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A Synergistic Role for IL-1 β and TNF α in Monocyte Derived IFN γ Inducing Activity

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Abstract

Although much is known about classic IFN γ inducers, little is known about the IFN γ inducing capability of inflammasome-activated monocytes. In this study, supernatants from LPS/ATP-stimulated human monocytes were analyzed for their ability to induce IFN γ production by KG-1 cells. Unexpectedly, monocyte-derived IFN γ inducing activity was detected, but it was completely inhibited by IL-1 β , not IL-18 blockade. Moreover, size-fractionation of the monocyte conditioned media dramatically reduced the IFN γ inducing activity of IL-1 β , suggesting that IL-1 β requires a cofactor to induce IFN γ production in KG-1 cells. Because TNF α is known to synergize with IL-1 β for various gene products, it was studied as the putative IL-1 β synergizing factor. Although recombinant TNF α (rTNF α) alone had no IFN γ inducing activity, neutralization of TNF α in the monocyte conditioned media inhibited the IFN γ inducing activity. Furthermore, rTNF α restored the IFN γ inducing activity of the size-fractionated IL-1 β . Finally, rTNF α synergized with rIL-1 β , as well as with rIL-1 α and rIL-18, for KG-1 IFN γ release. These studies demonstrate a synergistic role between TNF α and IL-1 family members in the induction of IFN γ production and give caution to interpretations of KG-1 functional assays designed to detect functional IL-18.

Keywords

Interleukin-1; caspase-1; TNF α ; KG-1 cell line; IFN γ

1. Introduction

Toll-like receptor mediated pathogen recognition by monocytes/macrophages leads to immediate NF κ B activation and expression of pro-inflammatory cytokines such as TNF α , IL-8, and IL-1 β [1–6]. Interleukin-1 β and IL-18 belong to the IL-1 family and are both synthesized by monocytic cells as biologically inactive precursors that undergo caspase-1-mediated proteolytic cleavage into biologically active forms [7–11]. However, unlike IL-1 β , IL-18 is constitutively expressed at the mRNA and protein level. Caspase-1 activation depends upon assembly of a multi-protein complex known as the inflammasome, which is classically induced upon recognition of pathogen associated molecular patterns (PAMPs), such as LPS, followed by a brief pulse with ATP [12–14].

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Interleukin-18 is mainly known for its IFN γ inducing capability, especially in synergy with IL-12 [15–20]. Although IFN γ is characteristically produced by NK, T and NKT cells, cells of the monocytic lineage have also been shown to constitutively and inducibly express IFN γ [21–27]. The human acute myeloid leukemic KG-1 cell line has been typically used in biological assays to assess the IFN γ inducing activity of IL-18 [28,29]. Specifically, the KG-1 bioassay has been used to study the IL-18 neutralizing capabilities of IL-18 antibodies [30–32] and human/virus-derived IL-18 binding proteins (IL-18 BP) [32–39], to study receptor-associated events [35,38,40,41] and signaling pathways [34,35,42,43] leading to IFN γ gene expression, and to measure the activity of endogenous IL-18 in biological samples [29,44]. Interestingly, IL-12 alone does not induce IFN γ production by KG-1 cells [29], and our preliminary experiments indicated that IL-12 does not synergize with IL-18 for IFN γ production in KG-1 cells.

The expression of many IL-1 β -induced inflammatory genes is synergistically enhanced by the LPS-induced cytokine, TNF α [9,45–49]. However, IL-1 β is not well-known for its IFN γ inducing capability. Only a few reports have implicated IL-1 β in IFN γ production in synergy with IL-2 or IL-12 in NK and T cells [18,50–55]. Moreover, KG-1 cells do not release significant amounts IFN γ in response to rIL-1 β (Fig. 1) and are typically used in bioassays to detect the activity of IL-18, not IL-1 β or IL-12 [28–44].

