IL-1β Augments TNF-α–Mediated Inflammatory Responses from Lung Epithelial Cells

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Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) mediate the development of numerous inflammatory lung diseases. Since IL-1β is typically activated in situations where TNF-α is produced, it was hypothesized that IL-1β alters TNF-α–induced proinflammatory epithelial cell function by altering TNF receptor shedding and surface abundance. In this study, the impact of IL-1β on TNF-α–mediated chemokine production as well as TNF receptor surface expression and shedding were investigated from mouse pulmonary epithelial cells (MLE-15). Interleukin-1β rapidly and persistently enhanced soluble and surface TNFR2. These effects were dependent on TNFR1 expression. TNFR2 small-interfering RNA (siRNA) shifted IL-1β responses, significantly increasing surface and shed TNFR1 implying IL-1β selectively modifies TNF receptors depending on cellular receptor composition. mRNA expression of both receptors was unaltered by IL-1β up to 24 h or in combination with TNF-α indicating effects were post-transcriptional. Interleukin-1β pretreatment enhanced TNF-α–induced macrophage inflammatory protein (MIP)-2 and KC mRNA expression as well as MIP-2 and KC protein levels at the same time point analyzed. Experiments utilizing siRNA against the TNF receptors and a TNFR1 neutralizing antibody demonstrated TNF-α induced MIP-2 through TNFR1, whereas both receptors may have contributed to KC production. These data suggest IL-1β modulates TNF-α–mediated inflammatory lung diseases by enhancing epithelial cell TNF receptor surface expression.

Introduction

THE PLEIOTROPIC CYTOKINE TUMOR necrosis factor-α (TNF-α) is a crucial mediator of inflammatory and fibrotic responses in the lung following toxicant exposure (Piguet and others 1990; Gozal and others 2002). Expressed by a wide variety of cell types including macrophages, fibroblasts, T cells, neutrophils, and epithelial cells (Aggarwal 2003), biological effects of TNF-α are transduced by two distinct receptors, TNFR1 (murine 55 kDa, TNFsfr1a) and TNFR2 (murine 75 kDa, TNFsfr1b). Both TNF receptors are also widely expressed, are inducible under certain conditions and are released from the plasma membrane by proteolytic cleavage, becoming extracellular soluble proteins (sTNFR) still capable of binding TNF-α. The transmembrane metalloprotease TNF-α converting enzyme (TACE), also known as a metalloprotease and disintegrin 17 (ADAM17), has been implicated as the sheddase responsible for the processing of both membrane-bound TNF receptors (Mullberg and others 1995; Black and others 1997; Reddy and others 2000; Schlondorff and others 2000).

Literature has shown that interleukin (IL)-1β influences TNF receptor regulation. (Winzen and others 1993) (Hultner and others 2000) Although its biological effects are transduced by binding to an independent receptor, IL-1R1, IL-1β activates similar signaling pathways as TNF-α to induce primarily proinflammatory gene expression (Kida and others 2005). However, effects of IL-1β on individual TNF receptor expression and shedding are not fully understood. For instance, in human airway epithelial cells in which TNFR2 is not constitutively expressed, IL-1β increased sTNFR1 (Levine and others 1996) while other authors showed IL-1β–induced sTNFR2 release from human gingival fibroblasts, which expressed both receptors, without modification of sTNFR1 (Ohe and others 2000). Holtmann and Wallach (1987) further demonstrated that IL-1β modified TNF-α signaling through alterations in TNF receptor mRNA expression. Finally, treatment of trachea isolated from mice and cultured with IL-1β showed constitutive expression of TNFR1 in the epithelium that increased after IL-1β exposure whereas TNFR2 was undetectable until stimulation. mRNA

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expression for both receptors were, however, detected constitutively in the parenchyma of control mice (Cardell and others 2008). These findings suggested IL-1β modulates pulmonary TNF receptor expression and shedding and thereby potentially impacts cell responsiveness to TNF-α.

In order to gain further insight into the role of IL-1β in the regulation of the two TNF receptors, the present study was designed to test the hypothesis that IL-1β–dependent alteration of pulmonary TNF receptor expression and localization enhances TNF-α signal transduction. Interleukin-1β–induced effects on endogenous mRNA expression, shed, cell-associated and surface TNFR1 and TNFR2 were studied in a murine lung epithelial type II-like cell line (MLE-15s) in which both receptor proteins are constitutively expressed and that respond to TNF-α with chemokine release and reduction in surfactant protein expression (Bachurski and others 1995). In addition, involvement of metalloproteases consistent with TACE in stimulant-induced shedding and surface expression was examined. Finally, receptor requirements for IL-1β–mediated alterations in TNF receptor surface expression and shedding as well as on TNF-α–induced production and alteration of both mRNA and protein levels of the two potent neutrophil chemo-attractants macrophage inflammatory protein (MIP)-2 and KC were determined. These studies suggest methods by which IL-1β can modulate TNF-α–dependent pulmonary inflammatory diseases.

Materials and Methods

Cell culture

Murine lung epithelial type II-like cell line (MLE-15) was cultured for 24 h on type I collagen, in Dulbecco's modified Eagle's medium-Ham's F12 (DMEM F-12) containing 10% heat-inactivated fetal bovine serum (FBS) and 20 μg per mL gentamicin at 37°C in a humidified 5% CO_2 atmosphere. After the growth period, cells were washed with Hanks Balanced Salt Solution (HBSS) and placed in DMEM-F-12 containing 0% FBS. After 24 h incubation, cells were once again washed with HBSS, placed in DMEM-F-12 containing 1% FBS and then incubated with or without TNF-α processing inhibitor-2 (TAPI-2) (100 μM; Peptides International, Louisville, KY), TNFR1 neutralizing antibody (5, 15, or 20 μg per mL, MAB430, R&D Systems, Minneapolis, MN) or Syrian Hamster control IgG (5, 15, or 20 μg per mL, sc-2713, Santa Cruz Biotechnology, Santa Cruz, CA) 1 h before exposure to 1, 5, or 10 ng per mL recombinant murine IL-1β (rmIL-1β; Biosource International Inc., Camarillo, CA). After 6, 24, or 30 h cell supernatant was removed, spun at 4°C, 1200 × g for 6 min to remove debris and then frozen for quantitation by enzyme-linked immunosorbent assay (ELISA). Cells were washed with HBSS and lysed in RIPA buffer (50 mM Tris, 1 mM EDTA, 150 mM NaCl, sodium deoxydodecholate, NP-40) with complete mini-protease inhibitor cocktail tablet (1 tablet per 10 mL; Roche, Mannheim, Germany), snap frozen in liquid nitrogen and stored at −80°C until analysis. For experiments involving TNF-α, cells were exposed to 5 ng per mL recombinant murine TNF-α (rmTNF-α; R&D Systems, Minneapolis, MN) for the indicated time before media removal described above and analysis of receptor expression described below.

