Biochimica et Biophysica Acta 1812 (2011) 431–438

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

ER stress impairs MHC Class I surface expression and increases susceptibility of thyroid cells to NK-mediated cytotoxicity

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ARTICLE INFO

Article history:
Received 6 August 2010
Received in revised form 9 December 2010
Accepted 16 December 2010
Available online 1 January 2011

Keywords:
ER stress
Natural killer cells
MHC-I expression
Thyroid autoimmunity

ABSTRACT

We recently reported that, in thyroid cells, ER stress triggered by thapsigargin or tunicamycin, two well known ER stressing agents, induced dedifferentiation and loss of the epithelial phenotype in rat thyroid cells. In this study, we sought to evaluate if, in thyroid cells, ER stress could affect MHC class I expression and the possible implications of this effect in the alteration of function of natural killer cells, suggesting a role in thyroid pathology. In both, a human line of fetal thyroid cells (TAD-2 cells) and primary cultures of human thyroid cells, thapsigargin and tunicamicin triggered ER stress evaluated by BiP mRNA levels and XBP-1 splicing. In both cell types, TAD-2 cell line and primary cultures, major histocompatibility complex class I (MHC-I) plasmamembrane expression was significantly reduced by ER stress. This effect was accompanied by signs of natural killer activation. Thus, natural killer cells dramatically increased IFN-γ production and markedly increased their cytotoxicity against thyroid cells. Together, these data indicate that ER stress induces a decrease of MHC class I surface expression in thyroid cells, resulting in reduced natural killer-cell self-tolerance.

1. Introduction

Major histocompatibility complex class I [MHC-I, also called HLA-I (human leukocyte antigens) in humans] molecules are cell surface glycoproteins expressed in virtually all nucleated mammalian cell types and are crucial in the regulation of cytotoxic effector functions of natural killer (NK) cells and T cells. MHC-I molecules present, indeed, antigens to cytotoxic T cells and are part of the recognition signals that regulate activation of NK cells. Usually antigenic peptides are generated by proteasomal degradation of cytosolic proteins and translocated from the cytoplasm to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). In the ER, the assembly of MHC class I α chain, β2-microglobulin and peptide is guided by chaperones [1], and antigenic peptides are found associated with the chaperones glucose-regulated protein (GRP)94 (GP96), protein disulfide isomerase (PDI) and calreticulin [2–6]. Functional class I-peptide complexes are then transported to the cell surface. A necessary condition for this complex process to occur properly is that MHC class I heavy chains must be glycosylated and correctly folded in the endoplasmic reticulum (ER). Failure to glycosylate or properly fold MHC class I heavy chains results in their slow or inefficient transport to the cell surface [7–11]. Cellular stress may result in a decrease of MHC class I expression on the cell surface, thereby altering the recognition of cells by the adaptive and innate immune system [12,13].

The single feature uniting different stress stimuli (heat shock, hypoxia, viral replication, abnormal proteins, starvation or transformation) is, indeed, that they all ultimately lead to accumulation of unfolded or misfolded proteins in the lumen of the ER [12,14]. The ER responds to the accumulation of unfolded proteins by activating intracellular signal transduction pathways, collectively called the unfolded protein response (UPR) [15,16]. Activation of these UPR transducers has pervasive effects on cellular protein economy: (i) attenuation of protein translation, (ii) increased degradation of ER proteins by ER associated degradation (ERAD), (iii) transcriptional activation of genes involved in the folding machinery of the ER and (iv) increased degradation of ER-localized mRNAs [15].

We hypothesized that protein misfolding and UPR execution may alter MHC-I plasmamembrane expression and consequently might be

Abbreviations: BiP, binding protein (GRP78); ER, endoplasmic reticulum; ERAD, ER associated degradation; GRP94, glucose regulated protein 94; HFE, hereditary hemochromatosis protein; HLA-I, human leukocyte antigen-I; IFN-γ, interferon-γ; IL-2, interleukin-2; MHC-I, major histocompatibility complex class I; NK, natural killer; PDI, protein disulfide isomerase; TH, thapsigargin; TN, tunicamycin; UPR, unfolded protein response; XBP-1, X-box binding protein 1

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doi:10.1016/j.bbadis.2010.12.013

relevant to NK-mediated immune responses. An important feature of NK cells is, indeed, their ability to distinguish target cells that differ only in their expression of MHC class I molecules, leading to the missing-self recognition by NK cells during the innate immune response [13]. NK cells can directly lyse target cells [17] and can also have regulatory roles; for example, they are significant early producers of interferon-γ (IFNγ), which can influence T-cell responses [18].

The NK lymphocytes play a major role in tissue immune surveillance, recognizing qualitative and quantitative alterations in the expression of MHC-I molecules on cells. Such alterations are described in tumors [19] and in viral infections [20]. However, recently, a role of NK cells in human autoimmune diseases has been recognized [21]. With regard to thyroid autoimmune diseases, in spite of the detailed characterization of the role of T and B lymphocytes and cytokines [22], scant evidences are present on the involvement of NK cells.

2. Materials and methods

2.1. Materials

DMEM, FCS, L-glutamine and BSA were from Invitrogen (Paisley, UK). Thapsigargin (TH) and tunicamycin (TN) were from Sigma-Aldrich (St. Louis, MO). Anti-MHC class I mAb (W6/32 class I) was purchased from DAKO (Milan, Italy) and isotype-matched labeled control was purchased from PharMingen (San Diego, CA).