In this report, we examined the ability of inflammasome-activated monocytes to induce IFN γ production by KG-1 cells. Importantly, we found a monocyte-derived, caspase-1 dependent, IFN γ inducing factor that was released within 4 h of LPS/ATP stimulation. Although LPS/ATP-stimulated monocytes released IL-18, this cytokine was not responsible for the detected IFN γ inducing activity. Via neutralization, immunodepletion and size-fractionation experiments we discovered that the detected IFN γ inducing activity was synergistically mediated by IL-1 β and TNF α , and that neither one of these cytokines alone possess IFN γ inducing activity in KG-1 cells. Finally, we found that rTNF α synergized not only with rIL-1 β , but also with rIL-18 and rIL-1 α for IFN γ production. The lack of IL-18-mediated IFN γ inducing activity in the monocyte conditioned media was likely due to sub-optimal levels of released IL-18, or to IL-18 being bound to an inhibitory protein. These findings suggest that common signaling pathways downstream of members of the IL-1 receptor (IL-1R) family may be crucial for the observed synergy with TNF α . Moreover, our results are unique in light of the facts that, IL-1 β and IL-1 α are not commonly known as IFN γ inducing factors, and IL-1 cytokines and TNF α have never been shown to synergize for IFN γ production.

2. Materials and Methods

2.1. Reagents

Purified *Escherichia coli* (*E. coli*) LPS (serotype 0111:B4) was obtained from Axxora (San Diego, CA) and cell culture tested adenosine 5'-triphosphate (ATP) disodium salt from Sigma-Aldrich (St. Louis, MO). Anti-human IL-1 β , IL-18 and IL-8 ELISA capture monoclonal Abs (clones 2805, 125-2H and 6217), IL-18 detection monoclonal Ab (clone 159-12B), IL-18 receptor (IL-18R) monoclonal Ab (clone 70625), IL-18 binding protein (IL-18BP), and human recombinant IL-1 β (rIL-1 β), rIL-18, rIL-1 α , rIL-8 and rIL-12 were purchased from R&D Systems (Minneapolis, MN). A TNF α capture monoclonal Ab (clone 2C8) was obtained from Advanced Immunochemical (Long Beach, CA), an IL-8 detection polyclonal Ab from Endogen (Rockford, IL), and an IL-12 neutralizing Ab (clone C8.6) and IFN γ ELISA kit from eBioscience (San Diego, CA). Recombinant TNF α was obtained from Knoll Pharmaceuticals (Whippany, NJ). Interleukin-1 receptor antagonist (IL-1ra) was purchased from Amgen (Thousand Oaks, CA). Interleukin-1 β and TNF α ELISA detection polyclonal Abs were developed in our laboratory. The caspase-1 inhibitor acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK) and caspase inhibitor negative control

benzyloxycarbonyl-Phe-Ala-fluoromethylketone (Z-FA-FMK) were purchased from Calbiochem (San Diego, California).

2.2. Cell Culture

Primary monocytes were isolated from human blood by density gradient using lymphocyte separation media (Cell Grow, Media Tech, Herndon, VA) followed by CD14 positive selection (Miltenyi Biotec, Auburn, CA), which yields >95% purity as determined by FACS. Isolated monocytes were cultured at 10^6 /ml or at 12.5×10^6 /ml in RPMI 1640 (Cambrex, East Rutherford, New Jersey), supplemented with 5% FBS (<0.0005 EU/ml) (HyClone, Logan, UT) and 1% penicillin/streptomycin (GIBCO, Grand Island, NY), in a 37°C in a humidified incubator. Monocytes were stimulated, or not, with *E. coli* LPS (10ng/ml) for 4 h and ATP (5mM) for the last 15 min of LPS stimulation, LPS alone, or ATP alone. The supernatants (conditioned media) from monocytes cultured at 10^6 /ml were harvested by centrifugation (5 min; 3,600 rpm) and analyzed for cytokine release by ELISA and IFN γ inducing activity in the KG-1 biological assay. Supernatants from monocytes cultured at 12.5×10^6 /ml were centrifuged twice (5 min at 3,600 rpm and 15 min at 13,200rpm), analyzed for cytokine release and IFN γ inducing activity and loaded on a size-exclusion column for fractionation. KG-1 cells (ATCC, Manassas, VA) were maintained in RPMI 1640 (Mediatech Inc, Herndon, VA), supplemented with 20% FBS (Atlas Biologicals, Fort Collins, CO) and 1% penicillin/streptomycin in a 37°C in a humidified incubator.

2.3. KG-1 Biological Assay

KG-1 cells were plated at a final cell density of 10^6 /ml in 96-well plates and incubated with test samples (100 μ l of monocyte conditioned media or column fractions), recombinant proteins (at the indicated concentrations), or both, in a total volume of 300 μ l. In selected experiments, neutralizing agents for IL-18 (IL-18 Ab – clone 125-2H, IL-18 BP, IL-18R Ab – clone 70625), IL-1 β (IL-1 β Ab – clone 2805, IL-1ra), TNF α (TNF α Ab – clone 2C8) or IL-12 (IL-12 Ab – clone C8.6) were used at the indicated concentrations to neutralize the activities of the respective cytokines prior to addition of ligand, or to neutralize IL-18/IL-1 receptors prior to addition of ligand to the KG-1 cells. KG-1 supernatants were harvested after 24 h and analyzed for IFN γ release by ELISA.

