Prevaccine Determination of the Expression of Costimulatory B7 Molecules in Activated Monocytes Predicts Influenza Vaccine Responses in Young and Older Adults

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Background. Innate immunity, including Toll-like receptor (TLR)–mediated expression of the B7 costimulatory molecules CD80 and CD86, is critical for vaccine immunity. We examined whether CD80 and CD86 expression vary with aging and predict response to the trivalent inactivated influenza vaccine.

Methods. One hundred sixty-two subjects between 21 and 30 years of age (the young group) or ≥65 years of age (the older group) enrolled before vaccination. We determined TLR-induced monocyte CD80/CD86 expression by flow cytometry and vaccine antibody responses by hemagglutination inhibition.

Results. The mean increase in TLR-induced CD80+ monocytes was reduced in older, compared with young, adults by 68% (P = .0002), and each decile increase of CD80+ cells was associated with an 8.5% increase in mean number of vaccine strains with a 4-fold titer increase (P = .01) and a 3.8% increase in mean number of strains with a postvaccine titer ≥1:64 (P = .057). Each decile decrease of CD86+ cells was associated with an 11% increase in the mean number of strains with a 4-fold increase (P = .002) and a 3.9% increase in the mean number of strains with a postvaccine titer ≥1:64 (P = .07).

Conclusions. CD80 and CD86 expression on activated monocytes is highly associated with influenza vaccine response. This approach prospectively identifies adults unlikely to respond to immunization who may benefit from alternative vaccines or antiviral prophylaxis during influenza outbreaks.

Influenza remains a significant global cause of morbidity and mortality affecting both the developed and developing worlds [1, 2]. Older adults (>65 years age) represent a particularly vulnerable population and make up 90% of the estimated 36,000 influenza-related deaths each year in the United States [3]. Several studies show that the use of the inactivated trivalent influenza vaccine causes a reduction in the incidence of influenza and all-cause mortality [4–9], and, in 2003, 292 million doses of influenza vaccine were distributed worldwide [10]. However, a recent study failed to corroborate the previously reported benefits of vaccination [11], and 2 meta-analyses reported only a modest benefit in community-dwelling older adults or health care workers [4, 12]. The observation that 30%–50% of older adults fail to mount protective antibody responses after influenza vaccination likely contributes to this limited efficacy [13–16].

The innate immune system, including the Toll-like receptors (TLRs) expressed on antigen-presenting cells, plays a critical role in activating the immune response...
to vaccination or infection [17–19]. TLRs recognize highly conserved components of pathogens (e.g., bacterial and mycobacterial lipopeptides, RNA and unmethylated DNA of viruses and bacteria, and lipopolysaccharide on gram-negative bacilli). TLR stimulation of monocytes or dendritic cells results in activation of the costimulatory B7 molecules CD80 (B7.1) and CD86 (B7.2), both ligands for the activating receptor CD28 and the inhibitory receptor cytotoxic T lymphocyte–associated protein–4 (CTLA-4) on T cells [20, 21]. However, little is known about the role of innate immunity in influenza vaccine responsiveness, particularly in older adults, who harbor a disproportionate share of the clinical morbidity from influenza each year. In this study, we determined the effects of CD80 and CD86 expression on strain-specific influenza vaccine responses and the influence of age on the expression of CD80 and CD86 on monocytes.

SUBJECTS, MATERIALS, AND METHODS

Subjects and study design. The objectives of this study were to evaluate whether the in vitro regulation of CD80 and CD86 expression on monocytes after stimulation predicted subsequent influenza vaccine response and to compare CD80 and CD86 responses between older and young adults. We enrolled older (≥65 years of age) and young (21–30 years of age) adults from influenza vaccination clinics organized by the Yale Health Services during the 2004–2005 influenza season. Immunocompromised subjects were excluded, including individuals with HIV infection, diabetes mellitus requiring medication, asplenia, other acquired or congenital immunodeficiencies, multiple sclerosis, malignancy (other than localized skin cancer or nonmetastatic prostate cancer), cirrhosis, renal failure requiring hemodialysis, and connective-tissue diseases, as well as pregnant women. Also, the current use of immunomodulating medications, including prednisone or other steroids, and self-reported symptoms of acute or recent infection were reasons for exclusion (figure 1). Heterogeneity was observed in sex and race distribution, but these and other covariates were accounted for in our multivariate model (see “Statistical analysis” below). This study was approved by the Human Investigations Committee at Yale University, and human experimentation guidelines of the US Department of Health and Human Services and those of Yale University were followed in the conduct of this study. Informed consent was obtained from all participants.

