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Priming of Qualitatively Superior Human Effector CD8+ T Cells Using TLR8 Ligand Combined with FLT3 Ligand

Anna Lissina*,†,†, Olivia Briceño*,‡,†, Georgia Afonso,‡,§,†, Martin Larsen,*,†, Emma Gostick,§, David A. Price,‖, Roberto Malloone,‡,§,‖ and Victor Appay*,†

The quality of Ag-specific CD8+ T cell responses is central to immune efficacy in infectious and malignant settings. Inducing effector CD8+ T cells with potent functional properties is therefore a priority in the field of immunotherapy. However, the optimal assessment of new treatment strategies in humans is limited by currently available testing platforms. In this study, we introduce an original model of in vitro CD8+ T cell priming, based on an accelerated dendritic cell coculture system, which uses unfractonated human PBMCs as the starting material. This approach enables the rapid evaluation of adjuvant effects on the functional properties of human CD8+ T cells primed from Ag-specific naive precursors. We demonstrate that a selective TLR8 agonist, in combination with FLT3L, primes high-quality CD8+ T cell responses. TLR8L/FLT3L-primecd CD8+ T cells displayed enhanced cytotoxic activity, polyfunctionality, and Ag sensitivity. The acquisition of this superior functional profile was associated with increased T-bet expression induced via an IL-12-dependent mechanism. Collectively, these data validate an expedited route to vaccine delivery or optimal T cell expansion for adoptive cell transfer. The Journal of Immunology, 2016, 196: 000–000.

Introduction

Cytotoxic activity, polyfunctionality, and Ag sensitivity predict CD8+ T cell efficacy against infected or dysregulated cells (1, 2). Potent cytotoxic activity is necessary for the elimination of diseased cells, and demonstrations of synergy between IFN-γ and TNF during the induction of permanent growth arrest in cancerous or infected cells have highlighted the importance of T cell polyfunctionality (i.e., the capacity to produce multiple effector cytokines simultaneously) (3). In addition, high levels of Ag sensitivity endow T cells with the capacity to recognize cognate peptide–MHC class I (pMHCI) complexes at low densities on the target cell surface, and thus to act swiftly against infected cells or to break the immune tolerance associated with cancer (4). However, the induction of de novo CD8+ T cell responses with such protective attributes in humans has proven difficult in the settings of vaccination and adoptive cell therapy.

Methods

Several methods have been developed to enhance the quality of Ag-specific CD8+ T cell responses in vitro (5, 6). dendritic cells (DCs) govern the nature of CD8+ T cell responses primed from naive precursors, via the provision of processed Ags in the form of pMHCI molecules, together with other important signals, including costimulatory interactions and inflammatory cytokines. Much effort has therefore been devoted to the modulation of DC function for the optimization of CD8+ T cell immunity (7). Adjuvants, such as TLR ligands, can further improve the immunogenicity of soluble protein and peptide Ags by mimicking pathogen-associated “danger” signals (8, 9). Combinatorial assessments of various modalities are therefore required to enhance the efficacy of immunotherapeutic interventions.

However, it is difficult to study the effects of potential adjuvants on human CD8+ T cell responses due to the lack of a suitable model. Although the widespread use of murine systems has greatly advanced our knowledge of TLR function and DC/T cell interactions, there are significant biological differences between mice and humans that complicate simple extrapolation between species. For instance, the cellular distribution of TLR8 is entirely different between humans and mice and is considered to be nonfunctional in the latter (10). As a consequence, the adjuvant properties of TLR8-selective agonists have not been fully assessed (11, 12). Moreover, traditional in vitro priming protocols that use human material rely on populations of inflammatory monocyte-derived DCs (moDCs) generated in a two-stage process from PBMC-purified CD14+ monocytes (13). In this setting, differentiation is achieved using a combination of GM-CSF and IL-4 prior to maturation with a mixture of inflammatory cytokines or LPS (14, 15). Although adequate for many purposes, this strategy has a number of limitations. In particular, the initial preparation of moDCs is laborious and time consuming. More importantly, it is limited to one subset of APCs, and no attempt is made to evaluate the role of conventional DCs and other resident blood cells (e.g., CD4+ T cells and NK cells) in the priming process.