2.4. ELISA

Sandwich ELISAs were used to measure cytokine release in the supernatants of monocytes and KG-1 cells [56,57].

2.5. Size-exclusion chromatography

A HiPrep16/60 Sephacryl S-100 HR chromatography column (Amersham, Piscataway, NJ) was equilibrated in RPMI and calibrated with known standards from a gel filtration low molecular weight calibration kit (Amersham, Piscataway, NJ). The conditioned media (1ml) from LPS/ATP-stimulated monocytes (10^7 /ml) from two donors were separately loaded on the column. Eluting fractions (120, 1.5ml each) were collected in glass tubes containing a final concentration of 0.1% BSA. The starting material (conditioned media loaded on column) and the eluting fractions were analyzed for the presence of cytokines (IL-1 β and IL-18) by ELISA, as well as IFN γ inducing activity in the KG-1 biological assay.

2.6. Immunodepletions

Anti-human TNF α (clone 2C8) and IL-18 (clone 125-2H) Abs (5 μ g/ml each) were used to immunodeplete the conditioned media from LPS/ATP-stimulated monocytes. Mouse IgG1 (clone 11711) (R&D Systems, Minneapolis, MN) was used as an isotype control for all 3 Abs. Monocyte supernatants were pre-cleared with protein A beads (Invitrogen, Carlsbad, CA) for

30 min, followed by overnight incubation with the respective Ab. Supernatants were then incubated with protein A beads (50 μ l/ml, 50% slurry) for 5 hours, followed by quick centrifugation to remove the Ab-bound beads. All incubations were done at 4°C, rocking. Immunodepleted supernatants were then sterilized with 0.22 μ m filters (Millipore, Billerica, MA) and assayed for TNF α and IL-18 by ELISA and for IFN γ inducing activity in the KG-1 bioassay.

2.7. Statistical analysis

Data are presented mean $\pm$ S.E.M. from ≥ 3 independent experiments. Comparisons were done by paired t-test with $p < 0.05$ defined as statistically significant.

3. Results

3.1. KG-1 cells release IFN γ in response to rIL-18, not rIL-1 β

The acute myelomonocytic KG-1 cell line has been typically used to measure the IFN γ inducing activity of IL-18, not IL-1 β [28–44]. We corroborated this by stimulating KG-1 cells with several doses of rIL-18 and rIL-1 β for 24 h. As expected, KG-1 cells released minimal amounts of IFN γ in response to rIL-1 β , compared to rIL-18 (Fig. 1). Furthermore, combining rIL-18 with rIL-1 β did not augment the levels of IFN γ released, compared to rIL-18 by itself.

3.2. Early release of IFN γ inducing activity by LPS/ATP-stimulated monocytes

Interleukin-1 β and IL-18 are known to be released by monocytes as a result of inflammasome and caspase-1 activation [12–14,58–61]. In an attempt to measure functional IL-18 in the supernatants of inflammasome activated monocytes, primary monocytes were stimulated, or not, with LPS (10 ng/ml) for 4 h and ATP (5 mM) for the last 15 min of LPS stimulation, LPS alone, or ATP alone. KG-1 cells were then incubated with the monocyte conditioned media for 24 h and IFN γ production was measured by ELISA. IFN γ inducing activity was detected in the conditioned media from monocytes that were stimulated with LPS in combination with ATP (Fig. 2A). This activity was significantly higher ($p < 0.005$) than that of monocytes stimulated with LPS only, suggesting it being due to the caspase-1 dependent cytokine, IL-18. Importantly, no IFN γ release was detected in the supernatants from ATP- or non-treated monocytes (Fig. 2A), or in the monocyte conditioned media prior to incubation with the KG-1 cells (data not shown), indicating that the KG-1 cells, not the monocytes, were the source of IFN γ .

