Subsisting H1N1 influenza memory responses are insufficient to protect from pandemic H1N1 influenza challenge in C57BL/6 mice

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The 2009 swine-origin pandemic H1N1 (pH1N1) influenza virus transmitted and caused disease in many individuals immune to pre-2009 H1N1 influenza virus. Whilst extensive studies on antibody-mediated pH1N1 cross-reactivity have been described, few studies have focused on influenza-specific memory T-cells. To address this, the immune response in pre-2009 H1N1 influenza-immune mice was evaluated after pH1N1 challenge and disease pathogenesis was determined. The results show that despite homology shared between pre-2009 H1N1 and pH1N1 strains, the effector memory T-cell response to pre-2009 H1N1 was generally ineffective, a finding that correlated with lung virus persistence. Additionally, pH1N1 challenge generated T-cells reactive to new pH1N1 epitopes. These studies highlight the importance of vaccinating against immunodominant T-cell epitopes to provide for a more effective strategy to control influenza virus through heterosubtypic immunity.

INTRODUCTION

Influenza remains a significant health and economic burden, despite the availability of vaccines and therapeutics. As a zoonosis, control is challenging, and novel strains often arise, some of which have the ability to productively infect humans (Beeler, 2009), such as the emergence of the highly pathogenic H5N1 strain of avian influenza in 2004–2005 (Suarez, 2010). More recently, the 2009 swine-origin H1N1 influenza virus (pH1N1) was transmitted from swine to humans, resulting in a pandemic.

Antibodies generated as a result of influenza infection or vaccination typically are protective against homotypic infections but often fail to cross-react effectively with novel strains possessing distinct subtypes of the haemagglutinin (HA) and neuraminidase (NA) proteins (Xie et al., 2011). Low levels of cross-reactivity with novel strains may exacerbate disease and enhance virus replication by a mechanism known as ‘original antigenic sin’ (Kim et al., 2009). Interestingly, some elderly individuals with antibodies against pre-2009 H1N1 strains, including the 1918 H1N1 virus, did not show an effective cross-reaction with the pH1N1 virus, yet did not develop substantial disease (Manicasamy et al., 2010; Reed & Katz, 2010; Wei et al., 2010). This result may be attributed to cross-protection mediated by T-cells to conserved internal viral proteins, such as the nucleoprotein (NP), which is known to confer heterosubtypic immunity (Skountzou et al., 2010; Tu et al., 2010).

Whilst antibody responses against pH1N1 have been studied with regard to cross-reactivity (Hancock et al., 2009), vaccination (Chen et al., 2011; Pascua et al., 2009) and pre-existing immunity (Chi et al., 2010; Gras et al., 2010), few studies have examined the contribution of pre-existing memory T-cells to the immune response against pH1N1 in a mouse model. Whilst the relatively conserved nature of T-cell epitopes probably confers a level of heterosubtypic immunity, it is possible that T-cell immunodominance to pH1N1 is different to that towards pre-2009 H1N1 influenza. To evaluate the memory T-cell response in this study, mice were primed with an H3N2 strain having HA and NA from A/Hong Kong/1/68 virus with the internal proteins from A/Puerto Rico/8/34 (X31). The T-cell response and subsequent pathology were evaluated following challenge with pH1N1 (A/California/4/2009) or a pre-2009 H1N1 (A/Puerto Rico/9/1934) influenza virus. Mice challenged with pH1N1 had inadequate heterosubtypic T-cell responses compared with homotypic T-cell responses, and adoptive transfer studies of H3N2-specific memory T-cells confirmed the presence of overlapping primary and memory T-cell responses to pH1N1 challenge. Subtle differences (1 amino acid) in NP and PA are known to affect MHC class I-restricted immunodominant epitopes (Moffat et al., 2009) and may be sufficient to modify the memory T-cell response against pre-2009 influenza and pH1N1 strains. This study showed how the 2009 pandemic H1N1 influenza virus may evade pre-existing H1N1 influenza virus memory T-cells, and provides important insights into the mechanisms that could contribute to the immunity and pathology, and provides future considerations for vaccination strategies against influenza.
Table 1. Amino acid comparison of immunodominant influenza epitopes

