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Molecular adjuvant IL-33 enhances the potency of a DNA vaccine in a lethal challenge model

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Abstract

Identifying new molecular adjuvants that elicit effective vaccine-induced CD8⁺ T cell immunity may be critical for the elimination of many challenging diseases including Tuberculosis, HIV and cancer. Here, we report that co-administration of molecular adjuvant IL-33 during vaccination enhanced the magnitude and function of antigen (Ag)-specific CD8⁺ T cells against a model Ag, LCMV NP target protein. These enhanced responses were characterized by higher frequencies of Ag-specific, polyfunctional CD8⁺ T cells exhibiting cytotoxic characteristics. Importantly, these cells were capable of robust expansion upon Ag-specific restimulation *in vivo* and conferred remarkable protection against a high dose lethal LCMV challenge. In addition, we demonstrate the ability of IL-33 to amplifying the frequency of Ag-specific KLRG1⁺ effector CD8⁺ T cells. These data show that IL-33 is a promising immunoadjuvant at improving T cell immunity in a vaccine setting and suggest further development and understanding of this molecular adjuvant for strategies against many obstinate infectious diseases and cancer.

Introduction

There is still a great need for effective vaccines against many chronic infectious including HIV, HCV, Tuberculosis and malaria. For these pathogens, it is known that T cell-mediated immunity is critical in either controlling, preventing or delaying the onset of disease (1).

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Thus, a crucial step in vaccine development for these infections requires producing cytotoxic T_H1 versus humoral T_H2 responses. Currently, licensed vaccines such as inactivated and recombinant protein or non-live vaccines predominately drive humoral immune responses (2). An overall shortcoming of these vaccines, especially non-live vaccines, is their inability to generate both effective T_H1 and $CD8^+$ T cell immunity, thus hindering their beneficial role in limiting or preventing diseases that require adaptive cellular immune responses (2,3). One way to improve the quality of immune responses during vaccination is to incorporate immunoadjuvants, which have been shown to help increase their T_H1 immune potency (2). However, it has been a challenge to discover immunoadjuvants that can amplify the induction of $CD8^+$ T cell responses. Notably, IL-33 has emerged as a proinflammatory cytokine capable of promoting both potent T_H1 and cytotoxic $CD8^+$ T cell immunity (4,5,6). Therefore, IL-33 has great potential to act as a potent molecular adjuvant in vaccines designed to boost $CD8^+$ T cell immune responses.

IL-33 is a member of the IL-1 cytokine family, which is released by necrotic cells or activated innate immune cells during trauma or infection (6,7). Therefore, it is considered to serve as the first line of defense against pathogens, by providing an endogenous “danger signal” that triggers inflammation and promotes cell-mediated immune response. Originally studied in the context of T_H2 immunity associated with inflammatory disorders (6,7), evidence has begun to unveil IL-33’s unappreciated ability to induce T_H1 and $CD8^+$ T cell-mediated immunity (4–6). We have recently reported that IL-33 can act as novel immunoadjuvant to induce both potent T_H1 and effective $CD8^+$ T cell responses in an anti-tumor DNA vaccine (5). Here we expanded the scope of these initial studies to evaluate the capacity of IL-33 to serve as a vaccine adjuvant to enhance and modulate cell-mediated responses against various models of infection that require $CD8^+$ T responses.

In the present study, we use the well-studied lymphocytic choriomeningitis virus (LCMV) model to investigate IL-33’s ability to facilitate the induction of antiviral and protective immunity and further elucidate its biological function on memory $CD8^+$ T cell expansion and differentiation in a vaccine setting. We hypothesize that IL-33 would have the capacity to improve the efficacy of DNA vaccines against a viral challenge, providing optimal effector function and protection. Here we show that the administration of IL-33 coadministered with a DNA vaccine against LCMV induces robust antigen-specific IFN- γ responses, enhances antigen (Ag)-specific polyfunctional $CD8^+$ T cell immune responses, increases the cytotoxic phenotype of the $CD8^+$ T cells, and provides substantial protective immunity against a high-dose lethal LCMV challenge. We also that inclusion of IL-33 can significantly amplify and expand the Ag-specific effector memory $CD8^+$ T cell responses. Furthermore, we provide evidence of IL-33’s ability to also enhance cell-mediated immune responses when co-delivered with an HIV DNA vaccine. These findings significantly highlight the important role of IL-33 as a potential future vaccine adjuvant with applicability in the treatment of a variety of chronic viral diseases that require potent T_H1 -type immunity for their prevention or control.