To circumvent these drawbacks, we developed an innovative model of human CD8+ T cell priming. This original in vitro approach is based on the rapid mobilization of DCs directly from unfractonated PBMCs, adapted from an earlier method designed to detect low-frequency memory T cell responses (15). Importantly, this strategy enables side-by-side studies of multiple test...
parameters in a standardized system with quantitative and qualitative readouts of the primed Ag-specific CD8+ T cell response. In this study, we used our new approach to compare conventional adjuvant combinations along side the largely uncharacterized ssRNA40 TLR8 ligand (TLR8L).

Materials and Methods

Flow cytometry reagents

Standard and CD8-null ELA/HLA-A2 tetramers were produced as described previously (16, 17). The CD8-null ELA/HLA-A2 complex incorporates a compound D227K/T228A mutation in the α3 domain that abrogates CD8 binding without impacting on the TCR docking platform (18). Directly conjugated mAbs were purchased from commercial sources as follows: 1) anti–CD8-allophycocyanin-Cy7, anti–CD45RA-V450, anti–CCR7-PE-Cy7, anti–CD107a-PE-Cy5, anti–IFN-γ-AlexaFluor 700, anti–TNF-PE-Cy7, and anti–granzyme-B-V450 (BD Biosciences); 2) anti–CD3-ECD (Beckman Coulter); 3) anti–CD28-AlexaFluor 700 (BioLegend); 4) anti–T-bet-Alexa Fluor 647 (eBiosciences); 5) anti–MIP-1β-FITC (R&D Systems); 6) anti–IL-2-allophycocyanin (Miltenyi Biotec); and 7) anti–perforin-BD48-FITC (Abcam). The amine-reactive viability dye Aqua (Life Technologies) was used to eliminate dead cells from the analysis. Intracellular staining for T-bet was performed using the Transcription Factor Buffer Set (BD Pharmingen), according to the manufacturer’s instructions. Intracellular staining for granzyme B and perforin-BD48 was compatible with this procedure. Staining with all other reagents was conducted according to standard protocols (19, 20).

Data were acquired using an LSR Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software version 9.3.7 (Tree Star).

Peptides

All peptides were synthesized commercially at >95% purity (Biosynthesis). The ELA-10 peptide (ELAGIGILTV; Melan-A/MART-1 residues 26–35) was used to pulse target cells in functional assays. The ELA-20 peptide (YTAAEELAGIGILTVILGVL; Melan-A/MART-1 residues 21–40) was used for in vitro priming. The ELA-20Ct peptide (GHGHSYTAAEELAGILTV; Melan-A/MART-1 residues 16–35) was tested in early presentation and stability experiments (Supplemental Fig. 1).

FIGURE 1. In vitro CD8+ T cell priming and the magnitude of TLR8L-primed Ag-specific cells. (A) Outline of the in vitro CD8+ T cell priming system using unfractionated PBMC samples. (B) Representative flow cytometry data showing the kinetics of ELA-specific CD8+ T cell priming in the presence of GM-CSF/IL-4 and a mixture of inflammatory cytokines. Plots are gated on viable CD3+ lymphocytes after aggregate exclusion. Numbers refer to percentages of ELA-HLA-A2 tetramer+ cells within the total CD8+ T cell population. (C) Response magnitudes for ELA-specific CD8+ T cell populations primed under different conditions from several healthy HLA-A2+ donor PBMC samples. Ag only (no maturation signal), cytokine mixture (TNF, IL-1β, PGE2, and IL-7), TLR4L (LPS), or TLR8L (ssRNA40) were each used in combination with either GM-CSF/IL-4 or FLT3L supplementation. Horizontal bars indicate median values. Statistical comparisons between groups were performed using the Wilcoxon signed rank test: *p < 0.05.

