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Influenza vaccine–induced human bone marrow plasma cells decline within a year after vaccination

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A universal vaccine against influenza would ideally generate protective immune responses that are not only broadly reactive against multiple influenza strains, but also long-lasting. Because long-term serum antibody levels are maintained by bone marrow plasma cells (BMPC), we investigated the production and maintenance of these cells after influenza vaccination. We found increased numbers of influenza-specific BMPC four weeks after immunization with the seasonal inactivated influenza vaccine, but numbers returned to near their pre-vaccination levels after one year. This decline was driven by the loss of BMPC induced by the vaccine, while pre-existing BMPC were maintained. Our results suggest that most BMPC generated by influenza vaccination in adults are short-lived. Designing strategies to enhance their persistence will be a key challenge for the next generation of influenza vaccines.

The goal of vaccination is to generate long-lasting protection against infection. Most vaccines in clinical use achieve this protection, at least in part, through the generation of pathogen-specific antibody responses. Antibody levels peak in the months following vaccination, followed by a decline to a plateau level which may be maintained for decades with minimal decline (1–3). Animal models have shown that these plateau antibody levels are maintained by non-dividing, bone marrow-resident long-lived plasma cells (4, 5). Studies of antibody synthesis rates have suggested that bone marrow plasma cells (BMPC) are likely to produce the majority of total serum IgG in humans as well (6, 7). Consistent with this, total and antigen-specific serum antibody levels correlate closely with BMPC numbers in humans (8, 9). Antibody titers and protective immunity decline rapidly following seasonal influenza vaccination (10, 11), suggesting that the vaccine may fail to elicit BMPC, or that these BMPC fail to become long-lived. To investigate these possibilities, we designed a clinical study of healthy adults (20–45 years) where we were able to obtain paired bone marrow samples before - and one month after - influenza vaccination, in conjunction with a full schedule of blood samples. In addition, we obtained a third bone marrow draw at approximately one year post-vaccination

from a number of individuals, allowing us to assess the long-term maintenance of vaccine responses in the bone marrow.

BMPC responses were measured in 53 volunteers that received the inactivated influenza vaccine between 2009 and 2018. A number of these 53 donors enrolled in multiple study years (see methods) and were treated as separate enrollments, for a total of 75 vaccine responses studied. In addition to providing blood samples on the day of vaccination (day 0) and on days 7, 14, 28, and 90 post-vaccination, the volunteers also underwent bone marrow aspiration procedures on days 0 and 28. In addition, a number of donors provided blood and bone marrow samples ~1 year post-vaccination, either at their re-enrollment for the following year's study, or as their final study timepoint.

We first examined influenza-specific immunity at baseline prior to vaccination. Using plasma cells enriched from the bone marrow aspirates, we performed ELISpot assays to quantify influenza-specific BMPC (Fig. 1A). Cells secreting influenza-specific antibodies (both IgG and IgA) were readily detectable in study volunteers prior to vaccination and were markedly more common than cells producing antibodies specific for tetanus toxin. Influenza-specific BMPC numbers were also 5–10 fold higher than we have observed for varicella

zoster virus (12). The percentage of influenza-specific IgG BMPC on day 0 followed a roughly log-normal distribution (Fig. 1B). The geometric mean percentage of IgG-secreting BMPC producing influenza-specific antibodies was 0.8% with an interquartile range (IQR) of 0.52% to 1.3%. Across the cohort, the percentage of influenza-specific IgG+ BMPC correlated significantly with blood influenza-specific IgG titers (Fig. 1C). These data are consistent with a key role for BMPC in maintaining humoral immunity to influenza. We also measured influenza-specific memory B cells (MBC) in the blood of the donors. Influenza-specific cells accounted for 0.93% (IQR: 0.58-1.5%) of IgG MBC in the blood (Fig. 1D). This MBC frequency correlated with blood antibody titers (Fig. 1E). Furthermore, there was a clear trend toward higher numbers of influenza-specific MBC in those donors with the most influenza-specific BMPC (Fig. 1F).