In addition, the monocyte conditioned media was assayed for IL-1 β , IL-18, TNF α and IL-8 release by ELISA. As expected, LPS/ATP stimulation induced significantly more IL-1 β ($p < 0.005$) and IL-18 ($p < 0.05$) release by monocytes compared to stimulation with LPS alone (Fig. 2B, i and ii). In contrast, release of the caspase-1 independent cytokines, TNF α and IL-8, was not different in response to LPS/ATP stimulation, compared to LPS alone (Fig. 2B, iii and iv). Furthermore, no significant cytokine levels were detected in the supernatants from ATP-stimulated or non-treated monocytes. Paradoxically, the amount of endogenous IL-18 in the monocyte supernatants (~ 1 ng/ml, Fig. 2B, ii) was significantly lower than the amount of rIL-18 required to induce IFN γ production by KG-1 cells (Fig. 1).

3.3. The IFN γ inducing activity was not due to IL-18, but was decreased with a caspase-1 specific inhibitor

To determine whether or not the monocyte-derived IFN γ inducing activity was due to IL-18, we neutralized the activity of this cytokine in the monocyte conditioned media prior to incubation with the KG-1 cells, or neutralized the IL-18 receptors on the KG-1 cells prior to incubation with the monocyte conditioned media. To our surprise, the IFN γ inducing activity

was not inhibited by IL-18 BP, a neutralizing IL-18 Ab, or a neutralizing IL-18R Ab (Fig. 3A). The IL-18 neutralizing agents were shown to be functional by their ability to completely inhibit rIL-18-induced IFN γ production (Fig. 3B).

Since KG-1 cells did not release IFN γ in response to rIL-1 β (Fig. 1), we decided to test whether the detected IFN γ inducing activity was caspase-1 dependent. Isolated monocytes were incubated with the specific caspase-1 inhibitor Ac-YVAD-CMK or the caspase inhibitor negative control Z-FA-FMK (25 μ M each), for 1 h prior to stimulation with LPS plus ATP. Consistent with a role for a caspase-1 dependent product, release of the monocyte-derived IFN γ inducing activity was inhibited by Ac-YVAD-CMK (~3.5-fold), not by Z-FA-FMK (Fig. 4A). Thus, the IFN γ inducing activity released by LPS/ATP-stimulated monocytes was caspase-1 dependent. As expected, caspase-1 inhibition with Ac-YVAD-CMK decreased monocyte IL-1 β release by ~3-fold and IL-18 release by ~10-fold (Fig. 4B and 4C). Importantly, Ac-YVAD-CMK treatment did not inhibit TNF α release (Fig. 4D), indicating that the inhibition of cytokine release was specific for caspase-1-dependent cytokines.

3.4. The IFN γ inducing activity can be inhibited with IL-1 β neutralizing agents

KG-1 cells do not release IFN γ in response to rIL-1 β (Fig. 1). However, the detected IFN γ inducing activity was caspase-1 dependent and independent of IL-18. Therefore, we decided to test the role of endogenous IL-1 β as the putative IFN γ inducing factor present in the conditioned media from LPS/ATP-stimulated monocytes. To our surprise the monocyte released IFN γ inducing activity was significantly ($p < 0.05$) and nearly completely inhibited by an IL-1 β neutralizing Ab and by the IL-1 receptor antagonist protein (IL-1ra) ($p < 0.05$) (Fig. 5). The lack of IFN γ inducing activity of rIL-1 β on KG-1 cells (Fig. 1), and the presence of IL-1 β -mediated IFN γ inducing activity in the monocyte conditioned media suggested the presence of a co-factor for IL-1 β -mediated IFN γ production by KG-1 cells.

3.5. The IL-1 β -dependent IFN γ inducing activity present in the monocyte supernatants is decreased upon size-exclusion fractionation

In order to test for the presence of an IL-1 β cofactor for IFN γ production, we measured the IFN γ inducing activity of monocyte-released IL-1 β before and after size-exclusion fractionation. The conditioned media from LPS/ATP-stimulated monocytes was fractionated by size-exclusion chromatography and the starting material (conditioned media) and eluting fractions were assayed for the presence of IL-1 β (Fig. 6A) and IFN γ inducing activity (Fig. 6B). As expected, IL-1 β eluted at a molecular weight of ~16 kDa. Consistent with the requirement for an IL-1 β cofactor, the specific activity (pg of IFN γ /pg of IL-1 β) of the IL-1 β positive fractions was dramatically decreased when compared to that of the starting material in two separate experiments (2 donors) (Table 1). The loss of specific activity suggested that the ability of IL-1 β to induce IFN γ production requires a synergistic factor, which differs from IL-1 β in molecular weight.

