

# Interferon- $\gamma$ Drives the Metalloproteinase-Dependent Cleavage of HLA Class I Soluble Forms From Primary Human Bronchial Epithelial Cells

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**ABSTRACT:** Activation of bronchial epithelial cells (BEC) and disruption of an intact epithelial barrier in a lung transplant recipient can lead to acute or chronic rejection, events that are associated with release of soluble human leukocyte antigen (sHLA) class I. Although we know that HLA is released from mitogen-activated *lymphocytes* in a metalloproteinase (MPase)-dependent fashion, the mechanism of release from *nonlymphoid tissue* is not well understood. To this end, we stimulated primary BEC with increasing amounts of the T-helper cell-1 cytokines, interferon  $\gamma$  (IFN $\gamma$ ), and/or tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and measured the quantity and forms of HLA class I release. We found that IFN $\gamma$ , but not TNF $\alpha$ , was able to stimulate a time- and concentration-dependent release of HLA/ $\beta_2$ m and  $\beta_2$ m-free heavy chain (HC) from the BEC. A portion (50%) of the HLA/ $\beta_2$ m release and

>90% of the  $\beta_2$ m-free HC release was mediated by a MPase. Western blot analysis supported the conclusion that a MPase-sensitive pathway produced 36 and 37 kDa cleaved forms, whereas the secreted 39 kDa form of  $\beta_2$ m-associated soluble HLA class I (sHLA/ $\beta_2$ m) was MPase-resistant. This adds to the growing understanding of the extracellular processing pathways of major histocompatibility complex class I that may be critical for both chronic rejection as well as immune regulation. *Human Immunology* 63, 893–901 (2002). © American Society for Histocompatibility and Immunogenetics, 2002. Published by Elsevier Science Inc.

**KEYWORDS:** bronchial epithelial cells; HLA; interferon  $\gamma$ ; metalloproteinase

## ABBREVIATIONS

APC	antigen presenting cell	IFN $\gamma$	interferon $\gamma$
BAL	bronchoalveolar lavage	kDa	kilodalton
BEC	bronchial epithelial cells	MPase	metalloproteinase
$\beta_2$ m	$\beta_2$ microglobulin	sHLA/ $\beta_2$ m	$\beta_2$ m-associated soluble HLA class I
ELISA	enzyme-linked immunosorbent assay	TCS	tissue culture supernatant
HC	heavy chain	TNF $\alpha$	tumor necrosis factor $\alpha$
HLA	human leukocyte antigen		

## INTRODUCTION

Human leukocyte antigen (HLA) class I proteins are constitutively expressed on the cellular membrane of all nucleated cells as a complex consisting of a polymorphic heavy chain (HC-44 kDa), a monomorphic light chain

( $\beta_2$  microglobulin,  $\beta_2$ m-12 kDa), and a 9 amino acid peptide. Soluble HLA proteins are thought to be generated by either membrane shedding (resulting in an intact 44 kDa HC), alternate splicing of the messenger RNA to excise the transmembrane domain (resulting in a 38–39 kDa HC), or metalloproteinase (MPase) cleavage (resulting in a 34–36 kDa HC). The susceptibility to MPase cleavage depends upon dissociation of the  $\beta_2$ m [1] and has been found to generate both peptide-conformed and nonconformed  $\beta_2$ m-free HC. Only the former can rapidly reassociate with  $\beta_2$ m to generate  $\beta_2$ m-associated soluble HLA class I (sHLA/ $\beta_2$ m) [2, 3].

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Stimulation with interferon  $\gamma$  (IFN $\gamma$ ), even for a short time, increases the cell-surface expression of HLA class I [4]. This upregulation resulted from effects on specific promoters of the HLA class I gene [5] and by increased expression of the transporter for antigen processing 1 and 2 [6], as well as the large multifunctional proteasome components 2 and 7, which cleave intracellular peptides for major histocompatibility complex I binding [7]. In contrast, the effects of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) on the surface expression of HLA are not uniform. In cultured airway epithelial cells (BET-1A), only IFN $\gamma$ , and not TNF $\alpha$ , was able to stimulate increased HLA expression [8], whereas in some cell types, like endothelial cells, TNF $\alpha$  acts synergistically with IFN $\gamma$  to upregulate the HLA class I expression [9]. Interestingly, in human melanoma cells, TNF $\alpha$  was shown to not only upregulate, but also stabilize the cell surface expression of HLA class I [10].

*In vitro*, both IFN $\gamma$  and TNF $\alpha$  have been found to drive the production of soluble HLA in a variety of cell types. He *et al.* [11] have illustrated that IFN $\gamma$  treatment of fibroblasts results in the release of a 38 kDa HLA HC, which they believe is the product of alternative mRNA splicing. *In vivo*, systemic treatment with IFN $\gamma$  upregulates the plasma level of soluble HLA [12].

