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Cellular distribution of proteasome subunit *Lmp7* mRNA and protein in human placentas

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SUMMARY

Human leucocyte antigen (HLA) class I antigen expression is closely controlled in placental trophoblast cells, which interface directly with genetically disparate maternal blood and tissues during pregnancy. In this study, the possibility that LMP7, a proteasome component that may be required for processing of class I-associated peptides, might be lacking or refractory to cytokine induction in trophoblast cells that fail to display HLA class I antigens was investigated. Analysis of *Lmp7* mRNA and protein in paraformaldehyde-fixed placentas by *in situ* hybridization and immunohistochemistry revealed that both HLA class I-positive and HLA class I-negative trophoblast cells contain *Lmp7* gene products. Consistent with these results, northern blot hybridization studies showed that HLA class I-positive (JEG-3) and HLA null (Jar) trophoblast-derived cell lines contain *Lmp7* mRNA. After 48 hr of exposure to HLA class I-modulating cytokines, *Lmp7* mRNA levels in JEG-3 cells were markedly increased by two interferons (IFN-β, IFN-γ) and tumour necrosis factor (TNF) whereas at the same time point, Jar cell *Lmp7* mRNA was modestly enhanced by IFN-γ. Collectively, the findings indicate that expression of HLA class I antigens in trophoblast cells is unlikely to be restricted by lack of *Lmp7* gene products and suggest that endogenous placental cytokines may have different influences on *Lmp7* mRNA levels in phenotypically distinct trophoblast subpopulations.

INTRODUCTION

Two subunits of a subset of proteasomes (multicatalytic proteinase complexes), LMP2 and LMP7, are derived from genes in the human leucocyte antigen (HLA) class II region of chromosome 6.[1–3] These cytosolic proteasomes function as extralysosomal adenosine triphosphatase (ATPase)-dependent systems, processing intracellular proteins into short peptides which are then transported via transporter for antigen processing (TAP) heterodimers into the endoplasmic reticulum and loaded onto assembling HLA class I antigens. As with other components of the HLA class I antigen-processing pathway, levels of LMP2 and LMP7 are elevated in response to interferon-γ (IFN-γ) and other IFNs.[4–6] While there are conflicting opinions as to whether or not LMP2 and LMP7 are required for efficient display of HLA class I antigens,[7] mice lacking the LMP7 subunit have low major histocompatibility complex (MHC) class I expression.[8]

Strict regulation of HLA antigen expression by trophoblast cells that form the fetal component of the materno-fetal interface during pregnancy is believed to be essential to maternal acceptance of the fetal semiallograft (reviewed in refs 9 and 10). Although HLA class II antigens are neither expressed nor inducible in any subpopulation of trophoblast cells, HLA class I mRNA and antigens are present in one subpopulation, the extravillous cytotrophoblast cells. These cells erupt from the placental villi into the maternal blood lacunae or decidua in early gestation tissues and form the chorion membrane at later stages.[11–17] By contrast, trophoblast cells contained within the villi, syncytiotrophoblast and villous cytotrophoblast cells, fail entirely to express levels of HLA class I antigens that can be detected with reagents such as the monoclonal antibody, W6/32, which identifies assembled heavy and light chains.[18–21]

Lack of HLA class I antigens might be due to a deficiency of one or another of the proteasome subunits coded within the class II region, as has been proposed for tumour cells lacking HLA class I antigens.[22] To investigate this possibility, we analysed trophoblast cell expression of the *Lmp7* gene at early and late stages of gestation by using *in situ* hybridization to detect specific mRNA, and immunocytochemistry to identify LMP7 protein in placental tissue sections. A second group of experiments was done to determine whether or not the *Lmp7* gene in trophoblast cells was responsive to cytokines known to enhance the rate of transcription of this gene. For these studies, HLA null Jar choriocarcinoma cells and HLA class I positive JEG-3 choriocarcinoma cells, both of which are derived from trophoblastic tumours, were used as models.