Assessment of immunogenicity. A serum sample was collected from each subject before vaccination and ∼35 days (range, 28–42 days) after vaccination. Serum samples were separated into aliquots and stored at a temperature of ∼20°C or lower until assayed. We performed hemagglutination inhibition (HAI) assays on pre- and postvaccine serum samples to determine antibody titers against each of the strains of influenza included in the vaccine by use of antigen reagents specific to the vaccine (A/New Caledonia/20/99-like [H1N1], A/Fujian/411/2002-like [H3N2], and B/Shanghai/361/2002-like); the laboratory was blinded with respect to age group and CD80/86 responses. We defined the seroconversion rate as the percentage of vaccine recipients who had an increase in serum HAI titers by at least a factor of 4 after vaccination, compared with pre-vaccination titers. Seroprotection rate was defined as the percentage of vaccine recipients with a serum HAI titer of at least 1:64 after vaccination.

Monocyte stimulation. Peripheral blood mononuclear cells (PBMCs) were isolated using the Histopaque (Sigma) gradient method. PBMCs were suspended in RPMI 1640 medium plus 10% fetal bovine serum (FBS), adjusted to a concentration of 2 × 10^6 cells/mL, and divided over 96-well plates. The following ligands in medium were added to PBMCs: Pam3CSK4 (10 μg/mL), lipoteichoic acid (LTA; 2 μg/mL), lipopolysaccharide (LPS; 1 μg/mL), flagellin (5 μg/mL), and poly(U) (0.5 μg/mL). RPMI 1640 medium plus FBS alone was used in control cultures. All ligands were obtained from Invivogen. Cells were incubated for 18 h with Golgi-stop (BD PharMingen), with brefeldin A present for the last 12 h for intracellular cytokine staining assays. Cell suspensions were stained with primary fluorescent-labeled antibodies for surface staining (CD11c, CD80, and CD86). Monocytes were identified by forward- and side-scatter gating, combined with surface-marker staining (CD11c). Thirty thousand total events were collected per sample. Additional samples were used for intracellular cytokine staining (interleukin [IL]–6 and tumor necrosis factor [TNF]–α). Data were acquired on an LSR II (Becton Dickinson) and analyzed using FlowJo software (version 7.2.1; Tree Star). All antibodies were obtained from eBioscience, except for anti–CD11c and anti-CD86 antibodies, which were from BD Biosciences PharMingen.

Statistical analysis. Proportions or means, where appropriate, were used to describe the demographic and clinical characteristics of each cohort at enrollment. To model both the variation in our sample of young and older adults as well as the correlation between ligand-specific stimulation [22], we employed a mixed effects model [23] to estimate the effect of age group on CD80 and CD86 levels. Specifically, we used an unstructured covariance structure that permitted each participant to have a unique correlation structure for each ligand stimulation; this accounted for the inherent variation of each participant. The full models tested for the fixed effect of age group (older vs. young); ligand; the interaction between age group and ligand; the covariates sex, race, medications or vaccination (aspirin, nonsteroid anti-inflammatory drug [NSAID], or statin use in the past 2 weeks and influenza vaccination in previous year), and comorbid conditions (heart disease, stroke, and peripheral vascular disease) by use of restricted maximum likelihood. Least-squared means were estimated for the fixed effects of age group and of the age group by ligand interaction, and the differences were tested.
Next, we estimated strain-specific seroprotection and seroconversion using multiple logistic regression forcing in age and strain-specific prevaccination titer and performed forward selection with the same covariates listed above [24] so that only covariates with a \( P \) value of \( < .05 \) were included. Then, we estimated the effect of age group on the number of strains with \( \geq 4 \)-fold titer increase or postvaccine titer \( \geq 1:64 \) using Poisson regression and controlling for covariates listed above [24, 25]. Subsequently, we used generalized estimating equations (GEEs) with a Poisson distributed error to estimate the association between the number of strains with \( \geq 4 \)-fold increase or a postvaccine titer \( \geq 1:64 \) with TLR-induced CD80 up-regulation or CD86 down-regulation, again with each of these models adjusting for correlations between ligand-specific stimulation, strain-specific prevaccination titer, and covariates selected as described above [26, 27]. This analysis was then repeated for each influenza strain using a GEE for a binary outcome [26, 27]. All statistical tests were 2-tailed, and \( P < .05 \) was considered to indicate statistical significance. All analyses used SAS (version 9.1; SAS Institute).