2.2. Tissues, cell cultures and treatments

Tissue specimens were obtained at surgery from the unaffected controlateral lobes of thyroid papillary carcinomas or from inter-nodular tissue of nodular goiters undergoing thyroidectomy. Briefly, tissues were chopped by scalpels in small pieces and digested by type IV collagenase (Sigma Chemical Co., St. Louis, MO; 1.25 mg/ml) in Ham's F-12 medium and 0.5% BSA overnight at 4 °C under rotation. Cells were pelleted by centrifugation at 150×*g* for 5 min, washed twice in BSA-Ham's F-12 medium (BSA/F12), seeded in petri dishes, and cultured in 5% CO_2 atmosphere at 37 °C in Ham's F-12 medium supplemented with 10% FCS. The medium was changed every 3–4 days, and the cells to be examined were harvested by treatment with 0.5 mM EDTA in calcium- and magnesium-free PBS containing 0.05% trypsin (trypsin/PBS). The follicular origin of the cultures was confirmed by the presence of cytokeratin and thyroglobulin, as assessed by flow cytofluorometry. The TAD-2 cell line, obtained by Simian virus 40 infection of human fetal thyroid cells, was a kind gift of Dr. M. Illario, University of Naples "Federico II" (Naples, Italy). TAD-2 cells were cultured in a 5% CO_2 atmosphere at 37 °C, in DMEM supplemented with 10% FCS. Medium was changed every 3–4 days. Cells were detached by 0.5 mM EDTA in calcium- and magnesium-free PBS with 0.05% trypsin. TH or TN (Calbiochem) was added to the medium for 30 min at a final concentration of 0.5 μM or 0.5 μg/ml, respectively. The medium was then replaced with medium without TH/TN until harvesting, as reported. Alternatively, TH or TN was left in the culture medium until harvesting, as reported.

2.3. RNA extraction, northern blot and semiquantitative reverse transcription-PCR

Total RNA extraction and northern blots were carried out as reported previously [23]. RNA was reverse transcribed to cDNA by using random hexamers and the ImProm-II reverse transcriptase system (Promega, Madison, WI). 10% of the cDNA synthesis reaction was submitted to semiquantitative PCR analysis by using Taq DNA polymerase (Promega). The following oligonucleotides were used: 5'-GCTTGTGATTGAGAACCAGG and 5'-GAGGCTTGGTGTATATATGG for XBP-1; 5'-ACCACCATGGAGAAGG and 5'-CTCAGTGTAGCCCAGGATGC for β-actin.

2.4. Preparation of polyclonal NK populations

Polyclonal NK cells were obtained from peripheral blood mononuclear cells (PBMCs), isolated by centrifugation on Lymphoprep (Nycomed Pharma) gradients from normal donor buffy coats obtained from the Blood Bank of the Medical School of the Federico II University of Naples. After separation, the PBMC were washed and incubated in complete medium, in a horizontally placed plastic flask, for 2 h at 37 °C in order to remove adherent cells. The recovered cells subjected to a depletion of CD3+ cells by using anti-CD3-coated magnetic beads (Dynal, Oslo, Norway) and a samarium cobalt magnet. The depletion procedure was repeated twice. Less than 2% of the remaining cells were CD3+, as assessed by fluorescence-activated cell sorter analysis (data not shown). The obtained polyclonal NK cells were used without pre-treatment or incubated for 24 h with 500 IU/ml of human recombinant IL-2 (IL-2) (Sigma-Aldrich).

2.5. Monoclonal antibodies, immunofluorescence and flow cytometry

Purified FITC-, PE-, and Cy-chrome-labeled mAb against IFN-γ (4 S. B3), CD3 (SK7), CD56 (MY31), CD107a (H4A3), MIC A/B (6D4) and isotype-matched controls were purchased from Becton Dickinson, (Mountain View, CA). Anti-HLA mAb (W6/32class I) was purchased from DAKO, Milan, Italy. Immunofluorescence, flow cytometry and data analysis were performed by FACScalibur apparatus and CellQuest software (Becton Dickinson, Mountain View, CA), as described [24]. Analysis gates were set such in order to exclude all the debris and apoptotic cells and to obtain that only viable cells were collected for the subsequent analysis.

2.6. Cytotoxicity assay

Cytotoxicity was measured using two different methods: (1) the conventional 4 h ^{51}Cr release assay. Target cells were labeled with $Na_2{}^{51}CrO_4$ (100 mCi/2 × 10^6 cells), and the percent of specific lysis was calculated as 100 × [(cpm in experimental wells) − (cpm in wells with target cells alone)]/(total cpm incorporated into target cells). The spontaneous ^{51}Cr release from target cells cultured alone was consistently less than 20% of the total cpm; (2) the evaluation of CD107a expression on NK effectors by using immune fluorescence and flow cytometry analysis, as described [25,26]. The expression of CD107a on the membrane of effector cells has been shown to finely correlate with target cell lysis, as compared with the classical ^{51}Cr-release assay [25,26] and it has been largely used as reliable marker for NK-mediated cytotoxicity [27]. This assay was used with primary normal human thyroid cells, in which the number of available cells was low (primary cells grow slowly and for a limited number of cell divisions). Moreover, the use of this assay allowed the simultaneous evaluation of interferon-γ (see next paragraph) and of the killing phenotype of NK effectors. Briefly, the polyclonal IL-2 activated NK effectors were stained with PE-conjugated anti-CD107a (BD) and incubated for 6 h, in a round bottomed microtiter plate (Falcon), with the appropriate cell line target by an effector/target (E/T) ratio of 1:1. After 1 h of stimulation, Brefeldin-A (5 μg/ml, Sigma Chem. Co., St. Louis, MO) was added. After the 6-h culture, the cells were stained as described in the *Detection of intracellular IFN-γ* paragraph.