Results

IL-33 elicits protection against a lethal LCMV challenge

The LCMV infection model has been extensively used to understand the role of virus-specific CD8⁺ T cell responses in the context of vaccine-elicited protection (8–10). Considering our recent novel finding that IL-33 can act as an immunoadjuvant to induce both anti-viral and anti-tumor CD8⁺ T cell immunity (5), we used the intracranial (i.c.) LCMV challenge model to further study the protective efficacy of IL-33. Three groups of C57BL/6 mice (B6) (n = 10) were vaccinated by electroporation (EP) one time with 10 µg of pLCMV-NP (NP) construct with or without 11 µg of mature IL-33 (mtrIL-33) construct. The empty vector pVAX was used as a negative control. The LCMV NP structural protein is recognized as a critical component and target for protective LCMV immunity since it is not a target for neutralizing antibodies (11). All animals were challenged 21 days post-vaccination (dpv) with a lethal 20×LD₅₀ dose of LCMV Armstrong (Fig. 1a) (10–13). Vaccinated animals with NP plus mtrIL-33 showed complete protection while the NP alone group achieved only 60% protection (Fig. 1b). On the other hand, all control pVAX vaccinated animals succumbed to infection. After showing that mice immunized using mtrIL-33 as an adjuvant exhibited 100% survival rate, we next sought to determine whether vaccinated mice with adjuvant could confer protection against an even higher lethal dose of LCMV challenge. Therefore, mice were challenged with a 40×LD₅₀ dose of LCMV Armstrong (11), 21 days post-single vaccination (Fig. 1c). Notably, animals receiving one immunization of NP plus mtrIL-33 yielded a significant 80% protection, while the NP alone group only conferred 10% protection against this highly lethal dose of LCMV (Fig. 1c). These data show that IL-33 elicits protection against a lethal LCMV challenge.

IL-33 significantly increases LCMV-specific CD8⁺ T cell responses

Considering that CD8⁺ T cell responses are essential for facilitating control against LCMV (8–13), we hypothesized that the IL-33 adjuvanted vaccine induced CD8⁺ T cells mediated antiviral protection. Thus, to better characterize the protective immune correlates driven by mtrIL-33, groups of mice (n = 4–5) were immunized once with NP either with or without mtrIL-33. The magnitude of NP-specific immune responses was measured 21 dpv in response to peptide re-stimulation (2.5 µg/ml) using the immunodominant epitope in the H-2^b background: D^bNP₃₉₆₋₄₀ (NP396) (11). Compared to NP alone-vaccinated mice, we found that co-immunization with mtrIL-33-encoding plasmid elicited stronger NP-specific T cell responses by greater than 2.5 fold (Fig. 2a); IFN-γ ELISPOT counts were ~2,500 spot-forming cells [SFCs] per 10⁶ splenocytes in the IL-33 vaccinated mice versus ~980 SFC/10⁶ splenocytes for the NP alone group. Next, we assessed the phenotypic and functional profile of vaccine-induced CD8⁺ T cells in response to NP396 peptide re-stimulation (2.5 µg/ml final peptide concentration). The gating strategy for intracellular cytokine flow cytometry analysis is depicted in supplementary Figure 1. Twenty-one days after vaccination there was a significant difference among vaccine groups in the frequency of CD8⁺ T cells producing effector cytokines (Figs. 2b,c). The NP vaccine coadministered with mtrIL-33 elicited a higher percentage of Ag-specific CD8⁺ T cells producing all three cytokines (Fig. 2b), and a significant number of the CD8⁺ T cells were polyfunctional (Fig. 2c). Compared with the NP alone vaccinated group, the NP+mtrIL-33 vaccinated group elicited substantially higher