Inhibition of TNFR1 and TNFR2 by siRNA

MLE-15 cells were plated on type I collagen and cultured in DMEM-F12 containing 10% FBS without antibiotic. After 1 day, cells were washed with HBSS and transfected with the indicated small-interfering RNA (siRNA) reagent using DharmaFECT (Dharmacon, Chicago, IL). Cells were transfected with 30 nM siRNA for TNFR2 (Ambion, AM16708, Austin, TX), 30 nM TNFR1 siRNA (Dharmacon, L-060201-01) or, as control, 30 nM luciferase siRNA (Dharmacon, D-001100-01-80). The ON-TARGET*plus* siCONTROL Non-Targeting siRNA 2 (30 nM, Dharmacon, D-001810-02-05) was also utilized as a control and produced results comparable to the luciferase control sequence. After 24 h, the cells were washed with HBSS and the medium was replaced with DMEM-F-12 containing 0% FBS. After 24 h incubation, cells were once again washed with HBSS and then exposed to DMEM-F-12 with 1% FBS containing 5 ng per mL rmTNF-α or 10 ng per mL rmIL-1β for 6 h. Culture media was isolated and spun as indicated above, and cells were assayed by fixed cell ELISA as described below.

Cell viability

Cell death was assessed by trypan blue exclusion assay performing cell counts by hemocytometer and light microscopy, or by lactate dehydrogenase (LDH) assay (Sigma) measured by Hewlett-Packard (HP-8453) UV-Visible spectrophotometer at 340 nm.

Quantitation of soluble murine TNFR1 and TNFR2, MIP-2, and KC

Utilizing sandwich ELISAs (R&D Systems), soluble receptors and chemokines were quantified in duplicate or triplicate for each medium sample. Where indicated, values were normalized to cell pellet total protein as determined by bicinchoninic acid assay (Pierce, Rockford, IL).

Fixed cell ELISAs for total and surface TNFR1 and TNFR2

To measure cell surface receptors, a modified ELISA protocol was applied to fixed adherent cells as previously described (Jones and others 2007; Sebag and Hinkle 2007). In order to ensure the antibodies were both sensitive and specific, fibroblasts were isolated from the lungs of wildtype and TNFR double knockout (KO) mice on a C57BL/6 background, cultured for several days, and assayed for surface TNFR1 or TNFR2 as described below in MLE-15 cells. The primary antibodies were then titrated to minimize background signal from the double receptor knockout fibroblasts. In experiments utilizing MLE-15s, cells were rinsed with phosphate-buffered saline (PBS), fixed for 10 min with 4% paraformaldehyde (Polysciences, Warrington, PA), and washed again with PBS for 5 min. Cells were blocked for 20 min with 5% nonfat dried milk in either RIPA buffer or PBS for total and surface detection of receptors, respectively, and then incubated for 2 h with RIPA or PBS containing 5% nonfat dry milk and goat-antimouse TNFR1 (R&D Systems, Cat. AF-425-PB) or hamster-antimouse TNFR2 (R&D Systems, Cat. MAB4262) at 0.5 μg or 1 μg per 0.5 mL,

respectively. Cells were then washed 3 times for 5 min each with 1× PBS, incubated with RIPA or PBS containing 5% nonfat dry milk and 1:5000 horseradish peroxidase–linked rabbit anti-goat secondary antibody (sc-2798, Santa Cruz Biotechnology, Santa Cruz, CA) or 1:1000 goat-antiSyrian hamster secondary antibody (sc-2493, Santa Cruz) for 45 min, washed 3 times with 1× PBS, and incubated for 5–20 min with BM Blue POD Substrate (Roche Applied Science, Indianapolis, IN). The reaction was terminated with 5% sulfuric acid, 300 μL were transferred to a 96-well plate, and the absorbance was read at 450 nm on a Spectromax spectrophotometer (Molecular Devices).

Isolation and analysis of TNFR1, TNFR2, MIP-2, and KC mRNA

MLE-15 cells were treated as described above and then cells were harvested in guanidine isothiocyanate buffer, immediately frozen on liquid nitrogen and stored at −80°C. RNA was extracted in acid/phenol facilitated by phase-lock gel, resuspended in RNase-free water, and quantified by absorbance at 260 nm. mRNA (0.5 μg) was used to synthesize first strand cDNA following the GeneAMP protocol (Applied Biosystems, Foster City, CA). Quantitative real-time polymerase chain reaction (PCR) was performed by commercial protocols with Assays-on-Demand Primer/MGB Probes (Taqman Probe/Primer sets, Applied Biosystems) for mTNFR1 (Mm01182929_m1), mTNFR2 (Mm00441889_m1), mMIP-2 (Mm00436450_m1), mKC (Mm00433859_m1) and ribosomal protein L32 (Mm00777741_sH). Standard curves were made with serial dilutions of a mixed pool of all cDNA assayed. All receptor and chemokine values were normalized to the rpL32 mRNA content of each sample.

Statistics

All values represent 2–4 test wells from each of 3–4 independent experiments and are expressed as means ± SEM. Means of 2 groups were compared by *t*-test. Differences among three or more experimental groups were analyzed by 1-way analysis of variance and Fisher's PLSD *post hoc* test using Statview Statistics Software (SAS Institute, Cary, NC). Differences were considered significant when $P < 0.05$. For calculations of percent change, treatment group values are expressed as percent difference compared to controls.

Results

Secretion of MIP-2 and KC increased in epithelial cells exposed to TNF-α

TNF-α–mediated chemokine secretion from MLE-15 cells was determined to be concentration-dependent (1–10 ng per mL, data not shown); however, at the 10 ng per mL concentration cell viability measured by trypan blue exclusion assay was notably decreased compared to control cells. Five nanogram per mL had no detectable effect on cell viability. In addition, the threshold for TNF receptor shedding was reached at 5 ng per mL (data not shown), thus this concentration was utilized for reproducibility and with minimal toxicity. Media samples isolated from mouse lung epithelial cells (MLE-15) following a 6 h exposure to 5 ng per mL

rmTNF-α were analyzed for the presence of the 2 chemokines macrophage inflammatory protein (MIP-2) and KC. These proteins were chosen as they are consistently induced in a TNF receptor–dependent manner in inflammatory lung diseases as produced by, for example, by infection or silica (MacEwan 2002; Pryhuber and others 2003; Wright and others 2004; Kida and others 2005). As shown in Figure 1A and B, both chemokines were increased in conditioned media of TNF-α treated, as compared to control, treated cells.

Effects of IL-1β pretreatment on TNF-α–mediated MIP-2 and KC

The proinflammatory cytokine IL-1β has been shown to co-operate with TNF-α to induce the expression of other cytokines, chemokines, and adhesion molecules (Le and Vilcek 1987; Dinarello 1994; Pan and others 1996; Doszczak and others 2008). In order to determine the effects of IL-1β on TNF-α–induced production of KC and MIP-2 in lung epithelial cells, MLE-15 cells were pretreated with rmIL-1β (10 ng/mL) for 24 h before washing and a further 6 h incubation in rmTNF-α (5 ng/mL). Analysis of mRNA levels was then performed using quantitative reverse transcriptase PCR. As shown in Fig. 2A and B, exposure to TNF-α for 6 or 30 h significantly increased mRNA expression of both chemokines compared to cells in which media was
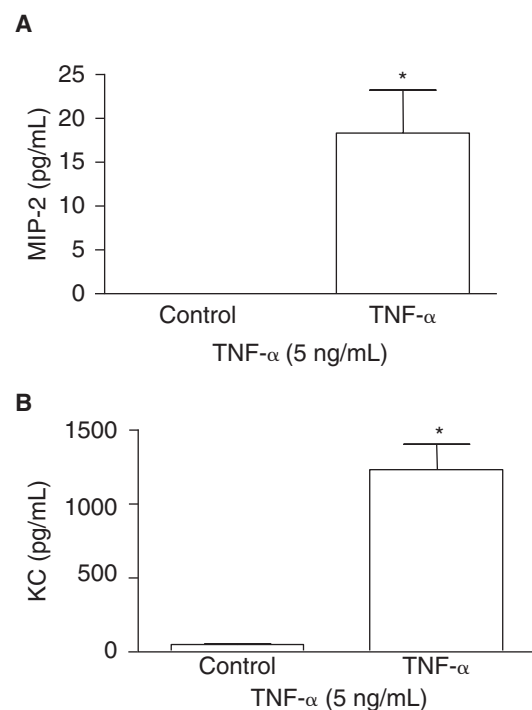