2.7. Detection of intracellular IFN-γ

Resting or IL-2-activated polyclonal NK cells (2×10^5) and TAD-2 or primary human thyroid cells, used as stimulators, were mixed at a ratio of 1:1 and incubated in a sterile 96-well plate (Falcon, Seattle, WA) for a 6 h period at 37 °C in RPMI 1640 medium and 10% FCS. In order to avoid any interference mediated by residual IL-2, the effectors were washed twice with complete medium before the co-culture. In order to promote intracellular cytokine retention, Brefeldin-A (5 μg/ml, Sigma-Aldrich)

was added during the incubation. Intra-cellular IFN-γ production was detected by using triple staining technique (FITC-conjugated anti-CD56, PE-Cy5-labeled anti-CD3 and APC-conjugated anti-Interferon gamma) and flow cytometry analysis. Briefly, after the incubation the culture was harvested, the cells were fixed, and permeabilised by using a cytokine staining kit, following the manufacturer's instructions (Caltag Laboratories, Burlingame, CA). A live gate was set such that only the NK $CD56^{+}$ lymphocytes were collected for analysis. In selected experiments, the staining for CD107a and IFN-γ on NK effectors was performed in the same experimental condition (see previous paragraph).

2.8. Statistical procedures

Data were analyzed with Statview software (Abacus Concepts) by one-factor analysis of variance. p values of less than 0.05 were considered statistically significant.

3. Results

3.1. Thapsigargin (TH) and tunicamycin (TN) activate the UPR in both TAD-2 and primary thyroid cells

As a first step, we set up the experimental conditions able to cause UPR activation in the ER of both TAD-2 and primary thyroid cells. To this aim, we tested the effect of the widely used ER stress-inducing agents TH and TN on the expression levels of the ER stress marker BiP and the splicing of mRNA encoding XBP-1. In particular, we treated TAD-2 and primary thyroid cells with a low TH/TN concentration that we have previously shown [28] to be effective to elicit a mild ER stress in thyroid cells that does not trigger the activation of apoptosis. We performed northern blots and RT-PCR assays. As shown in Fig. 1, treatments of 30 min with TH or TN, followed by 24 h in medium without TH/TN, increased BiP mRNA, in both TAD-2 (Fig. 1A, left panel) and primary thyroid cells (Fig. 1B, left panel) by ~6-fold. Furthermore, we observed an increase in the spliced active form of XBP-1 mRNA following TH and TN treatments in both TAD-2 (Fig. 1A, right panel) and primary thyroid cells (Fig. 1B, right panel). The increase of BiP mRNA and the appearance of the spliced active form of XBP-1 mRNA was similar when cells where incubated continuously for 24 h with TH/TN (data not shown).

3.2. ER stress affects MHC class I expression in TAD-2 cells

To test the effect of ER stress induction on MHC class I expression, TAD-2 cells were treated with TH or TN. Cells were incubated for 30 min (burst) with TH (THb) or TN (TNb) and the incubation medium was then replaced with fresh medium for 24 or 48 h. Alternatively, TH or TN treatments were left in the culture medium for