In transplant recipients, we have found soluble HLA class I (sHLA) of donor origin in the sera of heart and kidney transplant recipients during infection and rejection, but not during allograft quiescence [13, 14]. In contrast, the donor lung constitutively releases sHLA into both the airway (detected in the bronchoalveolar lavage fluid [15]) and into the vasculature (detected in the sera [16]). The baseline serum level of sHLA is intermediate between that of the liver (high) and heart or kidney (low) [16]. Similar to all other solid organ transplants, the level of soluble donor antigen in the sera increases when the lung allograft experiences rejection and/or infection [16].

Acute rejection of the lung, which involves the recognition of donor alloantigen by host T cells, as well as ischemic damage and reperfusion injury, are associated with the release of inflammatory cytokines TNF $\alpha$  and IFN $\gamma$  [17–19]. Given the impact of these cytokines on the regulation of HLA expression in parenchymal cells, we hypothesized that they could also activate the MPase, cleaving  $\beta_2$ m-free HC in lung cells. This could account for the increased release of sHLA during allograft rejection and for the spike of donor sHLA immediately after organ transplantation [14].

In lung allografts not undergoing acute rejection, the numbers of donor-derived leukocytes in the bronchoalveolar lavage fluid (BAL) may remain quite high: up to 60% of BAL-derived cells at 6 months post-transplant [20]. However, most donor leukocytes are rapidly re-

placed by host-type cells in cases of poor outcome [20]; yet these grafts release the largest amounts of donor-type HLA in the BAL and serum [15, 16]. During the rejection process, lymphocytes often infiltrate the allograft, but these are predominantly of recipient and not donor origin and therefore would not contribute to the increased pool of detected donor sHLA in the BAL and serum [15]. We speculated that the lung epithelium might be one of the sources of donor HLA class I proteins. To investigate this possibility, we stimulated cultured primary bronchial epithelial cells (BEC) with the inflammatory cytokines TNF $\alpha$  and IFN $\gamma$  and then measured the level and molecular weight forms of released HLA.

## MATERIALS AND METHODS

All reagents were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) unless otherwise noted.

### Isolation, Culture and Stimulation of Bronchial Epithelial Cells

Epithelial cells were derived from donor lung transplant surgical specimens and isolated by a modification of the method described by Schroth *et al.* [21] and Wu [22] *et al.* Primary BEC were grown to confluence on collagen VI-coated (Sigma C7521) tissue culture flasks or plates in supplemented BEGM (CC-3170, BEBM with 52  $\mu$ g/ml bovine pituitary extract, 0.5 ng/ml human recombinant epidermal growth factor, 0.5  $\mu$ g/ml hydrocortisone, 0.5  $\mu$ g/ml epinephrine, 10  $\mu$ g/ml transferrin, 5  $\mu$ g/ml insulin, 0.1 ng/ml retinoic acid, 6.5 ng/ml triiodothyronine, 50  $\mu$ g/ml gentamicin, and 50 ng/ml amphotericin-B, Biowhittaker, [Walkersville, Maryland, USA]). For stimulation assays, BEGM on confluent BEC in passage 2–4 was replaced with BEGM-4 (media without bovine pituitary extract, human recombinant epidermal growth factor, epinephrine, and hydrocortisone) for 24 hours. This media was removed and BEGM-4 was added with stimulators IFN $\gamma$  (Endogen, Woburn, Massachusetts, USA), TNF $\alpha$  (Promega Corporation, Madison, Wisconsin, USA), and/or the metalloproteinase inhibitor BB-94 (Batimastat, a kind gift of British Biotech Pharmaceuticals Limited, Oxford, United Kingdom) and cells were incubated for 1–4 days. For some experiments, the spent media was recovered daily and replaced with fresh lymphokine containing media.

### Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) for sHLA/ $\beta_2$ m and  $\beta_2$ m-free HC were performed as described previously [23]. Briefly, to detect sHLA/ $\beta_2$ m, mAb TP25-99 (which recognizes both  $\beta_2$ m-free and associated HLA [24], a kind gift of SangStat Medical Corporation,

Menlo Park, California, USA) was used as the capture antibody and rabbit antihuman  $\beta_2m$  (Accurate Chemical & Scientific Co., Westbury, New York, USA) and horseradish peroxidase coupled goat antirabbit IgG were used as the detection antibodies. To detect  $\beta_2m$ -free HC, HC-10 (which recognizes  $\beta_2m$ -free HC [25], a kind gift of H. Ploegh, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA) was used as the capture antibody and biotin-coupled TP25-99 and ExtrAvidin-HRP were used as the detection Abs. ELISA to detect IL-8 was performed with antibodies from R & D systems (Minneapolis, Minnesota, USA) following the recommended protocol. A tetramethylbenzidine (TMB) microplate substrate and TMB stop solution (KPL Laboratories, Gaithersburg, Maryland, USA) were used in all ELISAs. The plates were read at 450 nm with Bio-Tek ELx800 reader (Bio-Tek Instruments, Inc., Winooski, Vermont, USA). Data were analyzed using the KC3 software (Bio-Tek Instruments, Inc.).

