Synergistic action of pro-inflammatory agents: cellular and molecular aspects

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Abstract: Generation of an inflammatory response is a complex process involving multiple factors acting in parallel and in concert. Viruses, parasites, and bacteria, particularly lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, act cooperatively with the cytokine interferon (IFN)-γ to induce many of the genes involved in inflammation. In addition, these components synergistically induce secretion of tumor necrosis factor α (TNF-α), which also synergizes strongly with IFN-γ. The molecular mechanisms underlying the synergistic gene induction discussed in this review involve cooperative activation of transcription factors. IFN-γ-activated signal transducer and activator of transcription 1 and interferon regulatory factor-1 function synergistically with nuclear factor κB activated by LPS and TNF-α. In addition, cross-talk between the signal transduction pathways upstream of the activation of the transcription factors contributes to generation of the synergistic action. Cooperative activity of pro-inflammatory agents profoundly influences the immune response to infections and the efficiency of cellular clearance mechanisms. *J. Leukoc. Biol.* 67: 18–25; 2000.

Key Words: *synergy · inflammation · interferon-γ · tumor necrosis factor α · lipopolysaccharide · transcription*

INTRODUCTION

Redundancy and synergy are fundamental and ubiquitous phenomena in the immune system. The need for several independent stimuli to elicit a specific response allows the body to express highly potent substances such as cytokines, chemokines, radical generating enzymes, etc. only at the proper locations. Because most factors involved in elimination of infection can cause harm to the host itself if produced uncontrolled, the requirement for a dual stimulation for high-output production of specific products generates an efficient double-lock system that limits production of toxic doses to the sites where both stimuli are present. In recent years the molecular basis for the synergistic action of pro-inflammatory cytokines and microbial products has been extensively studied and our insight into this field has concomitantly increased. In this review some of the main players in pro-inflammatory responses will be described and the cellular and molecular aspects of synergy between these will be discussed in the context of how they affect the inflammatory process in infectious diseases.

LIPOPOLYSACCHARIDE (LPS), TUMOR NECROSIS FACTOR α (TNF-α), AND INTERFERON-γ (IFN-γ)

Mounting an inflammatory reaction to an invading pathogen is a complex process involving numerous factors, some of which are produced by the host and others that are delivered by the infectious agent. Among the host-derived products interleukin (IL)-1, IL-6, TNF-α, and interferons (IFNs) are important [1, 2], whereas bacterial LPS is the best-described microbially derived inducer of inflammation [3]. This discussion will focus on synergistic actions of pro-inflammatory agents, processes where notably IFN-γ, TNF-α, and LPS have been ascribed high activity.

LPS is a component of the cell wall of Gram-negative bacteria and is known as a potent activator of macrophages. A broad range of inflammatory reactions are induced by LPS, including cytokine production and cell migration as well as production of acute-phase proteins [3].

Intracellular signaling by LPS is a complex process that has as yet not been fully described. Cellular components with function as signaling LPS receptors have only recently been described. Toll-like receptor (TLR) 2 and 4, which are members of a larger family of TLR receptor proteins, have been shown to be signaling LPS receptors, working in a manner dependent on CD14 [4, 5]. Binding of LPS to the cellular receptor involves the LPS-binding protein (LBP). LBP binds LPS and this complex is recognized by CD14, which then presents the complex to TLR [4, 6].

The membrane proximal events in LPS signaling involve docking of MyD88 and IL-1 receptor-associated kinases (IRAKs) to the TLR, followed by recruitment of TNF receptor-associated factor 6 (TRAF6). TRAF6 then in turn associates with downstream kinases of the mitogen-activated protein (MAP) kinase kinase kinase family, resulting in activation of MAP kinase pathways and the IκB kinase (IKK) complex, ultimately leading

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to activation of the transcription factor nuclear factor (NF)-κB [4, 7, 8]. Other transcription factors activated by LPS include CCAAT/enhancer-binding protein β (C/EBPβ) and activator protein-1 (AP-1) [8]. As will be described below, NF-κB appears to be particularly important for the often observed synergistic action of LPS and IFN-γ. Among the responses triggered by LPS, production of TNF-α primarily by macrophages is pivotal. TNF-α is a powerful inducer of inflammatory responses possessing antitumor, antibacterial, and antiviral properties [9]. Many of these functions are, if not dependent on, then at least substantially augmented in the presence of other stimuli, as will be described below.