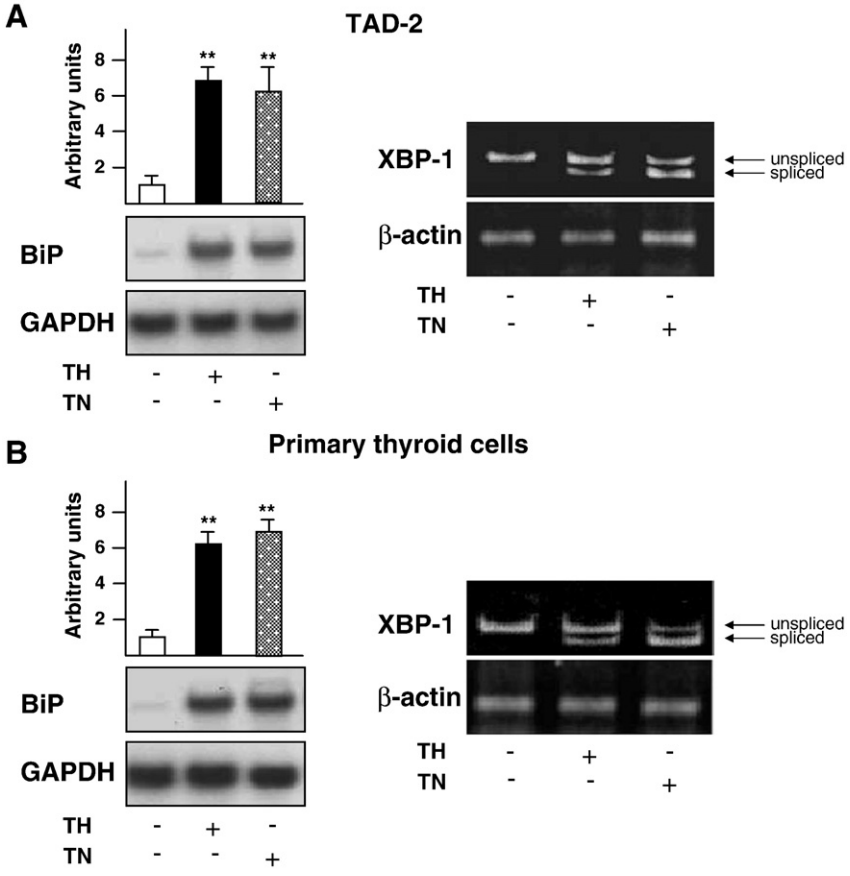

Fig. 1. Thapsigargin (TH) and tunicamycin (TN) induce ER stress in both TAD-2 and primary thyroid cells. (A) Left panel: TH and TN induce upregulation of mRNA encoding BiP in TAD-2 cells. Northern blot analysis of total RNA extracted from cells vehicle treated (Control) or treated with 0.5 μM TH or 0.5 μg/ml TN for 30 min, followed by 24 h in medium without TH or TN. GAPDH was analyzed on the same filters. The intensity of the band corresponding to BiP was quantified by phosphorimaging, and plotted on a bar graph with arbitrary units and represent the average + 1 SD of three different experiments. The asterisks represent statistically significant differences ($p<0.01$). Right panel: RT-PCR analysis for XBP-1 and β-actin of the same total RNAs used in (A left panel). (B) Left panel: TH and TN induce upregulation of mRNA encoding BiP in primary thyroid cells. Northern blot analysis of total RNA extracted from cells vehicle treated (Control) or treated with 0.5 μM TH or 0.5 μg/ml TN for 30 min, followed by 24 h in medium without TH or TN. GAPDH was analyzed on the same filters. The intensity of the band corresponding to BiP was quantified by phosphorimaging and plotted on a bar graph with arbitrary units and represent the average + 1 SD of three different experiments. The asterisks represent statistically significant differences ($p<0.01$). Right panel: RT-PCR analysis for XBP-1 and β-actin of the same total RNAs used in (B, left panel).

24 or 48 h. The expression of MHC class I molecules was determined by flow cytometry using antibody W6/32 that detects MHC (HLA)-A, B and C molecules. Mean and SD of the mean intensity of fluorescence of cells treated or not with TN and TH are shown (Fig. 2A and B, respectively, $n=3$). Both, continuative or burst treatments with TN and TH induced a significant ($p<0.01$) downregulation of MHC-I expression at both 24 and 48 h. Interestingly, a greater efficacy of TN in decreasing MHC-I expression (Fig. 2A and B) was paralleled by a greater ability of TN to affect the splicing of XBP-1 (Fig. 1). In Fig. 2 C is shown a representative experiment with TN.

3.3. Increased cytotoxic activity of NK incubated with TAD-2 cells undergoing ER stress

Since NK cells are potent killers, particularly of cells with reduced MHC-I expression [29,30] we investigated the susceptibility of TAD-2 cells, undergoing ER stress, to be lysed by NK cells. To this aim, TAD-2 cells have been treated with TH for 30 min followed by 24 h in medium without TH. Then, IL-2 activated polyclonal NK, obtained as outlined in Materials and methods, were incubated with TAD-2 cells for 4 h. As shown in Fig. 3, polyclonal NK cells incubated with TAD-2 cells previously treated with TH displayed a significantly ($p<0.01$) increased cytotoxic capacity, when compared to NK cells incubated with untreated TAD-2 cells. When resting (not IL-2 stimulated) NK cells were used, the increased cytotoxicity of NK cells towards ER stressed respect to unstressed TAD-2 cells was still present, albeit the absolute values were, as expected, lower (not shown). This result suggests that ER stress increases susceptibility of thyroid cells to NK-mediated killing.

3.4. Increased IFN-γ producing NK cells when incubated with TAD-2 cells undergoing ER stress

Besides their capability to lyse target cells, NK cells are also significant early producers of IFN-γ. Therefore, to extend a positive role of ER stress and reduced MHC-I expression on the increased activity of NK cells, we investigated IFN-γ production. As above, IL-2 stimulated NK cells were incubated for 6 h with TAD-2 cells previously treated or not with TH or TN for 30 min followed by 24 h in medium without TH/TN. As shown in Fig. 4, the percentage of