#### Flow Cytometry Analysis

To determine the surface expression of HLA, HLA-B7 expressing BEC cells were cultured for 24 h with IFN $\gamma$  and/or TNF $\alpha$  as above. Tissue culture supernatants were removed and cells were washed with phosphate-buffered saline (10 mM phosphate, 150 mM sodium chloride, pH 7.2). They were then detached during a 5-minute incubation with trypsin (0.25%)/ethylenediaminetetraacetic acid (1 mM). The trypsin was neutralized by washing the cells in 5 mls of RPMI 1640 (Biowhittaker) supplemented with 10% fetal bovine sera (HyClone Laboratories, Inc., Logan, Utah, USA). Cells were washed with wash buffer (Hank's balanced salt solution with 5mM HEPES and 0.1% bovine serum albumin, pH 7.4) and then incubated on ice with biotin-labeled anti-HLA-B7 mAb ME-1 (ATCC, [26]). After 30 minutes, cells were washed with wash buffer and incubated with StreptAvidin-allophycocyanin (Pharmingin-Beckton-Dickinson, San Jose, California, USA) for 25 minutes. Cells were finally washed with wash buffer and resuspended in wash buffer with 5  $\mu$ g/ml propidium iodide to discern live from dead cells. Mean fluorescence intensity of live cells were determined with a Calibur flow cytometer (Becton-Dickinson, San Jose, California, USA) and data was analyzed using CellQuest software (Becton-Dickinson).

#### Immunoprecipitation and Western Blot

Five milliliters of tissue culture supernatants (recovered on day 4) or plasma samples (4 ml) were immunoprecipitated overnight with either mAb HC-10 or w6/32 (which only recognized HLA/ $\beta_2m$ ) Sepharose-coupled beads. Bound proteins were separated on a 12% discontinuous polyacrylamide gel under reducing conditions following modifications of the Laemmli method [27] and

transferred onto 0.2  $\mu$ m nitrocellulose membranes (Bio-Rad Laboratories, Richmond, California, USA). Blots were blocked overnight at 37°C with 2% bovine serum albumin in phosphate-buffered saline and then incubated with the mixture of rabbit anti-HLA HC (a kind gift of H. Ploegh) and rabbit antihuman  $\beta_2m$  antibodies followed by incubation with horse-radish peroxidase-conjugated goat antirabbit antibodies. Blots were washed extensively between each of the incubation steps. Finally, TMB membrane substrate (KPL Laboratories) was added for 5–10 minutes. The blots were rinsed with dH<sub>2</sub>O and immediately scanned (SAPHIR, LinoType-Hell, Germany).

## RESULTS

### IFN $\gamma$ Induces the Release of HLA From Primary Bronchial Epithelial Cells

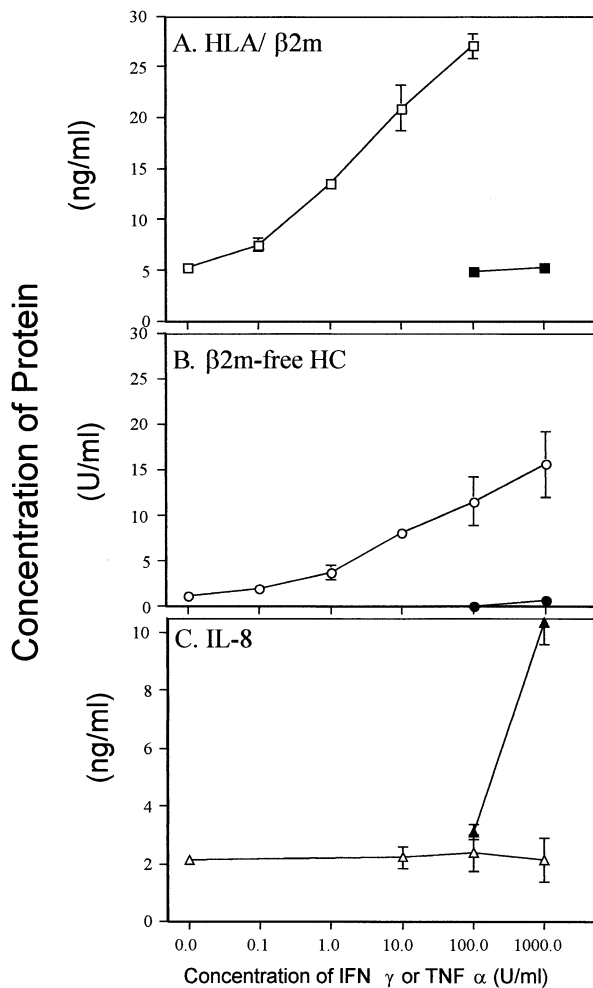
IFN $\gamma$  and TNF $\alpha$  are activating cytokines for BEC, inducing the release of a variety of chemokines including RANTES, IL-8, and fractalkine [28–30]. We first investigated whether these same cytokines could cause HLA class I release from cultures of primary BEC.