FIGURE 2. Greater cytotoxic potential of CD8+ T cells primed in vitro with an FLT3L/TLR8L combination. (A) Representative flow cytometry plots showing granzyme B (top panel) or perforin (bottom panel) expression by ELA-specific CD8+ T cells primed under different conditions. (B) Granzyme B (top graph) and perforin (bottom graph) expression by ELA-specific CD8+ T cells primed under different conditions from several healthy HLA-A2+ donor PBMC samples. Horizontal bars indicate median values. Statistical comparisons between groups were performed using the Wilcoxon signed rank test: *p < 0.05, **p < 0.01, ***p < 0.001. (C) Representative flow cytometry data from a fluorometric cytotoxicity assay showing the disappearance of ELA peptide-pulsed PBSE+ HLA-A2 B-LCL target cells relative to non–peptide-pulsed PBSE+ HLA-A2 B-LCL control cells in the presence of ELA-specific CD8+ T cells primed under different conditions. Numbers indicate the percentages of control (upper left) and target (upper right) B-LCL cells. (D) Specific lysis of HLA-A2+ B-LCL target cells presenting the indicated concentrations of exogenously loaded ELA peptide in the presence of ELA-specific CD8+ T cells primed under different conditions. A noncognate population of CD8+ T cells derived from the same HLA-A2+ donor and cultured under similar conditions to the ELA-specific CD8+ T cell population was used as a control. Error bars represent SD from the mean of two replicates.
PBMCs and cell lines

PBMC samples were obtained from healthy HLA-A2* individuals via standard protocols and cryopreserved before use in CD8+ T cell priming assays. The ELA-specific CD8+ T cell line Mel9916 was generated by in vitro priming and purified flow cytometric sorting of ELA/HLA-A2 tetramer+ events. An HLA-A2+ EBV–transformed B-lymphoblastoid cell line (B-LCL) was used to present the ELA-10 peptide in activation assays. The HLA-A2+ tumor cell line Me275 was used to present the naturally processed Melan-A/MART-1 epitope (AAGIGILTV; Melan-A/MART-1 residues 27–35). The HLA-A2+ Melan-A/MART-1 tumor cell line Me241 was used as a negative control.

In vitro priming of naive Melan-A/MART-1 Ag-specific CD8+ T cells

Naïve precursors specific for the HLA-A2-restricted Melan-A/MART-1 epitope ELA (ELAIGILTV; Melan-A/MART-1 residues 26–35) were primed in vitro using an accelerated DC coculture protocol. Thawed PBMCs were resuspended in AIM medium (Invitrogen), supplemented with either GM-CSF (1000 IU/ml; Milenyl Biotes) and IL-4 (500 IU/ml; InvivoGen) or FLT3L (50 ng/ml; R&D Systems), and plated out at 5 × 10^6 cells/well in a 24-well tissue culture plate (day 0). After 24 h (day 1), maturation of resident DCs was induced under one of the following conditions: 1) a standard mixture of inflammatory cytokines comprising TNF (1000 U/ml), IL-1β (10 ng/ml), IL-7 (0.5 ng/ml), and PGE_2 (0.1 μg/ml; R&D Systems) (15); 2) LPS (0.1 μg/ml; Invivogen); or 3) ssRNA40 (0.5 μg/ml; InvivoGen). The ELA-20 peptide was added together with the DC maturation reagents at a final concentration of 1 μM. A total of 10^6 EIAspecific CD8+ T cells were incubated with CD8-enriched PBMCs containing 5 × 10^6 ELA/HLA-A2 tetramer+ events from 10 healthy donors. Pie segments and colors correspond to the proportions of ELA-specific CD8+ T cells expressing the indicated number of functions, respectively. Polyfunctionality indices for ELA-specific CD8+ T cell populations, calculated from the data depicted in (B). Horizontal bars indicate median values. Statistical comparisons between groups were performed using the Wilcoxon signed rank test: *p < 0.05, **p < 0.001.