frequencies of NP-specific CD8⁺ T cells producing either IFN- γ alone (NP, 1.3%; NP+mtrIL-33, 2.3%), dual IFN- γ +TNF- α (NP, 0.76%; NP+mtrIL-33, 1.63%), or triple-positive IFN- γ +TNF- α +IL-2⁺ (NP, 0.20%; NP+mtrIL-33, 0.43%) in the spleens 21 dpv (Fig. 2c). Collectively, the enhanced Ag-specific CD8⁺ T cell response induced by IL-33 is indicative of IL-33's ability to provide substantial protection against LCMV challenge. We next characterized the cytotoxic potential of vaccine-induced CD8⁺ T cells. CD8⁺ T cells isolated from mice vaccinated with IL-33 showed a significantly higher frequency of antigen-specific (IFN- γ +CD107a⁺: 2.5%) degranulation compared to NP alone-vaccinated mice (IFN- γ +CD107a⁺: 1.2%) (Fig. 2d). We next evaluated the proliferative capacity of the CD8⁺ T cells by monitoring Cell Trace Violet dilution in splenocytes isolated from mice 21 dpv rechallenged *in vitro* with NP396 peptide re-stimulation. Figure 2E shows that mtrIL-33 vaccinated mice underwent significantly higher Ag-specific proliferation of CD8⁺ T cells, being ~2 fold greater than NP control group. Notably, there was an enrichment of effector memory CD8⁺ T cells (CD44⁺CD62L⁻) in the adjuvant-vaccinated group (Fig. 2f). There was no significant difference in the central memory CD8⁺ T cell (CD44⁺CD62L⁺) population (data not shown). Taken together, the inclusion of IL-33 elicits robust levels of NP-specific T cell immunity, especially enhancing CD8⁺ T cell immune responses.

To better understand the biological function of IL-33 on the induction of Ag-specific CD8⁺ T cells during the course of vaccination, we further characterized IL-33's ability to expand the Ag-specific effector memory CD8⁺ T cell population. To achieve this goal, we took advantage of the well studied D^bGP₃₃₋₄₁ MHC class I tetramer to follow Ag-specific CD8⁺ T cells as they develop after initial priming. Mice were vaccinated once with a LCMV glycoprotein LCMV-GP (GP) DNA vaccine and the frequency of D^bGP33-specific CD8⁺ T cells was monitored in the peripheral blood during the course of vaccination either with or without mtrIL-33 (Fig. 4a). Delivery of IL-33 expanded the number of D^bGP33 tetramer-specific CD8⁺ T cells in the peripheral blood (Fig. 3a). In peripheral blood lymphocytes (PBLs), the frequency of GP33-specific CD8⁺ T cell was significantly 2-fold higher at 18 and 21 dpv compared with the nonadjuvanted group (Fig. 3a). Similarly, the inclusion of IL-33 also increased the number of GP33-specific CD8⁺ T cells in the spleen 21 dpv (Fig. 3b) and the Ag-specific CD8⁺ T cells secreting IFN- γ , undergoing degranulation, and expressing the transcription factor T-bet (Figs. 3c–f). Additionally, all mice were boosted with the GP construct alone (21 days after initial immunization) to quantify the Ag-specific recall responses. Compared to control group, the IL-33 vaccinated group significantly increased the Ag-specific CD8⁺ T cells. Notably, the IL-33 immunized group GP33 tetramer-specific T cells were ~3-fold higher starting 3 days post-boost vaccination (d24) compared to the NP-vaccinated group (Fig. 3a). The significant difference in the amplification of the GP33-specific CD8⁺ T cells was still observed 10 days after DNA boost (d31). Consistent with figure 2, the current data further confirms IL-33's ability to induce the quality of Ag-specific CD8⁺ T cells which seemed to correlate with IL-33's adjuvant effect to mediate its antiviral protection as shown in Figure 1. Finally, since it has been demonstrated that effector-phenotype memory CD8⁺ T cells (T_{eff}) can mediate clearance of blood-borne pathogens (14–19), we next evaluated the ability of IL-33 to induce the differentiation of T_{eff} cells based on expression markers: CD44 and KLRG1 (Fig. 4b). The administration of mtrIL-33 resulted in a significant expansion in the percentages of

CD8⁺KLRG1⁺ T_{eff} cells in the PBLs, compared with the GP-only vaccinated group (Fig. 4c). We also evaluated the recall response of D^bGP33 tetramer-specific CD8⁺KLRG1⁺ T cells after DNA-GP boosting, 21 days after initial immunization and observed marked expansion of GP33-specific memory CD8⁺KLRG1⁺ T cells in both groups after boosting; however, the proportion of CD8⁺KLRG1⁺ T cells remained significantly higher in the mtrIL-33 adjuvant group (Fig. 4c). In summary, IL-33 significantly increased LCMV-specific CD8⁺ T cell immunity against two separate viral proteins and enhanced effector-memory CD8⁺KLRG1⁺ T cell subset differentiation.

