Vaccine 28 (2010) 3688–3695

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

A novel mitogen fusion protein against CD40$^+$ cells with potent vaccine adjuvant properties

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ARTICLE INFO

Article history:
Received 31 March 2009
Received in revised form 2 March 2010
Accepted 10 March 2010
Available online 30 March 2010

Keywords:
CD40
Fas
Fusion protein
Adjuvant
Mitogen
B-cell activation

ABSTRACT

A large number of infectious diseases caused by viral or bacterial infections are treatable and/or preventable by vaccination. In addition, ongoing research is aimed at the development of vaccines against other types of diseases, including almost all forms of cancer. The efficacy of a vaccine relies on the antigen-specific response by the entire repertoire of immune competent cells. Here, we have generated a powerful mitogen fusion protein, CD40L-FasL-IgFc, which stimulates CD40$^+$ cells robustly. We found that this specific cell activation is accompanied by increased expression of PRDI-BF1 (Blim-1) RNA, an indicator of terminal B-cell differentiation, in cultures stimulated with CD40L-FasL-IgFc. The addition of specific inhibitors of NF-κB and MEK1/2 partially suppressed the observed proliferative effects of CD40L-FasL-IgFc. When tested *in vivo*, the immune response to influenza HA vaccine was significantly increased by co-administration of CD40L-FasL-IgFc. Moreover, the co-administration of the cDNA expression plasmid encoding CD40L-FasL-IgFc significantly boosted the vaccine response. We now have a unique opportunity to evaluate our novel fusion protein adjuvant, and other similarly constructed fusion proteins, in both protein-based and genetic vaccines.

1. Introduction

Vaccination against infectious diseases represents one of the most important advances in modern medicine. As the cellular and molecular mechanisms of disease pathogenesis are elucidated, increasing numbers of diseases are likely to become treatable or preventable by using vaccine strategies. The field of neoplastic diseases is an area in which there has been cumulative evidence indicating that protective and therapeutic vaccine strategies are applicable [1–3]. For example, a vaccine against papillomavirus has been successfully applied to prevent cervical cancer in the female population [4,5]. Melanomas and breast cancers have also been identified as candidates for prevention by vaccination of cancer-specific antigens [6–9]. Other examples of diseases that are the subject of potential therapy or prevention by vaccination include parasitic infections such as malaria and Leishmania [10–12], non-infectious respiratory diseases such as asthma [13,14], and neurological diseases such as Alzheimer's [15,16].

In developing vaccine-therapies, the magnitude of immune responses against disease-specific antigens often decides the efficacy of the protection and cure. As a result, an effective and safe adjuvant, or a compound that facilitates the vaccine-specific immunological memory with minimal side effects, is required. The aluminum salts (Alums) have been the only vaccine adjuvant approved in the USA for use in humans [17]. To improve the efficacy of existing or newly developed vaccines, various compounds with immune stimulatory activity have been investigated for their potential as stronger adjuvants in both preclinical and clinical studies. Ongoing studies in adjuvant development target mainly compounds that stimulate native immune cells and B cells by "danger signaling" pathways, which are mediated by toll-like receptor function [18–20]. The activation of native immune cells by danger signaling increases the potential of antigen presentation and cytokine secretion by antigen presenting cells, which promotes the differentiation of Th1 type cells and T cytotoxic cells [21,22]. Both viral vaccine vectors and toll-like receptor ligands follow this

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0264-410X/$ – see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.vaccine.2010.03.014

paradigm and thus function as adjuvants [23–25]. The use of these adjuvants results in significant increases in humoral and cellular immune responses against either co-administered antigens or antigens that are loaded into viral vectors [26–30]. Thus far, studies using viral vector-mediated vaccine strategies for HIV-1 infection have not been able to effectively establish protective immunological memory [31]. It is possible that adjuvants targeting danger signaling are not sufficient to develop acquired immunity protective against particular types of pathogens. Therefore it would be valuable to develop adjuvants that directly stimulate differentiation of Th1 cells and T cytotoxic cells as well as promote vaccine antigen-specific B-cell affinity maturation.

To develop novel cell-reactive compounds, we began generating and testing multifunctional fusion protein constructs comprised of extracellular domains of death receptor ligands and co-stimulatory receptor ligands. We found that a particular fusion protein we created and characterized, CD40L-FasL-IgFc, indeed possesses powerful adjuvant properties. Specifically, CD40L-FasL-IgFc, when employed in cell proliferation assays, significantly stimulates non-T cells. The observed increased cell proliferation was accompanied by increased mRNA expression of PRDI-BF1 (Blimp-1), which is an indicator of terminal B-cell differentiation [32]. These effects brought about by our fusion protein were partially inhibited by specific inhibitors of NF-κB or MEK1/2. Taken together, the data suggests that co-clustering of CD40 and Fas by CD40L-FasL-IgFc induces B-cell activation and differentiation *in vitro*. When tested *in vivo*, the influenza HA vaccine response in mice was largely enhanced by co-administration of the fusion protein. We also found that the administration of the mammalian cDNA expression plasmid encoding our fusion protein proved to be equally useful as a DNA adjuvant, as its administration *in vivo* led to a significantly elevated vaccine response compared to the administration of vaccine alone. Therefore, the CD40L-FasL-IgFc fusion protein represents a powerful adjuvant that activates co-stimulatory receptors, thereby directly contributing to B-cell differentiation, antibody affinity and T cell maturation. Another advantage of this fusion protein system is that it can easily be modified to also encode vaccine antigen-specific peptides, thus creating a vaccine with the built-in adjuvant. Due to the discovery of the adjuvant activity of our fusion protein mitogen, we now have unique opportunity to test this potential vaccine platform as a protein and as a genetic vaccine.