Fluorometric cytotoxicity assay

ELA-10 peptide-pulsed HLA-A2+ B-LCL target cells were labeled with Pacific Blue succinimidyl ester (PBSE; 10 μM) to allow flow cytometric separation from unpulsed HLA-A2+ B-LCL control cells, which were left unlabeled (PBSE-). A total of 10^5 PBSE+ target and 10^5 PBSE- control cells were incubated with CD8-enriched PBMCs containing 5 × 10^6 primed ELA-specific CD8+ T cells for 12 h at 37°C. Control assays were set up in parallel either without effectors or with noncognate CD8+ T cells at the same E:T ratio (5:1). After harvesting, cells were stained with Aqua, anti-CD3-ECD, anti-CD8-allophycocyanin-Cy7, and anti-CD19-FITC prior to acquisition using an LSR Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software version 9.3.7 (Tree Star). Pie charts were constructed using SPICE software (National Institute of Allergy and Infectious Diseases), and polyfunctionality indices were calculated as described previously (22).

Comparisons between groups were performed using the Wilcoxon signed rank test in Prism 5 (GraphPad). A p value < 0.05 was considered significant.
Design of an accelerated DC coculture system to prime T cells in vitro

The experimental outline of our new priming approach, starting from unfractionated PBMCs, is summarized in Fig. 1A. As circulating human DCs are rare, precursors (in particular CD14+ monocytes) within the starting PBMC material were mobilized using GM-CSF/IL-4 and matured with a mixture of inflammatory cytokines including TNF, IL-1β, and PGE2. IL-7 was also added to facilitate the priming of naive T cells upon Ag-driven activation, as described previously during the optimization of our accelerated DC coculture protocol (15, 23). Alternatively, DC mobilization was achieved by exposure of PBMCs to FLT3 ligand (FLT3L), which has demonstrable T cell priming efficacy in animal studies (24). GM-CSF/IL-4 and FLT3L have been shown to act on various immune cell subsets and mobilize distinct populations of DCs (25–27). To ensure that sufficient numbers of Ag-specific CD8+ T cells were present in the naive pool for experimental purposes, we focused our analysis on the Melan-A/MART-1 epitope EAAGIGILTV26–35, which is recognized at remarkably high precursor frequencies in HLA-A2+ individuals (28, 29). The polyclonal composition of this naturally generated Melan-A/MART-1–specific repertoire presents distinct advantages over the TCR transgenic mouse models that are typically used to study Ag-driven adaptive immunity (30, 31). To ensure optimal immunogenicity, we used the heteroclitic sequence ELAGIGILTV (ELA) (32). Moreover, the ELA epitope was incorporated into a 20-mer synthetic long peptide (ELA-20) as a means of limiting Ag display to DCs with cross-presentation capacity (33–35). Prior to experimentation, we verified that the ELA-20 peptide required active processing. Specifically, we showed that ELA-20 was not presented directly by HLA-A2 (Supplemental Fig. 1A) and was not subjected to nonspecific cleavage by enzymes present in serum (Supplemental Fig. 1B).

Next, we evaluated the optimal parameters for ELA-specific CD8+ T cell priming in our system. An ELA-20 concentration of 1 μM consistently generated sufficiently large populations of primed cells for functional characterization (data not shown) and was chosen for all downstream assays. Primed ELA-specific CD8+ T cells peaked on day 10 (Fig. 1B), following identical kinetics and achieving comparable magnitudes with both the GM-CSF/IL-4 and FLT3L protocols (Supplemental Fig. 2A). Subsequent experiments were therefore conducted over this time frame. We also confirmed that primed ELA-specific CD8+ T cells originated from the naive (CCR7+CD45RA+) pool (Supplemental Fig. 1A) and was not subjected to nonspecific cleavage by enzymes present in serum (Supplemental Fig. 1B).
memory (CCR7^CD45RA^) compartment (Supplemental Fig. 2D, 2E). No differences in terms of differentiation status were observed between ELA-specific CD8^+^ T cell populations primed with either GM-CSF/IL-4 or FLT3L (Supplemental Fig. 2F).

