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Cutting Edge: Importance of IL-6 and Cooperation between Innate and Adaptive Immune Receptors in Cellular Vaccination with B Lymphocytes

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B lymphocytes are a potential alternative to dendritic cell immunotherapy, with the advantages of relative abundance in peripheral blood and the ability to function as APCs. Although B cells express multiple receptors that induce costimulatory molecules, B cell vaccine studies have focused primarily on CD40 stimulation. To optimize the potential efficacy of B cell vaccines (Bvac), we compared the capacity of differentially stimulated B cells to induce Ag-specific CD8+ T cell responses in vivo. CD40- or TLR7-stimulated B cell APCs demonstrated that Ag-presenting B lymphocytes stimulated the TNFR superfamily member CD40 up-regulate the expression of CD86 and proinflammatory cytokines. Consistent with this expression is the ability of peptide-pulsed B cells to induce CD8+ T cell responsiveness (4–7). Although these studies offer an important insight into the potential use of B cells in immunotherapy, it is generally believed that B cells are much less effective APCs than DCs, and thus not as attractive as candidates for cellular vaccines. However, we considered the possibility that the optimal signals for ex vivo activation of B cells as APCs have not been thoroughly investigated.

In addition to CD40, B cells can be activated through engagement of the BCR and innate immune receptors such as the TLRs. The stimulation of B cells through these receptors can induce the expression of costimulatory molecules and the production of proinflammatory cytokines such as IL-6 and TNF-α (8–11). Although BCR stimulation alone can in some circumstances induce B cell anergy, stimulation through the BCR together with any of the multiple TLRs expressed by B cells significantly enhances B cell effector functions, including cytokine production, Ab production, and surface molecule up-regulation (10, 11).

The use of TLR agonists as vaccine adjuvants is currently being tested (12, 13). Initial studies have focused upon agonists of the endosomal TLRs 7, 8, and 9. B lymphocytes readily respond to the TLR7 agonist R848, which has been shown to enhance B cell function alone and strongly synergize with BCR or CD40 stimulation (8, 14). To identify clinically relevant B cell vaccine (Bvac) stimulation strategies and to optimize the potential for B lymphocyte Ag presentation and immunotherapy, we have compared the CD8+ T cell stimulatory capacities of differentially stimulated Ag-pulsed B cells and extended this investigation to in vivo responses to an infectious pathogen.

Materials and Methods

Mice and cells

C57BL/6 and IL-6-deficient (C57BL/6 background) mice were purchased from The Jackson Laboratory and housed in a pathogen-free facility. All cells received support from an American Cancer Society postdoctoral fellowship (PF-07-067-01-LIB).

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§ Abbreviations used in this paper: DC, dendritic cell; Bvac, B cell vaccine; LM, L. monocytogenes; LMA, attenuated L. monocytogenes.
were incubated in BCM-10 medium (9). Use of mice in this study was in accordance with a protocol approved by the University of Iowa Animal Care and Use Committee (Iowa City, IA).

Cell isolation and stimulation
High density splenic B cells for use as Bvacs were purified by Percoll gradient isolation (15) followed by the removal of CD43− cells using magnetic isolation (Miltenyi Biotech). B cell purity was >99.3% as determined by CD19 expression analyzed by flow cytometry. CD11c+ cell contamination was undetectable.

Purified B cells were suspended at 2 × 10^6 cells/ml and stimulated with the following reagents: 2 μg/ml goat F(ab')2 anti-mouse μ-specific Ab (for BCR stimulation; Jackson ImmunoResearch Laboratory), 1 μg/ml R848 (TLR7 agonist; Alexis Biochemicals), 5 μg/ml rat anti-mouse CD40 mAb clone 1C10 (the 1C10 hybridoma was a gift from Dr. F. Lund, Trudeau Institute, Saranac Lake, NY); either in combination or individually as described in the figure legends. After 22 h of stimulation the OVA peptide SIINFEKL was added and the cells were incubated two additional hours. Cells were then washed with 20 ml of PBS and resuspended in PBS. OVA peptide-pulsed cells (2 × 10^6 cells/mouse) were adoptively transferred i.v. into naive C57BL/6 mice.

