Expert Review of Vaccines

ISSN: 1476-0584 (Print) 1744-8395 (Online) Journal homepage: informahealthcare.com/journals/ierv20

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To cite this article: Daniel O Villarreal & David B Weiner (2015) IL-33 isoforms: their future as vaccine adjuvants?, Expert Review of Vaccines, 14:4, 489-492, DOI: 10.1586/14760584.2015.1011135

To link to this article: https://doi.org/10.1586/14760584.2015.1011135

IL-33 isoforms: their future as vaccine adjuvants?

Expert Rev. Vaccines 14(4), 489–492 (2015)

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The identification and characterization of cytokine isoforms is likely to provide critical important new insight into immunobiology. Cytokine isoforms can provide additional diversity to their complex biological effects that participate in control and protection against different foreign pathogens. Recently, IL-33 has been identified as a proinflammatory cytokine having several different biologically active isoform products. Originally associated with Th2 immunity, new evidence now supports the role of two IL-33 isoforms to facilitate the generation of protective Th1 and CD8 T cell immunity against specific pathogens. Therefore, a better understanding of the IL-33 isoforms will inform us on how to utilize them to facilitate their development as tools as vaccine adjuvants for immune therapy.

The human immune system is no stranger to war. Tirelessly, it wages war against infectious agents that breach the bodies' barriers and threaten health and survival. With a highly diverse unknown enemy, the immune system has developed countless strategies to effectively control pathogenic intruders. One essential tactic is the release of chemical messengers known as cytokines to help mobilize and maximize immune defense. Cytokines are a group of proteins that promote a variety of biological functions that regulate, expand and target the innate and adaptive immune system. They facilitate inflammatory and proliferative responses, differentiation and crosstalk between immune cells, cells which perform the real-time fight against pathogens that enter the body [1]. Cytokine activity is highly pleiotropic as many cells can produce one to several cytokines, thus influencing many phenotypic traits of immune cells [1,2].

Along with the variety of the individual cytokines, there has recently been an appreciation for alternative splicing of cytokine genes, which result in multiple cytokine isoforms with different functional activities. This natural occurrence provides additional diversity to the cytokine spectrum. Alternative splicing of pre-mRNA contributes to the generation of many alternate gene products [3], whose functions range from antagonistic to agonistic with enhanced biologically active forms compared with the normal full-length unprocessed cytokine isoform. Several differentially spliced cytokine isoforms have been identified, such as IL-2, IL-4, IL-6, IL-7 as well as IL-10 isoforms [4,5]. In many of these cases, the isoforms act as competitive inhibitors of the primary isoform [5]. Recently, IL-33 has emerged as a cytokine containing several different isoforms [6–8]; however, in this case, most of the isoforms are actually more biologically active compared with their 'normal' counterpart [8]. Therefore, these alternatively spliced or processed IL-33 cytokine isoforms represent attractive candidates for further study as possible vaccine adjuvants or immune modulating therapeutics.

IL-33 is a member of the IL-1 cytokine family that is constitutively expressed by epithelial barrier tissue cells, although many other tissue cells and cell types can produce IL-33 [9]. IL-33 functions as an 'alarmin,' alerting the immune system to various stimuli, necrosis and/or tissue damage. Through its cognate receptor ST2, IL-33 activates a variety of immune cells downstream from its release. IL-33 can stimulate a

Keywords: adjuvant • cancer vaccine • CD8 T cell • cytokine • DNA vaccine • immunotherapies • IL 33 • isoform

 10.1586/14760584.2015.1011135 ISSN 1476-0584

plethora of immune cells, from hematopoietic stem cells to non-hematopoietic cells, leading to the production of various new molecules [10]. Although historically recognized as a cytokine mainly involved in driving Th2 immune responses, newer evidence supports that its functions exceed Th2 immunity and can also contribute to the development of Th1 and CD8 T cell immune responses [9,11–14]. These pleiotropic actions of IL-33 implicate it in diverse phases of the immune inflammatory process. This opens new avenues for harnessing the power of IL-33 as an immunostimulatory adjuvant for improving prototype vaccines against diseases that require Th1 protective immunity, such as TB, malaria, HIV and even cancer.