**Human CD8^+^ T cell priming with a selective TLR8 agonist**

In subsequent experiments, we used our validated system to study the effect of a recently available TLR8L (ssRNA40), compared in parallel with the standard mixture of inflammatory cytokines (TNF, IL-1β, PGE2, and IL-7), or with LPS, an extensively studied TLR4L. Either TLR4L or TLR8L was introduced during the DC maturation step, in lieu of the inflammatory cytokine mixture. Tetramer-based enumeration of ELA-specific CD8^+^ T cell populations expanded in parallel revealed no major differences in the magnitude of priming across the three maturation conditions tested (Fig. 1C). However, the GM-CSF/IL-4/TLR8L combination primed significantly fewer ELA-specific CD8^+^ T cells compared with the FLT3L/TLR8L combination, highlighting differences in the way that GM-CSF/IL-4 and FLT3L modulate subsets of APCs. To support this point, we subjected purified CD14^+^ monocytes to GM-CSF/IL-4 or FLT3L before introducing different maturation factors into the culture medium (Supplemental Fig. 3). As expected, GM-CSF/IL-4 but not FLT3L treatment resulted in the differentiation of monocytes into moDCs (with downregulation of CD14 from the surface of moDCs), TLR8L had no effect. The failure of GM-CSF/IL-4–induced moDCs to respond to TLR8L stimulation (despite expression of TLR8) provides one potential explanation for the poor T cell priming capacity of the GM-CSF/IL-4/TLR8L combination in our system. These findings highlight the limitations of moDCs and question the suitability of these in vitro–generated inflammatory cells for the study of potential adjuvants. Unfractionated PBMCs may therefore be a preferable alternative to isolated moDCs, the use of which would have overlooked the FLT3L/TLR8L effect on T cell priming. Notably, FLT3L/TLR8L–primed ELA-specific CD8^+^ T cells possessed significantly greater cytotoxic potential, as assessed by the expression of granzyme B and perforin, compared with their counterparts primed under other conditions (Fig. 2A, 2B). This observation was confirmed in APC lysis assays, where CD8^+^ T cells primed in the presence of FLT3L/TLR8L killed more than twice as many target cells loaded with 1 μM ELA-10 than CD8^+^ T cells primed under any other condition (Fig. 2C, 2D). Moreover, only FLT3L/TLR8L–primed CD8^+^ T cells were capable of eliminating targets presenting ELA-10 at a concentration of 10 nM.

**Qualitatively superior human CD8^+^ T cell responses primed with FLT3L/TLR8L**

Next, we assessed the ability of primed CD8^+^ T cells to deploy multiple effector functions in response to Ag encounter. In these assays, we measured the simultaneous induction of IFN-γ, MIP-1β, TNF, and IL-2 together with surface mobilization of CD107a (Fig. 3A). Although GM-CSF/IL-4 and FLT3L in conjunction with the cytokine mixture generated ELA-primed CD8^+^ T cells expressing similar levels of cytotoxic molecules (Fig. 2A, 2B), FLT3L treatment elicited greater frequencies of polyfunctional cells compared with GM-CSF/IL-4 (Fig. 3B, top row). Moreover, ELA-specific CD8^+^ T cells primed using the FLT3L/TLR8L combination displayed the highest levels of polyfunctionality (Fig. 3B). These differences were statistically significant in terms of the calculated polyfunctionality index across individual PBMC donors (Fig. 3C).