As illustrated in Figure 1, panels A and B, IFN $\gamma$  stimulates the cells to release both sHLA/ $\beta_2m$  and  $\beta_2m$ -free HC in a concentration dependent manner, beginning with as little as 0.1 U/ml IFN $\gamma$ . TNF $\alpha$  did not have the same effect on the release of HLA; there was no additional release of HLA over that seen in the resting media alone, even with 1000 U/ml of TNF $\alpha$ . In contrast, 1000 U/ml of TNF $\alpha$ , but not IFN $\gamma$ , induced a concentration-dependent release of IL-8 from the same cells (panel C).

Although TNF $\alpha$  did not affect the release of HLA from the BEC, we wanted to determine if incubation with TNF $\alpha$  resulted in an upregulation of the surface expression of HLA. BEC were cultured for 24 hours with TNF $\alpha$  or IFN $\gamma$ , washed, removed from the wells, and then tested with flow cytometry for expression of HLA-B7. As illustrated in Figure 2, 1000 U/ml of TNF $\alpha$  only modestly increased the surface expression of HLA-B7 over that in cultures without any stimulation, whereas IFN $\gamma$  at 10 U/ml, and to a greater extent 1000 U/ml, led to a dramatic increase in the surface expression of HLA.

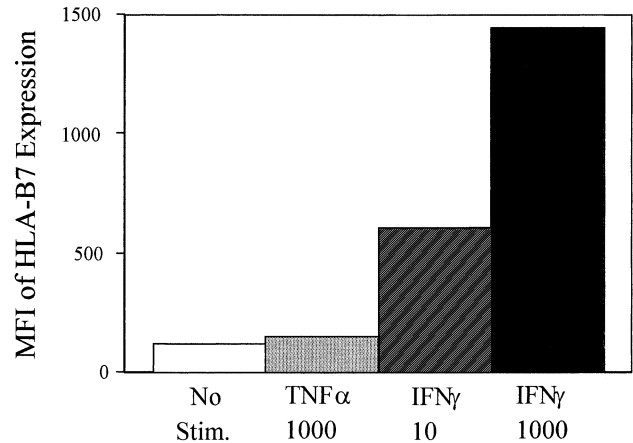
### Lack of an Additive Effect by TNF $\alpha$ on IFN $\gamma$ -Induced Release of sHLA

Although TNF $\alpha$  did not stimulate the release of sHLA from BEC per se, the combination of TNF $\alpha$  and IFN $\gamma$  have been demonstrated to be synergistic stimulants for upregulation of endothelial cell surface expression of HLA [9], as well as epithelial cell expression of a chemokine known as fractalkine (CXCL3) [31]. Therefore, we cultured BEC in media containing both IFN $\gamma$  and TNF $\alpha$  and determined the effect on the release of sHLA



**FIGURE 1** Interferon  $\gamma$  (IFN- $\gamma$ ) stimulates the release of human leukocyte antigen (HLA) from primary bronchial epithelial cells (BEC). BEC were cultured in the presence of increasing concentrations of IFN $\gamma$  (open symbols) or tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) (filled symbols). After 24 h, the expired culture media was removed and replaced with fresh media containing the same concentrations of stimulants. Tissue culture supernatants (TCS) were collected 24 h later from 2–4 individual cultures per test condition and the concentration of  $\beta_2$ m-associated soluble HLA class I (sHLA/ $\beta_2$ m) (A) or  $\beta_2$ m-free heavy chain (HC) (B) was determined by enzyme-linked immunosorbent assay (ELISA). To detect released IL-8 (C) tissue culture supernatant TCS was not changed, but was collected after 4 days of culture.

over that with IFN $\gamma$  alone. Each data line in Figure 3 represents the release of sHLA from cells cultured with a given concentration of TNF $\alpha$  (0, 100, or 1000 U/ml), but increasing concentrations of IFN $\gamma$  (0.01–1000 U/ml, x axis). As shown, there was no increase in the release of sHLA/ $\beta_2$ m when BEC were incubated with both IFN $\gamma$  and TNF $\alpha$  (filled symbols) as compared to IFN $\gamma$  alone (open symbols). There was, however, an  $\sim 33\%$  increase

