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Introduction

Influenza A viruses continuously evolve through mutations resulting in antigenic drift. Thus, circulating and predominant **viruses may be antigenically different from** the viral proteins used in the **current seasonal influenza vaccine** formulation. Hence, antibodies induced by the vaccine may poorly recognize circulating viruses. Immunosuppressed and elderly, immunosenescent people might be especially at risk. To evaluate the **cross-recognition ability** of antibodies induced in vaccinated **healthy and HIV-infected** individuals, we tested the antibody-binding capacity on a selection of H1 and H3 hemagglutinins (HA) including the **vaccine HA and predecessors and successors**. Further, we investigated the **amino acid changes** underlying poorly recognized variants and examined whether low cross-recognition relates to restricted **B cell receptor (BCR) repertoires**.

Methods

We analyzed cross-reactivity in a prospective 2007/2008 influenza vaccine cohort (24 uninfected, 31 HIV-infected individuals). A multiplex assay (Luminex) was employed to simultaneously assess the **IgG response against recombinant hemagglutinin** of **14 different influenza A viruses**. These included the 2007/08 vaccine strains H1N1/Solomon Islands/3/2006 and H3N2/Wisconsin/67/2005, and all strains included in previous (2000-2007), and subsequent (2008-2014) vaccine preparations. The magnitude (median fluorescence intensity, MFI) of each subject's IgG response against all 14 H1N1 and H3N2 was quantified longitudinally from day 0 to 28 post vaccination. Strain-specific responses were expressed as **relative response normalized to the vaccine strain response**. From 12 healthy subjects with a robust vaccine response, **memory B cells** ($CD19^+CD20^+IgD^-CD27^+CD38^-$) and **plasmablasts** (PB, $CD19^+CD20^-IgD^-CD38^{++}$) were **FACS-sorted** from cryopreserved PBMC prior to – and on day 7 after vaccination. **Total IgG BCR transcripts** of memory B cells (day 0 and 7) and PB (day 7) were amplified by multiplex PCR (BCR-PCR) and **sequenced** to test for a potentially skewed or restricted BCR repertoire. To determine **HA-specific B cell responses**, B cells of the same subjects were **directly labeled with HA**, FACS-sorted, cultured with IL-21 and CD40L to induce antibody production and tested via **Elispot**.

