Role of Cytokine Combinations in Biological Responses

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Cytokines are induced in response to exogenous or endogenous triggers and in most cases, several different cytokines are concomitantly produced. In addition, some cytokines induce the expression of other cytokines. Therefore, biological responses are usually the outcome of a cytokine combination. We are studying the biological activity of cytokine combinations at the molecular level. The present study clarifies the role of basal IL-1α in IFN-γ activities.

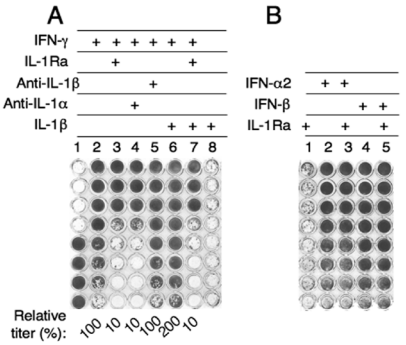

Fig. 1 *The effect of IL-1 inhibitors on the antiviral activity of IFNs. The antiviral titer of various Type I and II human IFNs was determined by a viral (VSV) cytopathic effect inhibition assay. Column 1 in Panel A is control WISH cells (lower four wells) and complete cytopathic effect of same cells following VSV challenge (upper four wells). (A) WISH epithelial cells were incubated with a serial twofold dilution of IFN-γ (4000 IU/ml), either alone or in the presence of exogenous IL-1β (2 ng/ml) or IL-1 inhibitors (IL-1Ra, 10 μg/ml; anti-IL-1α, 2 μg/ml or anti IL-1β, 2 μg/ml). The titer relative to IFN-γ alone (column 2) is given in percent at the bottom. (B) IFN-α2 (4000 IU/ml) or IFN-β (8000 IU/ml) were added to cells either alone or together with IL 1Ra, and the antiviral titer was determined as in A.*

Initially identified by its antiviral activity in non-immune cells, IFN-γ was found later to regulate a remarkable range of immune responses and is a master regulator of the Th1 response. Most cell types express the IFN-γ receptor and its binding activates STAT1, which translocates as a dimer to the nucleus, activating genes whose promoter contains the γ-activated sequence (GAS). Some of these IFN-γ induced genes are transcription regulators such as interferon regulatory factor (IRF)-1, which induces various genes, including those mediating the antiviral action of IFN-γ.

TNF and IL-1 enhance the transcriptional activity of IFN-γ. Additive or even synergistic induction of several pro-inflammatory genes, including ICAM-1, iNOS, various chemokine genes and hepatocyte growth factor have been reported. The additive and synergistic induction of genes by IFN-γ and IL-1/TNF is largely attributable to independent activation of the GAS and κB response elements, respectively. These two response elements are juxtaposed in many promoters of the above-mentioned genes. The importance of basal NF-κB in IFN-γ action has been studied to a limited extent. Since most cell types express constitutively low levels of IL-1α, we studied here its role in the biological activities of IFN-γ.

We found that IL-1 receptor antagonist (IL-1Ra), as well as anti-IL-1α, reduced the antiviral activity of IFN-γ by 90%, whereas anti-IL-1β had no effect on the antiviral titer of IFN-γ (Fig. 1A). IL-1α and IL-1β have no antiviral activity in the absence of IFN-γ. In contrast to IFN-γ, IL-1 inhibitors had no effect on the antiviral activity of Type I IFNs (Fig. 1B). These results indicate that endogenous IL-1α is critical for the antiviral activity of IFN γ.

To further confirm the critical role of endogenous IL-1 in the antiviral activity of IFN-γ, this activity was assessed in wild-type murine embryonic fibroblasts (MEFs) and in IL-1α/β double knockout MEFs. Similarly to WISH cells, IFN-γ was only 10% active in MEFs from one double knockout mouse as compared with wild-type C/57BL MEFs and 33% active in MEFs from another double knockout mouse.