**FIGURE 5.** TCR avidity and T-bet expression in primed CD8^+^ T cells. (A) Representative flow cytometry plots showing standard or CD8-null ELA/HLA-A2 tetramer staining of primed ELA-specific CD8^+^ T cells. Plots are gated on viable CD3^+^ lymphocytes after aggregate exclusion. Numbers refer to percentages of tetramer^+^ cells within the total CD8^+^ population. (B) Percentages of high-avidity ELA-specific CD8^+^ T cells primed using either GM-CSF/IL-4 or FLT3L in combination with a mixture of inflammatory cytokines (TNF, IL-1β, PGE2, and IL-7) or TLR ligands. These percentages were calculated from the corresponding CD8-null/standard ELA/HLA-A2 tetramer^+^ cell ratios. Data are averaged over four independent experiments. Error bars represent SD from the mean. (C) Representative flow cytometry plots showing intracellular T-bet expression (white histograms) by ELA-specific CD8^+^ T cells primed under different conditions. Gray histograms depict isotype control staining and vertical dotted lines indicate the mean fluorescence intensity (MFI) of T-bet staining for the weakest priming condition. (D) Intracellular T-bet expression by ELA-specific CD8^+^ T cells primed under different conditions. Horizontal bars indicate median values. Statistical comparisons between groups were performed using the Wilcoxon signed rank test: *p < 0.05, **p < 0.01, ***p < 0.001.

Ag sensitivity, a major determinant of CD8^+^ T cell functionality, was evaluated via cognate peptide titration in IFN-γ secretion assays. FLT3L/TLR8L–primed ELA-specific CD8^+^ T cells displayed the highest Ag sensitivity, with a typical EC50 value (7.45 × 10^{-9} M) ∼1 or 2 orders of magnitude lower compared with the GM-CSF/IL-4/TLR4L (5.77 × 10^{-8} M) or FLT3L/cytokine (1.36 × 10^{-7} M) combinations, respectively (Fig. 4A). IFN-γ production by GM-CSF/IL-4–cytokine–primed ELA-specific CD8^+^ T cells was negligible. These differences in Ag sensitivity between conditions extended across the full spectrum of effector functions, enabling FLT3L/TLR8L–primed CD8^+^ T cells to deploy multiple effector functions at ELA-10 peptide concentrations as low as 1 nM (Fig. 4B),
equivalent to the amount of Ag presented in physiological settings. Crucially, FLT3L/TLR8L-primed ELA-specific CD8+ T cells were indeed capable of mounting polyfunctional responses to an HLA-A2+ melanoma cell line naturally expressing the endogenous Melan-A/MART-1 Ag (Fig. 4C). In contrast, the same tumor cell line was not recognized efficiently by ELA-specific CD8+ T cells primed under other test conditions (Fig. 4C).

Mechanistic insights into TLR8L-mediated adjuvant effects

Next, we explored the mechanisms involved in the acquisition of superior functional attributes by FLT3/TLR8L-primed CD8+ T cells. It is established that TCR avidity, defined as the collective affinities of multiple monomeric TCR/pMHC interactions, can greatly influence Ag sensitivity and thereby dictate the functional profile of CD8+ T cells in response to cognate Ag (21, 36). Accordingly, we used standard and CD8-null ELA/HLA-A2 tetramers in parallel to quantify this parameter across different priming conditions (Fig. 5A). CD8-null tetramers enable the selective detection of high-avidity Ag-specific CD8+ T cells (17). Despite a small increase within the FLT3/TLR8L-primed CD8+ T cell population, no statistically significant differences in TCR avidity were detected across any of the test conditions (Fig. 5B).

The expression of effector molecules such as granzyme B, perforin, and IFN-γ is tightly controlled in CD8+ T cells by the T-box transcription factor T-bet (also known as Tbx-21) (37). We therefore assessed the intracellular expression of T-bet in CD8+ T cells upon priming. Significantly higher T-bet expression levels were detected in ELA-specific CD8+ T cells primed with FLT3L/TLR8L compared with other combinations (Fig. 5C, 5D). T-bet expression is regulated by IL-12 (38), which is known to be secreted by myeloid DCs upon TLR8 ligation (11). To assess the relationship between IL-12 levels and T-bet induction in our in vitro priming system, an anti–IL-12p70 blocking Ab was administered daily during the first 3 d of culture. This intervention led to a significant drop in T-bet levels in FLT3L/TLR8L-primed CD8+ T cells (Fig. 6A, 6B). Moreover, ELA-specific CD8+ T cells primed in the presence of the anti–IL-12p70 blocking Ab expressed considerably less granzyme B and perforin (Fig. 6C) and were also less polyfunctional (Fig. 6D).