**RESULTS**

**Participants.** During the 2004–2005 influenza season, 162 subjects were enrolled in 2 age groups: 21–30 years of age (\( n = 81 \)) and \( \geq 65 \) years of age (\( n = 81 \)). Baseline characteristics of these cohorts are summarized in table 1. Although medication use and comorbidities were significantly more prevalent in the older adult group, 75% of this group reported no major comorbid conditions.
Age-associated alteration in CD80/86 responses in monocytes after TLR stimulation. Immediately before vaccination, we tested the in vitro activation of costimulatory molecules on monocytes after stimulation of the TLRs (ligands in parentheses) expressed on monocytes: TLR1/2 (N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-Cys-[S]-Serl-[S]-Lys(4)trihydrochloride [Pam3CSK4]), TLR2/6 (LTA), TLR4 (LPS), TLR5 (bis(palmitoyloxy)-(2RS)-propyl-[R]-Cys-[S]-Serl-[S]-Lys(4)trihydrochloride [Pam3CSK4]), TLR2/6 (LTA), TLR4 (LPS), TLR5 (decrease of 29%, compared with that in young adults [P = .004]), and to poly(U), a homolog for single-stranded RNA engaging TLR7 and/or TLR8 (decrease of 87%, compared with that in young adults [P = .001]). Taken together, our results indicate that the induction of CD80 and CD86 costimulatory responses is defective in the context of aging.

Influenza antibody responses after vaccination. We performed HAI assays on serum samples of participants obtained before and 4–6 weeks after influenza vaccination. We found that older adults were less likely than young adults to have postvaccine titers ≥1:64 (which we defined as seroprotection) against the H1N1 vaccine strain (P = .004) and, overall, older adults manifested postvaccine titers ≥1:64 against a lower number of strains than young adults (P = .01) (figure 3A and 3C). On the other hand, older adults were less likely than young adults to show a 4-fold increase between pre- and postvaccine titers (which we defined as seroconversion) to the H1N1 vaccine strain (P = .008) but more likely to mount a 4-fold increase to the H3N2 vaccine strain (P = .002). Moreover, the number of strains to which subjects generated a 4-fold increase in titer was comparable between older and young groups (figure 3B and 3D). Notably, approximately a third of both young and older adults failed to seroconvert to any strain in the 2004–2005 vaccine (figure 3D).

Specifically, the down-regulation of CD86 was significantly impaired in the older group only in response to flagellin, a ligand for TLR5 (decrease of 29%, compared with that in young adults [P = .004]), and to poly(U), a homolog for single-stranded RNA engaging TLR7 and/or TLR8 (decrease of 87%, compared with that in young adults [P = .001]). Taken together, our results indicate that the induction of CD80 and CD86 costimulatory responses is defective in the context of aging.

### Table 1. Baseline characteristics of study subjects.

<table>
<thead>
<tr>
<th>Comorbidities$^a$</th>
<th>Young (n = 81)</th>
<th>Older (n = 81)</th>
<th>P$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stroke</td>
<td>0 (0)</td>
<td>4 (5)</td>
<td>.12</td>
</tr>
<tr>
<td>Heart disease$^d$</td>
<td>0 (0)</td>
<td>12 (15)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>PVD</td>
<td>0 (0)</td>
<td>3 (4)</td>
<td>.24</td>
</tr>
<tr>
<td>COPD</td>
<td>0 (0)</td>
<td>3 (4)</td>
<td>.24</td>
</tr>
<tr>
<td>PUD</td>
<td>1 (1)</td>
<td>5 (6)</td>
<td>.21</td>
</tr>
<tr>
<td>None of the above</td>
<td>80 (99)</td>
<td>62 (76)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Medications</th>
<th>Young (n = 81)</th>
<th>Older (n = 81)</th>
<th>P$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>0 (0)</td>
<td>45 (56)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>NSAID</td>
<td>28 (35)</td>
<td>7 (9)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Statin</td>
<td>0 (0)</td>
<td>27 (33)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Influenza vaccine in preceding year</th>
<th>Young (n = 81)</th>
<th>Older (n = 81)</th>
<th>P$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>54 (67)</td>
<td>79 (98)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%) of subjects, unless otherwise indicated. BMI: body mass index; COPD, chronic obstructive pulmonary disease; NSAID, non-steroidal anti-inflammatory drug; PUD, peptic ulcer disease; PVD, peripheral vascular disease.