IL-33 augments HIV-specific T cell-mediated responses

To determine whether mtrIL-33 adjuvant can enhance the vaccine potency for other pathogens requiring both T_H1 and CD8⁺ T cell responses, we assessed the Ag-specific T cell-mediated responses of mtrIL-33 co-delivered with a HIV DNA vaccine antigen (Fig. 5). B6 mice (n = 5) were vaccinated three times i.m. at two week intervals with 10 µg HIV Consensus clade C (ConC) alone or in combination with 11 µg of mtrIL-33 followed by EP. One week after final immunization Ag-specific immune responses were measured using cells derived from the spleen. Consistent with findings in the LCMV model (Figs. 2a–c), the inclusion of mtrIL-33 enhanced the numbers of HIV-specific IFN-γ secreting T cells (ConC, ~3,800 SFC) when compared with non-adjuvanted groups (ConC, ~2,300 SFC) (Figs. 5a). Furthermore, we characterized the cytokine-producing phenotype of the T cell population after immunization with the HIV DNA (Figs. 5b,c). In mice, the Ag-specific T_H1 response after HIV vaccination with mtrIL-33, consisted of significantly high frequency of polyfunctional double-positive (IFN-γ⁺TNF-α⁺) and TNF-α and IFN-γ single-positive CD8⁺ T cells in the spleen (Figs. 6b). Regarding CD4⁺ T cells, the Ag-specific T_H1 response after HIV plus IL-33 vaccination, consisted of high frequency of polyfunctional triple-positive (IFN-γ⁺TNF-α⁺IL-2⁺), double-positive (IFN-γ⁺TNF-α⁺) and IFN-γ single-positive CD4 T cells in the spleen (Fig. 5c). Overall, these findings significantly highlight the important role of IL-33 as an effective immunoadjuvant to be incorporated into future vaccines targeting an array of microbial infections.

Discussion

Interleukin 33 is a pleiotropic cytokine whose properties extend beyond T_H2 responses. We show here that IL-33 is capable of inducing and modulating T_H1 responses (6). However, the biological role of IL-33 as an adjuvant in vaccines remains to be further elucidated. The mature form of IL-33 delivered as an adjuvant remarkably augments LCMV-specific cell-mediated antiviral immunity and provides substantial protection against a high-dose lethal LCMV challenge.

The LCMV model of infection is well established with great applicability to investigations regarding vaccine-induced immunity. In a previous study, we showed that mice immunized once with a LCMV-NP DNA vaccine yielded 67% protection after a 20×LD₅₀ lethal dose of LCMV challenge (11). Therefore, we sought to investigate whether the inclusion of IL-33 after one immunization would increase its protective efficacy since a high frequency of LCMV-specific CD8⁺ T cells is considered a critical characteristic against an LCMV