FIG. 1. Tumor necrosis factor (TNF)-α enhances macrophage inflammatory protein (MIP)-2 and KC secretion. Media concentration of (**A**) MIP-2 or (**B**) KC was measured 6 h after treatment of MLE-15 cells with TNF-α (5 ng/mL). Means ± SEM, $n = 3$–5 individual experiments (2–4 wells/n). *$P < 0.05$ compared to control, medium-treated cells. MLE-15, murine lung epithelial type II–like cell line.

only changed at 24 h, or where no media change was made over 30 h C24/C6 or C30, respectively). The chemokine induction was not sustained after removal of TNF-α (see lane "TNF24/C6"). Interleukin-1β pretreatment, (IL-124/TNF6) significantly increased TNF-α–induced MIP-2 and KC. Interestingly, a 24-h pretreatment with TNF-α markedly blunted subsequent TNF-α–induced MIP-2 and KC consistent with desensitization. It can be noted that the pretreatment with TNF-α followed by a 6-h exposure to ligand downregulated surface expression of both receptors, especially TNFR1 (Fig. 6A), which may have contributed to the decrease in chemokine mRNA levels following sequential TNF-α treatment.

In order to determine whether changes in mRNA expression were parallel to protein levels, media from treated cells were collected and subsequently measured by ELISA for MIP-2 and KC (Fig. 2C and D). Tumor necrosis factor-α exposure resulted in increased expression of both

chemokines while IL-1β pretreatment further enhanced accumulation of both chemokines compared to TNF-α alone. Once again, similar to mRNA levels, the downregulation of MIP-2 and KC by two sequential exposures to TNF-α was also demonstrated (see lane "TNF24/TNF6") consistent with desensitization as previously described (Porteu and others 1991; Higuchi and Aggarwal 1994), although the chemokine levels remained greater than untreated control.

Past studies have shown IL-1β alters TNF receptor mRNA and release (Holtmann and Wallach 1987; Levine and others 1996; Ohe and others 2000; Kida and others 2005) but the reported effects varied by study. Since IL-1β enhanced TNF-α–induced mRNA expression and protein levels of MIP-2 and KC in MLE-15 cells, subsequent experiments examined the involvement of the 2 TNF receptor types in the upregulation of each chemokine as well as the effects of IL-1β on TNF receptor surface abundance and release.
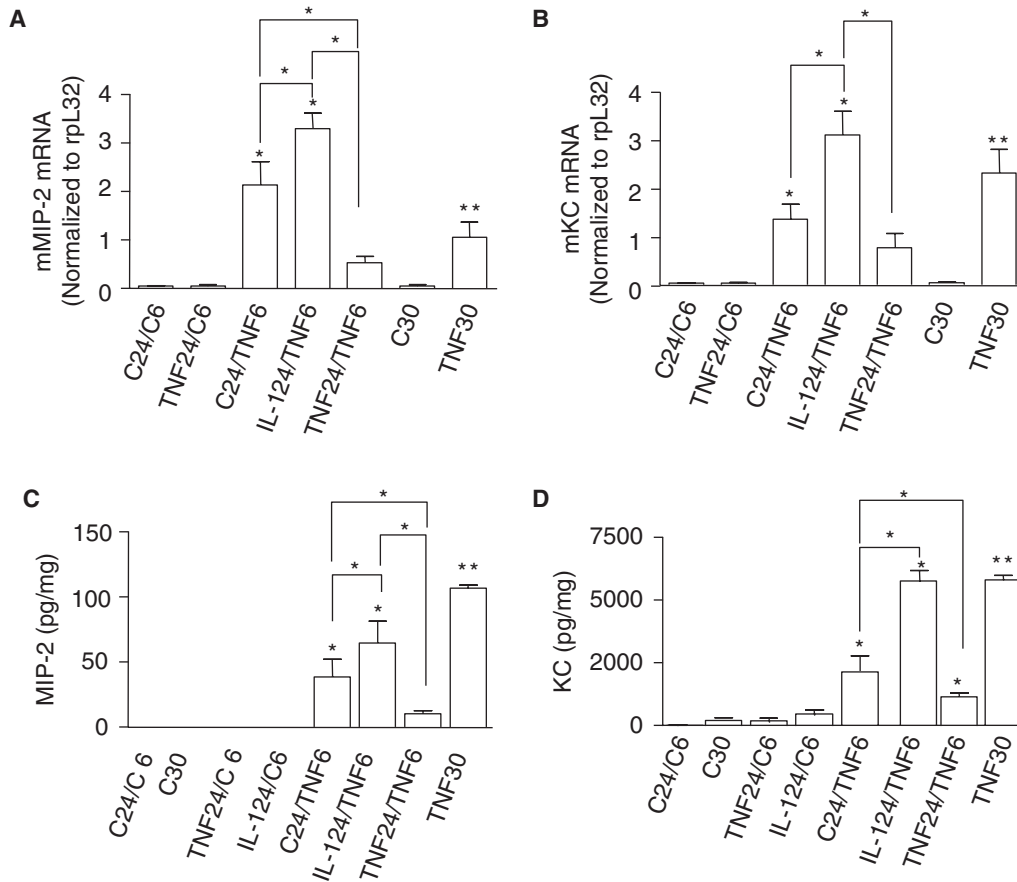

FIG. 2. Interleukin (IL)-1β enhances tumor necrosis factor (TNF)-α–mediated macrophage inflammatory protein (MIP)-2 and KC. MLE-15 cells were pretreated for 24 h with either vehicle, rmTNF-α (5 ng/mL, TNF24) or rmIL-1β (10 ng/mL), IL-124). Cells were then washed and treated with TNF-α or vehicle for an additional 6 h. One set of wells was treated with either vehicle or TNF-α for a continuous 30 h (C30, TNF30). At 30 h, all media were removed and the cellular fraction was analyzed by quantitative real-time polymerase chain reaction analysis for (**A**) MIP-2 or (**B**) KC mRNA expression. Media was then analyzed by sandwich enzyme-linked immunosorbent assay for protein levels of (**C**) MIP-2 and (**D**) KC. Means ± SEM, $n =$ 3–5 (2–3 wells/n). $*,** = P < 0.05$ compared to control-treated cells (C24/C6 for C24/TNF6, IL-124/TNF6 or TNF24/TNF6; C30 versus TNF30, respectively) or (brackets) cytokine-pretreated cells. MLE-15, murine lung epithelial type II-like cell line.