Discussion

We report in this study that human PBMCs mobilized with GM-CSF/IL-4 or FLT3L respond differently to maturation signals such as cytokines or TLR ligands, affecting the subsequent quality of the primed CD8+ T cell response. Combined use of FLT3L and TLR8L resulted in the priming of Ag-specific CD8+ T cells displaying robust effector functions, thereby showing that a selective TLR8 agonist can act as a potent adjuvant to prime functionally superior Ag-specific human CD8+ T cells. Collectively, our data indicate that TLR8L, through effects on FLT3L-mobilized DCs, triggers T-bet expression...
in primed CD8+ T cells via an IL-12-dependent mechanism. In turn, T-bet endows these CD8+ T cells with superior Ag sensitivity, robust cytolytic activity, and polyfunctionality. We also demonstrate that the enhanced Ag sensitivity of FLTL3/TLR8L-primed CD8+ T cells does not arise through the selection of T cells bearing high-affinity TCRs during the priming process. Instead, this gain in Ag sensitivity may be explained by a reduction in TCR/pMHC activation thresholds experienced by FLTL3/TLR8L-primed cells, leaving them poised for Ag-driven activation.

These findings were made possible by the development of an original in vitro priming model that offers several practical and theoretical advantages over existing systems. In addition to streamlining the search for more effective adjuvants, our approach is applicable to several notable challenges in the field. The use of unmanipulated PBMCs in particular will likely aid in the identification of immunization regimens suitable for individuals who are typically refractory to priming with standard vaccines, such as those at the extremes of age or with advanced HIV infection. Moreover, our priming model can be used to study the mechanisms underlying effective T cell priming (e.g., to identify the DC subsets or molecular pathways involved). For instance, preliminary data generated using our approach imply that DCs from the myeloid or molecular pathways involved. For instance, FLT3L was recently reported to mobilize a rare population of circulating mDC precursors (39). Finally, our in vitro priming system may also expedite the further testing of the adjuvant potential and inflammatory properties of selective TLR8 agonists in vivo. Recent work in transgenic mice expressing different levels of human TLR8 supports a role for this receptor in autoimmune inflammation, suggesting that TLR8L use may cause adverse effects in humans (42). However, inflammatory side effects might feasibly be averted through the specific targeting of such an agonist directly to lymph nodes, using delivery agents such as amphiphiles (i.e., linking the Ag and adjuvant to a lipopolysaccharide-binding tail) or nanoparticles (43, 44). These approaches could be called upon to decrease the systemic dissemination and potential toxicity of TLR8L while boosting CD8+ T cell priming efficacy. In light of our findings, investigating the adjuvant potential of other TLR8 (e.g., ssPoly(U) or the benzazepine analog TL8-506) or TLR7/8 (e.g., imidazoquinoline R848 or thiazoloquinoline CL075) ligands will likely be important. Differences in potency or unexpected antagonistic effects (e.g., between TLR7 and TLR8 signaling pathways) may be observed. Our in vitro priming system may also expedite the generation of potent T cells for adoptive cell therapy. It is notable in this respect that FLTL3/TLR8L-primed Ag-specific CD8+ T cells recognized naturally presented Ags on a tumor cell line and exhibited properties associated with in vivo efficacy. The present findings are therefore directly relevant to our understanding of effector T cell generation and the development of effective immunotherapy in humans.