We next examined whether seasonal influenza vaccination resulted in the production of new influenza-specific BMPC. Influenza-specific BMPC increased significantly to 1.9% (IQR: 1.2-2.9%) of IgG BMPC by day 28 post-vaccination (Fig. 2A and fig. S1A). The seasonal influenza vaccine contains hemagglutinin (HA) proteins from multiple viral strains; one from an H1N1 influenza A strain, one from an H3N2 strain, and either one or two HA proteins from influenza B strains. An HA protein similar or identical to that of the pandemic H1N1 strain A/California/7/2009 was included in the vaccine from the 2010-2011 season until the 2016-2017 season, allowing us to monitor responses against a single, consistent antigen in multiple seasons. IgG BMPC specific for this HA increased from 0.11% (IQR: 0.07-0.18%) to 0.36% (IQR: 0.18-0.76%) on day 28 (Fig. 2A and fig. S1B). We also measured influenza-specific IgA BMPC when plasma cell yields were high enough to run multiple antigen-specific ELISpot assays from each sample. Although we only obtained paired IgA data for six donors, there was a trend toward increasing numbers of IgA BMPC (Fig. 2A). This trend was significant in the unpaired analysis (fig. S1C), which included additional donors where IgA BMPC could be measured at day 0 or day 28 but not both. These increases were specific to plasma cells recognizing influenza vaccine antigens; no changes were observed in the percentage of cells secreting IgG specific for tetanus toxin (Fig. 2A and fig. S1C). Thus, we found no evidence of bystander effects of the vaccine on the frequencies of BMPC specific for unrelated antigens.

We have previously shown that influenza-specific antibody secreting cells (ASC) appear in the blood approximately one week after vaccination (13). Since bone marrow aspirate samples will unavoidably contain some level of contamination with peripheral blood, we wanted to confirm that influenza-specific ASC were not in circulation at the time of bone marrow aspiration. Influenza-specific IgG ASC were at very low levels or undetectable in the blood on day 0, apart from

in one donor with a recent history of an influenza-like illness (green symbols). ASC numbers peaked at day 7, declined sharply by day 14 and were again very rare or undetectable on day 28 (Fig. 2B). Similar results were seen for IgA and IgM ASCs (fig. S1, D and E). Thus, the percentages of influenza-specific BMPC on days 0 and 28 would not have been inflated by contamination with circulating ASC. The magnitude of the day 7 ASC response correlated with the increase in influenza-specific BMPC after vaccination (Fig. 2C). This is consistent with models suggesting that BMPC are derived from blood ASC that migrated to the bone marrow (14).

To determine whether the increase in influenza-specific BMPC was maintained, we obtained bone marrow samples from a subset of donors at a late timepoint (mean 325 days post-vaccination: range 220 to 457 days). The percentage of influenza-specific BMPC declined significantly between day 28 and the late sample (Fig. 3A). In subjects who re-enrolled in multiple seasons and were vaccinated a second time, influenza-specific BMPC typically increased after both immunizations, although there was substantial heterogeneity among donors (fig. S2). As shown in Fig. 3B, in the subset of donors who provided a long-term follow-up sample, there was a geometric mean 1.9-fold change (IQR: 1.3-3.2 fold) in influenza-specific BMPC between day 0 and day 28, similar to the overall cohort. However, by one year, the percentage of influenza-specific BMPC had declined to baseline. Analysis of serum antibody levels revealed similar trends (Fig. 3C). The level of decline in BMPC did not appear to correlate with the timing of the 1 year bone marrow aspirate (fig. S3), suggesting that the loss of newly generated influenza-specific BMPC happened largely within 7 months post-vaccination.