Involvement of individual TNF receptors in TNF-α–induced MIP-2 and KC

TNFR1 has been implicated as the main receptor by which TNF-α elicits its biological effects (Barbara and others 1994). To determine which TNF receptor types were required for TNF-α–mediated MIP-2 and KC production, MLE-15 cells were transiently transfected with siRNA specific for either receptor. Small-interfering RNA transfection and inhibitory efficiency was demonstrated by evaluation of the achieved percent reduction in receptor content within 3 compartments (total cell-associated, surface, and soluble) as shown in Table 1. A > 50% reduction in receptor content was achieved in all cases. Allowing 48 h for reduction in receptor protein, the transfected cells were then treated for 6 h with TNF-α. Macrophage inflammatory protein-2 and KC were then measured in isolated culture media. Lactate dehydrogenase assay showed transfections did not significantly affect cell viability (data not shown). TNFR1 downregulation by siRNA resulted in near total inhibition of TNF-α–induced MIP-2 (Fig. 3A). Reduction of TNFR2 expression resulted in significantly increased TNF-α–induced MIP-2 suggesting a potential inhibitory effect of TNFR2 for this chemokine. KC induction was blunted by TNFR1 siRNA but remained greater than control, suggesting a role for TNFR2 in KC production (Fig. 3B). Although induction of KC by TNF-α was slightly, but not significantly reduced following TNFR2 siRNA transfection, TNFR1 neutralizing antibody studies discussed below also suggest TNFR2-dependent KC production.

In order to dissociate the importance of the presence of TNFR1 from its ability to bind and transduce TNF-α signaling, the expression of MIP-2 and KC from epithelial cells exposed to TNF-α following pretreatment with a TNFR1 neutralizing antibody was determined and compared to the TNFR1 siRNA results (Fig. 3C and D). Compared to ligand alone, MIP-2 was not detectable in the media in the presence of the TNFR1 antibody with or without TNF-α, whereas KC, although significantly decreased by the antibody in a concentration dependent manner from peak TNF-α–induced levels, was 200 fold increased as compared to untreated controls, again suggesting a role for TNFR2 in KC induction. Control IgG demonstrated that the reduction in MIP-2 and KC was not a nonspecific effect of antibody presence. In fact, although MIP-2 was not affected, KC was significantly enhanced by the control IgG although the mechanism behind this increase is unknown. Perhaps Fc receptors expressed on MLE-15s were activated by the Fc fragment of control IgG

and thus upregulated expression of KC. Ultimately these data, consistent with the siRNA experiments, suggest that TNFR1 is required for MIP-2 upregulation whereas both receptors may induce KC.

IL-1β induces metalloprotease-dependent TNFR2 shedding and potentially surface expression of both receptors

We next tested the hypothesis that IL-1β enhanced TNF-α–mediated MIP-2 and KC secretion by altering TNF receptor expression and/or shedding. MLE-15 cells were exposed to increasing concentrations of IL-1β (1, 5, or 10 ng/mL) for 6 h and the TNF receptor concentrations in media and cell homogenate were quantified by ELISAs designed to detect the extracellular domains of the receptors. Similar to mouse primary type II cells, MLE-15 cells constitutively shed TNFR1 and TNFR2 (data not shown). Exposure to IL-1β did not markedly affect soluble TNFR1 concentration or total cell homogenate content; however, TNFR2 release and cell content were increased dose dependently by the interleukin, with both cell content and media accumulation of soluble receptor enhanced up to 2-fold as compared to untreated cells (Fig. 4A and B). These effects were not due to enhanced cytotoxicity as IL-1β exposure did not significantly change viable MLE-15 cell number either measured by trypan blue exclusion assay or LDH release compared to controls (data not shown).

We next investigated whether metalloproteases, which have been shown to cleave membrane-bound TNF receptors (Crowe and others 1995; Li and Fan 2005), are involved in IL-1β–induced receptor shedding and surface localization. MLE-15 cells were exposed to the general metalloprotease inhibitor TAPI-2 for 1 h before cytokine exposure. Subsequent surface expression of the receptors was measured by an ELISA developed to quantify plasma membrane receptors in fixed, nonpermeabilized cells (Jones and others 2007; Sebag and Hinkle 2007). Although TAPI-2 is not a specific inhibitor of the metalloprotease TACE, it has been demonstrated to ablate stimulant-mediated shedding of the TNF receptors in other studies (Crowe and others 1995; Mullberg and others 1995) and therefore was utilized in the following experiments. Analysis of culture media from TAPI-2–exposed MLE-15 cells showed the inhibitor completely prevented constitutive and cytokine-dependent solubilization of both receptors from MLE-15s (Fig. 4C). As shown in Fig. 4D and E, inhibition of release by TAPI-2 alone resulted in significantly increased constitutive surface presentation of TNFR1 and TNFR2. In the presence of TAPI-2, IL-1β significantly increased surface TNFR1 as compared to control and cytokine-treated cells suggesting that IL-1β does induce cellular production and surface localization of TNFR1 but surface accumulation is balanced by increased metalloprotease-dependent solubilization. Both exposure to TAPI-2 or IL-1β alone significantly induced surface TNFR2 expression on MLE-15 cells compared to controls. TNF-α processing inhibitor-2 and IL-1β had a more than additive effect on surface TNFR2 when given in combination (Fig. 4E). The data strongly suggest that IL-1β–dependent shedding and regulation of TNF receptor cell surface presentation is modified by metalloprotease activity.

TABLE 1. PERCENT REDUCTION OF TNFR1 AND TNFR2 BY siRNA AS COMPARED TO NONTRANSFECTED CONTROL CELLS

Receptor	Surface	Total cell	Soluble
TNFR1	57 ± 2.8*	74 ± 1.03*	88 ± 1.74*
TNFR2	64 ± 3.2*	56 ± 1.13*	62 ± 0.8

Means ± SEM, $n = 3$–4 (2–4 wells/n).
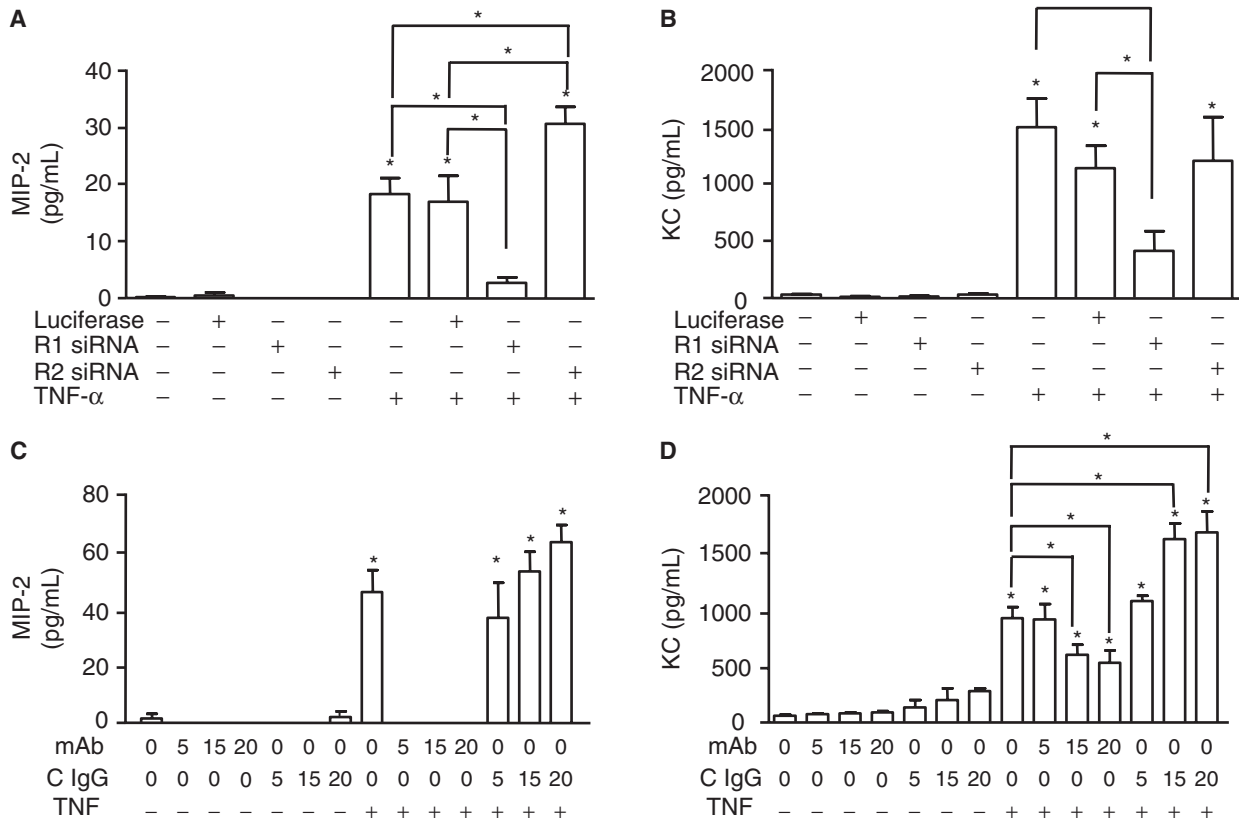*$P < 0.05$ versus both negative luciferase-control and nontransfected control cells.