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**Disclosures**

A.L., R.M., and V.A. are inventors of patent number EP14305080 entitled “Methods for testing T cell priming efficacy in a subject” filed on January 21, 2014. The other authors have no financial conflicts of interest.

**References**


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SUPPLEMENTAL DATA

Priming of qualitatively superior human effector CD8$^+$ T cells using TLR8 ligand combined with FLT3L

Running title: Enhanced T cell priming in humans

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Supplemental Figure 1. Surface presentation and stability of long versus short synthetic ELA peptides. A. IFNγ production by the Mel9916 ELA-specific CD8+ T cell line in response to HLA-A2+ B-LCL target cells pulsed with the optimal ELA-10 peptide or two different versions of the 20mer peptide (ELA-20 or ELA-20Ct) over a range of concentrations was measured via intracellular cytokine staining. The ELA-20Ct peptide, which contains the ELA epitope sequence at the C-terminal end, partially activated cognate CD8+ T cells without the need for processing and was subsequently eliminated from the study. The ELA-20 peptide did not bind directly to HLA-A2. B. The ELA-20 peptide was tested in a stability assay to determine if non-specific cleavage by enzymes present in serum could liberate the optimal ELA-10 epitope. IFNγ production by the Mel9916 ELA-specific CD8+ T cell line was measured as in (A). Prior to B-LCL pulsing, peptides were either handled normally or incubated with 10% v/v FCS for 24 hours at 37°C. Incubation with 10% v/v FCS reduced the antigenicity of the ELA-10 peptide by 1 log10 M, but did not generate functionally active fragments from the ELA-20 peptide.
Supplemental Figure 2. In vitro priming of ELA-specific CD8+ T cells from naïve precursors.

A. Comparison of ELA-specific CD8+ T cell priming kinetics from HLA-A2+ PBMC samples (n=10) using either GM-CSF/IL-4 or FLT3L in combination with a cocktail of inflammatory cytokines (TNF, IL-1β, PGE2 and IL-7). Error bars indicate SEM. B. Phenotypic profiles of purified naïve (top plot) or memory (bottom plot) CD8+ T cell subsets (black dots) overlaid on the phenotypic distribution of peripheral blood CD8+ T cells (grey density plot). CM, central memory; E, effector; EM, effector memory; N, naïve. Naïve (CCR7+ CD45RA+) and memory (CCR7+ CD45RA-) CD8+ T cell populations were isolated from HLA-A2+ PBMCs prior to reconstitution with CD8-depleted PBMCs in priming experiments. Unmanipulated PBMCs from the same donor were primed in parallel. C. Representative flow cytometry plots showing ELA/HLA-A2 tetramer+ CD8+ T cells on day 10 post-priming in the presence of either GM-CSF/IL-4 (top row) or FLT3L (bottom row) together with the inflammatory cytokine cocktail. Percentages of ELA/HLA-A2 tetramer+ cells within the total CD8+ gate are indicated. D. Representative phenotypic analysis of ELA-specific CD8+ T cells on day 10 post-priming. ELA/HLA-A2 tetramer+ CD8+ events are shown as black dots superimposed on density plots depicting the phenotypic distribution of
peripheral blood CD8$^+$ T cells. E. Phenotypic characterization of ELA/HLA-A2 tetramer$^+$ CD8$^+$ T cells primed from the donors shown in (A). Horizontal bars indicate median values. Statistical comparisons between groups were performed using the Wilcoxon signed rank test.
Supplemental Figure 3. Tracing the expression of CD14, HLA-DR and CD86 on purified monocytes, exposed to different maturation conditions. Expression of CD14, HLA-DR and CD86 on monocytes (initially purified with magnetic microbeads, on the basis of CD14 expression) differentiated in vitro using GMCSF/IL-4 (A) or FLT3L (B), and cultured for two days with ELA antigen alone (grey lines) or in the additional presence of the following maturation factors: cytokines (TNF-a, PGE2, IL-1b and IL-7; black lines), TLR4L (blue lines) or TLR8L (pink lines).