We next examined in detail the clonal composition of the B cell response to the vaccine by performing immunoglobulin heavy chain repertoire sequencing of BMPC and ASC from four donors. Three out of four (donors 35, 40, and 42) had a strong day 7 ASC response to the vaccine (fig. S4A), and all four donors showed serologic responses to each influenza strain in the vaccine (fig. S4B), particularly to the H1N1 component. To identify influenza-specific antibody families (or "clonotypes") in the repertoire sequencing data, we employed two strategies: production of monoclonal antibodies (mAbs) cloned from day 7 ASC (all four donors), and a proteomics-based approach where antibodies specific to the H1 HA protein were identified from the polyclonal serum (donors 35 and 42) (fig. S5A). We identified 15 clonotypes that were highly expanded (>0.5% of day 7 ASC sequences) and that were determined to be influenza-specific by either mAb cloning or proteomics analysis (fig. S5B and table S5). These antibody families already exhibited significant somatic hypermutation in their heavy chain variable region genes at day 7, indicating that they were likely derived from pre-existing memory B cells. All 15 clonotypes were represented

among both IgA- and IgG-expressing ASC although some clonotypes were enriched for one isotype or the other. We hypothesize that multiple memory B cells from each clonotype responded to the vaccine, with varying recruitment of IgA vs IgG memory cells. Interestingly, of the 11 H1-specific clonotypes that we identified, only one of them (clonotype 8 from donor 42) contributed detectably to H1-binding antibodies found in the serum on day 0 (fig. S5B). The other ten H1-specific clonotypes could only be detected in the serum starting at day 7 or day 14. This suggests either that there were few long-lived plasma cells belonging to these clonotypes prior to vaccination or that these pre-existing plasma cells produced antibodies that bound poorly to the pandemic H1 HA protein.

Out of the 15 influenza-specific clonotypes that were expanded in the day 7 ASC, 14 increased significantly among BMPC IgG sequences between day 0 and 28 post-vaccination ($P=0.0003$ by Wilcoxon signed-rank test; Fig. 4A). Thus, the same antibody families that contributed to the blood ASC response also contributed to the BMPC compartment, again consistent with the idea that blood ASC give rise to BMPC. Between day 28 and the late timepoint (approximately one year), the percentage of BMPC IgG sequences belonging to these clonotypes decreased sharply ($P=0.0017$). After 1 year, 11 out of the 14 clonotypes still made up a higher percentage of BMPC IgG reads compared to day 0, however this difference was not statistically significant ($P=0.068$). There was a wide variation in how well the initial increase in frequency of each clonotype among BMPC sequences was sustained (Fig. 4B). The median increase in frequency for these 14 clonotypes after 1 year was only 0.15-fold as high as the initial increase seen at day 28 (IQR: 0.01-fold to 0.29-fold). In other words, for most clones, 70-99% of the newly generated BMPC were lost over 1 year. As a separate strategy to measure clonotype abundances over time, we designed clone-specific qPCR probe/primer sets (fig. S5C). By this qPCR assay, we observed a similar trend where vaccine-specific antibody clonotypes expanded and then declined in BMPC RNA samples (median increase in frequency at 1 year was 0.09-fold of the initial increase at day 28; IQR 0 to 0.38-fold). Both the initial increase in clonotype frequency among BMPC and the long-term increase correlated with the representation of each clonotype among day 7 ASC sequences (Fig. 4, C and D), consistent with what we observed by ELISpot (Fig. 2C) and in keeping with the notion that blood ASC give rise to BMPC.

Our clonotype tracking results suggested that the contraction in influenza-specific BMPC between day 28 and 1 year which we saw by ELISpot was due to the loss of newly-generated cells. To determine whether pre-existing influenza-specific BMPC were more stable over time, we examined “non-recruited” influenza-specific clonotypes in the two donors whose H1-specific responses were profiled by serum proteomics analysis. These clonotypes were defined as HA-specific

antibody families that were detected in the serum and in IgG BMPC sequences on day 0, but which represented <0.1% of day 7 vaccination-induced ASC by antibody repertoire sequencing (tables S4 and S5). The majority of these clonotypes could be detected both IgG and IgA BMPC sequences (see table S5). The non-recruited clones showed no significant change in their abundances over one year in either donor (Fig. 4, E and F), indicating that they likely represented a stable population of long-lived plasma cells, likely generated by previous influenza infection or vaccination.