Amino acid sequences of the influenza internal proteins NP, PA and PB1 containing immunodominant epitopes, derived from genomic RNA sequence comparison between the priming strain X31 and challenge strains, H1N1 and pH1N1, are shown. The percentage amino acid identity is indicated. Where there is a difference in the immunodominant epitopes, the pH1N1 residue is shown in bold and underlined for the pH1N1.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Amino acid identity (%) relative to X31</th>
<th>Immunodominant epitope sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NP</td>
<td>PA</td>
</tr>
<tr>
<td>A/Puerto Rico/8/1934 (PR8; H1N1)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>A/California/4/2009 (pH1N1)</td>
<td>91.3</td>
<td>95.5</td>
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</tbody>
</table>

RESULTS

CD8+ T-cell epitopes are conserved between pH1N1 and pre-pandemic H1N1 influenza

To determine whether heterosubtypic immunity might participate in H1N1 cross-protection, CD8+ T-cell epitope homology between X31 (H3N2; priming strain) and pH1N1 (challenge strain) was compared with that of PR8, the prototypical H1N1 heterosubtypic challenge strain (Kees & Krammer, 1984; Liang et al., 1994; Rutigliano et al., 2010). The reassortant X31 virus (PR8 x A/ichi/68, H3N2) differs from PR8 by expression of H3 and N2, providing an ideal model for comparing memory T-cell responses (Rutigliano et al., 2010; Sipo et al., 2011). An amino acid comparison of immunodominant CD8+ T-cell epitopes of influenza, shown in Table 1, revealed that the highest degree of immunodominance was shown by NP (NP366), followed by the acid polymerase (PA224) and then the basic polymerase 1 (PB1703) (Crowe et al., 2003; Meijers et al., 2005). CD8+ T-cells that recognize these epitopes constitute the majority of the CD8+ T-cell response to influenza (Andreansky et al., 2005; Belz et al., 2001). pH1N1 has a high degree of homology to PR8/X31 (90–95%). The sixth amino acid of NP366 (M371V) is a T-cell receptor (TCR) contact residue (Kedzierska et al., 2008a; Price et al., 2000), and has been shown to affect immunity between PR8 and pH1N1 (Soboleski et al., 2011). This suggests that, whilst there is a high degree of homology, single amino acid differences in NP and PA epitopes may affect the expansion of heterosubtypic cross-reactive memory CD8+ T-cells recognizing the same antigen (Zhong et al., 2010).

Delayed virus clearance following challenge with pH1N1

Influenza has been known to undergo mutation of key amino acid residues with respect to neutralizing antibody epitopes (Hensley et al., 2009; Igarashi et al., 2009), suggesting the possibility that the amino acid differences in NP and PA might facilitate pH1N1 evasion of NP- and PA-specific memory CD8+ T-cells. To address this, X31-primed mice were challenged with a lethal dose (10 LD50) of PR8 (H1N1) or pH1N1. Lung virus titre was determined at days 1, 3, 5, 7 and 9 days post-challenge (Fig. 1). By day 3 post-challenge, a tenfold higher pH1N1 lung viral titre was evident compared with H1N1-challenged mice. H1N1-challenged mice had reduced lung viral titres up to day 3, and the virus was cleared by day 5 post-challenge (<102 TCID50 was the limit of detection). In contrast, lung viral titres in pH1N1-challenged mice remained significantly higher at day 3 (P=0.022) and day 5 (P=0.032) post-challenge. Notably, detectable lung viral titres persisted in pH1N1-challenged mice until day 7 post-challenge, i.e. 4 days longer than H1N1-challenged mice. Despite the greater lung viral burden in H1N1-challenged mice, none of the pH1N1 challenge mice succumbed to the lethal challenge, providing evidence of an effective memory response, albeit with delayed kinetics compared with the response to H1N1 challenge.