3.6. Interleukin-18 released by LPS/ATP-stimulated monocytes may be partially bound to an inhibitory protein

The conditioned media from LPS/ATP-stimulated monocytes and the column eluting fractions were also assayed for IL-18. It is noteworthy that the IL-18 eluted at both a high molecular weight (79 kDa) and a low molecular weight (19 kDa) peaks (data not shown). The presence of high molecular weight IL-18 suggested that IL-18 may be partially complexed to an inhibitory protein. Therefore, the low levels of IL-18 released by monocytes (Fig. 2B, ii), the high doses of rIL-18 required to induce significant IFN γ production by KG-1 cells (Fig. 1), as well as potential binding of IL-18 to an inhibitory protein may all explain the lack of IFN γ inducing activity of the monocyte-released IL-18 (Fig. 3A).

3.7. Supernatants from LPS-stimulated monocytes provide synergy to recombinant IL-1 β for IFN γ induction

To search for the putative IL-1 β cofactor we examined the ability of rIL-1 β to induce IFN γ production when added to conditioned media from LPS-, ATP- or non-treated monocytes, which have low or non-detectable levels of endogenous IL-1 β (Fig. 2B, i). Unlike the conditioned media from non- or ATP-treated monocytes, the media from LPS-stimulated monocytes provided synergy to rIL-1 β for IFN γ production by the KG-1 cells (data not shown). This synergy was completely inhibited when the conditioned media from LPS-stimulated monocytes was boiled for 5 min prior to incubation with the KG-1 cells (data not shown), indicating that the potential synergistic factor(s) is most likely an LPS-induced protein.

3.8. Interleukin-1 β synergizes with TNF α for IFN γ production

Tumor necrosis factor- α is a prominent pro-inflammatory cytokine released immediately following LPS stimulation that is known to synergize with IL-1 β in the induction of fever, inflammation and tissue damage [9,45–49]. Moreover, it is one of the primary response cytokines induced upon LPS stimulation [1–4]. Therefore, we tested the potential role of TNF α as the IL-1 β cofactor for IFN γ production by KG-1 cells. KG-1 cells were incubated with conditioned media from LPS/ATP-stimulated monocytes in the presence and absence of a TNF α neutralizing Ab, IL-1 β Ab, or both. Each of these conditions resulted in significant inhibition of IFN γ production ($p < 0.005$) (Fig. 7A).

We then immunodepleted endogenous TNF α from the conditioned media from LPS/ATP-stimulated monocytes (Fig. 7B) (immunodepletions were confirmed by ELISA - data not shown), and incubated KG-1 cells with the non-depleted and immunodepleted conditioned media. Immunodepletion of TNF α resulted in significant inhibition of IFN γ inducing activity ($p < 0.005$). Furthermore, rTNF α added to the TNF α -immunodepleted conditioned media restored the IFN γ inducing activity, and this effect was completely inhibited with IL-1ra. Importantly, rTNF α by itself did not induce IFN γ production at doses ranging from 0.1 to 100 ng/ml. As controls, immunodepletions done with an IL-18 Ab and IgG1 isotype control Ab (IgG1 Ab) (Fig. 7C) showed no significant inhibition of IFN γ inducing activity. All together, these results support a novel synergistic role for IL-1 β and TNF α in IFN γ production by the KG-1 monocytic cell line.

3.9. Fractionated IL-1 β recovers specific activity in the presence of rTNF α

To confirm that the loss of specific activity of fractionated endogenous IL-1 β (Fig. 5 and Table 1) was due to its separation from TNF α , we measured the IFN γ inducing activity of fractionated IL-1 β alone, plus rTNF α , or plus rTNF α and IL-1ra (Fig. 8). In agreement with our previous finding, fractionated IL-1 β acquired IFN γ inducing activity when combined with rTNF α . This IFN γ inducing activity was completely inhibited by IL-1ra.

3.10. rTNF α synergizes with rIL-1 β as well as rIL-1 α and rIL-18 for IFN γ production in KG-1 cells

Finally, we incubated KG-1 cells with rIL-1 β , rIL-1 α , rIL-18 and rTNF α (10 ng/ml each) or with different combinations of these cytokines and measured IFN γ released by ELISA (Fig. 9). Interestingly, rTNF α synergized not only with the caspase-1rIL-1 β , but also with rIL-18 and rIL-1 α , indicating that TNF α may be a synergistic factor for IFN γ production in combination with members of the IL-1 family in general.