Since the antiviral state is induced at the transcriptional level, we determined the effect of IL-1 inhibitors on IFN-γ-induced genes by expression array analysis. WISH cells were treated with IFN-γ, IL 1Ra, or their combination and total RNA was subjected to expression array analysis. The effect of IL-1Ra on gene expression by IFN-γ was maximal at 17 h. Of 370 genes induced at least 4-fold, we found a group of 65 genes whose induction was inhibited by at least 1/3 in the presence of IL-1Ra. Among these were some of the major IFN-γ-induced transcripts associated with the antiviral response, including Mx1, Mx2, 2′-5′ oligoA synthetase, ISG20 and GBP1 (Fig. 2). Additional known IFN-γ-induced genes unrelated to its antiviral activity were found to be IL-1-dependent. Among these were diubiquitin (UBD/FAT10), ICAM1 and MHCII (Fig. 2). Among the IFN-γ-induced genes that were not modulated by IL-1Ra, were complement components C4A and B, IL-15 receptor α chain, IFI 16b, IFI 41 and tryptophanyl synthetase. Semi-quantitative RT-PCR of RNA from IFN-γ-treated WISH cells as well as HaCaT keratinocytes (Fig. 3AB, respectively) confirmed the expression array data for many of these transcripts, including IRF-1, CIITA, HLA-DR, ICAM1, CXCL11, IL-32 (NK4) and FAT10 (UBD). Taken together, these results establish the existence of a group of genes whose induction by IFN-γ largely depends on endogenously expressed IL 1α.

IL-18BP is an IFN-γ induced gene whose expression is significantly elevated in various inflammatory diseases (2). Its basal expression is very low in non-hematopoietic cells and so far, only IFN-γ was identified as an IL-18BP inducer (3). Using RT-PCR, we found that IL-18BP induction in WISH cells was significantly dependent on endogenous IL-1 (Fig. 3). At the protein level, control cultures of HaCaT keratinocytes contained 0.39±0.03 ng/ml of IL-18BP as determined by ELISA. Upon induction with IFN-γ, its level rose to 2.77±0.024 ng/ml (P=0.0003, N=9), whereas no induction by IFN-γ was obtained in the presence of IL-1Ra

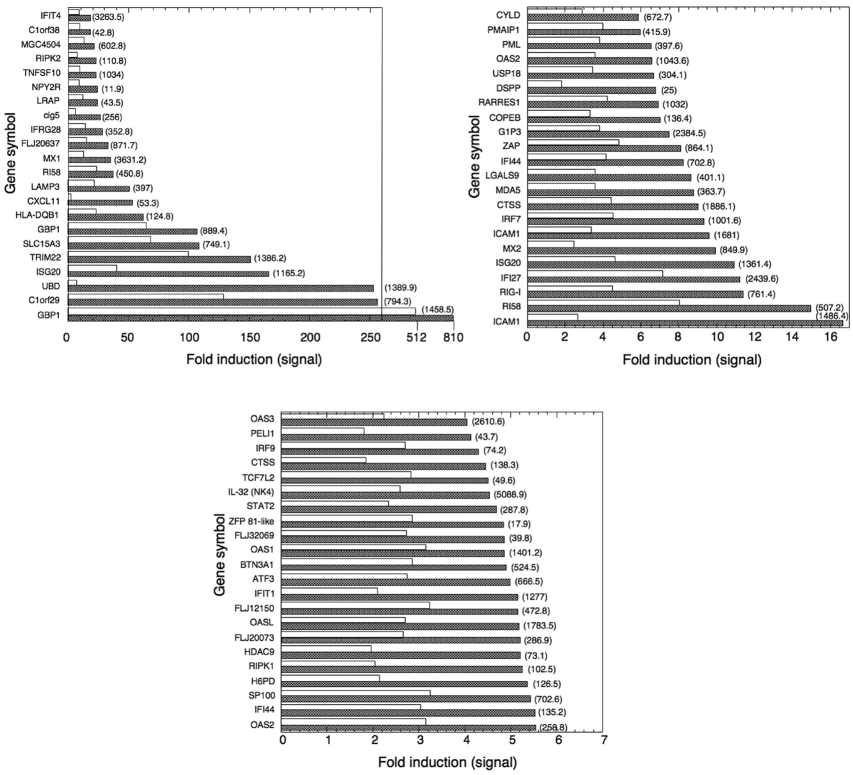

Fig. 2 *Expression array analysis of IFN-γ-treated human WISH cells. Human WISH cells were treated with either medium, IFN-γ (100 IU/ml), IL-1Ra (10 μg/ml) or their combination. Total RNA was isolated at 17 h and subjected to expression-array analysis (Affymetrix U133A chip). Fold induction of genes in cells treated with IFN-γ for 17 h in the absence (hatched bars) or presence (open bars) of IL-1Ra. The figures in parentheses are the average signal of two replicate assays. All signals and fold inductions shown were considered significant by the Affmetrix software (P<0.05).*

(0.35±0.003 ng/ml).