The kinetics of the memory T-cell response is delayed in pH1N1-challenged mice

In the absence of pre-existing neutralizing antibodies, antigen-specific T-cells are required for virus clearance...
(Epstein et al., 1998; Topham et al., 1996a, b). To address differences in the kinetics of virus clearance between H1N1- and pH1N1-challenged mice, the kinetics of memory T-cell reactivation was determined based on the influx of effector T-cells, defined as CD62Llo CD44hi (Ahmadzadeh et al., 2001; Cerwenka et al., 1999; Doyle et al., 1999) in the lung airways. At day 5 post-challenge, the majority (85%) of the CD8+ T-cells in the bronchoalveolar leukocytes (BAL) from H1N1-challenged mice were CD62Llo CD44hi effector cells, whereas for pH1N1-challenged mice, a lower proportion (55%) of CD62Llo CD44hi effector cells were in the BALs (Fig. 2a, left). At day 9 post-challenge, the frequency of CD62Llo CD44hi effector CD8+ T-cells in the BAL of pH1N1-challenged mice was similar to that in H1N1-challenged mice. The frequency of CD62Llo CD44hi effector CD8+ T-cells in the mediastinal lymph nodes (MLNs) was also lower (by approximately 30%) in pH1N1-challenged mice and remained so throughout the time course (Fig. 2a, right). It was notable that the peak response times of these T-cell subpopulations correlated with the time of virus clearance (Fig. 1; days 5 and 9 post-challenge, respectively).

CD62Llo CD44hi CD4+ effector T-cells in the BAL were similar in kinetics and frequency, regardless of the challenge virus strain (Fig. 2b). However, there was a slightly lower proportion of effector CD4+ T-cells isolated from the MLNs from days 5–9 after pH1N1 challenges. This finding may suggest that CD4+ T-cells are recognizing more conserved MHC class II epitopes between H1N1 and pH1N1; this hypothesis could explain why X31-primed mice are protected from a lethal pH1N1 challenge, which is consistent with a previous finding showing MHC class II (CD4+ HLA)-restricted epitopes were more conserved compared with MHC class I in another pH1N1 strain (Ge et al., 2010). The high percentage (>80%) of effector CD4+ T-cells may contribute to an antiviral response, perhaps through the elaboration of cytokines, until there is sufficient CD8+ T-cell to clear the infected cells.

Fig. 2. Effector T-cell responses are delayed after pH1N1 challenge compared with PR8 challenge. X31-primed mice were rested for 28 days and then challenged with PR8 or pH1N1. Representative contour plots of effector (CD44hi CD62Llo) CD4+ and CD8+ T-cells at days 5 and 9 post-challenge show the proliferative peak of T-cells in response to H1N1 and pH1N1 challenge, respectively. Gated plots of CD8+ (a) and CD4+ (b) T-cells show CD62L CD44 expression from BAL (left) and MLN (right) for PR8 and pH1N1 challenge. The mean percentage from three mice per experiment of CD4+ and CD8+ T-cells expressing the effector phenotype are indicated above the gate. Bar graphs showing the mean percentage ± SEM are also shown below the panels. Data are representative of two independent experiments.
Day 0

Inflammatory cell exudate in alveoli
Lymphoplasmacytic, histiocytic perivascular cuffs
Necrosis of bronchiolar epithelium

Day 5

Inflammatory cell exudate in alveoli
Lymphoplasmacytic, histiocytic perivascular cuffs
Necrosis of bronchiolar epithelium

Day 9

Lymphoplasmacytic, histiocytic perivascular cuffs

(b)

Pathology score (0–3)

<table>
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<tr>
<th>Days post-challenge</th>
<th>H1N1</th>
<th>pH1N1</th>
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<tbody>
<tr>
<td>5</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td>9</td>
<td>0.5</td>
<td>0.8</td>
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</table>

Pathology score (0–3)

<table>
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<tr>
<th>Challenge strain/days post-challenge</th>
<th>H1N1 day 5</th>
<th>pH1N1 day 5</th>
<th>H1N1 day 9</th>
<th>pH1N1 day 9</th>
</tr>
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<tbody>
<tr>
<td>H1N1 day 5</td>
<td>2.0</td>
<td>1.5</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>pH1N1 day 5</td>
<td>1.0</td>
<td>0.5</td>
<td>0.8</td>
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</tr>
<tr>
<td>H1N1 day 9</td>
<td>1.0</td>
<td>0.5</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>pH1N1 day 9</td>
<td>0.8</td>
<td>0.5</td>
<td>0.8</td>
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</tbody>
</table>
As the level of memory CD8\(^+\) T-cells determined by effector phenotype (Fig. 2) was inversely proportional to lung virus titre for H1N1- and pH1N1-challenged mice (Fig. 1), it is possible that the T-cells have different cytotoxic capacities towards H1N1- and pH1N1-infected cells. Thus, the level of memory CD8\(^+\) T-cell cytotoxicity generated against H1N1 or pH1N1 was evaluated. X31-primed mice were challenged with a lethal dose of H1N1 or pH1N1 and evaluated at day 5 post-challenge, i.e. the peak CTL response, using secondary \textit{in vitro} restimulation and