Signaling by TNF-α goes through two different receptors, TNF-receptor I (TNF-RI) and TNF-RII. TNF-RI is expressed ubiquitously, whereas TNF-RII is expressed mainly on myeloid, lymphoid, and endothelial cells [10]. Most of the functions induced by TNF-α are mediated via signaling through TNF-RI. The cytoplasmic domain of TNF-RI contains a so-called death domain. The death domain of TNF-RI interacts with an adapter protein, TNF receptor-associated death domain (TRADD), which in turn associates with two other adapter proteins, TRAF2 and Fas-associated death domain (FADD), as well as the kinase receptor interacting protein (RIP) [10]. Signals downstream of FADD, which are involved in apoptosis, include activation of caspase-8 and an acid sphingomyelinase [10, 11]. The association of TRADD, TRAF-2, and RIP, on the other hand, is responsible for TNF-α-induced NF-κB activation. TRAF-2 interacts with the NF-κB-inducing kinase NIK that activates IKK, leading to IκB phosphorylation and hence to IκB degradation and NF-κB activation [12]. In addition to NF-κB, TNF-α also activates the transcription factors AP-1 and interferon regulatory factor (IRF)-1 in some cell types [13, 14].

The third pro-inflammatory factor discussed in this review is IFN-γ, which is the prototypical T helper (Th) 1 cell cytokine but is also produced by natural killer (NK) cells [15]. The central biological actions described for IFN-γ are macrophage activation, antiviral activity, antiproliferative activity on tumor cells, production of free radicals, and induction of expression of adhesion molecules as well as of MHC class I and II [15].

Signaling from the IFN-γ receptor complex involves the tyrosine kinases Jak1 and Jak2, which become activated upon ligand binding [16]. The principal substrate for IFN-γ-activated Jak1 and Jak2 is signal transducer and activator of transcription 1 (STAT1). Tyrosine-phosphorylated STAT1 dissociates from the IFN-γ receptor complex and forms homodimers that translocate to the nucleus and induce transcription via binding to the gamma activating site (GAS) in IFN-γ-inducible promoters. One of the STAT1-induced genes is IRF-1 [17]. IRF-1 is itself a transcription factor that recognizes the sequence termed interferon stimulation response element (ISRE) and plays, like STAT1, a central role in IFN-γ-induced gene expression [18, 19].

A key issue when considering the biological activities of IFN-γ is its pronounced synergistic action with other stimuli, notably LPS and TNF-α. In the following this synergy will be discussed at the cellular and molecular levels.

IFN-γ SYNERGIZES WITH TNF-α AND INFECTIOUS AGENTS

Upon bacterial infection a range of host responses are induced leading to inflammation. LPS is known as the most potent bacterial component in activation of these processes, including extensive cytokine release, activation of leukocytes and migration into the tissue, radical production and degranulation, hence triggering fever and in extreme cases circulatory collapse, diffuse intravascular coagulation, and hemorrhagic necrosis. Many inflammatory events triggered by LPS are enhanced synergistically by IFN-γ, thus enforcing the antibacterial and potentially host-damaging reactions at the sites of bacterial infection [20, 21]. Taking advantage of the mouse knock-out approach, Car et al. Showed that the host response to LPS was severely impaired in IFN-γ receptor-deficient mice compared to the wild-type mice [22]. One of the genes induced by LPS and enhanced by IFN-γ is TNF-α. TNF-α plays a central role in mounting the inflammatory response induced by LPS [3]. For instance, studies in knock-out mice have shown that although mice with a targeted disruption of the TNF-α gene remain sensitive to high doses of LPS, they are resistant to the systemic toxicity of LPS on D-galactosamine sensitization [23]. As will be described below, TNF-α in turn synergizes with IFN-γ in induction of many genes (**Table 1**). It should be mentioned, though, that the processes initiated by LPS are numerous and many are independent of intermediary production of TNF-α [24].