infection (8–10,12,13). Here, the coadministration of IL-33 not only increased the magnitude of IFN- γ spot-forming NP396-specific CD8⁺ T cells, but also improved their polyfunctionality, increased the cytolytic phenotype of the CD8⁺ T cells, and their effector memory differentiation. As measured by IFN- γ ELISpot for the specific CD8 T cell epitope DbNP₃₉₆₋₄₀, the inclusion of IL-33 induced a 2.5-fold greater response compared to NP-alone immunization. IL-33 also induced greater responses compared with our previous LCMV-NP DNA vaccine study, where 25 μ g of pLCMV-NP administered i.m. twice at two week intervals only elicited ~1400 SCFs per 10⁶ splenocytes (11). Nevertheless, although our IL-33 adjuvant approach matched the immunogenicity of the 35 μ g of LCMV-NP dose that elicited 100% protection eventually after three immunizations (11), our IL-33-assisted DNA vaccination conferred 100% protection only after one immunization (Fig. 1). Furthermore, we show that IL-33 enhanced the polyfunctional CD8⁺ T cell populations secreting IFN- γ ⁺TNF- α ⁺IL2⁺, IFN- γ ⁺TNF- α ⁺ and IFN- γ ⁺ and elicited a greater Ag-specific CD8⁺ cytolytic degranulation (Fig. 2). We find this data in accordance with our previous findings that IL-33 can increase the Ag-specific cell-mediated immune response when coadministered with a DNA vaccine (5) and with others (4,6). The reasons behind the role of IL-33 to enhance the frequency of CD8⁺ T cells are not entirely clear. Nevertheless, recently Luzina et al. have shown in a vaccine delivery approach that mature IL-33 (the same isoform used in our vaccine) effect is ST2-dependent (30). Moreover, substantial research has shown the ST2 is important for IL-33 action as mice deficient in ST2 are entirely unresponsive to IL-33 (31). Therefore, the ability of CD8⁺ T cells to respond to IL-33 is a possible explanation for the augmentation, as activated differentiated CD8⁺ T cells can upregulate their cognate binding receptor ST2 (4,20) and therefore lead to more memory cells capable of responding to rechallenge. More importantly, consistent with the markedly enhanced cytotoxic CD8⁺ T cell activity and the higher levels of NP396-specific effector memory CD8⁺ T cells, the challenge studies using two different lethal doses of LCMV (Fig. 1) validate IL-33's potent adjuvant properties, ultimately to improving antiviral vaccine efficacy (4,5).

The inclusion of adjuvants in vaccine formulations has long been a method of improving vaccine efficacy, but how adjuvants alter the phenotype and differentiation of T cells still remain unknown. Current studies have shown that the characteristic profiles of Ag-specific T cell responses can be correlated with superior disease protection (15,16,32–34). Therefore, to examine the biological function of mtrIL-33 to expand and differentiate the CD8 T cells, we coadministered mtrIL-33 with an LCMV-GP DNA vaccine to monitor the DbGP33 tetramer-specific T cells after immunization. In accordance with our previous data (5), we show that mtrIL-33, similar to full-length IL-33, can significantly amplify GP33⁺CD8⁺ T cell responses in the peripheral blood in a vaccine setting. Nevertheless, although not significant, a similar trend was observed whereby the frequency of GP33⁺CD8⁺ T cells in the spleen was higher in the IL-33 vaccinated group (Fig. 3B). These results substantiated IL-33's cytokine property to mediate the expansion of CD8⁺ T cells. In addition, we show that IL-33 also induced significant expansion of Ag-specific CD8⁺KLRG1⁺ T cells in the periphery (Fig. 4c), as previously reported (4,5). These results can be explained by the ability of IL-33 to induce the trafficking of effector–memory T cells out of the spleen and into the periphery (Fig 4c), migrating to the site of infection to initiate immediate effector

function (21–23) and protective immunity as illustrated in figure 1. Moreover, the significant higher recall responses of the Ag-specific CD8⁺ T cells 8 days after boosting (Figs. 3a and 4c) was due to higher basal levels of responses which likely correlated with IL-33's adjuvant effect. We find these results in accordance with Olson et al demonstrating that this population can expand after boosting (15). These findings further support the notion that effector-memory CD8⁺ T cell responses can mediate potent protective immunity against certain pathogens (15,16). CD8⁺KLRG1⁺ T cells are usually considered as short-lived effector cells (19), and together, with recent reports demonstrating vaccines eliciting persistent effector-memory CD8 T cells may be essential for developing effective viral and cancer vaccines (32–34), suggest IL-33 as a suitable adjuvant for therapeutic vaccines. However, IL-33's role as a suitable adjuvant for preventative vaccines cannot be negated. On the contrary, new reports have suggested that KLRG1 expressing CD8 T cells may be capable of surviving long-term (15,16,21). And given that in this study IL-33 was administered in a prophylactic setting may also qualify IL-33's utility as a preventative vaccine adjuvant. Nevertheless, because preventive vaccines should induce long-lasting Ag-specific responses, we are currently pursuing the role of IL-33 to induce central memory CD8 T cells.