We then determined the role of IL-1α in induction of IL-18BP in vivo by comparing serum IL-18BP in IL-1α/β double deficient mice and wild-type C57/BL6 mice. Although both groups of mice had a similar basal level of circulating IL-18BP, significant induction of IL-18BP was obtained following IFN-γ administration only in the wild-type mice (P= 0.0004, N=8; Fig. 4). Taken together, these results indicate that endogenous IL-1α is essential for induction of IL-18BP by IFN-γ, as determined at the mRNA and protein levels, in vitro and *in vivo*.

IFN-γ signals through the Jak-STAT pathway and does not activate NF-κB directly. We hypothesized that endogenous IL-1α was critical for IFN-γ action by providing a basal level of NF-κB activity. Indeed, ammonium pyrrolidinedithiocarbamate (PDTC, 300 μM, 30 min), a specific inhibitor of NF-κB translocation to the nucleus, completely abrogated the induction of IL-18BP mRNA by IFN-γ. Furthermore, EMSA with a probe corresponding to the κB site of the IL-18BP promoter (bases -396 to -387 relative to the transcription start site) revealed the formation of a complex with nuclear extracts of IL-1α-treated WISH cells, which was abolished by IL-1Ra. This complex contained NF-κB, as shown by super-shifting with anti p65 antibodies.

To further establish the role of NF-κB as a co-activator of IFN-γ signaling, we employed exogenous TNF-α as an alternative inducer of NF-κB. As expected, nuclear extracts of TNF-α-treated WISH cells also formed a complex with the κB probe, which was inhibited by PDTC, but not by IL-1Ra. This TNF-α-induced complex was identified as NF-κB by super-shifting with anti p65. We then established that exogenously added TNF-α could replace the endogenous IL-1α in inducing the antiviral state by IFN-γ, enhancing the antiviral activity of IFN-γ by 32-fold. Taken together, these data suggest that basal IL-1α-activated NF-κB has a critical role in expression of a set of IFN-γ-induced genes, including those mediating its antiviral action.

IFN-γ was reported to induce membrane-associated IL-1α in various cells. We found that the basal level of cell-associated IL-1α was 10.5±1.6 and 2.9±0.4 pg/μg protein in WISH cells and HaCaT keratinocytes, respectively. At 24 h, IFN-γ induced IL-1α in these cells to 30.8±6.5 and 34.9±6.3 pg/μg, respectively (P=0.004 and 0.001, respectively, N=9). In contrast, the level of IL-1α in culture supernatants of WISH cells and HaCaT keratinocytes, either before or after 24 h treatment with IFN-γ was below the limit of detection.

Since most of the basal and IFN-γ-induced IL-α was cell-associated, we employed co-culturing experiments to determine if it was active as an integral-membrane protein. IL-1α was induced in human macrophage-like THP-1 (non-adherent) cells by treatment with IFN-γ for 1-17 h. The cells were then fixed with 1% paraformaldehyde and co-incubated for 6 h with either WISH

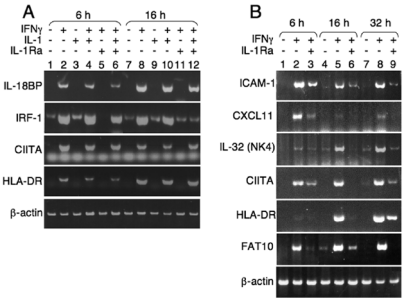

Fig. 3 *RT-PCR of select genes following induction with IFN-γ. Human WISH cells (A) or HaCaT keratinocytes (B) were treated with IFN-γ (100 IU/ml), IL-1β (2 ng/ml), IL-1Ra (10 μg/ml) or their combinations. RNA was isolated at the indicated times and subjected to semi-quantitative RT-PCR with specific primers. The number of cycles was adjusted to show maximal differences if any. RT-PCR of β-actin was used to control for sample loading.*