4. Discussion

In an effort to study the role of inflammasome-mediated events in the release of biologically active IL-18, we tested the IFN γ inducing capability of LPS/ATP-stimulated monocytes on KG-1 cells. As others had previously concluded, e.g. Pirhonen et al. [44], we surmised that the presence of antigenic IL-18 in our samples in conjunction with IFN γ induction in the KG-1 cell line proved that the detected IFN γ inducing activity was due to IL-18. However, to our surprise, 3 different IL-18 neutralizing agents did not inhibit the IFN γ inducing activity present in the monocyte conditioned media. Since rIL-1 β did not elicit a strong IFN γ response on KG-1 cells we decided to test whether the detected activity was due to a caspase-1 dependent or independent product of LPS/ATP stimulation. In agreement with ATP being an activator of caspase-1 in combination with LPS, the IFN γ inducing activity was proven to be caspase-1 dependent using a specific caspase-1 inhibitor.

Given the fact that IL-1 β and IL-18 are the most well-known caspase-1 dependent cytokines [7–11] and despite the fact that KG-1 cells did not release IFN γ in response to rIL-1 β , we decided to rule out the possibility of IL-1 β being responsible for the activity. To our surprise, we were able to nearly completely inhibit the monocyte-derived IFN γ inducing activity with 2 different IL-1 β neutralizing agents. Since KG-1 cells did not release IFN γ in response to rIL-1 β alone, we hypothesized that IL-1 β may require a synergistic factor to induce IFN γ release by KG-1 cells. Through size-fractionation, neutralization and immunodepletion experiments we uncovered for the first time that IL-1 β can synergize with TNF α for IFN γ production in KG-1 cells.

IL-1 β has been shown to synergize with TNF α for the induction of various early pro-inflammatory genes such as chemokines, endothelial adhesion molecules and iNOS. [9,45–49]. However, our results are unique in light of the facts that IL-1 β is not generally recognized as a potent IFN γ inducing factor, and that IL-1 β and TNF α have never been shown to synergize for IFN γ production. In the case of IL-1 β , only a few reports have implicated this cytokine in IFN γ production in synergy with IL-2 or IL-12 in NK and T cells [18,50–55]. IL-12 is a monocyte-derived cytokine and a secondary response gene product that is not likely to be released within 4 h of LPS/ATP stimulation of monocytes. However, prior to discovering the role of TNF α as the synergistic factor for IL-1 β -mediated IFN γ production, we stimulated KG-1 cells directly with rIL-1 β + rIL-12 and found no synergy between these cytokines (data not shown).

In the case of TNF α , this cytokine has been previously implicated in IFN γ induction in tumor macrophages as part of the tumor angiogenic switch [62]. Moreover, TNF and IL-12 are costimulators of IFN γ in SCID mice with listeriosis; *S. epidermis*-induced IFN γ production in whole blood is IL-18-, IL-12- and TNF α -dependent, but IL-1 independent; and *C. albicans*-induced IFN γ production in whole blood is IL-18-, IL-12- and IL-1 β -dependent, and to a lesser degree TNF α -dependent [63]. Therefore, the role of IL-1 cytokines and TNF α in IFN γ production had been previously suggested in neutralization experiments, however the strong synergy between these cytokines for IFN γ production has not been previously reported.

In summary, we have found a novel synergistic role for TNF α and IL-1 cytokines (IL-1 β/α and IL-18) in IFN γ production by KG-1 cells. Moreover, IL-18 released from LPS/ATP-treated monocytes was non-functional (did not induce IFN- γ release by KG-1 cells), due either to sub-optimal levels, as suggested by ELISA, or to it being bound to an inhibitory protein, as suggested by size-exclusion chromatography. Importantly, KG-1 cells should be used with caution in biological assays intended to measure functional IL-18 from biological samples. This is particularly true since both, IL-1 β and TNF α , are released concurrently with IL-18 upon pathogen challenge and IL-18 present in biological samples such as serum or conditioned media

may be bound to an inhibitory protein(s). In the case of IL-1 α , this cytokine might not have played a significant IFN γ inducing role, in synergy with TNF α , in the monocyte conditioned media since a specific IL-1 β neutralizing Ab resulted in almost complete inhibition of KG-1 IFN γ production (Fig. 5).

The KG-1 cell line has been typically used to measure the IFN γ inducing activity of IL-18 [28–44]. Moreover, TNF α has been shown to upregulate the expression of the IL-18R in KG-1 cells, making these cells even more sensitive to IL-18-induced IFN γ production [64]. Hence, KG-1 cells are often pre-treated with TNF α prior to incubation with IL-18 [33–38,41]. However, when we tested the role of TNF α in IL-1R expression upregulation, we did not detect any significant changes (data not shown). Therefore, the mechanism of synergy between IL-1 cytokines and TNF α most likely involves receptor upregulation in the case of IL-18, and well as another unknown mechanism in the case of IL-1 β/α and IL-18.

