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Cytokine Synergy: an underappreciated contributor to innate anti-viral immunity

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Abstract

Inflammatory cytokines, such as tumor necrosis factor and the members of the interferon family, are potent mediators of the innate anti-viral immune response. The intracellular anti-viral states resulting from treatment of cultured cells with each of these molecules independently has been well studied; but, within complex tissues, the early inflammatory response is likely mediated by simultaneously expressed mixures of these, and other, protective anti-viral cytokines. Such cytokine mixtures have been shown to induce potently synergistic anti-viral responses in vitro which are more complex than the simple summation of the individual cytokine response profiles. The physiological role of this 'cytokine synergy', however, remains largely unappreciated in vivo. This brief commentary will attempt to summarize the potential effects and mechanisms of anti-viral cytokine synergy as well as present several 'real-world' applications where this phenomenon might play an important role.

Keywords

cytokine synergy; innate immunity; anti-viral immunity; TNF; interferon

1. Introduction

Inflammatory cytokines, such as the type-I and type-II interferons (IFNs) and tumor necrosis factor (TNF), are small secreted proteins which, together with their cognate receptors and downstream signaling pathways, play a key role in the innate restriction of invading pathogens such as viruses. In past years, a tremendous amount of effort has been put into elucidating the molecular events triggered by the interaction of these cytokines with their target receptors [1, 2]. However, while it is technically straightforward to study the functions of these molecules independently, in vivo, the innate responses to invading pathogens are much more likely to involve simultaneous interactions of responding cells with complex cytokine mixtures. For example, for many years our lab has investigated the host tropism determinants of a rabbit specific leporipoxvirus called myxoma virus (MYXV) [3, 4]. MYXV infection is highly lethal in the European rabbit; however, all other tested species,

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including humans and mice, are able to readily control viral infection. In humans this control is mediated by the rapid secretion of multiple cytokines, including TNF and IFNα, from infected myeloid cells, such as macrophages, soon after virus challenge [5]. Cells adjacent to these activated myeloid cells are therefore subjected to a complex milieu made up of multiple cytokines. In the case of MYXV, none of the individual cytokines identified are sufficient to completely ablate viral replication in primary human cells; however, the combination of multiple cytokines together induces a unique cellular transcriptional program which displays significantly greater anti-viral effects than treatment with the individual cytokines alone can produce [6]. This "greater than the sum of its parts" response has been observed in a variety of other systems and has been termed 'cytokine synergy' [7]. Unfortunately, due to the complexity involved in analyzing the huge number of cytokine combinations that could potentially occur in vivo, the understanding of cytokine synergy has severely lagged behind the study of the anti-viral states induced by the individual proteins.

2. Anti-viral Effects of Cytokine Synergy

The phenomenon of anti-viral cytokine synergy can be traced back to a report by Wong et al in 1986 demonstrating that the replication of both RNA and DNA viruses could be synergistically blocked in a variety of cells by treatment with combinations of either TNF/IFN-β or TNF/IFN-γ [8]. Since then, a variety of groups have demonstrated that the replication of diverse viruses, including: varicella zoster (VZV) [9], herpes simplex virus-2 (HSV2) [8, 10–12], Epstein Barr virus (EBV) [13], vesicular stomatitis virus (VSV) [7], severe acute respiratory syncytia virus (SARS) [14], and others (reviewed in [15]), can be synergistically restricted by treating cells with various combinations of: TNF, IL1β, type-I IFN, and/or type-II IFN. Unfortunately, the molecular events mediating viral restriction by these synergistic anti-viral states are extremely complex and diverse and have therefore proven difficult to mechanistically deconstruct. For example, treatment of cells with TNF and IFN-γ inhibits human cytomegalovirus replication at a stage prior to viral early gene expression [8, 12] while a similar treatment allows murine cytomegalovirus to progress through DNA replication and late gene expression [16, 17]. In fact, various groups have observed restriction of viral replication following treatment with various combinations of cytokines at virtually every point in the viral replication cycle, including: binding/entry [8, 12, 18], early viral gene transcription [9, 19, 20], assembly [21], and/or egress [16, 17]. These data suggest that multiple mechanisms are likely involved in the restriction of viral replication by anti-viral synergistic cytokine responses. Additionally, the anti-viral effects of cytokine synergy have been shown to differ when slightly different doses of cytokines and/or virus are used [12, 22], or when the cytokines are added at various times in relation to either virus or each other [7] (and our unpublished observations). Significant new research will therefore be needed to deconstruct the molecular details of the complex modulatory effects of cytokine synergy on the replication of various viruses. This will be particularly true within the virus-infected host where the complete list of functional anti-viral cytokines induced in situ may not yet be determined.