Fig. 3. Lung pathology is more severe and viral antigen persists in mice challenged with pH1N1 compared with mice challenged with PR8. X31-primed mice were rested for 28 days and then challenged with PR8 or pH1N1. (a) Whole lungs from challenged mice were fixed, sectioned and evaluated for histopathology by haematoxylin and eosin (H&E) staining for various parameters that constitute pulmonary lesions. Panels are representative H&E stains of triplicate lung samples. Day 0 shows representative normal, uninfected tissue for both experimental groups. Specific pathological parameters are noted on each figure. Bars show the cross-section length in each panel. (b) The graphs below the panels indicate the comparative gross histopathological scores of the challenge groups (left graph) (in addition to scores associated with specific pathological parameters (right graph) at days 5 and 9 post-challenge. (c) Representative IHC images of influenza antigen (H1N1) on days 5 and 9 after challenge with PR8 or pH1N1 (n=3 per group). Day 0 shows representative normal tissue lacking influenza virus antigen for both experimental groups. The images and scores are representative of two independent experiments. Solid arrows indicate NP staining, whilst dashed arrows indicate scattered inflammatory cells. The circle indicates slight inflammation around a terminal bronchiole. A, alveolus; B, bronchiolae; TB, terminal bronchiole.
expansion. After in vitro expansion, CTL cytolysis was assessed by flow cytometry, but there were no detectable differences in cytotoxicity generated in response to pH1N1 or H1N1 challenge (data not shown). Thus, the intrinsic killing ability of CD8+ T-cells did not seem to be affected.

**Virus levels persist and are associated with pathology in pH1N1-challenged mice**

Histopathology of the lungs and airways following influenza infection results from a combination of events involving immune cells and virus replication (reviewed by La Gruta et al., 2007); thus, the level and timing of lung pathology was determined. Accordingly, lung histopathology was evaluated in X31-immune mice challenged with PR8 or pH1N1 at days 5 and 9 post-challenge, which were the peak T-cell response days for H1N1- and pH1N1-challenged mice, respectively. At day 5, mice challenged with PR8 or pH1N1 showed similar levels and types of inflammatory cells and exudates in the alveoli, but pH1N1-challenged mice had slightly more extensive necrosis of the bronchioles (Fig. 3a). Where inflammation was present, the infiltrates consisted mainly of lymphocytes with small number of macrophages, consistent with the high effector T-cell response (Fig. 2).

At day 9, pH1N1-challenged mice continued to exhibit pathological signs but with markedly higher levels of neutrophils in the alveoli compared with the PR8-challenged mice. At day 9, PR8-challenged mice showed attenuated pathology with minimal necrosis and inflammation, whereas pH1N1-challenged mice still had necrosis and inflammatory exudates consistent with the delayed virus clearance (Fig. 1). Although no substantial differences were evident by gross histopathology scores (scale of 0–3) between the challenge groups, pH1N1 challenge was associated with a consistently higher average score (Fig. 3b, left panel), and evaluation of specific pathological parameters (necrosis and alveolar exudates) revealed differences at day 9 post-infection, primarily lung exudate in the alveolar lumen (Fig. 3a, b).

The presence of viral NP antigen was evaluated by immunohistochemistry (IHC) (Fig. 3c). IHC of lung sections revealed little detectable NP antigen at day 5 after H1N1 challenge in the bronchioles and alveoli, but in pH1N1-challenged mice, terminal bronchioles and surrounding parenchyma showed bronchiolar epithelial cells with NP staining (Fig. 3c, solid arrows). The surrounding parenchyma displayed inflammatory infiltrate composed of macrophages, neutrophils and lymphocytes. Scattered inflammatory cells (Fig. 3c, dashed arrows) also exhibited strong nuclear staining. By day 9, NP was mostly undetectable, although there was slight inflammation around a terminal bronchiole (Fig. 3c, circled) with staining detectable. NP antigen was undetectable at day 9 after H1N1 challenge, a feature consistent with the virus titre findings.

