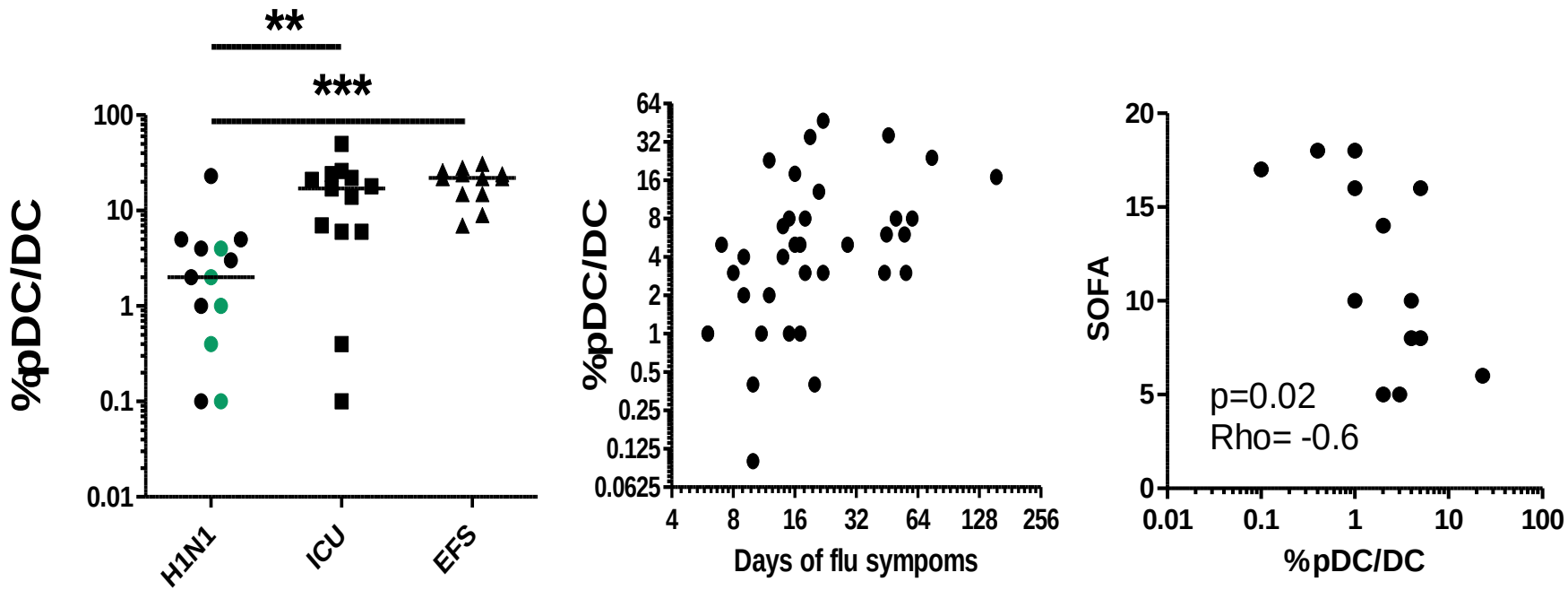


Figure 2 Histogram depicting the mean and median of the differences in gene expression levels between MV-NMV by intracellular signaling pathways. (< 0) means that expression in MV < expression in NMV; (> 0) means that expression in MV > expression in NMV).

n=19 patients with H1N1 pneumonia (18-65 years) : 12 with mechanical ventilation (MV), 7 without (NMV).
mRNA Microarrays Ingenuity Pathway Analysis 8.5 (IPA). Changes in gene expression verified with QRT-PCR

Bermejo-Martin et al, Crit Care 2010;14:R167

pDC levels are low during severe H1N1 influenza infection, particularly in most severe cases



%pDC determined by Flow cytometry on whole fresh blood at first sample in ICU. DC gating strategy : Lin-HLA-DR+ ; pDC : CD123+DC. HLA-DR-APC Cy7, Lin-FITC, CD123-PC5. Mann Whitney non parametric test (left panel), Spearman correlation test (right panel).

Guihot, personal data

Conclusion: Immune responses to influenza A(H1N1)v causing severe infection

- **Severe influenza A(H1N1)2009 infection :**
  - **Strong cellular CD4 and CD8 response** to influenza:
    Lung recruitment of CD8 T cells that differentiate with time of virus exposure
    Strong lung mobilisation of H1N1v-specific effector CD8 cells
  - **Strong HI and neutralizing antibody response**
- **Lethal cases :**
  - **'Cytokine Storm',** with **massive IFNγ production** in lung, with altered Ag presentation?
  - **Immune complex lung deposition** from low affinity specific Abs?
    With Pre-existing anti-influenza antibodies
  - **Low peripheral anti-influenza antibodies** which is predictive of death
    Reflecting the pulmonary translocation of specific antibodies

⇨ **Predictive marker identified for fatal fulminant influenza infection**

⇨ **Immune parameters to explore; Ab affinity, Ag presentation**

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