One molecular mechanism behind IL-33's potential to drive the formation and differentiation of effector memory CD8⁺ T cells is likely due to its ability to significantly increase T-bet expression in CD8⁺ T cells *in vivo* (Fig. 3f). Joshi and colleagues have shown that overexpression of T-bet is enough to induce the formation of KLRG1⁺ effector cells and that CD8⁺ T cells lacking T-bet are impaired in forming these cytolytic effector cells (23). Therefore, our data further support the notion that an increase in T-bet is associated with effector CD8⁺ T cell differentiation and their ability to have CTL phenotype. This finding also supports previous data showing that *in vitro* IL-33 can increase T-bet expression (20). Binfeng Lu and colleagues also reported that IL-33 promoted the effector CD8⁺ T cells synergistically with TCR and IL-12 signaling (20). Therefore, given that the expression of T-bet in T cells is known to be induced by a combination of T-cell receptor and IFN- γ signaling, could explain one underlining molecular mechanism by which IL-33 promotes effector-memory CD8⁺ T cells (19,23,24). Nevertheless, further studies are needed to further elucidate these mechanisms and how IL-33 alters the transcriptional programs to induce memory T cell differentiation.

The goal of incorporating immunoadjuvants in vaccines is to regulate, modulate, and enhance the desired adaptive responses to prevent disease acquisition and/or progression. Therefore, understanding the biological functions and immunological mechanisms of immunoadjuvants can allow us to better understand correlates of vaccine immunogenicity, ultimately resulting in the design of better vaccines. Here we demonstrate that the administration of IL-33 serves as potent adjuvant improving vaccine efficacy by augmenting the frequencies of effective Ag-specific CD8⁺ T cells, ultimately leading to significant antiviral protection in a widely used challenge model. This report also reveals new critical insight into the biological properties of IL-33 to perpetuate the differentiation of Ag-specific memory CD8⁺ T cells during vaccination. Consideration of these findings may facilitate the development of better vaccine as well as to improve immune responses against cancer.

Methods

Constructs

The DNA constructs encoding mature IL-33 (mtrIL-33), HIV (ConC) and LCMV-GP construct has been described (5,25–27). All constructs had highly efficient immunoglobulin E (IgE) leader sequence inserted at the 5' end of the gene. The constructs were commercially synthesized and optimized as described previously (27).

Animals

All animals were conducted and maintained in accordance with the NIH and the University of Pennsylvania Institutional Animal Care and Use Committee guidelines. Female C57BL/6 (H-2^b) 8-week-old mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Animal immunizations

Mice were immunized once intramuscularly (i.m.) in the tibialis anterior muscle. *In vivo* electroporation (EP) was delivered, with the CELLECTRA adaptive constant current EP device (Inovio Pharmaceuticals), at the same site immediately following vaccination as previously described (5,27). The mice were immunized with either 10 µg pVAX1 or 10 µg pLCMV-NP with or without 11 µg of mtrIL-33 construct. Three weeks after initial immunization, mice were sacrificed and splenocytes were harvested to measure immune responses. The LCMV-GP (GP) construct was administered at 10 µg. For the HIV immunizations, mice were immunized three times at two-week intervals with 10 µg of each construct (ConC) with or without 11 µg of mtrIL-33. One week after immunization, the mice were sacrificed and splenocytes were harvested to monitor immune responses. All studies were repeated at least two times.

LCMV Viral Challenge

For lethal challenge studies, immunized mice were challenged intracranial (i.c.) 21 days after initial vaccination with either 20×LD₅₀ or 40×LD₅₀ of LCMV Armstrong as previously described (11) in 30 µl of virus diluent (PBS with 20% FBS and 1× Anti-Anti (Invitrogen, Carlsbad, CA)). All mice LCMV challenged were housed in a BSL-2 facility and were observed daily for 21 days.