IL-33, similar to its relatives IL-1β and IL-18, is synthesized in a full-length form (human: 1–270 aa; mouse: 1–266 aa) and this form is usually found in the nucleus of cells. It can also be digested in a mature form (human: 112–270 aa; mouse: 110–266 aa), which typically occurs subsequent to release from the cells that produce it [9,15]. The processing and release of full-length IL-33 (proIL-33), in addition to how it is digested into mature IL-33 (mtrIL-33), is still a matter of debate [15]. However, mtrIL-33 cannot translocate into the nucleus because it lacks the nuclear localization signal found in proIL-33 [9,15]. Nevertheless, both isoforms are biologically active, indicating that IL-33 likely has dual-function – displaying both unique nuclear as well as extracellular effects.

Originally assuming that the mtrIL-33 was the biologically active isoform, most data concerning IL-33 signaling *via* ST2 was generated using recombinant IL-33 (mtrIL-33). Historically, mtrIL-33 was described as tipping the balance toward CD4 Th2-mediated immune responses; however, new evidence hints at a role in driving Th1 and CD8 T cell responses [9,11–14] particularly targeting intracellular pathogens. Two independent studies have shown that activated CD8 T cells can express ST2 and that exposure to mtrIL-33 – in synergy with TCR and/or IL-12 – enhanced IFN-γ production in effector CD8 T cells [11,12]. Subsequently, observation by Bonilla *et al.* [13] provided additional evidence that mtrIL-33 could drive a protective antiviral CD8 T cell response *in vivo*. However, Villarreal *et al.* [14] would be the first to demonstrate that the delivery of IL-33 as a molecular adjuvant in a vaccine setting could induce protective immunity. Villarreal *et al.* [14] optimized a DNA vaccine encoding mtrIL-33, and evaluated its efficacy when delivered as an adjuvant along with a DNA construct encoding the HPV16 antigens E6 and E7. In a murine model, intramuscular immunization of mtrIL-33 induced robust Ag-specific polyfunctional CD8 T cell responses, with a large proportion of this undergoing cytolytic degranulation. This vaccine plus adjuvant combination induced complete tumor regression in a therapeutic HPV murine tumor challenge model [14]. Recently, Luzina *et al.* [16] demonstrated in a vaccine delivery approach that mtrIL-33's effect is ST2-dependent; and therefore, the ability of $ST2^{+}$CD8 T cells to respond to immunoadjuvant mtrIL-33 is a possible explanation for the vaccine-induced augmentation, as activated IL-12-induced CD8 T cells can up-regulate the binding receptor ST2 [11,13]. Yang *et al.* [11] also demonstrated that CD8 T cells in the presence of IL-12 result in T-bet-dependent expression of ST2. Furthermore, it is possible that our selected HPV vaccine antigens, E6 and E7, which are potent Th1 antigens, may have facilitated the induction of Type 1 cytokines IL-12 and IFN-g secretion by APCs. Therefore, the favored Th1 cytokine milieu perpetuated by our selected antigen may have induced a favorable immune environment that allowed IL-33 to foster a greater Th1 cell-mediated immune response. Interestingly, a recent study examining the expression of IL-33 in cervical tissue of patients with different stages of HPV cervical disease showed that lower levels of IL-33 in cervical tissue were associated with more severe stages of HPV-induced dysplastic change [17], suggesting that this cytokine can have a protective Th1-type, anti-HPV response.