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MATERIALS AND METHODS

Tissues

Samples of first trimester and term placentas (*n*, three each) were obtained in co-operation with the Department of Obstetrics and Gynecology under a protocol approved by the institutional Human Subjects Committee. First trimester placentas (8–12 weeks of gestation) were obtained from patients undergoing elective pregnancy termination and term placentas were obtained from patients undergoing caesarean section to relieve fetal distress. The samples (0·5 to 1·0 cm^3) were fixed overnight at 4° in freshly prepared 4% paraformaldehyde solubilized in phosphate buffered saline (PBS) (pH 7·0). The tissues were then embedded in paraffin at low temperature. Five micron sections were cut for the *in situ* hybridization and immunohistochemical experiments.

Cell lines and cytokine treatments

Jar human choriocarcinoma cells were a gift from R. A. Patillo and JEG-3 human choriocarcinoma cells (HTB-36) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The cells were maintained as previously described in RPMI-1640 containing 10% fetal bovine serum (FBS), 2 mM glutamine and antibiotics.[23] To test for the ability of cytokines to alter *Lmp7* mRNA levels, subconfluent cultures were incubated in medium containing no cytokine (control), 1000 U/ml of human recombinant IFN-α or fibroblast IFN-β from Lee Biomolecular Research, Inc., San Diego, CA, 100 U/ml of human recombinant IFN-γ (Endogen, Boston, MA) or 1 U/ml of human recombinant tumour necrosis factor-α (TNF-α) (Endogen) for 48 h. Cells were collected by brief trypsinization of the cell layers, washed extensively, tested for viability by exclusion of trypan blue, pelleted and stored at −80° until used. Effectiveness of the cytokine doses was assessed by standard methods of flow cytometry. JEG-3 cells were incubated with 5 μg/ml of either mouse anti-human β2 m (AMAC, Inc., Westbrook, ME) or an equivalent concentration of normal mouse IgG and binding was detected using goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (Vector Laboratories, Burlingame, CA).

Northern blot hybridization

Detection of *Lmp7* mRNA by northern blot hybridization was performed as described earlier.[24] A fragment of the *Lmp7*-E2 (*Lmp7*) coding sequence (566 bp, bases 306–872) was subcloned into pSP72 (Promega, Madison, WI) and was used as a template for polymerase-directed synthesis of ^{32}P-labelled cRNA probe. The glyceraldehyde-3-phosphate dehydrogenase (G3PDH) DNA probe was synthesized from a 1·2 kb fragment of G3PDH (R.W. Allen, American Red Cross Blood Services, St. Louis, MO) cloned into pGEM3Z plasmid (Promega). Total cell mRNA was isolated using the guanidine isothiocyanate method,[25] fractionated on 1·2% agarose gels (10 μg RNA/lane), transferred to Nytran membranes (Schleicher and Schuell, Keene, NH) and hybridized with ^{32}P-labelled probes. After hybridization with *Lmp7* probe, membranes were stripped and rehybridized with G3PDH probe to assess the integrity of the RNA and equal loading of lanes. *Lmp7* and G3PDH mRNA hybridization signals were compared by scanning densitometry.

In situ *hybridization*

In situ hybridization using digoxigenin-labelled cRNA and a colorimetric detection system was performed as described before.[26] *Lmp7* cDNA subcloned into pSP72 was used as template for the polymerase-directed synthesis of sense and antisense orientation cRNA probes incorporating digoxigenin-labelled uridine triphosphate (UTP). Digoxigenin-labelled *Lmp7* probes were hybridized to paraformaldehyde fixed, paraffin-embedded tissue sections. Hybridization was detected by first incubating the tissue sections with a biotinylated monoclonal anti-digoxigenin antibody (Sigma Chemical Co., St Louis, MO), then with streptavidin conjugated to alkaline phosphatase from Sigma. Incubation in a BCIP substrate yielded a deep blue precipitate at the site of *Lmp7* cRNA–mRNA hybridization. The tissue sections were counterstained with methyl green, coverslipped and analysed by light microscopy as described earlier.[26]

Immunohistochemistry

Tissue sections taken from the same paraffin blocks that had been used for the *in situ* hybridization studies were immunostained to reveal LMP7 protein using methods that have been described.[24,26] LMP7 was identified by using rabbit antibody to human recombinant LMP7 (1:500 dilution), a generous gift from P. A. Peterson, The Scripps Research Institute, La Jolla, CA.[5] An equal concentration of normal rabbit serum was incubated with semi-serial sections of the same tissues to assess non-specific binding of the developing reagents. Binding was detected by using an avidin-biotin immunoperoxidase staining kit from Zymed (South San Francisco, CA), where antibody binding is indicated by the presence of a red precipitate. The tissue sections were counterstained with haematoxylin and evaluated by light microscopy.