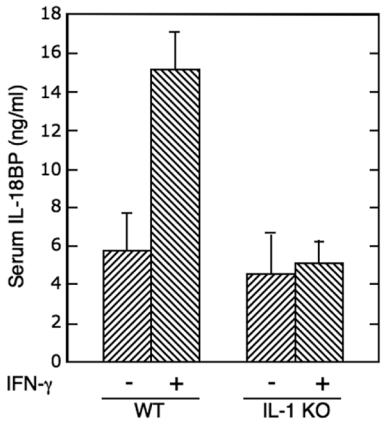

Fig. 4 *Serum IL-18BP in wild type and IL-1α/β double knockout mice following administration of IFN-γ. Wild-type and IL-1α/β double deficient C57BL6 mice (N=8 per group) were injected ip with murine IFN-γ (50,000 IU/mouse). Serum IL-18BP was determined before IFN-γ administration and 24 h after administration.*

cells or HaCaT keratinocytes in the presence or absence of IL-1Ra. After removal of the THP-1 cells, the extent of NF-κB activation in the WISH and HaCaT cells was evaluated by EMSA with a γ[32P]-labeled κB probe. Basal NF-κB activation was observed in cells that were co-cultured with untreated THP-1 cells and it was greatly induced when the cells were co-incubated with IFN-γ-treated THP-1 cells. Formation of NF-κB p65-containing complexes was reduced when the co-culturing was performed in the presence of IL-1Ra, thereby identifying integral-membrane IL-1α of the THP-1 cells as an important NF-κB inducer upon co-culturing with either WISH or HaCaT cells. Importantly, these data demonstrate that basal and IFN-γ-induced integral membrane IL-1α can act on neighboring cells to elicit biological activities in a juxtacrine manner.

Using EMSA with nuclear extracts of cells incubated for 15 min or 4 h with IFN-γ or IL-1α, alone or in combination, in the presence or absence of IL-1Ra, we found that formation of a complex between nuclear proteins and the GAS probe was not affected by either IL-1Ra or IL-1α. Therefore, it can be concluded that the GAS and NF-κB pathways are independent and hence the effect of endogenous IL-1α on the antiviral response of IFN-γ is not mediated through the Jak-STAT1 signaling pathway.

IRF-1 is an IFN-γ-induced transcription factor, which mediates many late IFN-γ-induced transcriptional activities. Previously, we have demonstrated that induction of IL 18BP by IFN-γ is abolished in IRF-1-deficient mice (3). We evaluated the effect of exogenous IL-1β on IRF-1 induction by IFN-γ in WISH and HaCaT cells. Treatment with IFN-γ alone induced IRF-1 only after 1 h as detected by immunoblotting of total cell extract. Pre-incubation of the cells with IL-1β for 1 h prior to addition of IFN-γ not only accelerated IRF-1 induction, resulting in detectable IRF-1 already after 30 minutes, but also increased its level. The activity of this elevated IRF-1 was verified by the observation of increased formation of IRF-1 nuclear complexes following IFN-γ in cell cultures supplemented with IL-1α, as determined by EMSA with an ISRE probe. Since induction of IRF-1 mRNA by IFN-γ was inhibited by IL-1Ra (Fig. 3A) and augmented by exogenous IL-1, we conclude that it requires activation of both the GAS and κB elements. This conclusion is consistent with the reported presence of κB elements in the IRF-1 promoter.

Many studies have demonstrated that TNF synergizes with IFN-γ, probably by eliciting the complementary NF-κB pathway. Here we show that NF-κB activation not only enhances IFN-γ activity, but is in fact essential for its basal activity. The critical role of basal IL-1 in IFN-γ action, combined with the ability of IFN-γ to increase the production of IL-1α, raises the possibility that cells such as keratinocytes, which express high levels of IL-1α, may enhance the responsiveness of infiltrating macrophages or dendritic cells to T-cell-derived IFN γ. Such a cascade of events may be the underlying mechanism of chronic skin inflammations. A role for IL-1 in psoriasis was confirmed recently by the finding of high levels of the IL-1-inducible chemokine IL-8 in psoriatic plaques as compared with non-lesional skin.

In conclusion, this study shows that the cell's response to a given cue is determined not only by its repertoire of receptors and transcription factors, but also by its collection of constitutively expressed cytokines and their associated signaling molecules.

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