In addition to LPS, bacterially derived DNA and mycobacterial lipoarabinomannan also trigger inflammatory responses and act synergistically with IFN-γ [25, 26]. As for LPS, TNF-α induction is at least partly responsible for the effect of bacterial DNA [25].

Not only bacterial substances act synergistically with IFN-γ. The mouse model of *Leishmania major* infection has shown that this parasite also augments many IFN-γ-induced functions. As described by Green, Nacy, and colleagues, autocrine TNF-α secretion by infected macrophages is largely responsible for the observed synergism [27, 28]. It is interesting that TNF-α is required for elimination of the parasite from IFN-γ-stimulated macrophages, thus showing that the potent antiparasitic action is mounted only at the sites of infection where both IFN-γ and TNF-α are present. This study thus provides an elegant example of how the action of a cytokine can be strongly amplified if allowed to act in concert with other appropriate cytokines.

Studies from our laboratory have shown that virus infections in some respects may show the same pattern as seen after *Leishmania major* infection. Using herpes simplex virus (HSV) type 2 we showed that infection of macrophages induced TNF-α secretion, which was enhanced synergistically by co-treatment with IFN-γ [29]. HSV infection and IFN-γ stimulation synergistically induced inducible nitric oxide (NO) synthase (iNOS) expression through a mechanism dependent on autocrine TNF-α secretion [29, 30]. Other studies from our laboratory have shown that HSV infection and IFN-γ synergistically induce IL-12 secretion from macrophages, but that this synergy seems to be independent of intermediary secretion of TNF-α or

TABLE 1. Synergistic Induction of Transcription by IFN-γ and TNF-α

Gene/promoter-induced	Involved IFN-γ-activated factor(s)	Involved TNF-α-activated factor(s)	Cell types analyzed	References
MHC I	IRF-1	NF-κB	Epithelial cells, neuroblastoma cells	[38, 41, 75]
β_2 microglobulin	IRF-1	NF-κB	Neuroblastoma cells	[38]
TAP1	STAT1	NF-κB	Epithelial cells	[76, 77]
MHC II[a]	CIITA	Unknown	Monocytes, epithelial cells	[78–80]
B7-1 (CD80)	Unknown	Unknown[b]	Fibroblasts	[81]
ICAM-1	STAT1	NF-κB	Epithelial cells	[35]
VCAM-1	IRF-1	NF-κB	Epithelial cells	[82]
E-selectin	Unknown	NF-κB	Epithelial cells	[83, 84]
IRF-1	STAT1	NF-κB	Fibroblasts, macrophages	[36, 45]
Mig	STAT1	NF-κB	Fibroblasts, macrophages	[36]
IL-6	IRF-1, SP-1[c]	NF-κB	Monocytes	[85]
IL-8	AP-1[d]	NF-κB	Gastric cells	[86]
IP-10	STAT1, p48	NF-κB	Fibroblasts, macrophages	[37, 87]
RANTES	Unknown[e]	NF-κB	Epithelial cells, fibroblasts	[36, 88]
gp91-phox	IRF-1, ICSBP	Unknown	Myeloid cells	[89, 90]
p67-phox	Unknown	Unknown	Myeloid cells	[89]
p47-phox	Unknown	Unknown	Myeloid cells	[89]
iNOS	STAT1, IRF-1	NF-κB	Macrophages	[18, 29, 33]
INDO	STAT1, IRF-1	Unknown	Fibroblasts	[31, 58]
HIV-LTR	Unknown	NF-κB	Macrophages, T lymphocytes	[91, 92]

[a] MHC class II gene expression is co-regulated with expression of invariant chain and DMA and DMB.
[b] The human B7.1 promoter contains an LPS-responsive NF-κB consensus sequence [93].
[c] Serine phosphorylation and DNA binding of SP-1 was increased in response to IFN-γ [85].
[d] AP-1 was found to be induced synergistically by IFN-γ and TNF-α [86].
[e] The murine RANTES promoter contains a functional ISRE motif [94, 95].

