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Co-administration of α-GalCer analog and TLR4 agonist induces robust CD8$^+$ T-cell responses to PyCS protein and WT-1 antigen and activates memory-like effector NKT cells

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ABSTRACT

In the present study, the combined adjuvant effect of 7DW8-5, a potent α-GalCer-analog, and monophosphoryl lipid A (MPLA), a TLR4 agonist, on the induction of vaccine-induced CD8$^+$ T-cell responses and protective immunity was evaluated. Mice were immunized with peptides corresponding to the CD8$^+$ T-cell epitopes of a malaria antigen, a circumsporozoite protein of *Plasmodium yoelii*, and a tumor antigen, a Wilms Tumor antigen-1 (WT-1), together with 7DW8-5 and MPLA, as an adjuvant. These immunization regimens were able to induce higher levels of CD8$^+$ T-cell responses and, ultimately, enhanced levels of protection against malaria and tumor challenges compared to the levels induced by immunization with peptides mixed with 7DW8-5 or MPLA alone. Co-administration of 7DW8-5 and MPLA induces activation of memory-like effector natural killer T (NKT) cells, i.e. CD44$^+$ CD62L$^-$ NKT cells. Our study indicates that 7DW8-5 greatly enhances important synergistic pathways associated to memory immune responses when co-administered with MPLA, thus rendering this combination of adjuvants a novel vaccine adjuvant formulation.

1. Introduction

Invariant natural killer T (*i*NKT) cells express an invariant TCR-α chain (Vα14Jα18 in mice and Vα24Jα18 in humans) and recognize endogenous and exogenous glycolipids, such as α-galactosylceramide (α-GalCer) and its analogs [1–4]. Their TCR is restricted to CD1d, a MHC-I-like molecule found on antigen presenting cells, such as dendritic cells (DCs) [5,6].

*i*NKT cells exert a very important role in bridging innate and adaptive immunity, which makes this subset an attractive target for the development of vaccine adjuvants [7,8]. *i*NKT cells have been shown to increase protective T-cell immunity upon activation with α-GalCer when co-administered with malaria vaccines [9]. This potent adjuvant effect is due in part to the fact that the activation of *i*NKT cells by α-GalCer also rapidly induces the full maturation of DCs in vivo and thereby acts as an adjuvant for both CD4$^+$ and CD8$^+$ T-cell immunity [7,10]. The Toll-like receptor (TLR) pathway has also been shown to participate in the interaction between *i*NKT cells and DCs [11]. Activation of the TLR pathway induces transcription of several components of the inflammatory response, such as factor NF-κB, interferon-regulatory factors (IRFs) and MAP kinases, which lead to the production of pro-inflammatory cytokines such as TNFα, IL-12, IL-6, and IL-1 by DCs [12–15]. Given the ability of the TLR pathway to shape humoral and cellular responses, TLR agonists have been proposed as vaccine adjuvants [14–18].

In an attempt to further increase the adjuvant effect of formulations based on TLR agonists, strategies that combine these with other potent activators of DCs and *i*NKT cells [19–21] have been evaluated. Given the cooperative effect of DCs and *i*NKT cells, focusing on these two is a promising strategy, as illustrated in Supplemental Fig. 1, for the development of better adjuvant formulations. In the present study, we intended to evaluate the combined effect of a potent glycolipid, 7DW8-5, α-

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GalCer analog [22], when utilized along with the agonist of TLR4, MPLA (monophosphoryl lipid A) (Supplemental Fig. 1). Our results demonstrate that the combination of 7DW8-5 and MPLA induces protective effector memory $CD8^+$ T-cell responses to an immunodominant epitope of a *Plasmodium yoelii* circumsporozoite protein (PyCSP) [23], as well as to HLA-A2-restricted epitopes of a Wilms Tumor antigen-1 (WT-1) [24].

2. Materials and methods

2.1. Mice

BALB/c mice at 6 to 8 weeks old were purchased from The Jackson Laboratory (Bar Harbor, ME). HLA-A2-transgenic mice on a C57BL/6 (B6) background were purchased from Taconic (Hudson, NY). Mice were kept in proper conditions as stated in the regulations and guidelines of animal care at the Comparative Bioscience Center animal facility at Rockefeller University.

2.2. Parasites

P. yoelii (17XNL strain) sporozoites were obtained from dissected salivary glands of infected *Anopheles stephensi* mosquitoes, 2 weeks after infectious blood meal as described [25,26]. The mosquitoes were maintained in the Insectary at New York University School of Medicine.

2.3. Immunization

BALB/c mice were immunized three to five times with 3-week interval by intra-muscular (i.m.) injection with PyCSP-derived peptide, SYVPSAEQI [23], at 20 μg with and without different adjuvants in varied concentrations and diluted in PBS. HLA-$A2^{+/+}$β2m- transgenic mice in B6 background were immunized three times with 3-week interval by i.m. injection with 20 μg of HLA-A2-restricted WT-1-derived peptides, WH (SLGEQQYSV) and WT (CMTWNQMNL) [24], with and without different adjuvants in varied concentrations and diluted in PBS.