ELISPOT assay

For mice vaccinated with DNA all spleens were processed and IFN-γ ELISpot assays were performed to determine the antigen-specific cytokine secretion. Spleens were collected in RPMI 1640 medium (supplemented with 10% FBS, 1× Antibiotic-Antimycotic, and 1× β-ME) and splenocytes were isolated by mechanical disruption of the spleen using a Stomacher machine (Seward Laboratory Systems, Bohemia, NY). The resulting mashed spleens were filtered using a 40µm cell strainer, treated with ACK lysis buffer for 5 minutes to lyse the RBCs, washed in PBS and then resuspended in RPMI medium for use in ELISpot or Flow Cytometry assay. The IFN-γ ELISPOT assays were conducted as previously described in detail (5,11,27). The measurement of LCMV-specific T cell responses were assessed by stimulating splenocytes with immunodominant LCMV epitope from the H-2^b

background (D^bNP₃₉₆₋₄₀₄ (NP396)) or (D^bGP₃₃₋₄₁ (GP33)) (Invitrogen). The HIV-specific T cell responses were measured by using pooled peptides (15-mers overlapping by 9 amino acids; 2.5 µg/ml final concentration). All peptides were synthesized from GenScript. Concanavalin A (Sigma-Aldrich, St. Louis, MO) was used as positive control and complete culture medium was used as negative control. Spots were enumerated using an automated ELISPOT reader (Cellular Technology, Shaker Heights, OH).

Flow cytometry

Lymphocytes were isolated and processed from the peripheral blood as previously described (5). Cells were stained with CD8, CD44, CD62L, KLRG1, and MHC class I peptide tetramer to LCMV-GP33 (KAVYNFATC) (Beckman Coulter) as described previously (28,29). Intracellular cytokine staining was performed after 5 hr of ex vivo stimulation with either LCMV epitope D^bNP₃₉₆₋₄₀₄ or D^bGP₃₃₋₄₁ peptide, HIV and Ag85B pooled peptides depending on the study as described (5,27). The following antibodies were used for surface staining: LIVE/DEAD Fixable Violet Dead Cell stain kit (Invitrogen), CD4 (FITC; clone RM4-5; ebioscience), CD8 (APC-Cy7; clone 53-6.7; BD Biosciences); CD44 (A700; clone IM7; Biolegend). For intracellular staining the following antibodies were used: IFN-γ (APC; clone XMG1.2; Biolegend), TNF-α (PE; clone MP6-XT22; ebioscience), CD3 (PerCP/Cy5.5; clone 145-2C11; Biolegend); IL-2 (PeCy7; clone JES6-SH4; ebioscience). All data was collected using a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR) and SPICE v5.2 (free available from <http://exon.niaid.nih.gov/spice/>). Boolean gating was performed using FlowJo software to examine the polyfunctionality of the T cells from vaccinated animals.

T cell proliferation assay

Splenocytes isolated from immunized B6 mice 21 days after initial immunization were labeled with Cell Tracer violet Violet (Molecular Probes) and pulsed with 10 µM peptide for 5 days. CD8 T cell proliferation was determined using flow cytometry to assess Cell Trace Violet dilution.

Statistical Analysis

Group analyses were completed by matched, two-tailed, unpaired *t*-test and survival curves were analyzed by log-rank Mantel-Cox test. For non-equally distributed samples nonparametric Mann-Whitney test was performed (Figures 2E and 2F). All values are mean ± SEM and statistical analyses were performed by GraphPad Prism (La Jolla, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. IL-33-elicited protection against lethal LCMV challenge

(a) Mice ($n = 10/\text{group}$) were immunized one time i.m. using EP with $10\ \mu\text{g}$ of empty vector control plasmid (pVAX) or $10\ \mu\text{g}$ of LCMV-NP with or without $11\ \mu\text{g}$ of mtrIL-33. At day 21 post-vaccination, mice were either challenged intracranial (i.c.) with (b) $20\times\text{LD}_{50}$ armstrong LCMV or (c) $40\times\text{LD}_{50}$ armstrong LCMV and animal survival was monitored for 21 days post challenge. Experiments were performed at least two times in independent experiments and data are representative of the result. *, $P < 0.05$; **, $P < 0.01$.