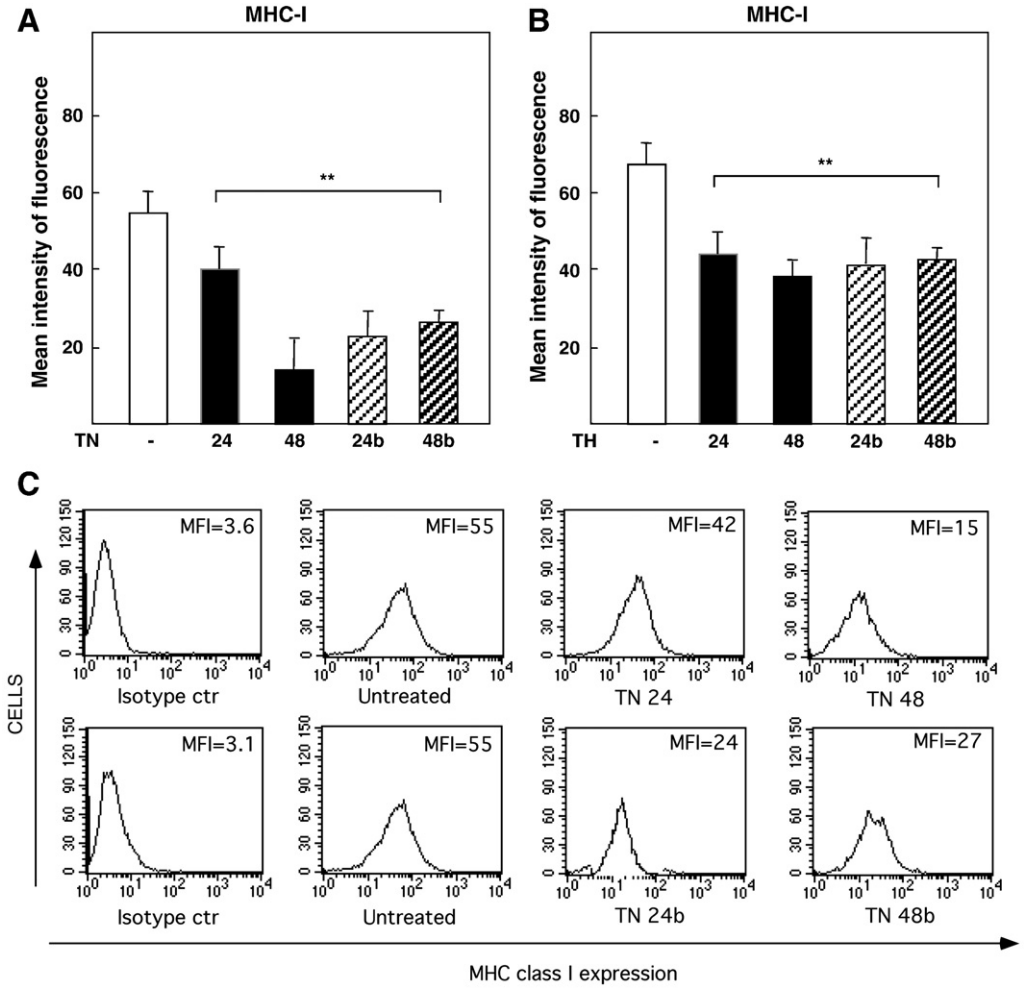

Fig. 2. TN and TH induce a decrease of MHC-I surface expression in TAD-2 cells. (A) TAD-2 cells were either treated continuously with 0.5 μg/ml TN for 24 or 48 h or treated for 30 min with 0.5 μg/ml TN and then the medium was replaced with fresh medium for 24 or 48 h (24b or 48b). At the indicated time points, cells were harvested and cell surface expression of MHC class I molecules was tested by flow cytometry using mAb W6/32 specific for MHC (HLA)-A, -B, -C. Mean and SD of the mean intensity of fluorescence of cells are shown ($n=3$). **, $p<0.01$. (B) TAD-2 cells were either treated continuously with 0.5 μM TH for 24 or 48 h or treated for 30 min with 0.5 μM TH and then the medium was replaced with fresh medium for 24 or 48 h (24b or 48b). At the indicated time points, cells were harvested and cell surface expression of MHC class I molecules was tested by flow cytometry using mAb W6/32 specific for MHC (HLA)-A, -B, -C. Mean and SD of the mean intensity of fluorescence of cells are shown ($n=3$). **$p<0.01$. (C) Data are representative of one out of three independent experiments with TN. Analysis gates were set such in order to exclude all the debris and apoptotic cells and to obtain that only viable cells were collected for the subsequent analysis.

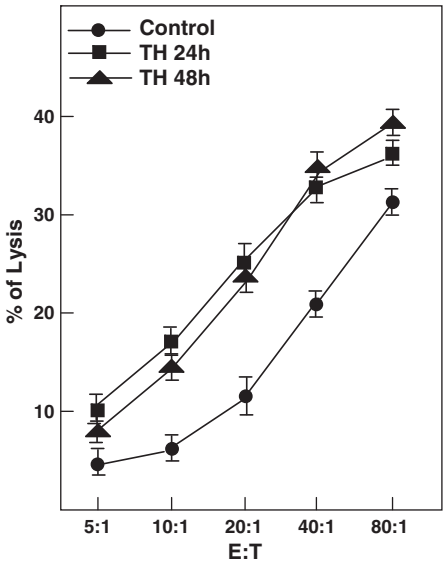

Fig. 3. ER stress significantly affects the NK lytic efficiency against TAD-2 cells. TAD-2 cells were treated for 30 min with 0.5 μM TH and then the medium was replaced with fresh medium for 24 or 48 h. Human IL-2 activated polyclonal NK cells were then used as effectors in a conventional 4 h ^{51}Cr release cytotoxicity assay against untreated (control) or treated (TH) TAD-2 cells. The percentage of lysis and E :T ratio was reported on the abscissas and ordinate axes, respectively. Data are reported as means of the results from three independent experiments. Standard deviations for each E/T ratio are indicated as error bars ($p<0.01$).

IFN-γ producing NK cells was significantly ($p<0.01$) increased by both TH and TN treatments when compared to untreated cells. Analogously to the cytotoxicity assays, the increased IFN-γ production caused by ER stressed TAD-2 cells was confirmed with resting NK cells, albeit with lower absolute values (not shown). These results show that interaction of NK cells with ER stressed thyroid cells increased not only cytotoxic activity but also cytokine production of NK cells.