IFN-α/β [Malmgaard and Paludan, unpublished results]. Together, these results show that IFN-γ and virus infection can synergistically induce many of the factors involved in host defense reactions and that the infection-induced synergy can be generated through mechanisms either dependent on or independent of autocrine/paracrine cytokine production.

In conclusion, infection of host cells quickly induces TNF-α secretion primarily from macrophages via a mechanism that is strongly amplified by IFN-γ. TNF-α then functions synergistically with IFN-γ to induce expression of genes involved in generating inflammatory processes (Table 1). Some substances, notably LPS, induce inflammatory reactions directly in addition to the indirect route via TNF-α secretion.

MOLECULAR MECHANISMS OF SYNERGISTIC GENE INDUCTION

In recent years major parts of the intracellular events leading to synergistic action of IFN-γ with TNF-α and LPS have been unraveled. **Figure 1** depicts a simplified illustration of the signal transduction events involved in generation of the synergistic responses. They will be described in more detail below.

As described above, IFN-γ induces activation of the transcription factor STAT1, which in turn triggers production of IRF-1. These two proteins independently activate transcription and are believed to be largely responsible for the transcription of IFN-γ-induced genes [15]. In addition to their independent action, recent studies have shown that a certain degree of cooperation between IRF-1 and STAT1 is observed in promoters containing binding sites for both factors [31–33]. Because IRF-1 is also induced by LPS and TNF-α in some cell types [14, 34], this cooperation between IRF-1 and STAT1 could possibly contribute to synergistic gene induction.

The major contributor to generation of synergistic promoter activation, however, appears to be synergistic action of STAT1/IRF-1 with NF-κB (Table 1). The vast majority of promoters induced synergistically by IFN-γ and LPS/TNF-α contain binding sites for STAT1 or IRF-1 and NF-κB (for references, see Table 1). By a range of different experimental approaches it has been shown that NF-κB strongly enhances transcription from promoters activated by IRF-1 or STAT1. By reporter gene assays using mutations or sequential elimination of full-length promoters or by using heterologous promoters, many studies have shown that the presence of functional κB sites alongside functional GAS or ISRE sites is sufficient for generation of synergy between TNF-α/LPS and IFN-γ [35–38]. Likewise, studies in knock-out mice have revealed that, depending on the gene, STAT1 or IRF-1 is essential for enabling IFN-γ to synergize with TNF-α or LPS [18, 36].

A number of studies have addressed in greater detail how the transcription factors induce the observed synergistic promoter activation. *A priori*, at least two scenarios can be envisaged. First, the transcription factors could bind independently to their respective sites on the promoter and generate a surface with enhanced interaction with the basal transcription machinery. Second, the transcription factors could interact physically on the promoter, thus forming a complex binding with higher avidity to the recognition sites than the transcription factors individually. Cooperativity between transcription factors to generate optimal interaction with the basal transcription machinery has been suggested for maximal induction of the IFN-β promoter (see below) [39, 40]. For the synergistic action of STAT1 and NF-κB, data seem to indicate that the two