**pH1N1 challenge induces a primary and cross-reactive memory T-cell response**

To evaluate the cross-reactive memory T-cell response, a pilot adoptive transfer study was performed to distinguish whether the T-cells responding against pH1N1 were from a memory pool or were a recently activated primary T-cell response. Donor mice (CD90.1) were primed with X31 and their memory T-cells transferred to naive congenic mice (CD90.2) and challenged with pH1N1 or PBS. At day 9 post-challenge, flow cytometry revealed that donor CD90.1+ T-cells (both CD4+ and virus-specific CD8+) were present at higher levels in PR8-challenged mice compared with pH1N1-challenged mice (data not shown). As this study was not statistically powered, the cross-reactivity of memory T-cells was evaluated by ELISPOT to determine the number of gamma interferon (IFN-γ) spot-forming units (s.f.u.). The resting (Fig. 4a) and effector (Fig. 4b) memory CD8+ T-cell response to pH1N1 immunodominant peptides (Table 1) was evaluated. Treatment with phorbol 12-myristate 13-acetate (PMA)/ionomycin induced a large number of s.f.u. compared with unstimulated cells, as expected. The resting CD8+ T-cell memory response to PR8/H1N1 NP was significantly (P=0.01) higher compared with that towards pH1N1 NP. The resting memory cell responses were similar between the PR8/H1N1 and pH1N1 PA. The difference in response to NP could be due to the Met-to-Val mutation occurring in the contact residue, which may have a more profound effect on the cellular response to the peptides. It is noteworthy that some cross-reactive CD8+ T-cells were able to respond to pH1N1 NP, which probably represents the effector CD8+ T-cell fraction that responded to pH1N1 in Fig. 2.

At day 5 post-challenge with PR8 or pH1N1, the level of IFN-γ expression in effector memory CD8+ T-cells in response to the same viral peptides was determined (Fig. 4b). As predicted, PMA/ionomycin treatment induced higher s.f.u. numbers compared with unstimulated cells. Although not significant, s.f.u. numbers for the controls were higher in H1N1-challenged mice, an effect that may be related to CD4+ and CD8+ T-cell bystander activation. Thus, this did not represent a strictly virus-specific CD8+ T-cell response. pH1N1-challenged mice had a higher s.f.u. response (P=0.008) to pH1N1 NP peptide compared with the response to PR8/H1N1 NP, a finding consistent with the hypothesis that a low frequency of X31 memory cells are responding to pH1N1 peptides and that a novel set of T-cells is responding to pH1N1 peptides. It was interesting to note that the response to PR8/H1N1 NP was comparable at day 5 (Fig. 4b), even though the NP response was higher in resting memory cells (P=0.01; Fig. 4a). In addition, the PR8/H1N1 challenge group mounted a more robust response (P=0.009) compared with that towards PR8/H1N1 PA, although there was no difference in the resting memory T-cells response. This could be due to skewing of the memory response towards PA and PB1 in secondary
responses over NP (La Gruta et al., 2006). Thus, subtle differences that may not be evident in a resting memory T-cell subpopulation can become exaggerated in effector memory T-cell responses. This may also explain why there was little difference between the challenge groups to PR8/H1N1 NP. Finally, stimulation with PB1 did not show any significant differences as expected because it is identical in both viruses.

**DISCUSSION**

This study showed that mice with T-cell immunity to pre-2009 H1N1 mounted a different response upon challenge with pH1N1 compared with homotypic challenge with H1N1 (PR8). Interestingly, X31-immune mice mounted a memory T-cell response against pH1N1, as well as a primary response to pH1N1, resulting in delayed virus