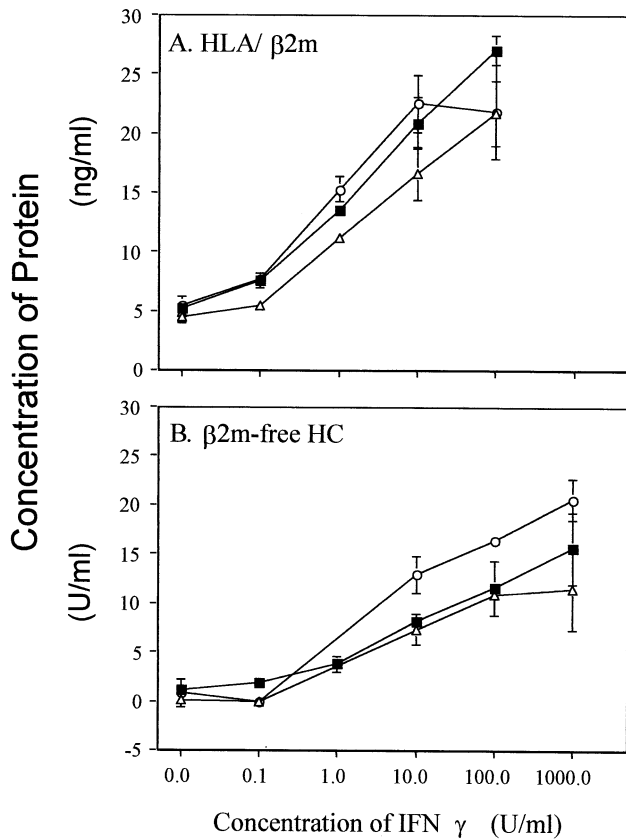


**FIGURE 2** Interferon  $\gamma$  (IFN $\gamma$ ), but not tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) upregulate the surface expression of human leukocyte antigen (HLA). Bronchial epithelial cells (BEC) were cultured for 24 hours without stimulators (open bar), with TNF $\alpha$  (1000 U/ml, gray bar), with IFN $\gamma$  at 10 U/ml (stripped bar), or with 1000 U/ml (filled bar) as described in methods. Cells were removed, washed, and incubated with biotin-labeled anti-HLA-B7 (ME-1) followed by SteptAvidin allophycocyanin. Mean fluorescence intensity (MFI) of live cells was determined by flow cytometry.

in the release of  $\beta_2$ m-free HC (panel B) at the highest dose (1000 U/ml) of added TNF $\alpha$ . In addition, there was no increase in the surface expression of HLA-B7 when the BEC were incubated with both cytokines together (data not shown).

#### Time Course of IFN $\gamma$ -Induced Release of sHLA From Primary BEC

Because the cellular response to cytokines often waxes and wanes over time, we wanted to determine the daily release of sHLA in response to various concentrations of IFN $\gamma$ . BEC cultured in 24-well plates were rested in BEGM-4 for 24 h as above and then incubated with fresh media containing IFN $\gamma$ , changing the media daily. Spent culture supernatants collected after each 24-hour period were tested in ELISA for sHLA/ $\beta_2$ m and  $\beta_2$ m-free HC. As illustrated in Figure 4, BEC without stimulation continued to release  $\sim 5$  ng/ml of sHLA/ $\beta_2$ m (panel A) and 1 U/ml of  $\beta_2$ m-free HC (panel B) throughout the test period (4 days). Cells stimulated with  $\geq 0.1$  U/ml IFN $\gamma$  manifested daily increases in release of sHLA/ $\beta_2$ m and  $\beta_2$ m-free HC. The most profound increases in the release of sHLA were observed with the higher doses of IFN $\gamma$ . Therefore, if cells were stimulated to release sHLA in response to IFN $\gamma$  (concentrations  $\geq 0.1$  U/ml), the release of sHLA increased daily, did not wane over the 4-day test period, and did not attain a steady-state release as seen without IFN $\gamma$  stimulation.