RESULTS

Cellular components of cross-sectioned human placental villi at early and late stages of pregnancy are shown schematically in Fig. 1. Two morphologically distinct subpopulations of trophoblast cells are readily distinguished, syncytiotrophoblast, an uninterrupted cell layer that is continuously exposed to maternal blood and cytotrophoblast cells, large cells with vesiculated nuclei. As gestation progresses, the cytotrophoblastic cell layer underlying the syncytium is depleted and the chorion membrane is formed by regression of the invasive cytotrophoblast cells. Centres of the villi contain Hofbauer cells (fetal macrophages), fibroblasts and fetal capillaries.

Figure 1 also indicates the HLA class I mRNA and antigen status of trophoblast cell subpopulations. In general, term syncytiotrophoblast and the cytotrophoblast cells in columns close to the villi are HLA class I $mRNA^-$ and do not contain immunohistochemically detectable HLA class I antigens. First trimester syncytiotrophoblast and villous cytotrophoblast cells may contain HLA class I mRNA but antigens are undetectable. Cytotrophoblast cells fanning out from the columns to form the trophoblastic shell distal to the villus as well as clusters of cells in the maternal blood lacunae and decidua are HLA class I $mRNA^+$/$antigen^+$. Class I genes expressed in these trophoblast cells include HLA-G and HLA-C.[13,14,16,17,27,28]

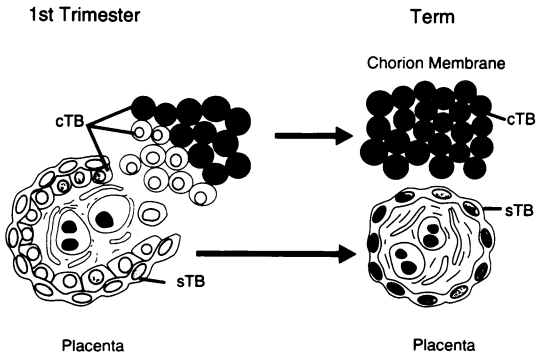

Figure 1. A schematic representation of the trophoblastic cell subpopulations in first trimester and term tissues. Cells lacking both HLA class I mRNA and protein are uncoloured, cells that often contain HLA class I mRNA but do not express detectable class I antigens are pale grey, and cells containing both HLA class I mRNA and protein are dark grey. cTB, cytotrophoblastic cells; sTB, syncytiotrophoblast. Mesenchymal cells and small blood vessels are located in the centres of the villi.

Identification of Lmp7 *mRNA and protein in first trimester human placentas*

Figure 2(a) shows that first trimester placentas contain *Lmp7* mRNA. Specific message is present in syncytiotrophoblast, villous cytotrophoblastic cells and extravillous cytotrophoblastic cells. As illustrated in the villus in the lower right hand corner of Fig. 2(a), some stretches of syncytiotrophoblast contained no detectable *Lmp7* mRNA. Within the cores of the villi, specific message was present in endothelial cells as well as in macrophage-lineage Hofbauer cells (large round cells with foamy cytoplasm). No signals were detected when semi-serial sections of the same tissues were hybridized with the sense orientation of the *Lmp7* cRNA (Fig. 2b).

Immunohistological examination of the same tissues identified specific protein in the same types of cells as those that contained *Lmp7* mRNA (Fig. 2c). No binding was detected when normal rabbit serum was substituted for the specific antibody (Fig. 2d).