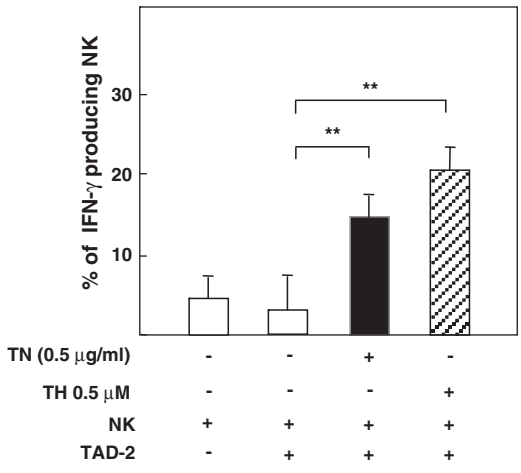

Fig. 4. Increased IFN-γ producing NK cells when incubated with TAD-2 cells undergoing ER stress. TAD-2 cells were treated for 30 min with 0.5 μM TH or 0.5 μg/ml TN and then the medium was replaced with fresh medium for 24 h. Then, TAD-2 cells were incubated with IL-2 activated polyclonal NK cells for a 6 h period. The flow cytometry analysis for the intracellular IFN-γ staining in NK cells is reported. Cells were stained with fluorescein isothiocyanate anti-IFN-γ monoclonal antibodies (mAbs). Mean and SD of the mean intensity of fluorescence of cells are shown ($n=3$). **$p<0.01$.

3.5. Decreased MHC-I surface expression by ER stress in primary thyroid cells and increased cytotoxic activity and IFN-γ production of NK cells incubated with primary thyroid cells undergoing ER stress

To strengthen the results obtained in TAD-2 cells, we decided to reproduce some of the above-mentioned experiments in proliferating primary normal human thyroid cells. As shown in Fig. 5A and B, similarly to TAD-2 cells, primary normal human thyroid cells treated for 30 min with TN and TH followed by 24 h incubation in TN/TH-free medium, showed a marked reduction of MHC-I surface expression ($p<0.01$), as evidenced by flow cytometry experiments.

In addition, this effect was accompanied by a significant increase ($p<0.01$) of the percentage of IFN-γ producing NK when primary human thyroid cells were treated with TN and TH and then incubated for 6 h with IL-2 activated polyclonal NK obtained as outlined in Materials and Methods (Fig. 6A and B). As with TAD-2 cells, similar results were obtained with resting NK cells, albeit, as expected, quantitatively lower (not shown).

To investigate the cytotoxic activity of NK cells when challenged with primary human thyroid cells, we took advantage of the recent assay of CD107a expression [29,30]. As shown in Fig. 6 C the expression of CD107a increased by 60% when NK cells were challenged with ER stressed primary cells respect to a challenge with unstressed primary cells. Of note, the increased expression of CD107a and increased production of IFN-γ were simultaneously evaluated in the same experiments.

Together these results suggest that ER stress downregulates MHC-I surface expression and NK-dependent self-tolerance also in normal human thyroid cells.

3.6. TH did not change the expression of stress-induced soluble major histocompatibility complex class I-related chains A/B (MIC A/B)

The MHC-I homologs, MICA/B, are described as activating ligands for the NK cell receptor NKG2D in human [31]. These stress-induced ligands appear able to induce NK cells by NKG2D engagement, and were described to be increased in various chronic diseases [31,32]. In order to ascertain if the cytotoxic NK phenotype against TH-treated thyroid cells could be dependent also on the increase of stress-induced ligands, we tested the effect of the drug in primary cells. As shown in Fig. 6D, the expression of MIC A/B on TH-treated primary thyroid cells appears to be unmodified. This result point to exclude a role for increased activating NK-Ligands on NK cytotoxicity toward drug-treated thyroid cells, underscoring the role played by MHC-I down-regulation.

4. Discussion

The ER provides the machinery to assist protein folding and to assure that only correctly folded proteins can move on along the secretory pathway. Many disturbances, including perturbation of calcium homeostasis or redox status, increased cargo protein synthesis or/and altered glycosylation, result in accumulation of unfolded proteins in the lumen and trigger the unfolded protein response (UPR). Elucidation of the mechanisms governing ER stress signaling has linked this response to the regulation of diverse physiologic processes as well as to the progression of a number of diseases, such as diabetes, cystic fibrosis, Alzheimer disease and cancer. We have shown that perturbing the endoplasmic reticulum homeostasis of rat thyroid cell lines alter the folding pathway of thyroglobulin [33–38], the major biosynthetic product of thyrocytes, leading to ER stress and UPR activation [28,39]. We have also shown that, in these conditions, thyroid cells execute a dedifferentiation program, involving tissue-specific proteins and epithelial tissue differentiation and organization [28]. We hypothesized that ER stress could affect another important process in thyroid cells, that is, MHC-I surface expression. This, might, in turn, affect NK function. In this