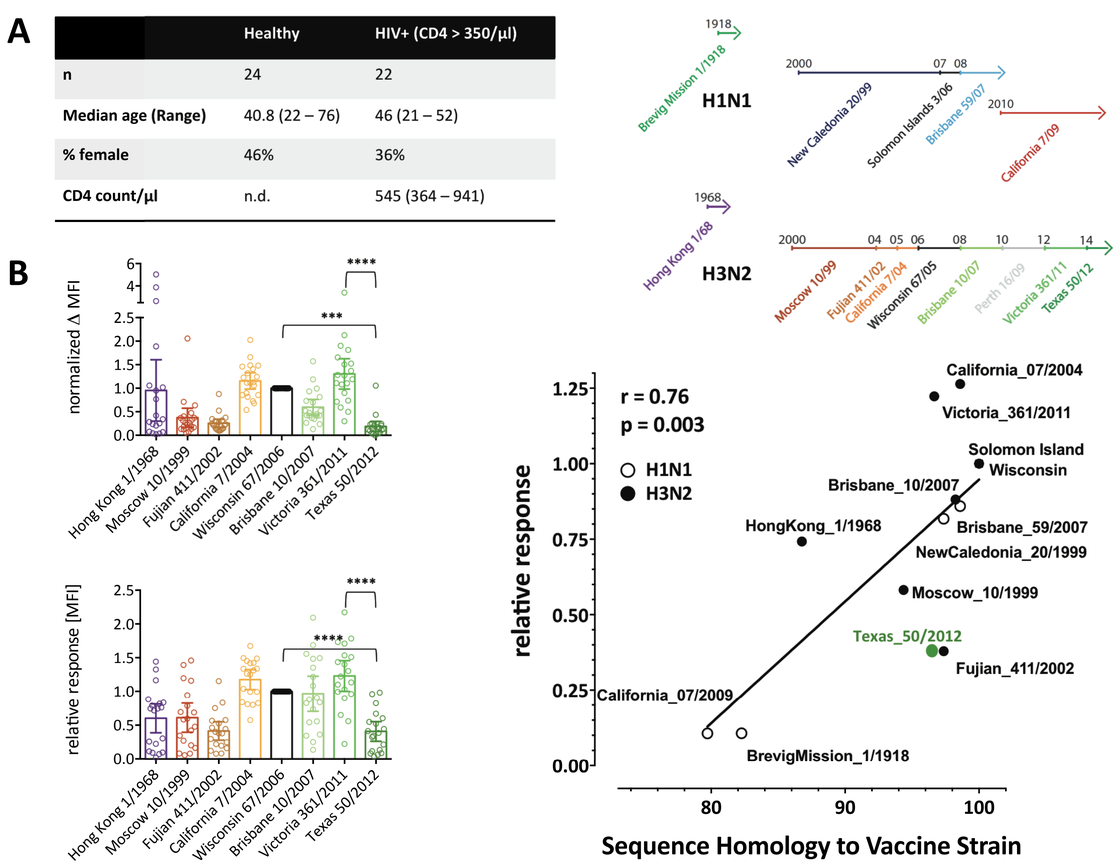

Figure 1 – Antibody cross-reactivity only partly correlates with overall sequence homology. Key characteristics of the cohorts (**A**). Tested H1N1 and H3N2 HA depicted in the order they were included in the vaccines. 2007/08 Vaccine strains are shown in black (**A**). Relative responses against H3N2 HA of healthy donors (top) and HIV-infected individuals (bottom). Response against the vaccine strains (Wisconsin) is set to 1, median relative intensity is depicted for the other strains. Key statistics are indicated for Wisconsin, Victoria and Texas strain (Friedman test). For H1N1, California 2009 and Brevig Mission were poorly recognized, whereas the response to the others were comparable (data not shown). High HA sequence homology generally associates with good cross-reactivity. Notable exceptions are Texas_50/2012 and Fujian_411/2002 (**B**).

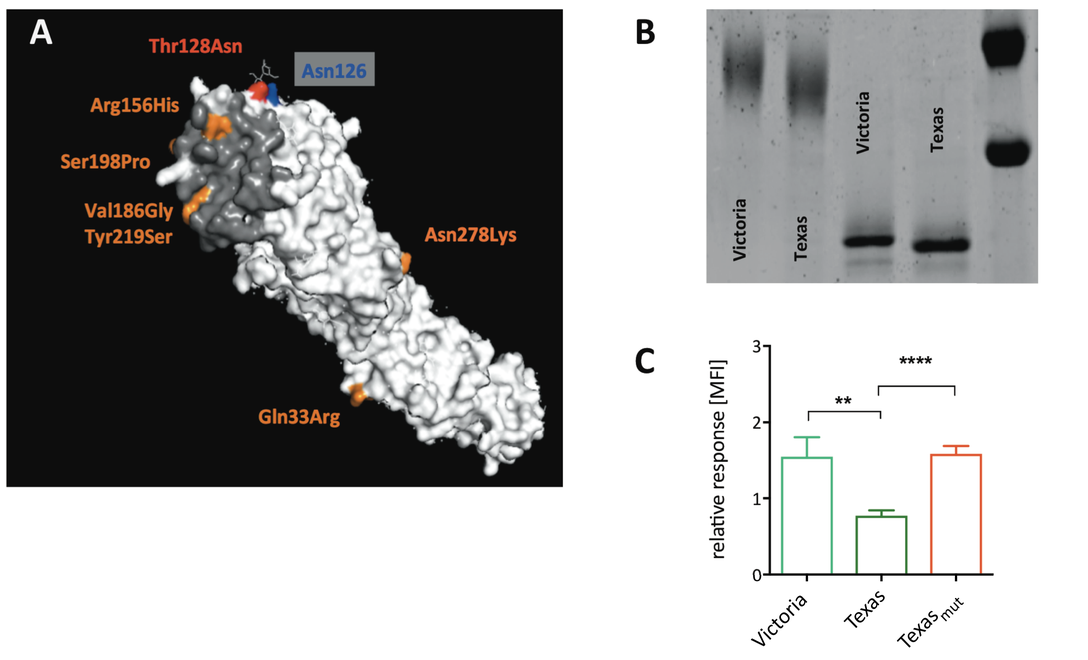

Figure 2 – A single amino acid change resulted in loss of cross-recognition between Victoria and Texas strain. Amino acid changes (orange) between Victoria and Texas. T128N (red) potentially disrupts glycosylation at N126 (blue). In grey: Receptor-binding site (**A**). Mobility shift in PAGE shows loss of glycan structure in the Texas HA (left). Deglycosylated proteins are shown as controls (right)(**B**). Antibody cross-reactivity is restored in $Texas_{mut}$ (Texas background with N128T mutation) (**C**).