3. Mechanisms of Synergy

A report by Peng et al [23] proposed two distinct mechanistic forms of synergy, coined 'synergy by cooperative action' and 'synergy by independent action' (Figure 1). 'Synergy by independent action' occurs when two cytokines induce distinctive sets of host response genes whose combined effector functions synergistically inhibit viral replication. In contrast, 'synergy by cooperative action' occurs when treatment with two cytokines synergistically enhances the expression levels of anti-viral genes normally induced to lower levels by one, or both, of the individual cytokines. For example, treatment of primary human fibroblasts with IFN-β has been shown to induce expression of OAS1, as one of a much larger set of

interferon stimulated genes (ISGs), while treatment of these same cells with IFN-γ induces a related but distinct set of ISGs that includes expression of INDO. In 'synergy by independent action' treatment of fibroblasts with both cytokines would induce expression of both OAS1 and INDO to similar levels seen by the single cytokine treatments; however, simultaneous expression of both ISGs would synergistically inhibit viral replication. In contrast, in 'synergy by cooperative action' treatment with both cytokines would induce expression of OAS1 and/or INDO to much higher levels than are observed following treatment with either cytokine alone, thus improving each individual protein's ability to block viral replication.

We have recently defined a third potential mechanism of synergy related to 'synergy by cooperative action' which we have termed 'synergy by cooperative induction'. In this form of synergy, treatment of cells with multiple cytokines induces a unique set of response genes that are not induced by either cytokine alone [6] (Figure 1). Induction of these new genes can then inhibit viral replication through novel effector mechanisms not present following treatment with the individual cytokines.

Importantly, these mechanisms are not thought to be mutually exclusive and it is therefore likely that two or even all three mechanisms might occur within a single cell. Additionally, it is not known whether synergy occurs *in addition* to the responses mediated by the individual cytokines or *instead of* these responses. Therefore, cells reacting to treatment with multiple cytokines have the potential to induce a myriad number of possible responses including: a response to one or both individual cytokines, induction of one, two or all three forms of anti-viral synergy, or simultaneous but distinct responses to the individual cytokines and one or more synergistic responses.

To date, very little has been reported about the molecular mechanism(s) mediating any of these forms of synergy. 'Synergy through cooperative action' and 'synergy through cooperative induction' appear to occur at the transcriptional level [6, 23, 24] and are therefore likely mediated by changes in the intensity or duration of the intracellular signaling cascades triggered by the inducing cytokines. Whether these forms of synergy are caused by increased or prolonged activation of the same transcription factors mobilized by the individual cytokines, the activation of new transcriptional cascades, or the induction of new target genes whose expression requires multiple transcriptional pathways to be active, however, remains unknown. In contrast, 'synergy by independent action' occurs post-transcriptionally and its effectiveness in the context of a given induced anti-viral state likely depends of a large array of variables including: cell type, which cytokines were used, and which virus is being restricted. Unfortunately, very few of these variables have been explored in a meaningful fashion.

A variety of other mechanisms to explain the synergistic inhibition of viral replication have also been proposed, such as one cytokine altering expression of the receptor for a second cytokine [25, 26] and a role for possible secondary secretion of soluble factors [27]; however, the breadth and impact of these potential mechanisms remains difficult to evaluate.

4. Looking Forward

Most of the work on the synergistic anti-viral effects of TNF and IFN's has focused on the ability of this phenomenon to restrict viral replication in vitro. However, while a variety of groups have shown that treatment of cells with TNF and IFN's can synergistically restrict the replication of a wide variety of viruses in culture (reviewed in [15]) relatively little work has been done to determine the practical implications of this phenomenon.

One potential practical use for cytokine synergy could be to improve current cytokine based therapies. For example, soluble IFN is frequently used clinically as both an anti-viral [28] and/or anti-cancer agent [29, 30]. Unfortunately, this treatment is highly toxic when used at clinically effective concentrations. A recent report by Sainz et al, however, demonstrated that treatment with IFN-α in combination with IFN-γ provides significantly improved protection to mice subjected to a lethal challenge with herpes simplex virus type-1 at much lower IFN dosages then normally required [19]. This suggests that current cytokine based therapies might be improved through an understanding and application of synergistic cytokine interactions.

Cytokine synergy might also play a key role in determining the species tropism of certain viruses. For example, MYXV, which is currently under investigation in our lab as a potential oncolytic agent for the treatment of a variety of human malignancies [31–37], is highly infectious in rabbits [38] but is unable to replicate or cause pathology in vivo in any known non-rabbit species [38, 39]. In cultured primary murine fibroblasts this restriction is caused by the rapid induction of the canonical type-I IFN response [40]. Genetic compromise of this interferon response, for example through abrogation of STAT1 signaling, renders murine cells permissive to MYXV infection and causes mice to become susceptible to lethal MYXV infection following intracranial injection [40]. In primary human fibroblasts, however, treatment with IFN-β alone retards, but does not completely block MYXV replication [6]. In these cells, a complete blockade of MYXV replication can be accomplished only by treatment with combinations of cytokines, such as IFN-β/IFN-γ or IFN-β/TNF, which are likely both secreted from local infected myeloid cells, particularly macrophages. This suggests that complete restriction of MYXV in humans, which occurs following deliberate injection of live virus into volunteers [39], and could also occur following bites by MYXV-bearing mosquitoes or during MYXV-based oncolytic virotherapy, might require synergistic cytokine responses.