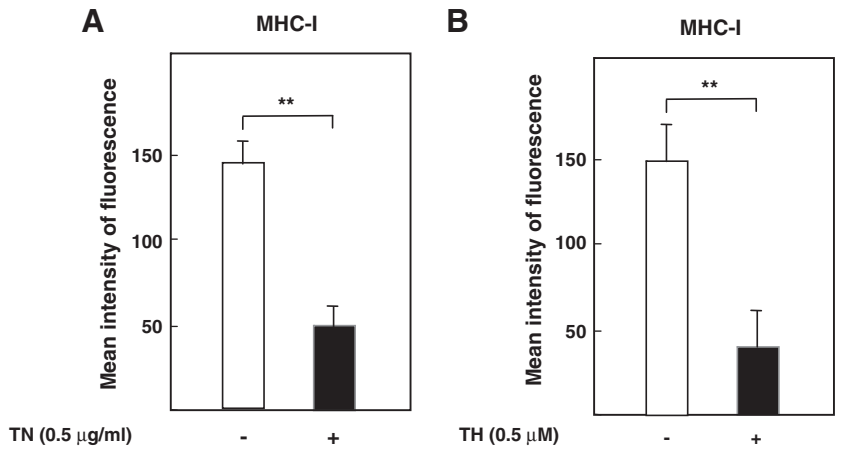

Fig. 5. Decreased MHC-I surface expression by ER stress in primary thyroid cells. Primary human thyroid cells were treated for 30 min with 0.5 μg/ml TN (A) or 0.5 μM TH (B) and then the medium was replaced with fresh medium for 24 h. Then, cells were harvested and cell surface expression of MHC class I molecules was tested by flow cytometry using mAb W6/32 specific for MHC (HLA)-A, -B, -C. Mean and SD of the mean intensity of fluorescence of cells are shown ($n=3$). $^{**}p<0.01$. Analysis gates were set such in order to exclude all the debris and apoptotic cells and to obtain that only viable cells were collected for the subsequent analysis.

study, we report for the first time that, besides tissue-specific differentiation, ER stress, induced by TH/TN, negatively affects surface expression of MHC-I proteins. Furthermore, we show that ER stress-induced downregulation of MHC-I was accompanied by increased NK activation, monitored by increased cytotoxic activity and increased production of IFN-γ.

It is well known that an important feature of NK cells is their ability to distinguish target cells that differ only in their expression of MHC class I molecules. The recognition and lysis of MHC class I low target cells by NK cells is aptly referred to as missing-self recognition [40]. This recognition occurs frequently in virus-infected and cancer cells [40]. It is thought that the downregulation of MHC class I molecules by virus-infected cells is a strategy to evade cytolysis by CD8+ T cells and that missing-self recognition by NK cells is a counter-response by the immune system. Importantly, viral infection or transformation of target cells does not seem to be a prerequisite for missing-self recognition. Even untransformed, uninfected target cells can be lysed by NK cells if the target cells fail to express a full complement of normal self MHC class I proteins [41]. This may well be the case of stressed normal cells, when the stress is able to reduce surface expression of MHC-I molecules. We have shown a similar occurrence in thyroid cells experiencing ER stress. However, missing-self recognition may operate in conjunction with stimulatory recognition. To lyse target cells or to produce effector cytokines, NK cells, similar to T cells, may be triggered by stimulatory receptors [41]. We have begun to address this possibility by evaluating the expression of MICA/B, activating ligands for the NK cell receptor NKG2D [31], on ER stressed primary thyroid cells. We detected no variations on MICA/B following ER stress, and this result underscores the role played by MHC-I down-regulation. Of course, this result does not exclude that other activating ligands may be involved.

Interestingly, a link between ER stress, decreased MHC-I expression and immunological anomalies is suggested by recent findings on haemochromatosis, an inherited autosomic recessive disease of iron metabolism characterized by an increased intestinal iron absorption that leads to iron overload of several organs and to immunological anomalies [42,43]. The expression of the mutated HFE (the genetic defect in haemochromatosis) triggers an UPR and decreases MHC-I surface expression in 293 T cells [44].

Recently, a role of NK cells in human autoimmune diseases has been recognized [21]. In several cases, an activated NK phenotype secondary to KIR (activating receptors of NK cells)-MHC-I genotypes has been found in human autoimmune diseases. For example, scleroderma and rheumatoid arthritis are associated with the presence of activating KIR2DS2 [45,46]. However, with regard to thyroid autoimmune diseases, in spite of the detailed characterization of the role of T and B lymphocytes and cytokines [22], scant evidences are present on the involvement of NK cells. Thus, it is know that thyroid cells from patients with autoimmune thyroid diseases but not from healthy controls upregulate MHC-II expression [47,48] and that MHC-I and TAP are overexpressed in autoimmune thyroid diseases but not in normal glands [47,49]. These results suggest a more efficient priming of TCD4+ and TCD8+ cells in response to autoantigens. The key cytokine involved in these alterations was IFN-γ, either in vitro [50,51] and in vivo [52].

Apparently in contrast with these consolidated findings, our data, indicating a critical role for ER stress in decreasing MHC-I molecules and in regulating both NK cytotoxicity and IFN-γ production in thyroid cells, may support a role of NK cells in autoimmune thyroid diseases.

However, the role in thyroid autoimmunity of NK and of TCD4+ and TCD8+ cells, sustained by a downregulation or upregulation of MHC-I, respectively, may be only apparently in contrast and not mutually exclusive. NK cells may be activated early in the autoimmune response, by MHC-I downregulation on thyroid cells, and may start to damage them and to produce IFN-γ. Interestingly, viral infections (that are vaguely associated with autoimmunity) are able to cause ER stress, and other environmental factors of thyroid autoimmunity [53], may also be able to cause ER stress and MHC-I downregulation. This may lead to IFN-γ-driven increased MHC-I, MHC-II and TAP expression on thyroid cells. Then, a more efficient priming of TCD4+ and TCD8+ cells will play a role in autoimmune disease progression.

Interestingly, increased [54], unchanged [55], and decreased [56] NK activity have been reported in thyroid autoimmune diseases, raising the possibility that in these reports different stages of the diseases may have been analyzed.