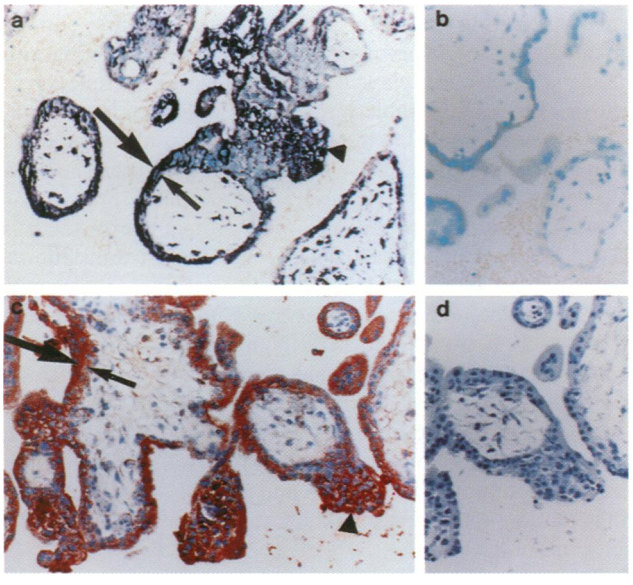

Figure 2. First trimester placenta. (a) Hybridization with the antisense orientation of the *Lmp7* cRNA. Positive hybridization signals are present in all subpopulations of trophoblast cells and in mesenchymal cells in the centres of the villi. Note that some stretches of syncytiotrophoblast do not contain *Lmp7* transcripts. (b) Hybridization with the sense orientation of the *Lmp7* cRNA reveals no hybridization signals. (c) Immunohistochemical staining with rabbit anti-LMP7 shows that specific protein is present in all subpopulations of trophoblast cells as well as in mesenchymal cells. (d) Negative results were obtained when normal rabbit serum was substituted for the specific antibody. Large arrows, syncytiotrophoblast; small arrows, villous cytotrophoblastic cells; arrowheads, extravillous cytotrophoblastic cells. Original magnifications, ×152.

Identification of Lmp7 *mRNA and protein in term human placentas*

Figure 3(a) demonstrates *Lmp7* mRNA in a term placenta. Specific message is abundant in syncytiotrophoblast and is also detectable in endothelial cells and stromal cells. Villous cytotrophoblastic cells and extravillous cytotrophoblastic cells were not identified in samples from late gestation placentas. No signals were detected when semi-serial sections of the same tissues were hybridized with the sense orientation of the *Lmp7* cRNA (Fig. 3b).

Immunohistological examination of the same tissues with rabbit anti-LMP7 identified specific protein in the same cells as those that contained *Lmp7* mRNA (Fig. 3c). No binding was detected when normal rabbit serum was substituted for the specific antibody (Fig. 3d).

Cytokine regulation of the Lmp7 gene in trophoblast cell lines

The identification of *Lmp7* mRNA and protein in placentas

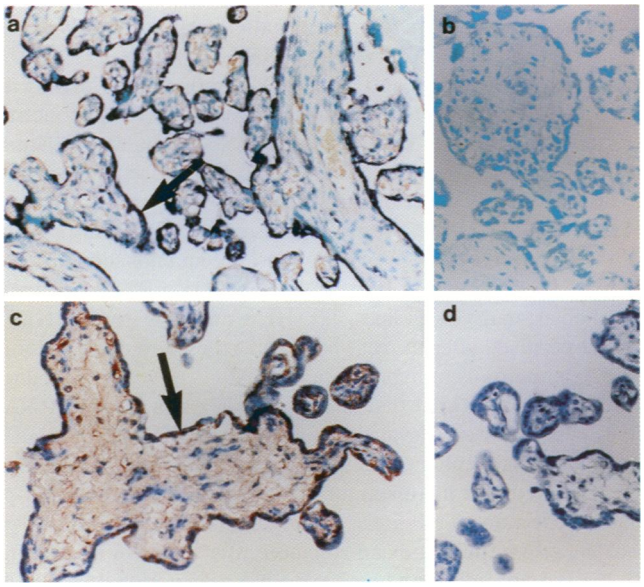

Figure 3. Term placenta. (a) Hybridization with the antisense orientation of the *Lmp7* cRNA. Positive hybridization signals are present in syncytiotrophoblast (arrows) and in mesenchymal cells in the centres of the villi. (b) Hybridization with the sense orientation of the *Lmp7* cRNA reveals no hybridization signals. (c) Immunohistochemical staining with rabbit anti-LMP7 shows that specific protein is present in syncytiotrophoblast (arrows) as well as in mesenchymal cells. (d) Negative results were obtained when normal rabbit serum was substituted for the specific antibody. Original magnifications, ×152.