In recent years, it has become clear that humans are capable of generating protective antibody responses directed against epitopes on the influenza HA and NA proteins that are broadly conserved across diverse viral strains. This has raised hopes that a “universal” influenza vaccine targeting these conserved regions could be developed. If such a vaccine elicited protective antibody responses that persisted for at least several years, it would eliminate the need for annual immunization, which would be a major improvement on current vaccines. The data presented here adds to our understanding of why humoral responses to influenza vaccine are not well maintained. We show that serum influenza-specific titers correlate with BMPC numbers prior to vaccination, supporting a key role for these cells in maintaining antibody levels. Vaccination does lead to the generation of new BMPC, and some of these persist for at least one year. However, most of the newly generated BMPC are lost within a year, showing that localization to the bone marrow is not enough to determine longevity. Several steps are required for a BMPC to become a long-lived plasma cell: the cell must reach the appropriate survival niche and successfully compete for space there, and the cell must undergo changes in gene expression and metabolism that promote longevity (15–20). Thus, our data suggests that most vaccine-induced BMPC fail at one or more of these steps.

The decline in BMPC and antibody levels observed here are likely not unique to the influenza vaccine. One study of children receiving the hepatitis B vaccine showed declines in antibody titers of 85-90% from peak levels in the first year post-vaccination (21). On the other hand, in a study of adults given a first booster of the tetanus, diphtheria, and acellular pertussis vaccine (Tdap), IgG antibody levels to the vaccine antigens declined only 40-66% in the first year and another 15-25% in the following year (22). This is a considerably slower rate of decay than we observed, suggesting that there is room for improvement for influenza vaccines.

A few findings from this study may be helpful for designing next-generation influenza vaccines capable of eliciting longer-term immunity. First, the blood ASC response after vaccination was highly predictive of the overall BMPC response, both in the short-term and in the long-term, and thus may be a useful marker for screening vaccine candidates.

Since ASC generation after influenza vaccination can be increased through the use of adjuvants (23–25), it may be useful to assess whether adjuvants are able to improve long-term BMPC responses in humans. Second, most of the pre-existing H1-specific antibody families detected in the serum on day 0 were not detected in the day 7 ASC response, and most of the H1-specific clonotypes that *were* recruited did not contribute appreciably to serum H1-specific titers prior to vaccination. This may reflect antibody feedback, in which pre-existing serum antibodies binding to the vaccine block B cells recognizing the same epitopes from acquiring antigen (26–30). Designing strategies to overcome this effect will be critical for boosting long-term numbers of BMPC targeting conserved epitopes.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

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References (31–42)

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Fig. 1. Analysis of human influenza-specific bone marrow plasma cells (BMPC) prior to vaccination. (A) Example ELISpot data using magnetically-enriched BMPC, with detection of cells secreting total or antigen-specific IgG or IgA. (B) The number of BMPC producing influenza-specific IgG prior to vaccination is expressed as a percentage of the total number of IgG-secreting BMPC. (C) Correlation between pre-vaccination influenza-specific IgG BMPC and blood influenza-specific IgG levels as measured by ELISA. (D) Influenza-specific IgG memory B cells (MBC) from peripheral blood are shown as a percentage of total IgG MBC prior to vaccination. (E) Correlation between influenza-specific IgG MBC and blood IgG titers prior to vaccination. (F) Correlation between the percentages of influenza-specific IgG BMPC and MBC. The data shown in (B) to (F) is for all study donors for which day 0 influenza-specific BMPC and MBC percentages were measured ($n=59$ including repeat study participants; $n=43$ unique donors). The means and standard deviations of the log-transformed data are indicated by red lines in (B) and (D). In (C), (E), and (F), Pearson correlation analysis was performed on the log-transformed BMPC, MBC, and ELISA data. The green dots represent a donor with evidence of recent influenza infection who was excluded from statistical calculations.