FIG. 3. Analysis of individual tumor necrosis factor (TNF) receptor involvement in TNF-α–induced macrophage inflammatory protein (MIP)-2 and KC secretion. As described in Materials and Methods, MLE-15 cells were treated with vehicle or 5 ng per mL TNF-α for 6 h, 48 h after transfection with small-interfering RNA (siRNA) sequences targeted to TNFR1 or TNFR2 (**A** and **B**), or 1 h after treatment with a TNFR1 neutralizing antibody (mAb at 0, 5, 15, or 20 µg/mL) or with the same concentration of control IgG (C IgG) (**C** and **D**). Media was then analyzed for (**A** and **C**) MIP-2 or (**B** and **D**) KC. Means ± SEM, $n = 3$ independent experiments with 2–4 wells per n. *$P < 0.05$ compared to nontreated, control IgG-treated cells or (brackets) cytokine alone. MLE-15, murine lung epithelial type II–like cell line.

Effects of reciprocal TNF receptors on the modulation of IL-1β–induced alterations in TNFR surface expression and shedding

To further characterize the mechanisms of IL-1β effects on individual TNF receptors, surface expression and/or shedding were assayed in MLE-15 cells transiently transfected with siRNA targeted against each TNF receptor. The percent reduction achieved is shown in Table 1. TNFR1 or TNFR2 suppression by siRNA did not alter constitutive surface abundance or shedding of the other receptor (Fig. 5). Compared to negative control, luciferase-siRNA transfected, and nontransfected, IL-1β–treated cells, suppression of TNFR1 significantly reduced the interleukin-mediated increase in surface and shed TNFR2 (Fig. 5A and B). In contrast, reduction of TNFR2 expression resulted in significantly increased IL-1β–induced surface and shed TNFR1 compared to nontransfected and luciferase-siRNA transfected, IL-1β–exposed cells (Fig. 5C and D). These studies demonstrate IL-1β–induced TNFR2 surface localization and shedding requires TNFR1 expression while suggesting that surface and shed TNFR1 may be counter-regulated by the presence of TNFR2.

IL-1β induces surface TNF receptors and TNF-α– induced surface TNFR2 is modified by IL-1β

As shown, exposure of MLE-15 cells to IL-1β for 24 h before TNF-α stimulation significantly enhanced chemokine production compared to TNF-α alone. Further experiments demonstrated TNFR1 to be instrumental in both MIP-2 and KC production while TNFR2 was sufficient for KC alone. However, although exposure to IL-1β for 6 h increased surface and shed TNFR2, TNFR1 had not changed. Therefore, effects of IL-1β on TNF receptors were analyzed at time points relevant to the combined IL-1β/TNF-α treatments used for the chemokine studies. As shown in Fig. 6A, (lane "IL-1 24") TNFR1 surface expression was increased by IL-1β at 24 h. These effects were subtle and transient, as analysis of TNFR1 after 30 h of treatment with IL-1β showed no difference compared to controls (lane "IL-1 30"). Similar to 6 and 24 h of exposure to IL-1β significantly increased surface and shed TNFR2 compared to control-treated cells (Fig. 6B, lane "IL-1 24" and data not shown). However, in contrast to TNFR1, IL-1β–induced surface TNFR2 was still significantly increased after 30 h of exposure, suggesting a rapid and sustained process (Fig. 6B, lane "IL-1 30"). These data show that
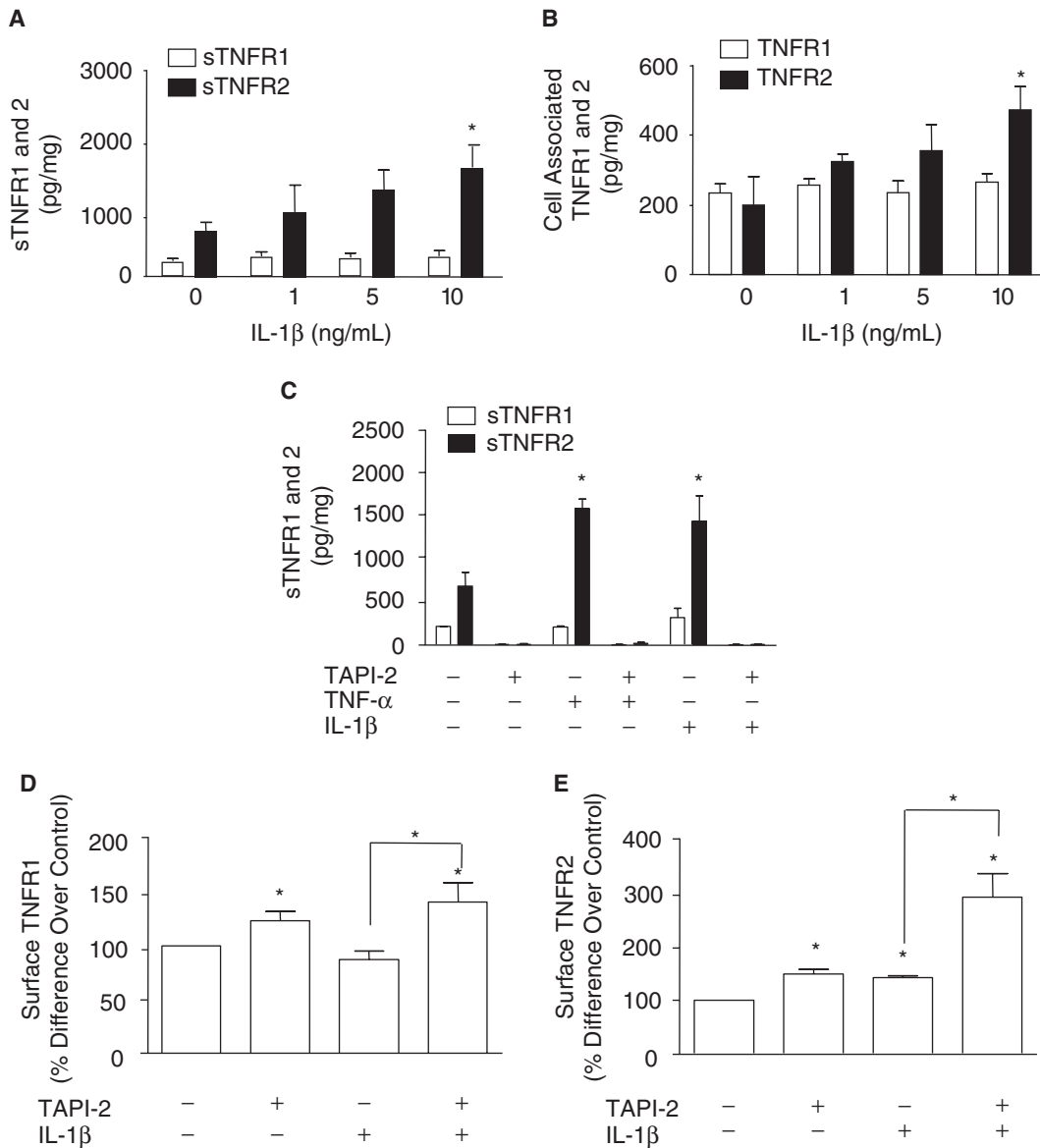
FIG. 4. Metalloprotease-dependent interleukin (IL)-1β–mediated shedding of tumor necrosis factor (TNF) receptors. MLE-15 cells were exposed to vehicle, or increasing concentrations (1, 5, or 10 ng/mL) of rmIL-1β. After 6 h, the (**A**) soluble and (**B**) cell-associated content of the 2 TNF receptors was determined as described in Materials and Methods. In (**C–E**), MLE-15 cells were incubated for 1 h with 100 µM TAPI-2 before exposure for 6 h to vehicle, 5 ng/mL rmTNF-α or 10 ng/mL rmIL-1β. Surface expression of (**C**) TNFR1 and (**D**) TNFR2 were determined by enzyme-linked immunosorbent assay (ELISA) on paraformaldehyde fixed, nonpermeabilized cells, while (**E**) soluble TNFR1 and TNFR2 levels were determined by sandwich ELISA. Surface expression is measured as percent difference compared to control. Means ± SEM, n = 3–4 independent experiments (2–4 wells/n). *P < 0.05 compared to control treated or (brackets) cytokine-alone treated cells. MLE-15, murine lung epithelial type II–like cell line.