2. Materials and methods

2.1. Cells

The long term culture of human Burkitt lymphoma line Raji, Chinese hamster ovary cell (CHO), mouse T cell lymphoma line EL4 and human T cell lymphoma line Jurkat was maintained in RPMI 1640 with 10% FCS. Peripheral blood mononuclear cells (PBMCs) from normal healthy human adults were separated using Ficoll-Paque Plus (Amarsham Biotech. Piscataway, NJ, USA). Peripheral blood lymphocyte (PBL) non-T cells were negatively purified using pan-T magnet beads (M-450, Dynal, Lake Success, NY, USA). PBL-B cells were negatively purified using RosetteSep (StemCell Tec. Vancouver, BC, Canada). The purity of human PBL-T cells was greater than 97% by CD3 staining and flow cytometry. The residual of T cells in non-T cell subsets was less than 5% by flow cytometry studies.

2.2. Plasmid construction, transfection and protein purification

Fusion proteins were prepared by PCR methods using various specific primers.

CD40L-IgGFc—the extracellular domain of human CD40L (amino acid residues 46–261) was amplified from human thymic RNA (Clontech B-D, Palo Alto, CA, USA) using the following primers: forward 5′-CTTCATAGAAGGTTGGACAAGATA-3′ and reverse 5′-GAGTTTGAGTAAGCCAAAGGACGT-3′. The signal peptide of Oncostatin M (Locus NM_020530, amino acid residues 1–25) [33] was amplified using the following primers: forward 5′-ATGGGGGTACTGCTCACACAGAGG-3′ and reverse 5′-CATGCTCGCCATGCTTGGAAACAG-3′. These PCR products were subsequently utilized in a second round of PCR. Primers encoding the 3′ sequence of the Oncostatin M fragment (5′-AGCATGGCGAGCATG-3′) and the 5′ portion of human CD40L (5′-CTTCATAGAAGGTTG-3′) were designed to overlap; annealing of the PCR products yielded a hybrid template. The template encoding the chimeric construct was selectively amplified using external primers specific for the 5′ region of Oncostatin M and the 3′ region of human CD40L. Each primer containing appropriate restriction sites (NheI/XhoI) for subcloning into the mammalian expression vector *PCIneo* (Promega, Madison, WI). The resulting PCR fragment was ligated into the *PCIneo* vector, and transfected into *Escherichia coli* competent cells, which were selected on ampicillin-containing agar plate (OncoM-hCD40L/PCIneo). Next, the hinge, CH2 and CH3 domains (amino acid residues 219–447) of human IgG1 were amplified by RT-PCR from total human peripheral lymphocytes RNA, using the following primers: forward 5′-AAACTCGAGTTTGAGTAAGCCAAAGGACGTGAAGCCAGTGC-3′ and reverse 5′-CGTCTAGATCATTTACCCGGAGACAGGGAGAG-3′, introducing the XhoI and XbaI sites, respectively (sites are underlined). This PCR product was cloned into *OncoM-hCD40L/PCIneo* after digestion and purification. The resultant product was coded as *OncoM-CD40L-IgFc/PCIneo*. This fusion protein was detected as a ∼50 kDa molecular mass in reduced SDS-PAGE and was designated as CD40L-IgFc in text.

hFasL-IgGFc—Chimeric Ig molecules expressing the extracellular portion of the human FasL gene and the human IgG1 constant domains were created as follows: the extracellular domain of human FasL (amino acid residues 108–281) was first amplified by RT-PCR from total RNA of human thymus, using the following primers: forward 5′-CCGCTCGAGCAGCTCTTCCACCTACAG-3′ and reverse 5′-GGCCTCGAGCTTATATAAGCCGAAAAACGTC-3′, including the XhoI sites, respectively (sites are underlined). External primers encoding the 5′ portion and the 3′ portion of Oncostatin M, FasL and IgG1 were used to amplify the *OncoM-hCD40L-hFasL-IgFc/PCIneo*. Each primer contained appropriate restriction sites for subcloning into the *PCIneo* vector, yielding *OncoM-hFasL-IgFc/PCIneo*. This fusion protein was detected as a ∼45 kDa molecular mass in reduced SDS-PAGE and was designated as FasL-IgFc in text.

hCD40L-hFasL-IgGFc—the extracellular domain of human FasL (amino acid residues 108–281) was cloned in frame at the 3′end of the hCD40L in the *OncoM-hCD40L-IgFc/PCIneo* (the resultant product coded as *OncoM-CD40L-FasL-IgFc/PCIneo*). This fusion protein was detected as a ∼80 kDa molecular mass in reduced SDS-PAGE and was designated as CD40L-FasL-IgFc in text.

All constructs were confirmed by DNA sequencing. The plasmid DNA designated as *pCIneo-CD40L-FasL-IgFc* was purified by cesium chloride gradient ultracentrifugation.

CHO cells were transfected by electroporation with these vectors and the transfectants were selected in RPMI 1640, 10% FCS containing 0.5 mg/ml G418 (Invitrogen, Carlsbad, CA, USA), and high producer cells were selected by RT-PCR or specific ELISAs to FasL and CD40L.

Culture supernatants from each transfectant were passed over Protein G columns. Fusion proteins were acid-eluted and dialyzed against PBS. In some experiments the protein was adsorbed for 2 h at 4 °C with Polymixin B beads (Sigma, Oakville, ON, Canada) to eliminate endotoxin. The presence of each protein was assessed by SDS-PAGE/human IgG-specific Western blotting and Coomassie Blue staining.