Listeria monocytogenes (LM) infections
Two strains of LM expressing the OVA peptide SIINFEKL were used in these experiments, virulent LM and attenuated LM (LMA). Mice were infected i.v. with either 1 × 10^7 or 5 × 10^6 CFU virulent LM (5 × LD50) in PBS (16). On the indicated days the mice were sacrificed and liver samples were collected, weighed, and homogenized in 7 ml of 2% Igepal and plated at log dilutions in PBS on tryptic soy agar plates containing 100 μg/ml streptomycin. Plates were incubated at 37°C and colonies per gram of liver were enumerated.

LMA was injected i.v. at 1 × 10^7 per mouse in sterile PBS. Four days postinfection the mice were sacrificed, spleens were collected, homogenized, and depleted of RBC, and the resulting splenocytes were pulsed with or without 1 μg/ml OVA peptide for 6 h at 37°C. Cells were then stained for IFN-γ-producing CD8+ T cells as described below.

T cell recall responses and intracellular flow cytometry
Splenocytes harvested from infected or vaccinated mice were pulsed with or without OVA peptide for 6 h at 37°C in the presence of 0.5 μg/ml GolgiPlug (BD Biosciences). Cells were then stained for expression of CD8α (mAb 53.6.7; BD Biosciences) and Thy1.2 (mAb 53.21; BD Biosciences) followed by intracellular staining for IFN-γ (mAb XMG1.2, BioLegend) as previously described (17).

Expression of the IL-6R subunit CD126 on purified CD8+ T cells was monitored using an anti-CD126 mAb (clone 715A7) purchased from BioLegend followed by a FITC-conjugated goat anti-rat IgG2b Ab (SouthernBiosciences). In brief, 1 × 10^6 purified CD8+ T cells at 100 μl per sample were incubated with either 1 μg of anti-CD126 mAb or rat IgG2b isotype control mAb (Southern-Biosciences) for 30 min at 4°C. Cells were then washed three times in 5 ml of FACS buffer (PBS containing 1% FBS and 0.01% NaN3). Cell pellets were then resuspended at 100 μl and incubated with 1 μg of goat anti-rat IgG2b mAb for 30 min at 4°C, washed three times in 5 ml of FACS buffer, and analyzed by flow cytometry.

Cytokine multiplex
B lymphocytes were isolated and stimulated as described above. Supernatants were collected after 24 h of stimulation and analyzed in a 20-cytokine multiplex ELISA (BioSource) according to the manufacturer’s protocol. Analyte amounts were determined using the multiplex reader Luminex 2000 (Bio-Rad).

CD8+ T cell proliferation assay
Flat-bottom, 96-well plates were coated with 1 μg/ml anti-CD3ε Ab (clone 145-2C11; eBioscience) in PBS (17). CD8+ T cells were harvested from the spleens of naive C57/B6 mice using negative MACS separation (Miltenyi Biotech) were seeded at 1 × 10^5 per well in anti-CD3 Ab-coated 96-well plates. Recombinant mouse IL-6 (eBioscience) was added to wells in triplicate. For testing of conditioned medium, 50 μl of supernatants collected from stimulated B cells (see above) with or without anti-IL-6 Ab (clone 20F3.11) after 24 h were added to CD8+ T cells (final volume of 200 μl). After 72 h of stimulation 5 μl of [3H]thymidine was added to each well and further incubated for 12 h. Cells were then harvested and a scintillation counter used to evaluate [3H] incorporation into DNA.