A third role for cytokine synergy could be in preventing cellular malignant transformation. It has been shown that the ability to induce a synergistic transcriptional program has been specifically lost in a wide variety of human cancer cells [24]. Additionally, while the proliferation of normal primary cells can be completely blocked by treatment with various cytokine combinations, these same combinations have significantly reduced cytostatic effects on virtually all transformed cells [24]. While highly preliminary, these data suggest that escape from the anti-proliferative effects of cytokine synergy might be an important step towards cellular transformation. Additionally, the ability of the induced synergistic anti-viral state to potently restrict viral replication, combined with its apparent absence or compromise in so many transformed cells, provides a molecular mechanism to explain the phenotypic ‘oncotropism’ of many oncolytic virus candidates, including MYXV.

Clearly, cytokine synergy has the potential to play a major role in a variety of practical applications. Despite being identified over 20 years ago, however, a multitude of questions remain unanswered. In the future, we hope additional attention is paid to the role cytokine synergy plays in cellular processes, particularly oncogenic transformation and the mediation of anti-pathogen defenses, and that its potential clinical applications are explored more thoroughly.

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Highlights

- Simultaneous treatment with multiple cytokines produces synergistic antiviral affects
- The mechanisms of cytokine synergy remain unknown
- Cytokine synergy could play a key role in a variety of real world applications

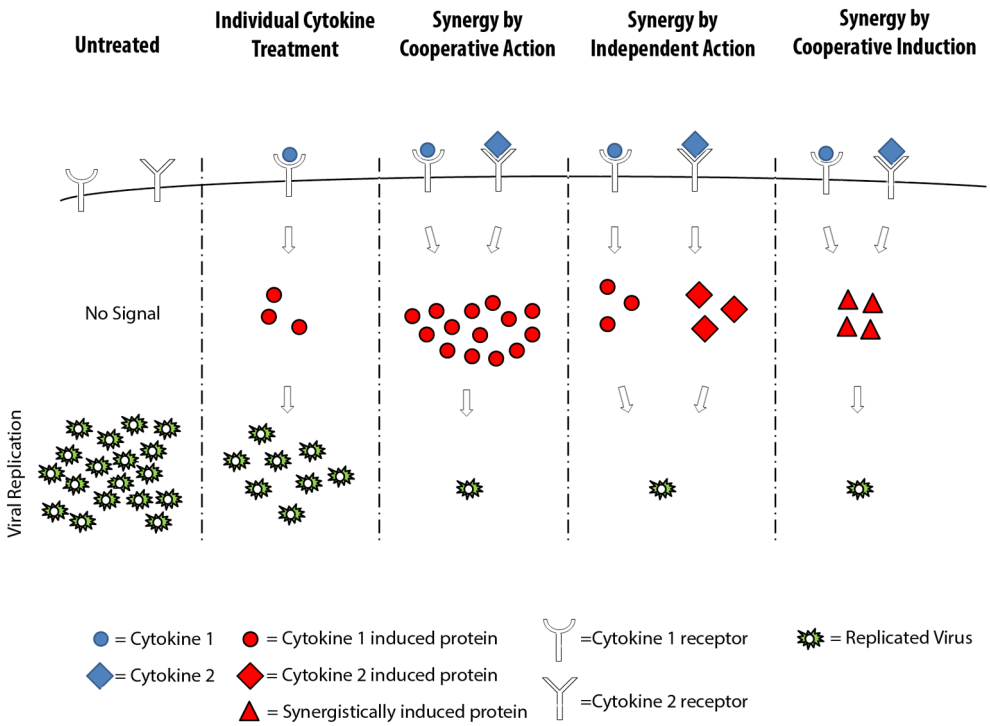

Figure 1. Mechanisms of anti-viral cytokine synergy

Three distinct, but non-exclusive, mechanisms of cytokine synergy have been proposed. 'Synergy by cooperative action' in which treatment with multiple cytokines enhances the level of expression of one or more anti-viral proteins. 'Synergy by independent action' in which multiple anti-viral proteins induced to a low level functionally synergize to block viral replication. "Synergy by cooperative induction' in which treatment with multiple cytokines induces expression of anti-viral proteins not induced by treatment with the individual cytokines. In each case, the anti-viral effects of the cytokine combination are more potent than any of the individual cytokines alone. Each response can occur individually or in combination with any or all other potential responses.