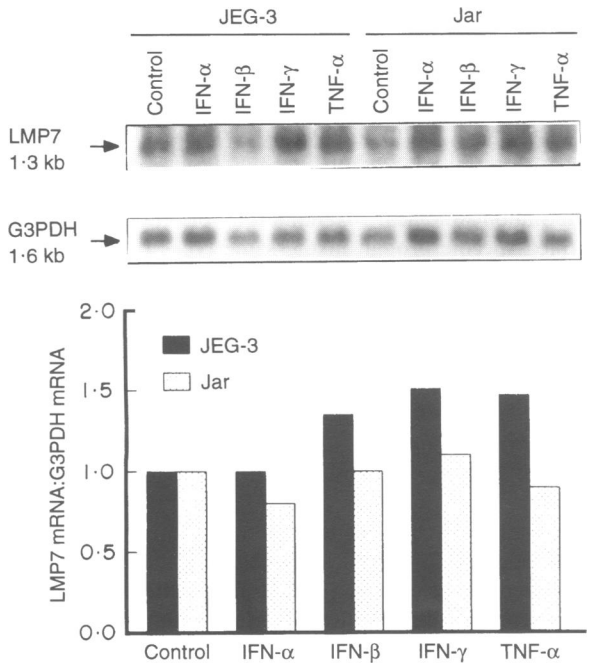

Figure 4. Northern blot hybridization analysis of *Lmp7* and G3PDH mRNA in JEG-3 and Jar cells treated with no cytokine (Control), IFN-α, IFN-β, IFN-γ or TNF-α for 48 hr. The blot was first hybridized to reveal *Lmp7* mRNA, then was stripped and rehybridized for G3PDH mRNA. Relative proportions of *Lmp7*:G3PDH mRNA were obtained by scanning densitometry.

raised the question of whether or not endogenous placental cytokines might promote expression of this gene. Therefore, experiments were done to identify alterations in steady state levels of *Lmp7* mRNA by IFN-α (1000 U/ml), IFN-β (1000 U/ml), IFN-γ (100 U/ml) and TNF-α (1 U/ml), all of which have been identified in human trophoblast cells.[29,30] These concentrations of cytokines effectively stimulated light chain (β2m) expression in JEG-3 cells, as assessed by fluorescence intensity in flow cytometry experiments, and had no adverse effect on the viability of JEG-3 or Jar cell layers, as assessed by visual inspection of the cell layers and trypan blue exclusion (data not shown).

Figure 4 shows *Lmp7* and G3PDH mRNA in two trophoblast-derived cell lines, Jar (HLA null) and JEG-3 (HLA class I positive), after the cells had been exposed to cytokines for 48 h. Comparisons of hybridization signal intensities by scanning densitometry indicated that steady-state levels of *Lmp7* mRNA in JEG-3 cells were elevated by exposure to IFN-γ, IFN-β and TNF-α. A slight enhancement of Jar cell *Lmp7* mRNA (+10%) was observed after treatment for 48 hr with IFN-γ whereas IFN-β had no effect and TNF-α was slightly inhibitory (−10%). IFN-α was entirely ineffective as an enhancer; after 48 hr of treatment, IFN-α did not influence *Lmp7* mRNA levels in JEG-3 cells and had a depressive effect in Jar cells.

DISCUSSION

The results of the *in situ* hybridization and immunohistochemical analyses presented here show clearly that human placental cells of diverse lineage and with different HLA class I profiles express the *Lmp7* gene. Because *in situ* hybridization studies to detect transcripts from this gene have not been previously reported, it was not possible to compare our results with those of others. Yet matching patterns for specific mRNA and specific protein indicated that hybridization signals were correctly identified, and other types of routine controls were negative (Figs 2b and 3b).

The presence of *Lmp7* transcripts in human placentas was not completely unexpected; previous northern blot hybridization analyses have demonstrated *Lmp7*-specific transcripts in human placentas[24] and various tissues in mice contain *Lmp7* mRNA.[31] However, it was surprising to find abundant *Lmp7* mRNA and protein in both HLA class I-positive and class I-negative placental cells. We anticipated that the *in situ* hybridization and immunohistochemical experiments might reveal patterns of *Lmp7* gene expression that matched patterns of HLA class I antigen expression, as appears to be the case with *Tap-1* mRNA (K. F. Roby, D. Gershon & J. S. Hunt, unpublished data). Both *Lmp7* and *Tap-1* transcripts are low to absent in HLA class I-deficient human small cell lung carcinoma cells.[22] The findings reported here indicate that deficiencies in *Lmp7* gene products are unlikely to limit expression of HLA class I antigens in either trophectoderm-derived trophoblast cells or in inner cell mass-derived stromal cells. Nor were any patterns observed that would suggest differential usage by cells that produce novel class I antigens (trophoblast) and conventional class I antigens (stromal cells).