![Graphical representation](image-url)
clearance and corresponding to exacerbated lung histopathology compared with PR8 challenge. One explanation may be that PR8-challenged mice have a higher frequency of cross-reactive memory T-cells compared with pH1N1 challenged mice. This explanation is supported by the findings in this study where, after challenge, a higher proportion of memory T-cells recognized cross-reactive immunodominant epitopes and were able to expand and traffic to the airways to exert their effector functions, as evident by the higher number and proportion of effectors (CD44hi CD62Llo) T-cells. These findings were corroborated by results from adoptive transfer studies showing memory T-cells from X31-immune mice being present at higher levels in PR8-challenged mice compared with those in pH1N1-challenged mice. The presence of cross-reactive memory T-cells responding to pH1N1 was supported by the survival of mice following lethal challenge, as X31-primed mice were challenged with an equal lethal dose of PR8 or pH1N1. Substantial differences in the kinetics of the T-cell response and in CTL cytotoxicity were expected, but no detectable differences in the ability of memory CD8+ T-cells generated in response to PR8 and pH1N1 challenge were observed. This suggested that the intrinsic CTL cytotoxicity was comparable in response to pH1N1- and PR8-infected cells, and is consistent with previous studies that have dissected the difference in T-cell clonotypes (Kedzierska et al., 2008b; Zhong et al., 2007) and TCR usage (Kedzierska et al., 2006), and whose findings reveal very small differences in T-cells responding to the different viruses and their epitopes. Future studies will investigate the properties of memory CD8+ T-cells in the response to pH1N1 and homotypic and heterosubtypic responses to influenza vaccination and challenge.

The difference in the level of CD4+ effector T-cells was less pronounced between PR8- and pH1N1-challenged mice. One possibility is that a higher proportion of the memory CD4+ T-cells are able to recognize conserved pH1N1 MHC class II-restricted epitopes. It is likely that effector CD4+ T-cells contribute to protection against the lethal influenza challenge in this model, as has been shown previously for other influenza studies (Topham et al., 1996a, b).

The primary T-cell response following pH1N1 challenge may be explained by several mechanisms including by the level of viral antigen in the lungs. Established memory CD8+ T-cells have been shown to suppress clonal expansion of naïve CD8+ T-cells specific for identical and similar epitopes from the same pathogen (Stevenson et al., 1999). This threshold may have been sufficient so that memory CD8+ T-cell activation did not occur, allowing antigen-presenting cells to present antigen to naïve T-cells in pH1N1-challenged mice. This was supported by virus titre (TCID50) and antigen clearance (IHC) findings, where virus antigen persisted at least 4 days longer compared with the H1N1 challenge.

It would have been optimal to compare the immune response in X31-primed mice with naïve mice challenged with pH1N1 as a positive control for a primary response, but naïve mice do not survive beyond day 7 following lethal challenge (data not shown). Accordingly, a lower dose of pH1N1 challenge using 1 LD50 (70 p.f.u.) was investigated but yielded very low lung virus titres after intranasal infection, thus preventing comparisons (data not shown). However, the results presented here provide evidence that small differences in T-cell response can be attributed to differences in the sequence between PR8 and pH1N1. Whilst the overall homology was high (90%), the difference in the immunodominant epitopes as shown in Table 1 (H-2Dβ NP366 and H-2Dβ PA224) affected the CD8+ T-cell response when challenged with pH1N1. The ELISPOT results (Fig. 4) highlight this method as a useful indicator of T-cell cross-reactivity for examining immunodominant epitopes when comparing the T-cell response to closely related viruses, as the IFN-γ expression in response to immunodominant viral peptides showed some remarkably different responses. Whilst the ELISPOT may serve as a useful indicator of cross-reactivity, it does not indicate to what extent the CD8+ T-cells are cross-reactive, i.e. TCR avidity to MHC peptide. This is important, as different avidities induce different responses including cytokine secretion (La Gruta et al., 2004), and subtle changes in immunodominant epitopes in pH1N1 can affect the host response and lead to increased immune evasion, as has been observed for protection against H5N1 avian influenza (Kreijtz et al., 2008; Lee et al., 2008).

Although antibodies have a significant role in neutralizing influenza virus, studies evaluating pre-2009 influenza virus antibody cross-reactivity against pH1N1 have concluded that there are generally no cross-reactive antibodies, although there may be cross-protective antibodies (Hancock et al., 2009; Wrammert et al., 2011) such as antibodies against NP (LaMere et al., 2011; Miyoshi-Akiyama et al., 2010; Mizuike et al., 2011) and M1 (Sipo et al., 2011). As these proteins are not expressed on the cell surface, T-cells dominate the response against them. To our knowledge, this is the first study to evaluate the role of pre-2009 memory T-cell responses against these pandemic influenza virus proteins. The study results presented here emphasize the need to generate cross-protective memory T-cells following vaccination, and the need to consider pre-existing T-cell responses when designing vaccination strategies against influenza virus.