IL-1β modestly upregulated surface expression of TNFR1 and TNFR2 over 24 h of incubation.

Effects of IL-1β pretreatment on TNF-α–mediated surface TNF receptor expression were also analyzed. Tumor necrosis factor-α treatment decreased surface TNFR1 regardless of pretreatment (Fig. 6A) while surface TNFR2 increased (Fig. 6B). Interestingly, 2 exposures to 5 ng per mL TNF-α (TNF 24/TNF 6) resulted in a significant decrease in surface TNFR1 compared to continuous exposure to TNF for 30 h (Fig. 6A). These data suggest pretreatment for 24 h to TNF-α resulted in sensitizing MLE-15 cells such that a second exposure further augmented the ligand-induced decrease in surface TNFR1. Continuous exposure to TNF-α may result in desensitization of the effects on TNF receptors such that TNFR1 expression recovers toward baseline while intermittent ligand allows resetting and potentially additive reduction in surface TNFR1. The same seems to be true for TNFR2 when exposed to multiple
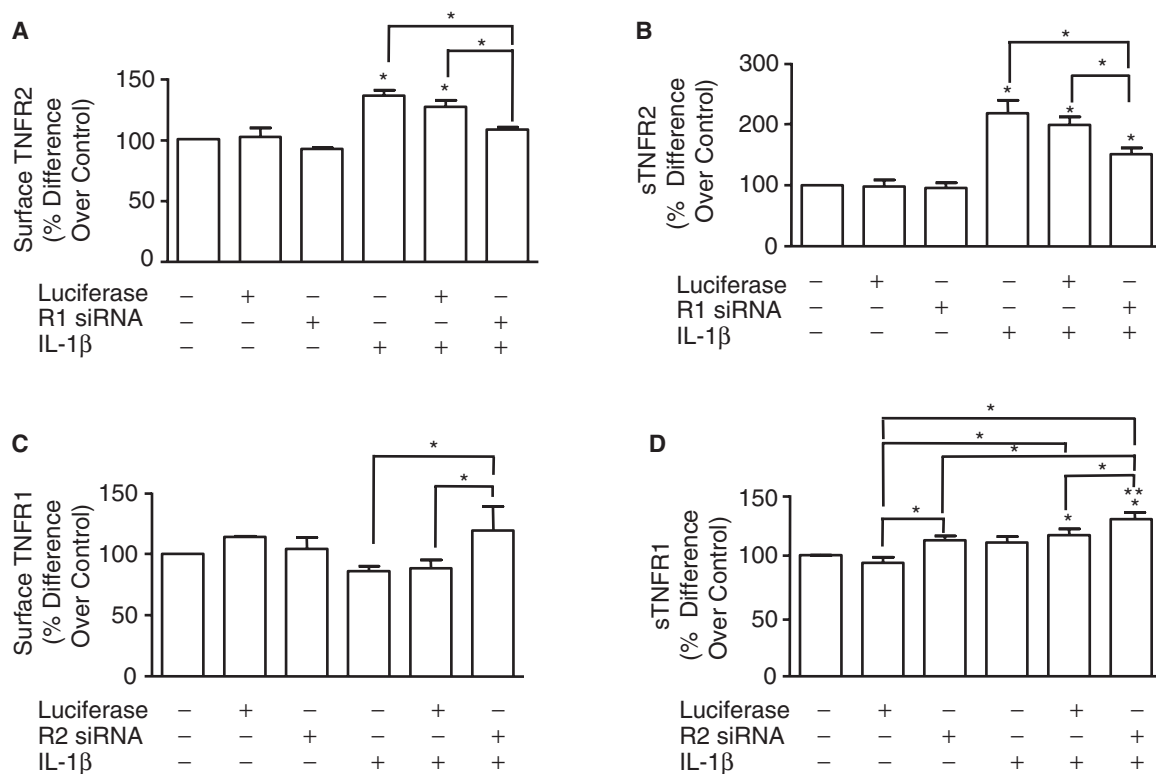
FIG. 5. Effects of small-interfering RNA (siRNA)-mediated receptor suppression on tumor necrosis factor (TNF) receptor membrane localization and shedding. MLE-15 cells were transfected with siRNA sequences targeted to each receptor or, as control, to luciferase as described in Materials and Methods. After treatment for 6 h with vehicle or 10 ng per mL rmIL-1β, media soluble receptor content and surface receptor expression were analyzed by sandwich enzyme-linked immunosorbent assay (ELISA) or fixed cell ELISA, respectively. (**A**) Surface TNFR2, (**B**) soluble TNFR2, (**C**) surface TNFR1, and (**D**) soluble TNFR1. Means ± SEM, $n = 3–4$ independent experiments (2–4 wells/n). *$P < 0.05$ compared to control-treated cells or (brackets) cytokine-treated cells and for (**D**) **$P < 0.05$ IL-1β alone versus TNFR2 siRNA + IL-1β. MLE-15, murine lung epithelial type II–like cell line.

versus continuous TNF-α. When MLE-15 cells were pre-treated with TNF-α, followed by a second 6 h TNF-α exposure, TNFR2 membrane expression returned to baseline, potentially contributing to the desensitization of the cells to TNF seen in Figure 2. Interestingly, pretreatment with IL-1β augmented both the TNF-α–mediated reduction in surface TNFR1 and increase in surface TNFR2 much as it augmented chemokine accumulation at this time (Fig. 6A and B).