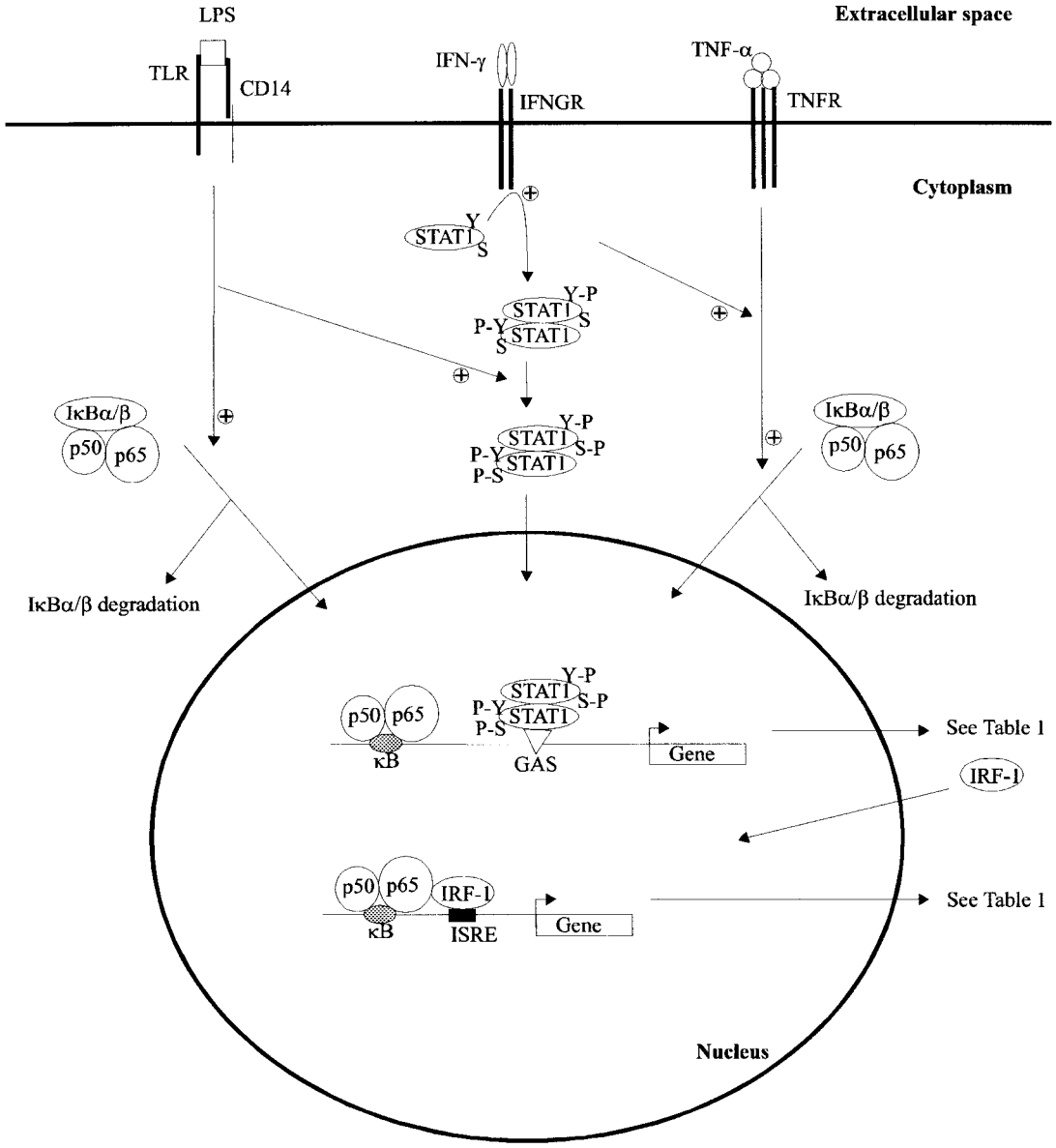

Fig. 1. Molecular mechanisms for synergistic gene induction by IFN-γ and LPS or TNF-α. (1) NF-κB activated by TNF-α or LPS acts synergistically with STAT1 activated by IFN-γ. (2) IFN-γ-induced IRF-1 also acts cooperatively with NF-κB. (3) IFN-γ enhances NF-κB activation induced by low concentrations of TNF-α in some cell types. (4) LPS and TNF-α synergize with IFN-γ in IRF-1 induction in some cell types. (5) LPS induces phosphorylation of STAT1 on serine 727, thus enhancing its *trans*-activating potential. See text for more detailed descriptions.