Importantly, our results support the conclusion that signaling events and induced genes common to the IL-1R and the IL-18R, which both belong to the IL-1R family, may be crucial in the synergy between members of the IL-1 family of cytokines (IL-1 α/β and IL-18) and TNF α for IFN γ production in the KG-1 cell line. Alternatively, TNF α may upregulate cell surface expression of the IL-1R in a similar manner as it upregulates expression of the IL-18R in KG-1 cells. Studies are underway to investigate primary cell types of the monocytic lineage, which may behave in a similar manner as KG-1 cells, and the potential mechanism of synergy between IL-1 family members and TNF α in KG-1 cells at the receptor, intracellular signaling, or gene induction level.

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IFN γ inducing activity

Figure 1. KG-1 cells release IFN γ in response to rIL-18, not rIL-1 β

KG-1 cells (10^6 /ml) were incubated with rIL-18, rIL-1 β , or both (6.25, 12.5, 25, or 50 ng/ml each). KG-1 supernatants were harvested after 24 h of stimulation and analyzed for IFN γ release by ELISA. Results are shown as mean $\pm$ S.E.M. (n = 3–20)

A. IFN γ inducing activity **B. Cytokine release by monocytes****i. IL-1 β** **ii. IL-18** **iii. TNF α** **iv. IL-8** **Figure 2. Monocyte release of IFN γ inducing activity and proinflammatory cytokines in response to LPS/ATP stimulation**

Primary monocytes (10^6 /ml) were stimulated, or not, with LPS (10 ng/ml) for 4 h and ATP (5mM) for the last 15 min of LPS stimulation, LPS alone, or ATP alone. (A) KG-1 cells (10^6 /ml) were incubated with the monocyte conditioned media for 24 h. The KG-1 supernatants were then harvested and analyzed for IFN γ release by ELISA. Results are shown as mean $\pm$ S.E.M. **, $p < 0.005$ (n = 16) (B) Monocyte conditioned media was also assayed for IL-1 β (B, i), IL-18 (B, ii), TNF α (B, iii) and IL-8 (B, iv) release by ELISA. Results are shown as mean $\pm$ S.E.M. *, $p < 0.05$; **, $p < 0.005$. (A: n = 16), (B, i: n = 13; B, ii: n = 4; B, iii: n = 7; B, iv: n = 5). N.T., no treatment.

A. IFN γ inducing activity **B. IFN γ inducing activity**

Figure 3. The IFN γ inducing activity released by LPS/ATP-stimulated monocytes cannot be inhibited with IL-18 neutralizing agents

(A) KG-1 cells (10^6 /ml) were incubated with conditioned media from monocytes (10^6 /ml) that were stimulated with LPS (10 ng/ml, 4 h) and ATP (5 mM, last 15 min) in the presence and absence of either IL-18 binding protein (IL-18 BP) (2 and 10 μ g/ml), IL-18 Ab (IL-18 Ab) (2 and 10 μ g/ml), or IL-18 receptor Ab (IL-18R Ab) (2 and 10 μ g/ml). (B) KG-1 cells were stimulated with recombinant IL-18 (rIL-18) (50 ng/ml) in the presence and absence of either IL-18 BP (2 μ g/ml), IL-18 Ab (2 μ g/ml), IL-18R Ab or (2 μ g/ml). KG-1 supernatants were harvested after 24 h and IFN γ release was measured by ELISA. Results are shown as mean $\pm$ S.E.M. (A: IL-18 BP: n = 5–12; IL-18 Ab: n = 4–11; IL-18R Ab: n = 4–8), (B: n = 3–13).

Caspase inhibition

A. IFN γ inducing activity B. IL-1 β

C. IL-18

D. TNF α

Figure 4. The monocyte-derived IFN γ inducing activity is caspase-1-dependent

Monocytes were pre-treated with Ac-YVAD-CMK or Z-FA-FMK (25 μ M each) for 1 h prior to LPS (10ng/ml, 4 h) plus ATP (5mM, last 15 min) stimulation. (A) KG-1 cells (10^6 /ml) were incubated with the monocyte conditioned media for 24 h, followed by IFN γ ELISA on the KG-1 supernatants. In addition, the monocyte conditioned supernatants were assayed for IL-1 β (B), IL-18 (C), and TNF α (D) release by ELISA. Results are shown as mean $\pm$ S.E.M. (A: n = 5), (B: n = 3–7), (C: n = 3), (D: n = 4). N.T., no treatment.