Analysis of mRNA for TNFR1 and TNFR2 (Fig. 6C and D, respectively) by quantitative real-time PCR demonstrated that although TNFR1 surface levels decreased after exposure to TNF-α for 6 h, mRNA abundance of the receptor did not significantly change compared to control-treated cells or between individual treatment groups. Notably, prolonged TNF-α or IL-1β treatment induced TNFR1 mRNA. In contrast, similar to surface abundance, TNFR2 mRNA levels were significantly increased compared to control-treated cells after stimulation with TNF-α for 6 h. Two, or sustained, exposures to TNF-α significantly reduced TNFR2 mRNA concentration back to baseline compared to one 6 h exposure whereas TNFR1 mRNA remained unaltered. Interleukin-1β pretreatment did not significantly alter TNF-α–induced TNFR1 or TNFR2 mRNA compared to TNF-α alone. And

mRNA samples from double TNF receptor knockout mice were added as a negative control for mRNA analysis of the 2 receptors.

Discussion

Mouse alveolar epithelial type II–like cells (MLE-15), in which both TNF receptors are constitutively expressed and shed by metalloproteases, were utilized to show the inflammatory cytokine interleukin-1β (IL-1β)–mediated augmentation of TNF receptor presentation correlated with a significant increase in mRNA and protein expression of TNF-α–induced, TNFR1-mediated MIP-2 and KC, the latter potentially induced by both receptors. The IL-1β effects were TNF receptor-specific and dependent. TNFR2 release and plasma membrane abundance was rapidly increased after 6 h exposure to IL-1β, an effect dependent on TNFR1 expression as it was blocked by TNFR1 siRNA. Reduced expression of TNFR2 actually increased interleukin effects on surface TNFR1, significantly increasing membrane abundance and shed receptor levels compared to IL-1β alone or luciferase siRNA transfected, IL-1β–treated. These studies demonstrate IL-1β–induced TNF receptor modifications that are dependent on relative cellular receptor composition. Together
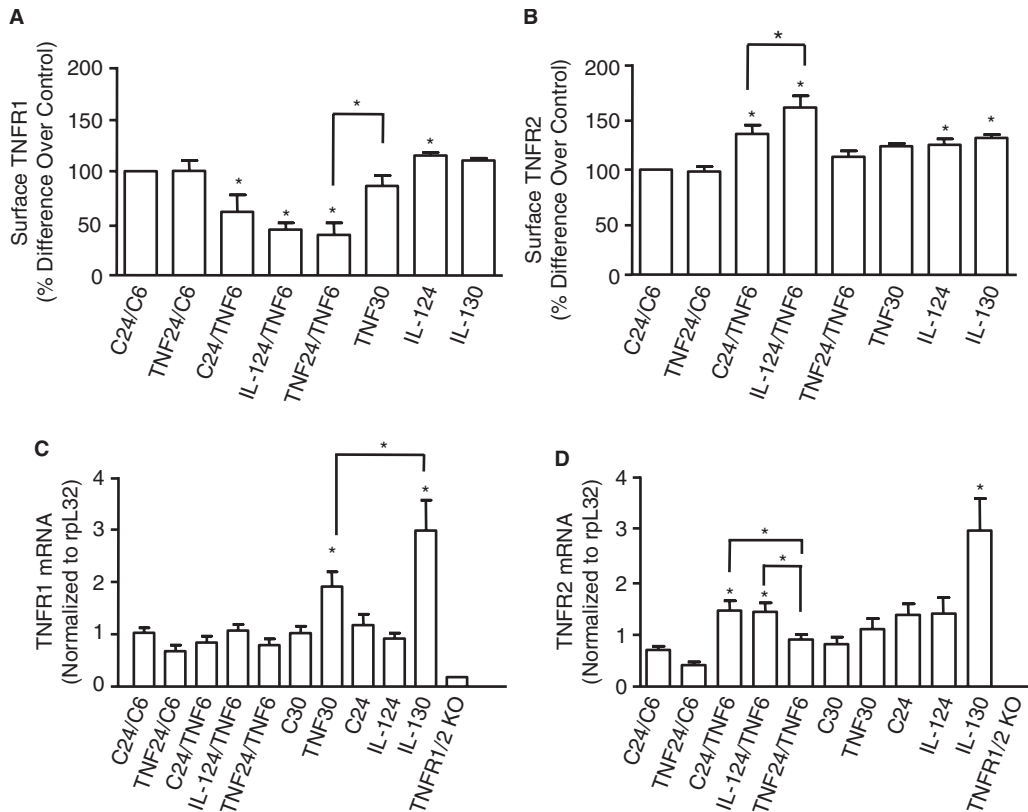
FIG. 6. Alterations in tumor necrosis factor (TNF) receptor cell surface expression dependent on duration of treatment and on prior exposure to interleukin (IL)-1β or TNF-α. MLE-15 cells were treated for 24 h with either vehicle, 5 ng per mL rmTNF-α or 10 ng per mL rmIL-1β. After this time, cells were washed and then treated with 5 ng per mL TNF-α or vehicle for an additional 6 h. Sets of wells were treated for a continuous 24 or 30 h with TNF-α or IL-1β (TNF24, TNF30, IL-124, IL-130). (**A**) Surface TNFR1 and (**B**) surface TNFR2 were then measured by fixed cell enzyme-linked immunosorbent assay (ELISA). (**C**) TNFR1 and (**D**) TNFR2 were measured by quantitative real-time polymerase chain reaction. For **A** and **B**, means ± SEM, $n = 3$ independent experiments (2–3 wells/n). For **C** and **D**, means ± SEM, $n = 2$–3 independent experiments (3–6 wells/n). *$P < 0.05$ compared to control-treated cells or (brackets) cytokine-treated cells. MLE-15, murine lung epithelial type II–like cell line.

these results suggest that during inflammatory conditions where both IL-1β and TNF-α are upregulated, lung epithelial cell chemokine production in response to acute TNF-α effects are enhanced by IL-1β activity.

Interleukin-1β also, however, appeared to augment TNF-α negative feedback on TNFR1 (Fig. 6). In the MLE-15 cells, TNF-α exposure suppresses cellular TNFR1 content (data not shown). Pretreatment with IL-1β reinforced this autoregulation, suggesting that while in the short-term IL-1β augments TNF-α–mediated induction of chemokines, in the longer term, IL-1β may contribute to control and downregulation of the TNF-α response. Thereby, the cytokines coordinately impact the development and resolution of lung inflammatory diseases.

Signaling through two distinct membrane-bound receptors (TNFR1 and TNFR2), past studies have shown that the majority of TNF-α biological responses can be attributed to TNFR1 while TNFR2 may enhance TNFR1 signaling through a process called ligand passing (Tartaglia and others 1993; Pan and others 1996). In addition, the two receptors share many intracellular signaling intermediates, including NF-κB, and thus together may enhance inflammatory

mediator production (Weiss and others 1998). In the current study; however, an siRNA-mediated reduction in TNFR2 augmented TNF-α induction of MIP-2 and of IL-1β induction of surface and shed TNFR1. Therefore, these data suggest that the cellular balance of the two receptors plays an important role in determining cell-specific responses to TNF-α and that the TNF receptor balance determines, and can be altered by, cellular response to another pleiotropic cytokine, IL-1β.