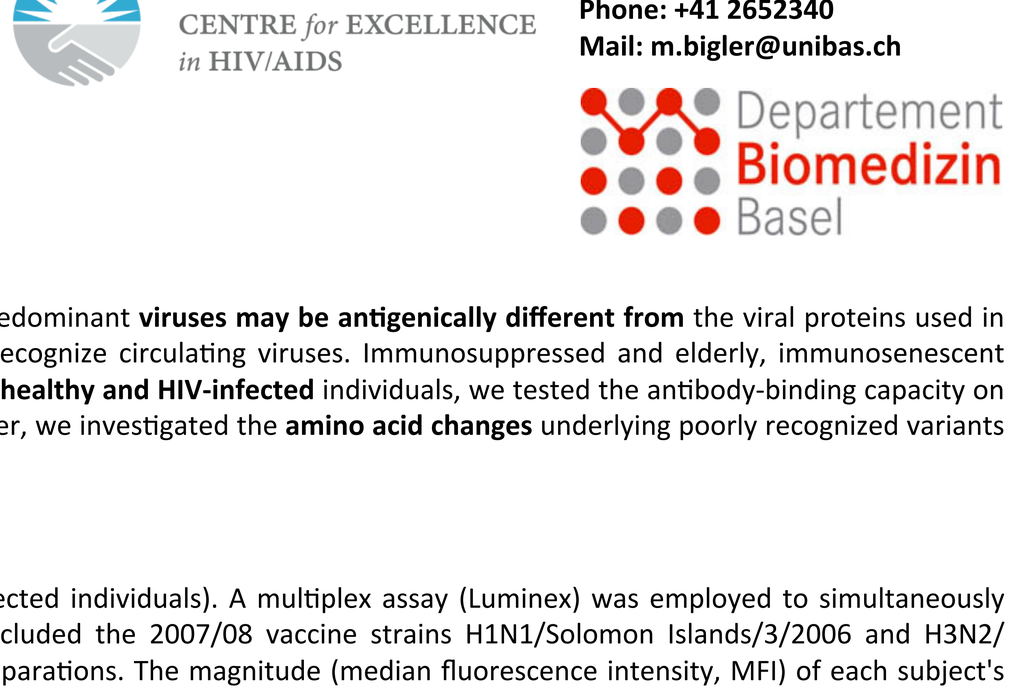

Figure 3 – Restricted memory BCR repertoire in subjects with low cross-reactivity towards Texas. There was considerable variation in strain cross-recognition of Texas vs. Victoria (healthy subjects shown). Individuals were grouped into Texas 'high' and 'low' cross-responders (**A**). Memory B cells (B_{mem}, gated on $CD19^+CD20^+IgD^-$) and Plasmablasts (gated on $CD19^+CD20^+$) were sorted as depicted (**B**). BCR sequencing revealed higher clonal overlap (= more restricted repertoire) in memory B cells of individuals with low Texas cross-recognition (light blue) as compared to those with good recognition (red) (**C**).

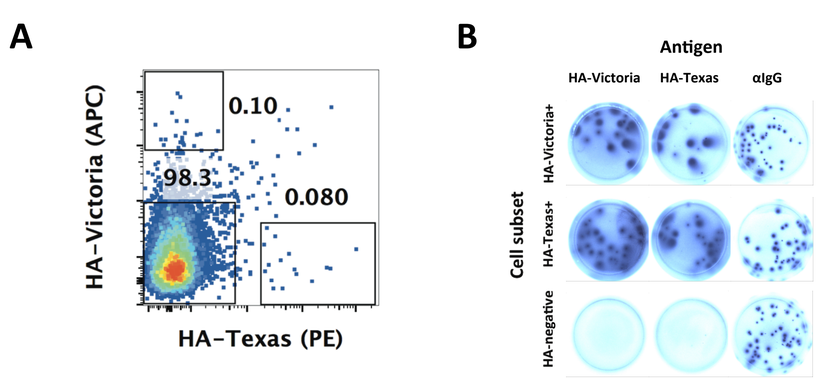

Figure 4 – HA-labeling identifies cognate antigen-specific B cells and can be used for BCR sequencing of single or cross-recognicing B cell clones. B cells can be co-stained with two different HAs (**A**). After sorting and 4 days incubation in IL-21 and CD40L, HA-specific antibodies are produced, as assessed in B cell (IgG) ELISPOT. Anti-IgG coated wells serve as a positive control (**B**).

Results

Antibodies induced against the vaccine strains cross-recognized various non-vaccine strains. **Cross-reactivity correlated positively with sequence homology** to the vaccine strain (r=0.76, p=0.003), and showed a very similar pattern in healthy and HIV-infected individuals (**Fig. 1**). Notably, cross-recognition between the closely related HAs of **H3N2 A/Victoria/361/2011 and A/Texas/50/2012** was low in healthy (median ΔMFI -52%), and extremely poor in HIV-infected individuals (-69%). To test for potential **antibody binding escape** we reverted a single amino acid of A/Texas/50/2012 potentially **restoring an N-linked glycosylation site**. This sufficed to **rescue** the response (**Fig. 2**). BCR repertoire analysis suggested that individuals with **low cross-recognition** of this variant had a significantly **more restricted memory BCR repertoire** (before and after vaccination) compared to those with high cross-recognition (**Fig. 3**). B cells can be labeled directly with fluorochrome-coupled HA, sorted and differentiated to plasmablasts specific for their cognate antigen (**Fig. 4**).

Conclusions and Outlook

- ✪ Generally, cross-recognition potential of anti-Influenza antibodies decreases with increasing genetic distance
- ✪ However, antigenic changes driven by single amino acid mutations can occur and severely impact antibody cross-recognition
- ✪ Individuals with restricted memory B cell repertoires seem to be more vulnerable to escape mutations
- ✪ HA-specific B cells can be sorted using different hemagglutinins enabling in vitro characterization of the BCR repertoire