Results and Discussion

B cell vaccines and induction of CD8+ T cell responses
We hypothesized that different types of stimulation of B lymphocytes used in vaccines may result in different effectiveness of B cells as APCs and, hence, induce different levels of CD8+ T cell secondary responses. Mice that had received differently stimulated Bvacs were challenged with attenuated LM expressing the OVA peptide SIINFEKL. Splenocytes of these mice were analyzed for OVA-specific CD8+ T cells 4 days postinfection. All Bvac-treated mice displayed higher levels of Ag-responsive CD8+ T cells than the PBS-treated group (Fig. 1). These data indicate that the various Bvacs sensitized mice to Ag, resulting in an enhanced secondary T cell response upon challenge. Although all Bvac groups were able to generate secondary responses, there were differences in the magnitude of the responses induced by the different groups (Fig. 1). Of particular note, B cells stimulated through CD40, the reported method for activating B cells, induced the lowest T cell secondary response of all groups tested, indicating that other stimulation strategies may be more effective for Bvac.

The Bvac BCR plus TLR7 treatment resulted in the most effective vaccine for inducing T cell secondary responses. This result is consistent with previous reports of B cell-enhanced cytokine production and surface molecule up-regulation upon BCR and TLR dual stimulation (8, 10, 11). The treatment of B cells with the TLR7 agonist R848 alone (BvacTLR7) resulted in a higher T cell response than the BvacCD40 group, suggesting that the use of TLR agonists may be an effective and practical
alternative to CD40 stimulation. Bvac$^{BCR + TLR7}$ was an effective B cell vaccine, although its ability to induce a secondary response was ~40% of that of DC vaccine (supplemental Fig. 1). However, this comparison does not consider the difficulty of isolating DCs for clinical use.

To focus on optimizing Bvac effectiveness, we limited our treatment groups to stimulation via BCR plus TLR7, CD40, or TLR7 alone. These groups were chosen as representatives of the most effective stimulation (BCR plus TLR7), the published standard (CD40) (18), and a clinically viable option or TLR7 alone. These groups were chosen as representatives of treatment groups to stimulation via BCR plus TLR7, CD40, or TLR7 alone. To determine whether enhanced secondary responses of CD8$^+$ T cells to Bvac-treated groups resulted in enhanced protection from an infectious pathogen, Bvac-treated mice were challenged with recombinant, 5 × LD$_{50}$ virulent LM expressing the OVA peptide SIINFEKL. Three days postinfection the clearance of bacteria from the liver of these animals was analyzed. Bvac-treated groups displayed better clearance than controls (Fig. 2). Bvac$^{BCR + TLR7}$ treatment reduced bacterial burden by an average of 97% (1.41 × 10$^5$ CFU/g liver) compared with naive mice (4.17 × 10$^6$ CFU/g liver), whereas Bvac$^{CD40}$ and Bvac$^{TLR7}$ treatments reduced the burden by 93% (2.74 × 10$^5$ CFU/g liver) and 84% (6.79 × 10$^5$ CFU/g liver), respectively. Although there were no statistical differences in bacterial burden between Bvac-treated groups, the trend toward lower bacterial burdens was consistent with the level of CD8$^+$ T cell activation induced by the individual vaccines as seen in Fig. 1. Interestingly, despite a lower OVA-specific CD8$^+$ response, the clearance of pathogen in Bvac$^{BCR + TLR7}$ treated mice was similar to that of DC vaccine-treated mice (supplemental Fig. 2).

Role of IL-6 production in cellular vaccine efficacy

T cell activation by APCs is dependent upon presentation by peptide-loaded MHC molecules (signal 1) as well as costimulatory molecules (signal 2). We considered the possibility that the difference between Bvac groups might be attributed to differential B cell expression of MHC class I or costimulatory molecules. However, no difference in either MHC class I or CD86 expression was seen between variously treated B cell groups at the time of Bvac administration (data not shown). Because no detectable differences existed between groups with regards to signals 1 and 2, we focused our attention on signal 3, cytokine production.