Previous studies have shown alveolar epithelial cells respond to inflammatory stimuli by increasing expression of chemokines such as MIP-2 and KC. These two potent neutrophil chemo-attractants belong to the C-X-C family of chemokines that are considered to be murine homologues of human IL-8. In addition, they share many functional properties with the human interleukin; both are rapidly upregulated and promote inflammation by recruiting and activating leukocytes (Driscoll 1994; Pryhuber and others 2003). Tumor necrosis factor-α, a key pleiotropic cytokine that is released after exposure to environmental toxicants and microbial pathogens, has been implicated in the upregulation of lung MIP-2 and KC *in vivo* as well as in isolated alveolar type II

cells and type II-like cell lines (Smith and others 1998; Weiss and others 1998; Calkins and others 2001; Wright and others 2004). Consistent with previous studies (Barrett and others 1998; Barrett and others 1999), exposure of MLE-15 cells to TNF-α led to increased MIP-2 and KC chemokine production. Our findings indicate endogenous TNFR1 in MLE-15 cells induces the expression of both MIP-2 and KC. These findings are consistent with previous observations in which bronchoalveolar lavage fluid-derived MIP-2 and neutrophils were decreased in TNFR1 KO mice exposed to pseudomonal endotoxin (Skerrett and others 1999). However, while TNFR2 aids in KC production the receptor also plays a negative role in regulating TNF-α–induced MIP-2. Macrophage inflammatory protein-2 might be expected to be enhanced in TNFR2 KO mice where TNFR1 remains intact, however this has not yet been reported.

Because exposure of MLE-15 cells to IL-1β increased inflammatory mediator production in response to TNF-α, TNF receptor surface localization and shedding after stimulation with the interleukin were studied. Exposure of MLE-15 cells to IL-1β resulted in a rapid increase in surface expression and release of TNFR2 at 6 h after exposure which was sustained at 24 and 30 h. These IL-1β–mediated TNFR2 effects were similar to those of TNF-α, which also rapidly increased and sustained surface and shed levels, as well as results seen in human gingival fibroblasts, where IL-1β selectively enhanced TNFR2 release without modifying TNFR1 (Fig. 6B) (data not shown) (Higuchi and Aggarwal 1994; Ohe and others 2000). However, in human airway epithelial cells, which did not spontaneously express TNFR2 (Shimomoto and others 1995; Levine and others 1996; data not shown), IL-1β induced TNFR1 release. In our model, surface and shed interleukin-mediated TNFR1 remained unchanged at the 6 h time point, although 24 h exposure to IL-1β resulted in increased surface, but not shed TNFR1. Indeed, only after suppression of TNFR2 expression by siRNA did IL-1β induce surface and shed TNFR1 suggesting the composition of cell receptors determines the interleukin effects on receptor surface expression and release.

Literature has shown TNF-α and the two receptors are cleaved by metalloproteases consistent with TACE and/or a regulator of TNFR1 shedding (ARTS-1) possibly to downregulate cell surface receptors and sensitivity to TNF-α (Reddy and others 2000; Cui and others 2002). However, past studies have shown independence in the regulation of shedding of the two receptors with the mechanisms behind the differences unclear. For instance, TNF-α induces TNFR1 internalization and activation of certain signaling events, or internalization and subsequent degradation while increasing solubilization of TNFR2 (Kull and Cuatrecasas 1981; (Smith and others 1990) Higuchi and Aggarwal 1994; Schneider-Brachert and others 2004). *In vivo*, it has been found that both receptors are shed constitutively and more markedly during disease states (Engelmann and others 1989; Pinckard and others 1997; Peschon and others 1998; Ziegenhagen and others 2000; Parsons and others 2005) although the mechanisms and functions of the solubilization remain unclear. *In vitro* experiments showed cells treated with elastase shed only TNFR2 but not TNFR1 (Porteu and others 1991) whereas experiments demonstrated exposure of various cell lines to the obligate intracellular human pathogenic bacterium

Chlamydia trachomatis resulted in enhanced TNFR1 release without TNFR2 modification, thereby downregulating TNFR1 immune responses and allowing for infection (Paland and others 2008).

In this study, the inhibitor TAPI-2 increased surface TNF receptor abundance indicating metalloprotease involvement in spontaneous receptor turn-over. Interestingly, addition of TAPI-2 in the presence of IL-1β resulted in an additive increase in surface TNFR2 expression indicating IL-1β upregulates surface TNFR2 independent of metalloprotease activity. In addition, although there were no changes in soluble TNFR1 levels after interleukin addition, inhibition of shedding by TAPI-2 resulted in a significant increase in IL-1β–induced plasma membrane TNFR1 abundance. This finding indicated that in addition to turn-over of surface TNFR1 by internalization and degradation (Higuchi and Aggarwal 1994; Levine and others 2005), metalloproteases also play a role in limiting spontaneous and cytokine-induced surface TNFR1. Interleukin-1β appears to increase TNFR1 production and/or translocation to the membrane but any tendency to increase TNFR1 concentration is off-set by metalloprotease-dependent receptor turn-over.

The metalloprotease TACE has also been shown to modulate shedding of TNF-α (Crowe and others 1995). Soluble TNF-α has been demonstrated to bind with higher affinity to TNFR1 whereas transmembrane (tm) TNF-α has higher affinity for TNFR2, therefore inhibition of shedding by TAPI-2 may lead to altered signaling through the two TNF receptors. In past studies, tmTNF-α has been demonstrated to induce cell death through "reverse signaling" while soluble TNF-α has been shown to induce proliferation and proinflammatory properties through "forward signaling" (Zhang and others 2008). As such, inhibition of TNF-α release could therefore potentially lead to ligand binding to membrane receptors via cell–cell contact or autotropic signaling and thus result in decreased MIP-2 and KC secretion while inducing cytotoxic effects.

Stimulation with IL-1β before TNF-α enhanced the latter's effects on surface TNF receptors (Fig. 6A and B). However, messenger RNA analysis of the two receptors showed the IL-1β–mediated changes in TNF-α–induced surface receptors did not correlate with alterations in mRNA levels, suggesting the cytokine's effects were post-transcriptional. In fact, only modification of TNFR2 by TNF-α was transcriptionally regulated whereas TNFR1 mRNA levels did not differ between treatment groups. This observation is consistent with TNFR2 expression being under an inducible promoter, allowing for rapid, sustained changes in abundance. In contrast, TNFR1 gene expression is under largely a noninducible, housekeeping promoter. This study also suggests that the reduction in surface expression seen after TNF-α exposure (Fig. 6A) was due to receptor internalization and downregulation and not altered transcription (Rothe and others 1993; Skerrett and others 1999; Cui and others 2002).

In conclusion, these results suggest that during inflammatory responses where TNF-α and IL-1β are rapidly upregulated, TNF-α–mediated inflammatory responses from epithelial cells may be at least transiently enhanced by IL-1β in association with, and potentially due to modification of TNF receptor expression and shedding. Thus the 2 cytokines interact to alter the pathogenesis of lung inflammatory diseases.

Acknowledgments

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