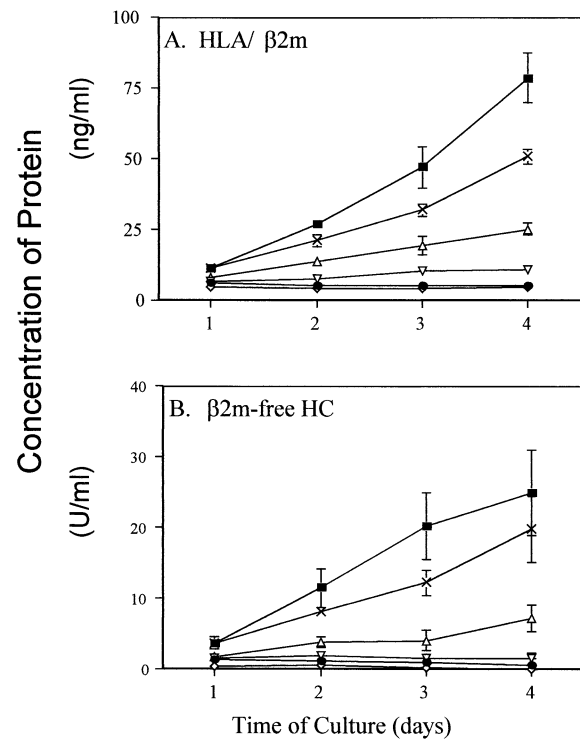


**FIGURE 3** Co-incubation of bronchial epithelial cells (BEC) with tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and interferon  $\gamma$  (IFN $\gamma$ ). BEC were cultured with increasing concentrations of IFN $\gamma$  (0–1000 U/ml, x axis) as described in Figure 1, either without TNF $\alpha$  (filled symbols) or in the presence of both IFN $\gamma$  and TNF $\alpha$  (100 U/ml = open triangles; 1000 U/ml = open circles). Data represents 2–4 independent cultures at each condition tested.

#### Role of the MPase in HLA Class I Release From BEC

A MPase cleaves  $\beta_2$ m-free membrane-bound HLA class HC [1] releasing a 36–37 kDa molecule that can reassociate with  $\beta_2$ m if the HC retains its peptide and conformation [2, 3] (*i.e.*, sHLA/ $\beta_2$ m) or otherwise, if the HC has released its peptide it remains  $\beta_2$ m-free ( $\beta_2$ m-free HC). We have previously illustrated that the MPase cleavage pathway accounts for the majority of sHLA/ $\beta_2$ m (conformed 36 kDa) and  $\beta_2$ m-free HC released from lymphocytes after stimulation with pokeweed mitogen [23].

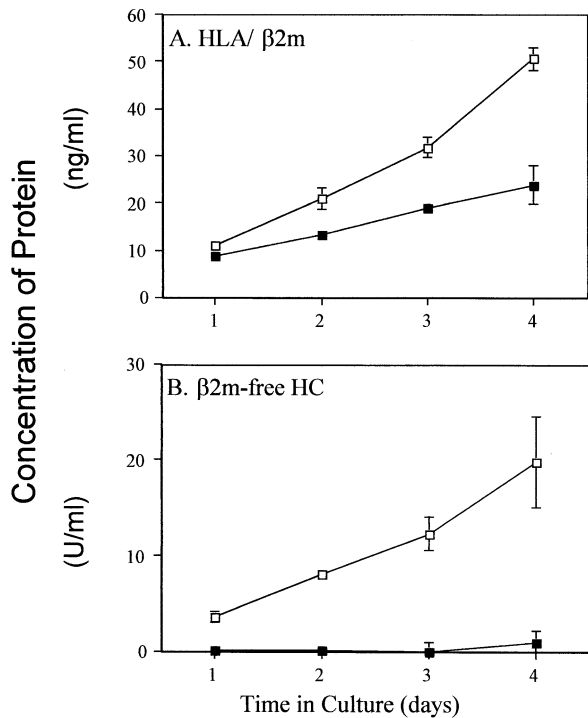
To determine if the metalloproteinase pathway is involved in the release of HLA from BEC stimulated with IFN $\gamma$ , we incubated cells with increasing concentrations of IFN $\gamma$  as above, but added an inhibitor of metalloproteinases, BB-94 (Batimastat, 10  $\mu$ M). As indicated in Figure 5, the addition of BB-94 inhibited 20%–60% of



**FIGURE 4** Release of human leukocyte antigen (HLA) increases through at least day 4 of culture. Cells were stimulated with increasing concentrations of interferon  $\gamma$  (IFN $\gamma$ ) (● = 0 U/ml; ◇ = 0.01 U/ml; ▽ = 0.1 U/ml; △ = 1 U/ml; × = 10 U/ml; ■ = 100 U/ml), tissue culture supernatants (TCS) were collected every 24 hours and fresh media with IFN $\gamma$  was added. The concentration of  $\beta_2$ m-associated soluble HLA class I (sHLA/ $\beta_2$ m) (A) or  $\beta_2$ m-free heavy chain (HC) (B) in the TCS was tested in enzyme-linked immunosorbent assay (ELISA). Data represents four independent cultures at each condition tested.

the release of sHLA/ $\beta_2$ m and all of the release of  $\beta_2$ m-free HC.