$^a$ Based on t tests for normally distributed characteristics and Fisher’s exact tests for categorical characteristics.

$^b$ For aggregate percentage nonwhite.

$^c$ Totals may exceed 100%, because some subjects listed >1 comorbidity.

$^d$ Includes coronary arteriosclerosis, congestive heart failure, and arrhythmias.

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Association of CD80/86 responses with influenza vaccine responses. We found that prevaccine determination of TLR-induced CD80 and CD86 expression on stimulated monocytes was associated with antibody response to influenza vaccination. Specifically, for each subject, the percentages of TLR-induced CD80+ monocytes were categorized into deciles; each decile increase in CD80+ cells was associated with a 8.5% increase in the mean number of strains with a 4-fold increase between pre- and postvaccine HAI titers (P = .01) (figure 4A) and a 3.8% increase in the mean number of strains with postvaccine titers ≥1:64 (P = .037) (figure 4B) for a strain in the vaccine.

The extent of CD86 down-regulation also associated with the count of strains with a 4-fold increase in titer, with each decile decrease in CD86+ monocytes after TLR stimulation associated with a 11.0% increase in the mean number of strains with a 4-fold increase (P = .002) (figure 4D); each decile decrease of CD86+ cells was associated with an 3.9% increase in the mean number of strains with a postvaccine titer ≥1:64 (P = .07) (figure 4C). Both CD80 and CD86 results were obtained through multivariable analysis and were adjusted for significant covariates. On an individual strain level, significant associations were noted in the multiple logistic regression models for strains H1N1 (for 4-fold increase, P = .03 for CD80 and P = .0008 for CD86; for postvaccine titer ≥1:64, not significant for both), H3N2 (for 4-fold increase, not significant for both; for postvaccine titer ≥1:64, not significant for CD80 and P = .004 for CD86), and B (for 4-fold increase, P = .006 for CD80 and not significant for CD86; for postvaccine titer ≥1:64, not significant for both). TLR-induced TNF-α and IL-6 responses to any ligand did not predict vaccine responses (data not shown; [30]).

DISCUSSION

We found that determination of the expression of the costimulatory molecules CD80 (B7.1) and CD86 (B7.2) on in vitro activated monocytes before vaccination is associated with antibody responses to influenza immunization. In particular, the extent of TLR-induced CD80 up-regulation predicted both seroconversion and seroprotection to influenza vaccination.
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Figure 4. Prediction of influenza vaccine responses by CD80/86 expression. Data are the mean percentage ± SE of Toll-like receptor–induced change in CD80+ (A and B) or CD86+ (C and D) monocytes in subjects with a postvaccine titer ≥1:64 (A and C) or with a 4-fold increase in titer (B and D) for none, 1, 2, or all of the vaccine strains in the 2004–2005 vaccine (A/New Caledonia/20/99-like, A/Fujian/411/2002-like, and B/Shanghai/361/2002-like).

These associations remained highly significant after adjustment for potentially confounding covariates such as sex, age, prevaccination HAI titer, comorbid conditions, and medications (including aspirin, NSAID, and statin use). Thus, in the present study, prevaccine assessment of TLR-induced CD80 and CD86 expression on monocytes predicted vaccine response, although a formal demonstration of prediction remains to be done in an independent cohort. In addition, we observed an age-associated alteration in CD80 up-regulation after TLR engagement in monocytes. Although TLR-dependent responses could be specifically diminished in older individuals, it is also possible that our results reflect a generalized, age-associated defect in costimulatory responses, and further studies using TLR-dependent and TLR-independent stimuli will be required to address this issue. Nonetheless, given the essential role of costimulatory signals in the activation of T cells, it is attractive to speculate that decreased CD80 expression on antigen presenting cells may contribute to the diminished responses observed in older adults to other vaccines in addition to influenza. Defective costimulation may also contribute to the increased morbidity and mortality to infectious diseases in older adults.