transcription factors bind DNA independently [36], thus supporting the first possibility described above as the mechanism. The information available at present is, however, too limited for definitive conclusions. By contrast, the mechanism underlying the synergistic action of IRF-1 and NF-κB has been extensively studied and there is evidence by different experimental approaches that IRF-1 and NF-κB interact physically [14, 41]. In one study Drew et al. tested *in vitro*-translated IRF-1 for its ability to interact with the NF-κB subunits p50 and p65 fused to glutathione S-transferase [41]. By this approach they showed that IRF-1 interacts with both p50 and p65 *in vitro*. Furthermore, it was shown that the amino-terminal 115 residues of IRF-1, which constitute the DNA-binding domain, are sufficient to mediate the interaction with both NF-κB subunits. Neish and co-workers showed by DNase footprinting that NF-κB and high-mobility-group I(Y) protein, HMGI(Y), which enhances binding of NF-κB to promoters [42], facilitated binding of IRF-1 to the vascular cell adhesion molecule 1 (VCAM-1) promoter [14]. In the same study it was also shown that *in vitro*-translated IRF-1 interacted with p50 and HMGI(Y). Very recently the first report of *in vivo* interaction between IRF-1 and NF-κB has been published [43]. This elegant work from Lowenstein's laboratory showed that IRF-1 and NF-κB co-immunoprecipitate in a stimulus-dependent manner, and by electrophoretic mobility shift assay it was shown that the interaction by one of the factors with its cognate recognition site was enhanced by an adjacent binding site for the other.

The recently published structure of the IRF-1/ISRE complex shows that IRF-1 recognizes DNA with an accompanying bend of the DNA, which might assist in the interaction of IRF-1 with neighboring transcription factors [44]. It is interesting that κB and ISRE sites are often juxtaposed in promoters induced

synergistically by IRF-1 and NF-κB, so physical interaction is likely to play a functional role. A theoretical model based on the structures of IRF-1 and the NF-κB p50 homodimer complexed to their respective binding sites in the IFN-β promoter shows that the DNA bending imposed by IRF-1 actually brings IRF-1 into closer contact with NF-κB [44]. In another study it was shown that binding of NF-κB and IRF-1 to the iNOS promoter imposes a bend of the DNA as determined by cyclization assays [43]. This bend was dependent on both the ISRE element and the downstream κB site of the iNOS promoter, suggesting that interaction between the two transcription factors generates a loop structure within the promoter region. The creation of bends and loops within promoters brings together DNA binding factors that interact with distantly located recognition sites. These transcription-factor-dense promoter regions then in turn interact with the basal transcriptional machinery and thereby contribute to synergistic effects on gene transcription. Such multi-factor transcription initiation complexes, generally termed enhanceosomes, have long been thought to be important for maximal and sustained transcription, and strong evidence is now mounting that virus-induced IFN-β expression is established by generation of an enhanceosome complex involving multiple transcription factors, histone acetyl transferases, general transcription factors, and cofactors [39]. The stability and function of this pre-initiation complex depends on the presence of all these factors and maximal transcriptional output is achieved only in the presence of all factors [39, 40].

Altogether, these data seem to indicate that the synergy between LPS- or TNF-α-activated NF-κB and IFN-γ-induced IRF-1 is at least partly due to physical interaction, which strengthens DNA binding of both factors and enhances the cooperation with the basal transcription machinery.

Recent reports have elucidated new aspects of the signaling leading to synergistic gene expression. Studies on regulation of IRF-1 expression have shown that this transcription factor is synergistically induced by IFN-γ and TNF-α in some cell types [36, 45]. Given that TNF-α also activates NF-κB and that IRF-1 and NF-κB synergize as described above, enhanced IRF-1 expression will further augment the degree of synergism between IFN-γ and TNF-α.

Another study from Baldwin's laboratory showed synergistic activation of NF-κB by TNF-α and IFN-γ [46]. Using concentrations of TNF-α that induced only sub-maximal levels of NF-κB activity, they showed that IFN-γ synergistically enhanced the activation of NF-κB. As to the mechanism of the observed phenomenon, the authors were able to show that enhanced IκBβ degradation was observed on co-stimulation and likely represents the molecular explanation for the synergy. In a subsequent report from the same laboratory it was shown that the double-stranded RNA-activated protein kinase PKR is involved in the synergistic activation of NF-κB by TNF-α and IFN-γ [47].

A study by Kovarik et al. showed that LPS augments IFN-γ-induced STAT1 activity independent of NF-κB activation [48]. It has previously been described that phosphorylation of serine 727 of STAT1 enhances the *trans*-activating function of the transcription factor [49], and the authors found that this was the mechanism by which LPS brought about its effect on STAT1.