To further prove that the released HLA was proteolytically derived, we did immunoprecipitations of the TCS with antibodies against the sHLA/ $\beta_2$ m (mAb w6/32, which recognizes a conformational epitope formed by  $\beta_2$ m bound to HC [32]) or against the  $\beta_2$ m-free HC (mAb HC-10, which recognizes only  $\beta_2$ m-free HC [25]). Cells were stimulated with 10 U/ml IFN $\gamma$  for 4 days without changing the media to generate sufficient quantities of sHLA for analysis. Precipitated proteins were separated on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blotted with antibodies against the HLA-HC and  $\beta_2$ m. The majority of the  $\beta_2$ m-free HC precipitated with mAb HC-10 from the cultures stimulated with IFN $\gamma$  migrated as a doublet with an apparent molecular weight of 36–37 kDa (Figure 6, left panel). Confirming the ELISA results, we found that both bands were absent in



**FIGURE 5** The metalloproteinase pathway is involved in the release of human leukocyte antigen (HLA) from bronchial epithelial cells (BEC). Cells were cultured with 10 U/ml of interferon  $\gamma$  (IFN $\gamma$ ) alone (open symbols) or with IFN $\gamma$  plus 10  $\mu$ M of BB-94 (filled symbols). As in Figure 4, the media was changed every 24 h and the concentration of  $\beta_2$ -associated soluble HLA class I (sHLA/ $\beta_2m$ ) (A) or  $\beta_2m$ -free heavy chain (HC) (B) in the tissue culture supernatant (TCS) was tested in enzyme-linked immunosorbent assay (ELISA). Data represents four independent cultures at each condition tested.

the TCS from BEC stimulated with IFN $\gamma$  in the presence of BB-94. Interestingly, unlike the proteins which are precipitated from human plasma, there was no intact, 44 kDa, HLA-HC precipitated from any of the cultures tested. In Figure 6, right panel, we observed 39 kDa conformed sHLA/ $\beta_2m$  proteins representing alternately spliced products and, as expected, the intensity of this band was not affected by the presence of BB-94. In contrast, the 36–37 kDa doublet band of peptide-conformed (based on the w6/32 mAb reactivity) sHLA/ $\beta_2m$  was almost completely suppressed by the MPase inhibitor, consistent with the partial inhibition of total sHLA/ $\beta_2m$ -release measured by ELISA in BEC cultures treated with BB-94.

## DISCUSSION

We illustrate here that soluble HLA is actively released from primary bronchial epithelial cells and that at least one of the primary mechanisms of release is the same as

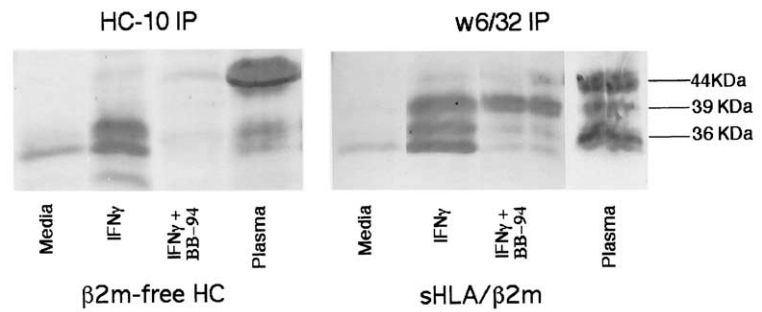
that for human leukocytes, namely, the metalloproteinase pathway. We also illustrate that IFN $\gamma$  is a potent stimulator of this release, but that another inflammatory cytokine, TNF $\alpha$ , which induces the BEC to release IL-8, has little or no effect on the release of sHLA class I.

The differential effect of IFN $\gamma$  versus TNF $\alpha$  on sHLA release raises questions about the mechanism of release. It is clear from our data that metalloproteinases are involved here (Figures 5 and 6) and previous studies have demonstrated that  $\beta_2m$  must dissociate from the HLA class I HC to allow for MPase-dependent release of sHLA [1]. Therefore, the increased MPase-dependent release of sHLA may simply be due to the IFN $\gamma$ -induced upregulation of the surface expression of the noncleavable  $\beta_2m$ -associated HLA-HC (Figure 2) followed subsequently by passive dissociation of  $\beta_2m$  from the HLA class I HC. Alternatively, IFN $\gamma$  might also affect the MPase expression or activity. Interestingly, TNF $\alpha$  and IFN $\gamma$  appear to have opposite effects on the expression of matrix metalloproteinase-9 in BEC [33], with IFN $\gamma$  inhibiting the expression. A more relevant recent finding is that the cell-membrane-bound metalloproteinase, ADAM17, originally identified as the enzyme that cleaves a cell-bound TNF $\alpha$  precursor to release the mature soluble form [34], can cleave the chemokine fractalkine [35]. However, unlike sHLA class I, where IFN $\gamma$  was the primary stimulator of release and where only a modest increase in the release of  $\beta_2m$ -free HC was detected when TNF $\alpha$  was included with the IFN $\gamma$ , fractalkine release is driven by TNF $\alpha$  and the combination of IFN $\gamma$  and TNF $\alpha$  have a synergistic effect on its surface expression and release [31]. Therefore, the stimulatory effect of IFN $\gamma$  on the release of sHLA class I may involve other MPases besides ADAM-17.