Our results describe a new age-associated defect in the innate immune system, and they are the first, to our knowledge, to link a marker of innate immune function in monocytes to human vaccine responses. In the adaptive immune system the age-associated loss of CD28 expression on CD8+ T cells has been previously reported to prospectively predict human influenza vaccine response [31, 32]. Because CD28 is the activating receptor on T cells for both CD80 and CD86, this further emphasizes the crucial role of costimulation in vaccine immunity [33]. Notably, we observed divergent responses in these B7 family molecules after TLR engagement in monocytes, with up-regulation of CD80 and decreased expression of CD86. The down-regulation of CD86 has been previously reported in human monocytes as resulting from the TLR-induced expression of the anti-inflammatory cytokine IL-10 [28, 29]; thus, our findings suggest potentially distinct roles for CD80 and CD86 in modulating the costimulatory signals linking the innate and
adaptive immune responses. The signal transduction mechanisms mediating TLR-dependent CD80 and CD86 expression remain incompletely understood, and whether the distinct responses we have observed reflect preferential engagement of either of the known CD80 and CD86 receptors on T cells, the activating CD28 or the inhibitory CTLA-4 receptor, remains to be determined [34].

In the present study, we chose not to employ a restrictive enrollment protocol such as SENIEUR, which utilizes clinical and laboratory data that results in the exclusion >70% of subjects >65 years of age [35, 36]. The use of multivariable mixed effect modeling to account for heterogeneity in our cohort allowed for adjustment for potential confounders while simultaneously using information from all TLR ligand stimulations. As a result, we believe our findings are not only statistically robust but also more representative of the community-dwelling human population. However, although most of our older cohort reported no comorbid conditions, we did not formally evaluate our older participants for phenotypes associated with frailty [37], and further studies will be required to determine whether frailty or comorbidity conditions influence the CD80- and CD86-dependent effects on vaccine responsiveness we have observed. Nevertheless, it is noteworthy that we observed abnormal CD80 or CD86 responses in both young and older adults and that the associations of CD80 and CD86 expression with vaccine responsiveness were robust in both age groups (figure 4). Therefore, the effect of costimulation on vaccine antibody response we observed may be generalizable to all adults. Furthermore, our results highlight limitations in the immunogenicity of the trivalent inactivated influenza vaccine, with a substantial proportion of both young and older adults in the present study failing to mount a 4-fold increase in HAI titer to any of the vaccine strains (figure 3D). The utility of costimulatory responses as a predictor of protection in young adults against influenza infection may be especially important given the predilection of potential pandemic avian influenza strains to affect younger adults [38, 39].

The ongoing burden of morbidity and mortality from seasonal influenza, the potential global impact of pandemic influenza strains, and the uncertain potential for bioterrorism lends urgency to identify individuals who will respond poorly to immunization for future vaccine development. At present, the trivalent inactivated influenza vaccine remains the mainstay of influenza disease prevention, despite data suggesting only modest benefits for community-dwelling older adults and health care workers [4, 12]. The use of high-dose influenza vaccine in at-risk older adults has been suggested as an approach to enhance relatively poor antibody responses [40]. The ability to prospectively distinguish those unlikely to respond to conventional doses of vaccine through the assessment of costimulatory responses could facilitate the identification of individuals who may benefit from this and other vaccine formats aimed at augmenting immune responses. This information could be ascertained before immunization; this would be preferable to determination of antibody responses by HAI titer, which can only be determined 4–6 weeks after vaccination and requires specialized antigens specific to each year’s vaccine. The addition of costimulatory molecules or TLR ligands to vaccines has been suggested as one approach to increasing immunogenicity [41–43]; our results provide a framework for the biological plausibility of these approaches. Moreover, evaluation of innate immune responses could be employed as a strategy to stratify need for alternative vaccine use or for antiviral prophylaxis of vulnerable individuals in influenza outbreaks or a global pandemic.

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