Collectively, the data available on the molecular mechanisms underlying the synergistic activity of IFN-γ and TNF-α/LPS suggest cooperation between IFN-γ-activated STAT1/IRF-1 and TNF-α/LPS-activated NF-κB as the major players, but also indicate that other mechanisms might be involved. It is important that, of the mechanisms described above, few are mutually exclusive and independent of each other. This opens up the possibility that generation of synergy involves several different mechanisms that cross-talk and thereby might amplify each other.

BIOLOGICAL IMPLICATIONS

Concerning the biological implications of the above-described synergistic interactions between pro-inflammatory agents, numerous studies have shown that IFN-γ acts synergistically with LPS and TNF-α in antitumor, antibacterial, and antiviral activities.

Early studies clarified that IFN-γ and TNF-α exert a synergistic effect on tumor cell killing [50]. The exact mechanism is not known but MHC class I and NO, both known to be induced synergistically by IFN-γ and TNF-α, have been shown to be involved [51, 52]. IFN-γ and TNF-α have also been found to act in concert on clearance of several bacterial infections. Many studies have demonstrated that IFN-γ and TNF-α act synergistically in inhibition of multiplication of intracellular bacteria like *Mycobacterium tuberculosis* and *Listeria monocytogenes* [53–55]. NO has been shown to be involved in clearance of infections by *M. tuberculosis*, *Salmonella typhimurium*, and *Staphylococcus aureus* [56], whereas it does not seem to be an essential part of the defense against *L. monocytogenes* [54, 55, 57]. In a study by Mackenzie et al., IFN-γ and TNF-α inhibited growth of Group B streptococci in a synergistic manner [58]. The growth restriction could be reverted by addition of L-tryptophan, indicating that the L-tryptophan-depleting enzyme indolamine 2,3-dioxygenase (INDO), which was synergistically induced by IFN-γ and TNF-α, was largely responsible for the antibacterial effect.

Work by Green and co-workers has shown that IFN-γ and LPS cooperatively enhance killing of intracellular *Leishmania major* in macrophages and that this cooperative action is dependent on autocrine TNF-α secretion [27, 28, 59]. Other reports have shown similar results with other intracellular parasites like *Schistosoma mansoni*, *Toxoplasma gondii*, and *Trypanosoma cruzi* [50, 60, 61]. As to the antiparasitic mechanism, the data available are ambiguous. Some studies point to NO production as the major effector mechanism [27, 59], whereas others suggest MHC class I induction or production of free oxygen radicals or INDO as principle players in clearance of parasite infections [60, 62, 63]. This probably reflects that synergistic generation of antimicrobial activity involves multiple redundant mechanisms and that differences in the experimental protocols can expose a specific effector mechanism to play a particularly large role in parasite clearance in one set-up but not in another.

With respect to viral infections, there are also a number of

reports describing synergistic effects of IFN-γ and TNF-α on viral clearance [64–67]. In one study the two cytokines were found to exert a strong synergistic antiviral effect against HSV, which was dependent on IFN-β secretion [64], suggesting NK cell activity, MHC class I, 2'-5' oligoadenylate synthetase, or PKR as central players in virus elimination. Several other studies have shown that iNOS, which is induced synergistically by IFN-γ and TNF-α, is important in clearance of HSV infections [68–71]. Synergistic antiviral activity of IFN-γ and TNF-α is also found against other viruses including murine cytomegalovirus, adenovirus type 2, and pseudorabies virus [65–67].

Apart from enhanced expression of specific genes, another important contribution to the synergistic effect on clearance of infections by pro-inflammatory agents can be ascribed to the temporal aspect. Dual stimulation has in many studies been found to result in not only enhanced but also sustained expression of, e.g., IL-1, TNF-α, and endothelial adhesion molecules [72–74]. With respect to infections, such prolonged expression of pro-inflammatory cytokines and adhesion molecules is potentially important in order to obtain complete clearance of the infecting microbes.

In conclusion, a growing amount of data clearly show that the synergistic effect of pro-inflammatory agents on a specific set of genes leads to enhanced clearance of infections and more efficient tumor surveillance. The need for more than one stimulus to mount a strong response generates control mechanisms, allowing to focus a vigorous response, with inherent cytotoxic potentials, to the desired locations, thus sparing healthy tissues.

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