METHODS

RNA isolation and sequencing. Viral RNA was extracted using an RNeasy kit (Qiagen). NP, PA, and PB1 gene segments for sequencing were amplified using the One-step RT-PCR kit (Qiagen) according to the manufacturer’s instructions. Purified sequencing products were loaded onto an ABI 3130XL genetic analyser (Applied Biosystems) and separated by capillary electrophoresis through an 80 cm capillary array. The resulting sequence traces were trimmed and assembled using Sequencer software (Genecodes). Nucleotide coding sequences were translated into the corresponding amino acid sequences using the online ExPaSy Proteomics Server provided by the Swiss Institute of Bioinformatics. The amino acid sequences of X31 and H1N1 were...
then aligned with the pH1N1 sequence using the National Center for Biotechnology Information’s influenza virus sequence database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html).

**Influenza viruses and infection of mice.** Influenza strains H1N1 (A/Puerto Rico/8/1934) and X31, a recombinant virus with the HA and NA (H3N2) derived from A/Hong Kong/1/68 but with the internal proteins (e.g. NP, PA, PB1) from Puerto Rico/8/1934, were propagated in 9-day-old embryonated chicken eggs as described previously (39). Allantoic fluids were extracted and stored at −80 °C until use. Mouse-adapted influenza A/California/04/2009 (pH1N1) was grown in vitro by infecting Madin–Darby canine kidney (MDCK) cells in minimal essential medium (MEM) supplemented with 1-glutamine and 1 μg TPCK-treated trypsin (Worthington) ml−1 at an m.o.i. of 0.01. Three days after infection, cell-culture supernatant was collected and stored at −80 °C.

For infections, 8–10-week old female C57BL/6 mice (National Cancer Institute) were anaesthetized with 2,2,2-trichloroethanol (Avertin) (Tripp et al., 1997). Subsequently, the mice were instilled intranasally with 1000 p.f.u. X31 diluted in 50 μl PBS. At 28 days after priming with X31, the mice were challenged in the same manner with 10 LD_{50} of H1N1 (1000 p.f.u.) or pH1N1 (700 p.f.u.). All mouse work was approved by the Institutional Animal Care and Use Committee of the University of Georgia.

**Cell preparation and flow cytometry.** At days 5 and 9 after H1N1 or pH1N1 challenge, mice were euthanized and BALs and MLNs were collected to prepare single-cell suspensions. Cell numbers from the BAL and MLN samples were enumerated using a Z2 Coulter Counter (Beckman-Coulter). To analyse cells by FACS, 10^6 BAL or MLN cells were enumerated using a Z2 Coulter Counter or pH1N1 challenge, mice were euthanized and BALs and MLNs were collected and stored at −80 °C until use. Mouse-adapted influenza A/California/04/2009 (pH1N1) was grown in vitro by infecting Madin–Darby canine kidney (MDCK) cells in minimal essential medium (MEM) supplemented with 1-glutamine and 1 μg TPCK-treated trypsin (Worthington) ml−1 at an m.o.i. of 0.01. Three days after infection, cell-culture supernatant was collected and stored at −80 °C.

For infections, 8–10-week old female C57BL/6 mice (National Cancer Institute) were anaesthetized with 2,2,2-trichloroethanol (Avertin) (Tripp et al., 1997). Subsequently, the mice were instilled intranasally with 1000 p.f.u. X31 diluted in 50 μl PBS. At 28 days after priming with X31, the mice were challenged in the same manner with 10 LD_{50} of H1N1 (1000 p.f.u.) or pH1N1 (700 p.f.u.). All mouse work was approved by the Institutional Animal Care and Use Committee of the University of Georgia.

**Histopathology and IHC.** Lungs from mice challenged with pH1N1 or PR8 were perfused and fixed in cold 10 % (w/v) neutral-buffered formalin. The lungs were treated with paraffin and sectioned as described previously (Smith et al., 2011). Briefly, 5 μm sections of lungs were stained with H&E. Influenza antigen was examined by IHC of the same tissue sections. Sections were blocked with 1 % BSA in PBS and treated with proteinase K (Dako) to minimize non-specific staining and expose the epitopes. Subsequently, sections were incubated with goat anti-influenza A H1N1 antibody (1 μg ml^{-1}; Meridian Life Science) and then incubated with a biotinylated rabbit anti-goat antibody (Dako). Finally, streptavidin–HRP complex (Dako) was added for 10 min with colour development carried out using the HRP substrate diamino benzidine for 10 min. The cells were assessed by microscopy, and histopathology and IHC images and pathology scores were evaluated in a blinded manner by an independent pathologist.