Figure 2. IL-33 induces potent antigen-specific effector CD8⁺ T cells

C57BL/6 mice (n = 4–5) were immunized once by i.m. followed by EP with 10 μ g of NP with or without 11 μ g of mtrIL-33. Splenocytes were harvested 21 days post vaccination to assess the cellular immune responses. ELISpot and ICS assays were stimulated for 18 hours and 5 hours, respectively, with a final concentration of 2.5 μ g/ml. Mice that received 10 μ g pVAX only served as a negative control. (A) The ability of T cells to produce IFN- γ in response to D^bNP396 epitope (CD8⁺) was determined by IFN- γ ELISpot assay. (B) Flow cytometry was used to determine the total cytokine (IFN- γ , TNF- α and IL-2) frequencies of the D^bNP396-specific CD8⁺ T cells induced by mtrIL-33. (C) Column graph shows multifunctional subpopulations of single-, double- and triple-positive CD8⁺ T cells releasing the cytokines IFN- γ , TNF- α and IL-2. (D) The antigen-specific cytolytic degranulation T cells induced by mtrIL-33 adjuvant were measured by degranulation marker expression, CD107a and producing IFN- γ . Dot plots show representative examples of each mouse group. (E) Splenocytes from mice were labeled with cell tracer violet and stimulated with D^bNP396 peptide. Cell tracer signals on CD8⁺ T cell population were detected by flow cytometry to measure proliferation. (F) After proliferation the % of effector CD8⁺ T cells upon stimulation with NP^DNP396-specific peptide (2.5 μ g/ml) was assessed. Dot plots show representative figure of CD62L⁻CD44⁺ CD8⁺ T cells after stimulation. Data shows the SEM

of two independent experiments repeated at least two to three times. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with NP.

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Figure 3. IL-33 amplifies the percentage of antigen-specific CD8^+ T cells

C57BL/6 mice ($n = 4-8$) were immunized once with $10 \mu\text{g}$ LCMV-GP (GP) construct with or without mtrIL-33 and the Ag-specific CD8^+ T cell population was monitored during the course of vaccination. (A) Kinetics $\text{D}^b\text{Gp33}(\text{Tet}^+)$ -specific CD8^+ T cells in the blood following DNA vaccination with a prime at day 0 and boost with GP alone (red arrow) on day 21 post vaccination (dpv). The cells are gated on live $\text{CD8}^+\text{CD44}^+$ T cells. (B-F) At day 21, spleens ($n = 4$ per group) were harvested and antigen-specific responses were monitored *ex vivo* with GP33 peptide. (B) Frequency of $\text{D}^b\text{GP33}$ CD8^+ T cells at 21 dpv. (C) $\text{IFN-}\gamma$ ELISpot assay used to measure the GP-specific T cells producing $\text{IFN-}\gamma$ on 21 dpv. (D) Frequency of GP-specific $\text{IFN-}\gamma^+\text{CD8}^+$, (E) $\text{CD107a}^+\text{CD8}^+$, and (F) $\text{Tbet}^+\text{CD8}^+$ T cells at 21 dpv. Data shows the SEM of two independent experiments repeated at least two times. *, $P < 0.05$; **, $P < 0.01$; no statistical difference in Fig 3 B, C, E.

Figure 4. IL-33 enhances the expansion of CD8⁺KLRG1⁺ effector-memory T cells

(A) Vaccine immunization schedule in mice. B6 mice ($n = 4-8$) were immunized once with 10 μ g of GP plasmid with or without 11 μ g of mtrIL-33 construct and boosted with only GP (red arrow) at 21 days after initial vaccination. Ag-specific responses in the blood were monitored as indicated. (B) Gating strategy used to identify the D^bGp33⁺CD44⁺KLRG1⁺CD8⁺ T cell population in the blood following vaccination (C) Kinetics of the CD8⁺KLRG1⁺ T cell population after DNA vaccination. Experiments were repeated two times. Population were gated on D^bGp33⁺CD8⁺CD44⁺KLRG1⁺ *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 5. IL-33 enhances potent HIV-specific cell-mediated immune responses

B6 mice ($n = 5/\text{group}$) were immunized three times i.m. at two week intervals with 10 μg of HIV Consensus Clade C (ConC) alone or in combination with 11 μg of mtrIL-33. One week after last immunization mice were sacrificed and spleens were processed to monitor the vaccine induced immune responses. (A) IFN- γ ELISpot were performed to detect antigen specific cells secreting IFN- γ after vaccination. (B & C) Multiparameter flow cytometry was used to determine the percentages of both the polyfunctional CD8⁺ and CD4⁺ T cell cytokine profiles. The column chart shows the percentage of HIV-specific T cells displayed as triple, double and single positive secreting cytokines. Pie charts show the relative proportion of each cytokine subpopulation. Experiments were performed at least two times with similar results. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with ConC group.