A. IFN γ inducing activity

Figure 5. The IFN γ inducing activity released by LPS/ATP-stimulated monocytes can be inhibited with IL-1 β neutralizing agents

KG-1 cells (10^6 /ml) were incubated with conditioned media from monocytes (10^6 /ml) that were stimulated with LPS (10 ng/ml, 4 h) and ATP (5 mM, last 15 min) in the presence and absence of either IL-1 β Ab (IL-1 β Ab) (2 and 10 μ g/ml), or IL-1 receptor antagonist (IL-1ra) (2 and 100 μ g/ml). KG-1 supernatants were harvested after 24 h and IFN γ release was measured by ELISA. Results are shown as mean $\pm$ S.E.M. *, $p < 0.05$ ($n = 4$).

A. IL-1 β

B. IFN γ inducing activity

Figure 6. The monocyte-derived IL-1 β -dependent IFN γ inducing activity decreases upon size fractionation

Monocytes ($12.5 \times 10^6/\text{ml}$) were stimulated with LPS (10 ng/ml, 4 h) and ATP (5 mM, last 15 min). Monocyte conditioned media (1 ml) was fractionated by size-exclusion chromatography (as described in the Materials and Methods section). The starting material (conditioned media loaded on column) and eluting fractions were analyzed for IL-1 β by ELISA (A) and IFN γ inducing activity in the KG-1 biological assay (B). Data is representative of two separate experiments with monocytes obtained from two different donors.

Figure 7. Recombinant TNF α synergizes with endogenous IL-1 β for IFN γ production by KG-1 cells (A) KG-1 cells (10^6 /ml) were incubated with conditioned media from monocytes (10^6 /ml) that were stimulated with LPS (10 ng/ml, 4 h) and ATP (5 mM, last 15 min) in the presence and absence of either TNF α Ab, IL-1 β Ab, or both (10 μ g/ml each) ($n = 3-9$). (B) Conditioned media from LPS/ATP-stimulated monocytes were immunodepleted of TNF α . KG-1 cells were incubated with the non-depleted and immunodepleted conditioned media ($n = 7$). In addition, rTNF α (0.1, 1, 10, 100 ng/ml) ($n = 3-5$), or rTNF α (10, 100 ng/ml) plus IL-1ra (10 μ g/ml) ($n = 3$) were added to the immunodepleted supernatants prior to incubation with KG-1 cells (10^6 /ml). (C) Supernatants from LPS/ATP-stimulated monocytes were immunodepleted of IL-18. Mouse IgG1 was used as an isotype control for the IL-1 β , TNF α and IL-18 Abs. KG-1 cells were incubated with the immunodepleted or non-depleted supernatants ($n = 3$). (A – C) KG-1 supernatants were harvested after 24 h and IFN γ release was measured by ELISA. Results are shown as mean $\pm$ S.E.M. **, $p < 0.005$.

A. IFN γ inducing activity

Figure 8. rTNF α synergizes with fractionated IL-1 β for IFN γ production by KG-1 cells
 KG-1 cells (10^6 /ml) were incubated with monocyte-derived fractionated IL-1 β (Fig. 6) in the presence or absence of rTNF α (10 ng/ml), or rTNF α plus IL-1ra (10 μ g/ml). KG-1 supernatants were harvested after 24 h and IFN γ release was measured by ELISA. Results are shown as two separate experiments with endogenous IL-1 β derived from monocytes obtained from two different donors.

IFN γ inducing activity

Figure 9. rTNF α synergizes with rIL-1 β , rIL-1 α and rIL-18 for IFN γ production by KG-1 cells
 KG-1 cells (10^6 /ml) were incubated with either rIL-1 β , rIL-1 α , rIL-18, or rTNF α (10 ng/ml each) and different combinations of these cytokines. KG-1 supernatants were harvested after 24 h and IFN γ release was measured by ELISA. Results are shown as mean $\pm$ S.E.M. (n = 3).

Table 1Specific activity^a of IL-1 β before and after size-fractionation.

Donor	Before	After	Fold Difference (Before/After)
1	0.01	0.002	5
2	0.06	0.005	12

^aSpecific activity = pg IFN γ /pg IL-1 β