Does this Th1-cytokine-driven release of sHLA/ $\beta_2m$  and  $\beta_2m$ -free HC from BEC have a positive or negative impact for the lung allograft recipient? The answer most likely depends on the following: first, the nature of the peptides that are bound by the sHLA, especially those that remain bound to the conformed  $\beta_2m$ -free HC during MPase cleavage, second, the nature of the antigen presenting cell (APC) that will present these peptides, third, the nature of the class I mismatches between donor and recipient and hence potential allopeptides that may be derived from the  $\beta_2m$ -free HC for presentation to "indirect pathway" T cells, and fourth, the proportion of effector to regulatory antigen-specific T cells, which will be stimulated by the peptide-pulsed APC.

It is clear that release of HLA is a routine *in vivo* function. These proteins are continuously produced and their production is dramatically upregulated under conditions of inflammation. Why? The upregulated surface expression makes sense in light of an infection, but why would a cell spend energy when activated to upregulate

**FIGURE 6** Western blot analysis of human leukocyte antigen heavy chain (HLA-HC) supports the role of a metalloproteinase in the release of HLA from bronchial epithelial cells (BEC). Cells were stimulated with 10 U/ml of interferon  $\gamma$  (IFN $\gamma$ ) alone or with IFN $\gamma$  plus 10  $\mu$ M BB-94 for 4 days without changing the media. Soluble HLA in the tissue culture supernatant (TCS) was precipitated with either HC-10 (to precipitate  $\beta_2$ m-free HC [left panel]) or with w6/32 (to precipitate sHLA/ $\beta_2$ m [right panel]). Human plasma from a nontransplanted donor was used as the positive control for HLA bands.



the release of a protein which is not a chemokine? One proposed explanation would be to deliver high affinity immunodominant peptides to lymph node APC via cross-priming. This idea presupposes that sHLA/ $\beta_2$ m 36 kD forms are ones with high affinity peptides, which remain conformed even after  $\beta_2$ m dissociates. Once the HC/peptide is cleaved by the MPase, it reassociates with  $\beta_2$ m outside of the cell [1, 3]. These “high affinity” peptide charged sHLA/ $\beta_2$ m could then be picked up by dendritic cells, stripped of the high affinity peptides which are then represented in lymph nodes and spleen to T cells, amplifying the immune response against the original pathogen. In the case of an allograft with similar HLA-I, the MPase cleavage pathway would also function in such a “cross-priming” fashion to propagate dissemination of high-affinity minor H peptides for response by host T cells. However, if the HLA-I proteins are mismatched with those of the recipient, propagation of the peptide-conformed HLA-I and nonconformed  $\beta_2$ -free HC may elicit either alloimmunity or allotolerance to peptides derived from the donor HLA-HC, depending on the balance between effector and regulatory T-cell responses. The uptake of donor-type  $\beta_2$ m-free HC by host APC might trigger an indirect response to HLA-I allopeptides presented on host class II molecules to CD4<sup>+</sup> T cells. Potentially the same process of HLA cleavage from autologous cells and uptake by APC could expose previously cryptic self-HLA peptides to self-reactive T cells, thus evoking an autoimmune response [36], which could have a negative impact on the allograft.

It is of interest to compare the above “indirect pathway” scenario with the direct interaction of peptide-conformed sHLA class I multimers with CD8<sup>+</sup> T cells causing allo-specific or peptide-specific activation induced cell death, either through interaction with the T cell receptor [37, 38] or with the CD8 molecule [39]. These effects of sHLA-I on CD8<sup>+</sup> T cells require relatively high doses of sHLA protein (0.5–4  $\mu$ g/ml). In contrast, as little as 0.5 ng/50  $\mu$ l (10 ng/ml) of donor HLA-B proteins has been found to trigger indirect pathway regulation of delayed-type sensitivity (Jankowska-Gan, *et al.*, this issue). In the former type of regulation,

activated (but not resting) direct pathway CD8<sup>+</sup> T cells undergo apoptosis after encountering sufficiently high levels of the intact sHLA/peptide/ $\beta_2$ m complex in monomer or multimer form. In the latter type of regulation, sHLA-A,B proteins are being processed to allopeptides by APCs, which induce both T regulator cells and effector cells, the former causing dominant suppression of the latter. Such regulator T cells can also cause bystander or linked suppression of responses to potent recall antigens like tetanus toxoid or Epstein–Barr virus [40]. This immunoregulation appears to be driven by transforming growth factor  $\beta$  and/or interleukin-10 in that blocking these cytokines abrogates the regulation [40].

In conclusion, we have found that bronchial epithelial cells can easily be stimulated by the Th1-cytokine IFN $\gamma$  to release high levels of sHLA largely by a MPase-cleavage pathway. The effect of these proteins in transplant recipients may, in certain instances, be detrimental to allograft survival or, alternatively, may feed a regulatory pathway in the recipient immune system leading to allograft acceptance.

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