Differences between groups could be attributable to variations in acute phase cytokine production, such as IL-1α/β, TNF-α, or IL-6. Although no detectable IL-1 was produced by the Bvac groups (data not shown), both TNF-α and IL-6 were produced in measurable amounts (Fig. 3). TNF-α was produced at low levels and did not correlate with the Bvac performance (Fig. 1). In contrast, IL-6 production correlated well with vaccine efficacy (Fig. 3). To ascertain whether IL-6 played a causal role in CD8$^+$ T cell responses, CD8$^+$ T cells were analyzed for IL-6 receptor (CD126) expression and proliferative responses to rIL-6. Negatively isolated splenic CD8$^+$ T cells expressed the IL-6R-specific subunit CD126 (Fig. 4) and demonstrated an enhanced proliferative response to TCR engagement in the presence of IL-6 (Fig. 4). Consistent with previous findings, proliferative enhancement occurred in an IL-6 dose-dependent manner, indicating a direct effect of IL-6 on CD8$^+$ T cells (19).

To further determine the effects of Bvac-produced IL-6, CD8$^+$ T cells were stimulated with anti-CD3 mAb and treated with conditioned medium from differentially stimulated B cells. The incubation of CD8$^+$ T cells with medium from CD40-, TLR7-, and BCR plus TLR7-stimulated B cells enhanced the proliferative effects of TCR stimulation (Fig. 4). Importantly, the stimulatory capacity of the different supernatants was consistent with our previous CD8$^+$ T cell activity and protection results, as conditioned medium from BCR plus TLR7 stimulated B cells induced higher levels of CD8$^+$ T cell proliferation than that from TLR7 or CD40 alone. The ability of each supernatant treatment to enhance T cell proliferation was partially reduced by the addition of anti-IL-6 Abs, indicating the importance of IL-6 but also suggesting that other factors produced by B cells may contribute to CD8$^+$ T cell proliferation (Fig. 4 and supplemental Fig. 3). These results demonstrate that the production of IL-6 is an important component of a cellular vaccine.
To ascertain whether IL-6 produced by the Bvac played a causal role in T cell responses in vivo, BvacBCR plus TLR7 derived from IL-6-deficient mice were compared with BvacBCR plus TLR7 derived from IL-6-sufficient mice. The T cell response induced by the IL-6-deficient Bvac was reduced by ~40% (Fig. 5). Again, these data demonstrate the importance of the production of IL-6 during CD8+ T cell responses to cellular vaccines.

The importance of B cells as APCs has come to be evident over recent years. B cells have been implicated in multiple sclerosis through the direct enhancement of T cell responses in the absence of Ab production (20, 21). In infectious diseases such as AIDS and bacterial meningitis, B cells have been shown to be important as innate immune cells, again independent of Ab production (22, 23). An important knowledge gap in Bvac usage has been determining the optimal B cell stimulation to produce APCs able to induce an efficacious T cell response. There are three required interactions associated with successful T cell activation: signal 1 (MHc:peptide complex); signal 2 (costimulatory molecules and their receptors); and signal 3 (cytokines and receptors). Although other signal 3 cytokines such as IL-2 are well defined, this is the first report demonstrating an important in vivo requirement for IL-6 as a signal 3 cytokine for CD8+ T cells and reveals another important consideration in the design of Bvacs that promote optimal T cell activation and proliferation.

Although data using DCs as cellular vaccines are promising, clinical use of cellular vaccines has yet to yield substantial gains. The requirements of high cell numbers and effective cellular expansion and activation remain substantial obstacles in effective DC-based immunotherapy (24). B lymphocytes display many of the characteristics desired for use in immunotherapy: 1) easily isolated in high numbers; 2) no requirement for differentiation in vitro; 3) ability to present Ag to T cells; and 4) responsive to stimulation using experimental and medicinal compounds. Gaining knowledge about the properties of B cells as APCs will advance our clinical capabilities for cancer or infectious disease immunotherapy.

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Disclosures
The authors have no financial conflict of interest.

References