The presence of abundant *Lmp7* mRNA and protein in HLA class I negative trophoblast cells strongly supports the postulate that proteasomes containing this component perform functions during placental development that are entirely unrelated to processing of peptides destined for loading into the clefts of classical class I antigens. One likely function is destruction of undesirable intracellular polypeptides; ubiquinated proteins targeted to these proteasomes would be expected to undergo rapid and selective breakdown as has been shown in other types of cells.[1,2] However, the ubiquitin–proteasome system is not limited to destruction of protein substrates; it is clearly involved in limited proteolysis and post-translational processing to generate biologically active peptides (reviewed in Ref. 3). Recent studies on processing of nuclear transcription factors such as NFκB and IκBα highlight the importance of proteasomes in post-translational regulation of cell activation by cytokines such as TNF-α[32] and point to a role for proteasomes in degradation of unstable c-Jun.[33] It is, therefore, of interest that trophoblast cells in both early and late gestation placentas contain TNF-α mRNA and protein[30] as well as other inflammation-associated cytokines that normally induce the transcription factors.[34]

Treatment of cells with IFN-γ causes proteasome subunits X and Y to be replaced with LMP7 and LMP2.[35,36] It appears that in the placenta, substitution with LMP7 takes place very early in gestation. This might be due to encounter with endogenous cytokines such as IFNs and TNF-α which are present in first trimester placentas[29,30] and, as discussed below, are capable of enhancing *Lmp7* levels in trophoblast cell lines. Whether or not proteasomes containing LMP7 might augment the capacity of the placenta to resist infection by influencing the profile of peptides presented by trophoblast cell HLA class I antigens is not known. However, one of the novel class I antigens expressed by trophoblast, HLA-G, is recognized by $CD8^+$ T lymphocytes.[37]

Regulation of *Lmp7* gene expression in trophoblast cells, as defined by the outcome of our experiments on trophoblast-derived choriocarcinoma cells, does not differ from regulation in other types of cells. Levels of specific mRNA in both JEG-3 and Jar cells were, as expected, elevated 48 h after exposure to IFN-γ. The upregulation of *Lmp7* mRNA in JEG-3 cells was considerably more robust than in Jar cells, which is the reverse of the situation observed after 24 h of exposure to IFN-γ.[24] Thus, the kinetics of *Lmp7* mRNA enhancement differ in the two phenotypically distinct cell lines, with JEG-3 cells responding more slowly to IFN-γ than Jar cells. Whether or not this difference has any biological significance is not known. Aki *et al.* reported similar findings in human colon carcinoma cells (SW620) and monocytic leukemia cells (J111), with the former being induced earlier than the latter.[6] This group failed, as did we, to enhance *Lmp7* mRNA levels with IFN-α. Our results could not be attributed to inactivity of the cytokine or to a generalized unresponsiveness of the trophoblast cells. In other studies, we have shown that HLA class I antigens on JEG-3 cells are efficiently up-regulated by the same IFN-α preparation (Y. Yang & J.S. Hunt, unpublished data). Hager *et al.* have recently reported that purified, Sendai virus-induced human trophoblast IFN-α induce HLA class I antigens on human term cytotrophoblast cells,[38] suggesting that this cytokine influences other genes involved in antigen expression. JEG-3 cells were responsive to a broad range of modulators, with IFN-β and TNF-α as well as IFN-γ promoting increased steady-state levels of *Lmp7* mRNA. These results contrasted with those reported for SW620 and J111 cells, where neither IFN-β nor TNF-α enhanced *Lmp7* mRNA.[6] However, both cytokines augmented IFN-γ effects. Therefore, it seems likely that many factors, including cell lineage, may play a part in determining the impact of cytokines on *Lmp7* gene expression.

In summary, we here present the first report on the cellular distribution of *Lmp7* mRNA and protein in a normal organ and supply new data relevant to regulation of HLA class I antigen expression in trophoblast cells. Furthermore, we provide evidence in support of the postulate that LMP7 performs major functions in human placentas that are likely to be separate and distinct from generation of HLA class I-associated peptides.

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