**Adoptive transfer of congenic memory cells.** A congenic mouse model was used to assess the source of T-cells (primary or memory) responding to pH1N1 or PR8 challenge by adapting methods described previously (Topham et al., 2001; Turner et al., 2001). Briefly, CD90.1^+ C57BL/6 (donor) mice were primed via intranasal infection with 10^5 p.f.u. X31 and rested for 28 days. The mice were sacrificed to extract single cells from the MLNs and spleen. CD3^+ T-cells were enriched by negative selection using an AutoMACS magnetic cell separation kit (Miltenyi Biotec). Post-enrichment analysis by flow cytometry confirmed that the cells were >98 % CD3^+ T-cells. Memory cells were sorted from CD3^+ T-cells by using FACS Aria II cell sorter (BD Biosciences). Post-sort analysis revealed that the sorted cells were >90 % memory CD3^+ T-cells. The cells were washed and resuspended in PBS for intraperitoneal injection into CD90.2^+ congenic C57BL/6 mice (10^6 memory T-cells per recipient). Mice were then allowed to rest for 1 day after the adoptive transfer and then challenged with 0.1 LD_{50} H1N1 or pH1N1. At day 9 post-challenge, the mice were sacrificed and the cells from MLNs, spleen and BAL were immunostained and analysed by flow cytometry for CD90.1, CD90.2, CD8^+, IFN-γ, CD44 and CD62L expression and CD8^+ T-cell epitope specificity by MHC class I tetramers.

**ELISPOT.** ELISPOT was used to assess the frequency of IFN-γ expressing memory CD8^+ T-cells responding to virus immunodominant peptides as previously described (Andreansky et al., 2005). Briefly, ELISPOT plates (Millipore) were coated with 10 μg ml^{-1} of anti-mouse IFN-γ antibody (capture antibody; BD Biosciences) overnight at 4 °C. Splenocytes were extracted and plated (5 x 10^{5}–1.25 x 10^{6} cells per well) and cultured in complete RPMI. Cells were stimulated with 10 μM H1N1- or pH1N1-derived NP, FA and PB1 peptides (Table 1), in addition to an irrelevant peptide (GFP300–308, HYLSTQFL), no peptide (medium/cells only), or PMA and

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IONOMYCIN AT 25 AND 1.25 MG ML⁻¹, RESPECTIVELY, USED TO STIMULATE THE INTRACELLULAR PRODUCTION OF CYTOKINES FOR MAXIMUM INFN-Ɣ INDUCTION. ALL PEPTIDES WERE MANUFACTURED BY NEW ENGLAND PEPTIDES. TWO DAYS AFTER CULTURE, CELLS WERE REMOVED AND INCUBATED WITH BIOTINYLATED ANTI-INFN-Ɣ (DETECTION ANTIBODY; BD BIOSCIENCES) AT ROOM TEMPERATURE FOR 1 H. FINALLY, STREP-IALevin–ALKALINE PHOSPHATASE (THERMO SCIENTIFIC) WAS ADDED FOR 30 MIN AND THE INFN-Ɣ SPOTS WERE DEVELOPED USING A ONE-STEP BCIP/NBT KIT (KPL). THE NUMBER OF SPOTS WAS ENUMERATED.

STATISTICS. ALL DATA POINTS WERE ASSESSED FOR STATISTICAL SIGNIFICANCE USING A ONE-TAILED STUDENT’S t-TEST WHERE APPROPRIATE TO EVALUATE THE DIFFERENCE IN MEANS BETWEEN CHALLENGE GROUPS/TREATMENTS. EXACT P VALUES ARE LISTED WHEN SIGNIFICANT (P ≤ 0.05). ALL STATISTICAL ANALYSES WERE PERFORMED USING GRAPH PAD PRISM SOFTWARE (GRAPH PAD SOFTWARE). THE NUMBER OF INDEPENDENT EXPERIMENTS IS INDICATED FOR EACH EXPERIMENT IN THE FIGURE LEGENDS.

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REFERENCES


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