IL-33 can also impact T helper responses. We observed that DNA immunoadjuvant mtrIL-33 augmented vaccine-induced Ag-specific polyfunctional CD4 T cell responses [14], likely important in immune expansion. Although for years it was assumed that IL-33 mainly played a role in CD4 Th2 immunity (given the early findings of the selective expression of ST2 by Th2 but not Th1 cells), these results intimated that mtrIL-33 *in vivo* might directly enhance Th1 CD4 T cell responses. The ability of IL-33 to expand the frequency of Th1 CD4 T cells is not yet entirely clear. The fact that IL-33 has been shown to activate NK cells [18] could be an explanation by which IL-33 together with these innate cells drives Th1 CD4 immunity. Given the reports showing mtrIL-33 signaling is dependent on ST2 receptors [16], it is likely that only activated Th1 CD4 T cells can express ST2 receptor, and in synergy with IL-12, local IL-33 will significantly augments a Th1 response. A similar concept demonstrated by Bonilla *et al.* [13] reported that only 20% of activated, virus-specific CD8 T cells express ST2 receptor. However, this aspect requires further study. Overall, IL-33 as a DNA adjuvant can broadly expand T cell responses. Given these significant findings, we went on to observe in a preclinical setting, that mtrIL-33 would also increase the Ag-specific CD4 and CD8 T cell responses when co-delivered with a DNA vaccine encoding either a flu, HIV or tuberculosis antigens [Unpublished Data]. In addition, the delivery of adjuvant mtrIL-33 with a DNA vaccine encoding the lymphocytic choriomeningitis virus nucleoprotein can provide 100% protection against a lethal intracranial challenge of lymphocytic choriomeningitis virus after only a single immunization [Unpublished Data]. In these studies, mtrIL-33 improved both the magnitude of the T cell responses as well as enhanced the rapidity with which they were induced. Taken together, the data on IL-33 support the concept that mtrIL-33 might be an effective vaccine adjuvant with great future applications in the treatment of chronic viral infections and cancer vaccine therapies.

Unlike its cousins IL-1β and IL-18, which both require protease processing to become active, IL-33 is more akin to IL-1a, with its full-length, pro-form (proIL-33) being active *in vivo* [15]. Because mtrIL-33 represents an artificially truncated form of the protein, further attention needs to be drawn to the

nuclear function of proIL-33 as this is likely another primary function by which IL-33 exerts its immunomodulatory effects [14,16]. Luzina *et al.* [16] showed that unlike mature IL-33, proIL-33 induces inflammation (not Th2 mediated) *in vivo*, in an ST2-independent fashion. Thus, as previously mentioned, we explored the adjuvant properties of proIL-33 and demonstrated that the delivery of proIL-33 as an immunoadjuvant induced potent tumor immunity and complete tumor regression in an established TC-1 therapeutic tumor-bearing model [14]. Notably, we demonstrated that proIL-33 ability to expand the Ag-specific CD8 T cells in the periphery likely correlated with tumor regression [14]. Given a lack of available adjuvants that induce optimal CD8 T cell responses, the effects of IL-33 are enlightening. Nevertheless, additional studies that involve IL-33 receptor knockout mice will help confirm whether proIL-33 Th1 adjuvant properties are dependent or independent of ST2. Although CD8 T cell responses were important, we also examined effects on B cell responses. Antibody binding and function also appeared to be improved [14]. This validated its study not only as an ideal adjuvant for enhancing effective therapeutic vaccines but also for improving prophylactic vaccines. The dual-function property of proIL-33, to act as a cytokine and as a nuclear transcription factor, may explain the increase in antibody responses by proIL-33. Its nuclear localization may have effects on modulating the humoral immune responses. Thus, the specific transcriptional targets of nuclear IL-33 are still unknown and must be further investigated. In addition, these effects may not have been driven solely by proIL-33, but also by processed proIL-33 (aka mtrIL-33) as processing of the adjuvant did occur *in vitro*. Two bands of IL-33 were detected after transfection with proIL-33 in human rhabdomyosarcoma cells [14]. Other possibilities for this outcome include induced expression of different splice variants of proIL-33 or the generation of different processed mtrIL-33 isoforms. Indeed, recent studies have demonstrated the existence of several human full-length active splice variants [6–9]. Tsuda *et al.* [7] demonstrated the existence of multiple splice variants of IL-33 dependent on the cell type expressing IL-33. Furthermore, Lefrancais *et al.* [8] reported that neutrophil-specific proteases in neutrophils could cleave full-length human IL-$33_{1–270}$ to generate several mature processed variants (IL-$33_{95–270}$, IL-$33_{99–270}$, IL-$33_{109–270}$) with enhanced biological activity (tenfold) compared with proIL-33. They also demonstrated that murine IL-33_{1-266} can be similarly cleaved by these same neutrophil proteases (cathepsin G and elastase), generating two isoforms of mtrIL-33 (IL-$33_{102–266}$ and IL-$33_{109–266}$) [8]. Such data suggest that proteolytic processing of released proIL-33 may be essential for generating diverse potency of IL-33 isoforms depending on the cell types expressing IL-33. Future studies comparing signaling patterns and immune modulation induced by these different full-length splice variants or processed mature IL-33 isoforms will be useful for providing insight into their potential in vaccination systems as well as providing evidence regarding the role of these isoforms in disease pathogenesis.