Fig. 2. Increase in human influenza-specific plasma cells in the bone marrow after vaccination. (A) The percentage of IgG- or IgA-secreting BMPC specific for the indicated antigens was measured by ELISpot on the day of vaccination (d0) and 28 days later (d28). Influenza = trivalent or quadrivalent inactivated influenza vaccine ($n=46$ IgG responses measured from 34 unique donors; $n=6$ IgA responses from 6 unique donors); pdm H1 HA = recombinant hemagglutinin protein from pandemic H1N1 strain A/California/07/2009 ($n=20$ responses from 14 unique donors); tetanus = tetanus toxin C fragment ($n=6$ responses from 6 unique donors). P-values shown are for paired, two-tailed *t* tests using the log-transformed data. The means and standard deviations of the log-transformed data are indicated in red. (B) The blood IgG antibody secreting cell (ASC) response to the influenza vaccine was measured by ELISpot. Data are shown for $n=45$ vaccine responses from 35 unique donors. The dotted line represents the lower limit of detection for the assay. (C) Pearson correlation between the magnitude of the IgG ASC response at day 7 and the increase in influenza-specific BMPC, which is calculated as the percentage of influenza-specific IgG BMPC at day 28 minus this percentage at day 0. Green symbols and lines in (A) to (C) indicate a donor with evidence of recent influenza infection who was excluded from the statistical analyses.

Fig. 3. Decline of human influenza-specific BMPC at one year after vaccination. (A) The percentage of influenza-specific IgG BMPC is shown over time in volunteers who returned for a third bone marrow aspiration approximately one year post-vaccination. P-values shown are for paired, two-tailed t tests of the log-transformed data. (B) The same data are plotted as in (A), except the percentages of influenza-specific BMPC at day 28 and 1 year are normalized to the day 0 percentage to visualize the fold-change relative to pre-vaccination levels in each donor. Numbers above the points indicate the geometric means of the fold-changes, with the interquartile ranges in parentheses. (C) Influenza-specific blood IgG ELISA titers at day 28, day 90, and 1 year were normalized to the day 0 titer and are plotted as in (B). The same $n=18$ responses from 15 unique donors are shown in all panels.

Fig. 4. Longitudinal tracking of human influenza-specific antibody lineages (clonotypes) after vaccination. Clonotypes were tracked over time in BMPC and ASC immunoglobulin sequences. 15 clonotypes comprising at least 0.5% of day 7 blood ASC IgG sequences were considered to have been “recruited” into the vaccine-specific response. Clonotypes are named by their frequency among ASC sequences (e.g., “35-2” = clonotype with second-highest read count in IgG sequences from day 7 ASC from donor 35). (A) The percentage of BMPC IgG sequences belonging to each recruited clonotype is shown on day 0, day 28, or 1 year post-vaccination. (B) For the 14 recruited clonotypes that increased in the bone marrow between days 0 and 28, the long-term increase after one year is shown as a proportion of the initial increase. A clonotype that neither expanded nor contracted between day 28 and 1 year would have a value of 1 on this scale, while a clonotype that declined to its starting frequency would have a value of 0 on the scale. Values above 1 indicate that the clonotype expanded among BMPC sequences between day 28 and 1 year, and values below 0 indicate clonotypes that comprised a lower percentage of IgG reads at one year compared to day 0. (C) Correlation between the percentage of day 7 ASC IgG sequences belonging to each clonotype and the initial increase in that clonotype among BMPC IgG transcripts between day 0 and day 28. (D) Correlation between the percentage of day 7 ASC IgG sequences belonging to each clonotype and the long-term increase of these clonotypes among BMPC sequences between day 0 and 1 year. (E) Persistence of pre-existing, “non-recruited” H1 HA-specific clonotypes in donor 35. Clonotypes were defined as pre-existing if they were found in the BMPC repertoire sequencing data at day 0 and confirmed to be influenza specific by serum proteomics analysis or monoclonal antibody production. Clonotypes were defined as “non-recruited” if they accounted for less than 0.1% of day 7 IgG, IgM, and IgA ASC sequences. (F) Persistence of pre-existing, “non-recruited” H1 HA-specific clonotypes in donor 42. P-values shown in (A), (E), and (F) are for the Wilcoxon signed-rank test. In (C) and (D), Pearson correlation was performed using the non-transformed data and the best-fit linear regression lines are shown.