2.4. Cell lines

To prepare antigen-presenting cells (APCs) for the ELISpot assay, A20.2J cells (mouse B cell lymphoma) were grown to 1.0×10^6 cells/mL in complete RPMI-10 medium supplemented with 10% fetal bovine serum (FBS), antibiotics and 10 mM HEPES and kept at 37 °C in 5% CO_2 in an incubator. EL-4 expressing HLA-A2 were grown in complete DMEM supplemented with 10% FBS, antibiotics and 10 mM HEPES and kept at 37 °C in 5% CO_2. Cells were washed, resuspended in supplemented media at a concentration of 1×10^7 cells/mL and loaded with PyCSP-derived and WT-1-derived peptides in the previous section, as well as HIV gag (TLNAWVKVV) mock peptide as negative control, at 20 μg/mL and incubated for 2 h at 37 °C. After the incubation, cells were irradiated with 8000 rad (12 min) using an OPD irradiator. After irradiation, cells were washed one more time and resuspended in complete media at 1×10^6 cells/mL.

2.5. Generation of a C1498 cell line co-expressing WT-1 and HLA-A2

The WT-1-C1498 murine leukemia cell line, which is syngeneic to C57BL/6 mice, was established via transfection of C1498 with murine WT-1 cDNA [27,28]. The HLA-A2.1 (HHD) gene, which encodes an interspecies hybrid MHC-class I gene linked to a human β2-microglubulin (β2m), was amplified from AAV-A2 vector [29] and subsequently subcloned into pLPCX vector (Moloney MLV-based retroviral vector, Clontech, Mountain View, CA). The recombinant retrovirus encoding for the hybrid HLA-A2-β2m molecule was produced by transient transfection of the ectopic packaging cell line Platinum-E (Plat-E, Cellbiolabs, San Diego, CA), using Lipofectamine 2000 transfection reagent (Invitrogen, Life Technologies, San Diego, CA). Viral supernatants were harvested 48 and 72 h after transfection, concentrated and purified. Retroviral supernatants were then loaded onto Retronectin-coated, non-tissue culture treated 24-well plates according to the manufacturer's instruction (Takara Bio Inc., Otsu, Japan). For transduction, mouse WT-1-C1498 cells were seeded and incubated for at least 48 h with the viral particles. Two days after transduction, the double positive population of HLA-A2 and β2m was sorted out using FACsAria Cell Sorter (BD Biosciences Immunocytometry Systems, Franklin Lakes, NJ) (Supplemental Fig. 2A) and cloned by limiting dilution in the presence of Puromycin (1 μg/mL). WT-1 expression stability was verified by both flow cytometric analysis (Supplemental Fig. 2A) and RT-PCR (Supplemental Fig. 2B), as described [27].

2.6. Sporozoite challenge and assessment of parasite burden in the liver

P. yoelii sporozoite challenge was performed as described [25,26]. Briefly, immunized as well as naïve mice were injected with 1×10^4 live *P. yoelii* sporozoites via tail vein. Forty-two hours after the challenge, when the parasites fully matured in the hepatocyte, livers were collected from the mice, and RNA was extracted. The parasite burden in the liver was determined by measuring parasite-specific ribosomal RNA using 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Parasite burden was described as a ratio of the absolute copy number of parasite ribosomal RNA to that of mouse GAPDH mRNA.

2.7. Tumor challenge

WT-1^+ HLA-$A2^+$ C1498 tumorigenic cell lines were grown in complete RPMI 1640 (Sigma Aldrich) supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) and pyruvic acid (1%) at 37 °C. The selection was performed with puromycin (100 μg/mL). To induce tumor growth, 3×10^6 WT-1^+ HLA-$A2^+$ C1498 cells in 100 μL PBS were injected subcutaneously into the right flank of the HLA-A2 transgenic mice immunized previously with peptides and adjuvants alone or in combination. Tumor challenge was performed 14 days after vaccination. Tumor growth was monitored up to 50 days after subcutaneous injection of WT-1^+ HLA-$A2^+$ C1498 cells.

2.8. ELISpot assay

The numbers of PyCSP-specific, IFN-γ-secreting $CD8^+$ T-cells among splenocytes obtained from immunized BALB/c mice and WT-1-specific IFN-γ-secreting $CD8^+$ T-cells in the splenocytes of immunized HLA-A2 transgenic mice were determined by an ELISpot assay as previously described [23,25,26], with some modifications. Briefly, after splenocytes were prepared from spleen collected from mice 14 days after immunization, they were co-cultured the peptide-loaded APCs for 24 h at 37 °C on the ELISpot plate pre-coated with IFN-γ antibody, as previously described. Then the ELISpot plate was incubated with biotinylated anti-mouse IFN-γ antibody, followed by incubation with avidin-conjugated with horseradish peroxidase. Finally, the spots were developed after adding ELISpot substrate (Biolegend, San Diego, USA), as described [23,25,26]. The assays were run in duplicates for each mouse sample and 5×10^5 splenocytes/well were incubated with irradiated peptide-loaded or unloaded APCs at the ratio of 2:1 (splenocyte:APC). In order to identifying the number of IFN-γ-secreting $CD8^+$ T cells for each well, the mean number of spots (for duplicates) counted in the wells incubated with splenocytes together peptide-loaded APCs was subtracted by the mean number of spots (for duplicates) counted in the wells incubated with splenocytes and unloaded APCs.

2.9. Assessing memory phenotypes and $tetramer^+$ T-cells by a flow cytometric analysis

Upon lysing red blood cells, splenocytes were prepared as abovementioned. After washing the cells twice, splenocytes were