Despite these positives, a large body of evidence documents a darker side of IL-33: its pathogenic role in Th2-mediated diseases when IL-33 is administered *via* the mucosal route into naïve mice [16,19–21]. IL-33 also plays a key role in promoting mucosal immunity against parasites through the expansion of Type 2 innate lymphoid cells and other innate cells that drive Th2 inflammatory responses [10,22]. In addition, data is emerging that shows that IL-33 may also expand regulatory T cells [23], a mechanism known to dampen vaccine-induced responses. But that has not been observed with DNA gene adjuvants, where no induction of T regulatory cells or Th2 driven responses was observed [[14], Unpublished Data]. Why one method of IL-33 administration elicits Th2 responses, whereas another skews T cell responses toward a Th1/CD8 axis, is unclear. The reasons for the differences may be attributed to variations in the routes of immunization in which the surrounding microenvironment (targeting cells and cytokine network) potentiate different outcomes. Although the mucosal sites are normally rich in Tregs, mast cells, basophils, eosinophils and innate lymphoid cells, which are all Th2-driven-mediated cells, it is likely that residential and local immune cells in the muscle may favor a Th1 response during vaccination. However, the key cell types in the muscle site that could be expressing ST2 and responsible for mediating the vaccine-induced responses have not been investigated. Therefore, the route of delivery should be carefully studied to maximize the desired T cell phenotype in a particular immune setting to better target a particular disease. Furthermore, the nature of the adjuvant properties could have also been because of the vaccine antigen activating APCs to trigger IL-12, which will support IL-33's IFN-γ-inducing activity, and without it, a Th2 response will dominate. Thus, fully understanding the IL-33 signaling system, especially its cytokine contributions as a nuclear modulator versus extracellular molecule, will open new chapters on how to harness the power of all IL-33 isoforms in future vaccines and cancer therapies. Continued study of both ST2- and IL-33-deficient mice will help add more information to our current understanding of the biological functions of IL-33 and its many isoforms. Overall, the immune system may never win the war against disease, but with effective adjuvants like IL-33, it might just avoid some mortal battles.

Financial & competing interests disclosure

DB Weiner has grant funding, participates in industry collaborations, has received speaking honoraria, and fees for consulting. This service includes serving on scientific review committees and advisory boards. Remuneration includes direct payments or stock or stock options and in the interest of disclosure therefore he notes potential conflicts associated with this work with Pfizer, Bristol Myers Squibb, Inovio, Touchlight, oncosec, Merck, VGXI, and possibly others. Licensing of technology from his laboratory has created over 100 jobs in the private sector in the biotech/pharma industry. The other authors declare no competing financial interests.

No writing assistance was utilized in the production of this manuscript.

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