Besides thyroid autoimmunity ER stress-induced MHC-I down-regulation may be present in thyroid cancers, affecting the immune response toward cancer cells. We plan to pursue this additional aspect of our findings.

Moreover, in the case described here, an activated NK phenotype is not secondary to cell surface modifications of NK cells [45,46], but to modifications of the target cells, a mechanism particularly relevant for organ-specific autoimmune diseases. Thus, a better understanding of the connection of the UPR with autoimmune thyroid diseases, including its role in MHC-I antigen presentation pathway and possible implications for new clinical approaches, deserves to be further investigated.

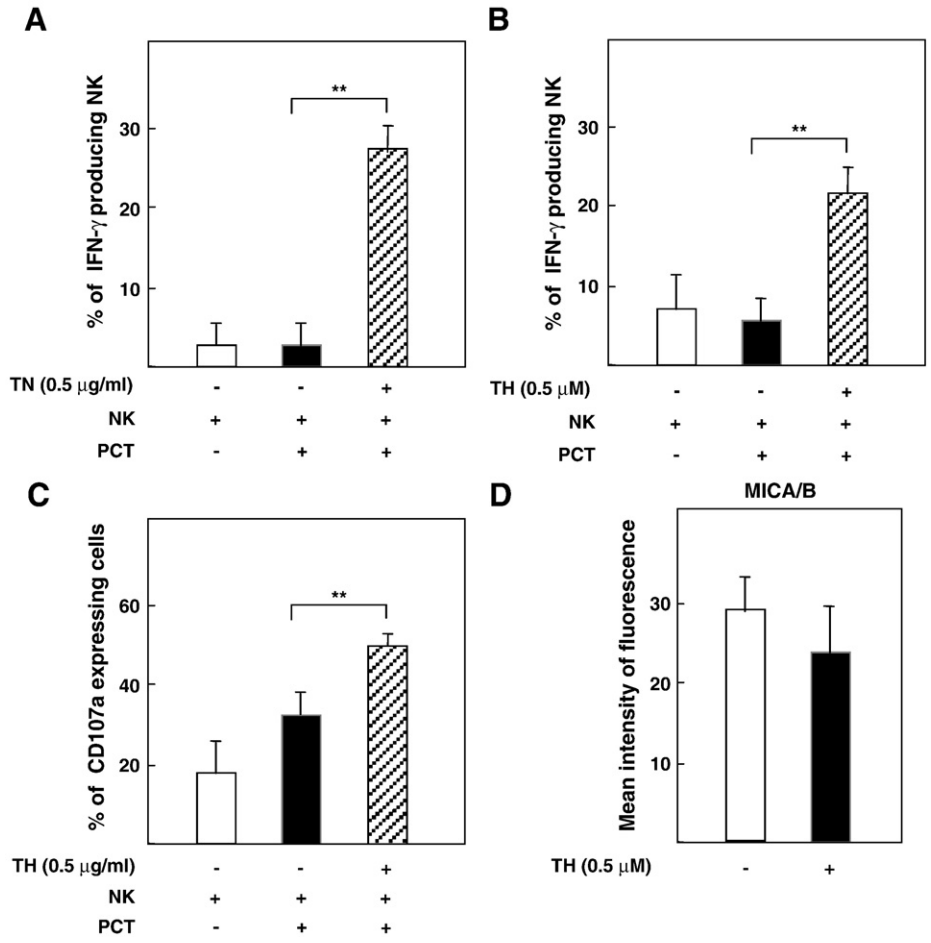

Fig. 6. Increased IFN-γ production and CD107a expression of NK cells incubated with primary thyroid cells undergoing ER stress. Unchanged expression of MICA/B in primary thyroid cells undergoing ER stress. Primary human thyroid cells were treated for 30 min with 0.5 μM TN (A) or 0.5 μM TH (B) and then the medium was replaced with fresh medium for 24 h. Then, cells were incubated with IL-2 activated polyclonal NK cells for a 6-h period. The flow cytometry analysis for the intracellular IFN-γ staining in NK is reported. Cells were stained with fluorescein isothiocyanate anti-IFN-γ monoclonal antibodies (mAbs). Mean and SD of the mean intensity of fluorescence of cells are shown ($n=3$). $^{**}p<0.01$. (C) Primary human thyroid cells were treated for 30 min with 0.5 μM TH and then the medium was replaced with fresh medium for 24 h. Then, cells were incubated with IL-2 activated polyclonal NK cells for a 6-h period and cell surface expression of CD107a molecules was tested by flow cytometry using PE-conjugated anti-CD107a antibodies. Mean and SD of the mean intensity of fluorescence of cells are shown ($n=3$). $^{**}p<0.01$. (D) Primary human thyroid cells were treated for 30 min with 0.5 μM TH and then the medium was replaced with fresh medium for 24 h. Then, cells were harvested and cell surface expression of MICA/B molecules was tested by flow cytometry using anti-MICA/B 6D4 antibodies. Mean and SD of the mean intensity of fluorescence of cells are shown ($n=3$). $^{**}p<0.01$.

Acknowledgments

This work was supported by the PRIN 2004062075_004 to B. Di Jeso and partly by the European Foundation for the Study of Diabetes (EFSD), the European Community's PREPOBEDIA (201681), grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC), and from the Ministero dell'Università e della Ricerca Scientifica (PRIN and FIRB: RBIP0689BS). The financial support of Telethon-Italy to F. Beguinot is also gratefully acknowledged.

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