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ORIGINAL ARTICLE

Interleukin 10 decreases MICA expression on melanoma cell surface

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Natural-killer group 2, member D (NKG2D) binds to a variety of ligands, including the major histocompatibility complex (MHC) class I chain-related proteins (MIC) and UL16-binding proteins (ULBP). It is regarded as a co-activating receptor on NK cells, having an important role in the cell-mediated immune response to tumours. We studied the influence of interleukin (IL)-10 on the regulation of MIC and ULBP expression on melanoma cells, and its effect on the cytotoxic function of NK cells *in vitro*. Here, we show that, in the presence of IL-10, FMS mel and BL mel cell lines decreased MICA and ULBP2 surface expression, whereas MHC class I did not change substantially on the cell surface. MICA mRNA levels decreased in IL-10-treated FMS and IL-10-transduced BL cell lines. Interestingly, we observed that MICB surface expression and its mRNA levels increased upon IL-10 treatment in a melanoma cell line. These changes in NKG2D ligands surface expression patterns owing to IL-10 treatment resulted in an effect on lysis susceptibility mediated by lymphocyte-activated killer cells, as tumour cell lines that displayed a higher decrease of MICA on their surface had lower levels of lysis. In addition, expression of CD107a was downregulated on the surface of NK cells following stimulation with IL-10-treated FMS cells. Our results suggest a novel function for IL-10 in the modulation of NKG2D ligand expression and in the control of cytotoxicity mediated by NKG2D/NKG2D ligand axis.
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Natural-killer group 2, member D (NKG2D) is a co-activating receptor expressed on NK cells.[1] It has also been identified on γδ T, αβ $CD8^+$ T cells and in NKT cells in human beings.[2] NKG2D binds to a variety of ligands that resemble the major histocompatibility complex (MHC) class I proteins.[3–5] In human beings, the UL16-binding proteins (ULBP),[6,7] also denominated retinoic acid early transcript 1,[8] and MHC class I chain-related proteins A and B (MICA and MICB, respectively)[3,4] have been described. Ligands for NKG2D (NKG2DL) are rarely detectable on the surface of healthy cells, but they can be upregulated by cellular malignant transformation, viral infection and cellular stress, among other stimuli, such as chemotherapeutic drugs.[8–11]

NKG2D and its ligands have a critical role in tumour immune surveillance.[5,12] In fact, NKG2D-deficient mice are defective in establishing a response against spontaneous malignancies.[13] In addition, there is evidence of an early induction of NKG2DL surface expression during spontaneous tumour genesis.[14] However, during the immunoediting process, tumour cells can interfere with NKG2D-mediated activity by different mechanisms, such as expression of tumour-derived NKG2DL by exosomes and release of soluble forms of NKG2DL by metalloproteases or alternative splicing.[2,15–18] These molecules interact directly with NK cells and $CD8^+$ T cells in an NKG2D-dependent manner, and strongly reduce the NKG2D-mediated cytotoxic function of NK and $CD8^+$ T cells.[19–21] Moreover, intracellular sequestration of immature forms of MICA in the endoplasmic reticulum leads to a significant reduction of NKG2DL expression on the tumour cell surface.[22] Hence, manipulation of NKG2DL expression on tumour cells may improve or worsen the effectiveness to evade the immune response. Furthermore, cytokines such as interferon gamma (IFN-γ) and transforming growth factor beta (TGF-β) have been found to have negative regulator properties. For instance, INF-γ downregulates NKG2DL expression and impairs NKG2D-mediated cytolysis of MHC class I-deficient melanoma cells by NK cells,[23,24] whereas TGF-β suppresses MICA and ULBP2 surface expression on malignant glioma cells.[25]

Interleukin 10 (IL-10) is a pleiotropic cytokine.[26] *In vitro*, this cytokine inhibits monocyte-MHC class II expression,[27] and affects human leukocyte antigen-A2 surface expression in some melanoma cell lines.[28] Also, IL-10 is produced by several human carcinoma

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lines,[29] and its mRNA is detected in solid tumours.[30] Increased IL-10 levels in the serum have been reported in patients with cancer, such as malignant melanoma.[31,32] Therefore, IL-10 contributes to immunosuppressive and anti-inflammatory effects by inhibiting the anti-tumour immune response.[33] In addition, the preferential expression of IL-10 in metastatic vs primarily lesions of melanomas has been established, indicating an increased metastatic potential of IL-10-secreting melanoma cells.[34]

As malignant melanoma expression of NKG2DL has been shown *in vitro* and *in situ*,[35–37] we decided to study the influence of IL-10 in the regulation of MICA/B and ULBP expression and its effect on *in vitro* cytotoxic functions of lymphocyte-activated killer (LAK) cells. In this work, we characterized the NKG2DL profile expression on melanoma cell lines. NKG2DL were detected at different levels on the surface of melanoma cells. We also show here that, in the presence of IL-10, tumour cells presented decreased MICA surface expression and mRNA levels. This response had an effect on NKG2D-dependent killing of melanoma cells by cytotoxic NK cells, as evaluated by chromium-release assays. Furthermore, cell-surface mobilization of CD107a (LAMP-1), a marker of intracytoplasmic cytolytic granules, was downregulated on NK cells stimulated with IL-10-treated melanoma cells. Therefore, our results suggest that IL-10 could have a facilitating role in the escape of immune surveillance by melanoma cells by regulating the expression of NKG2DL on the surface of malignant cells.

RESULTS

NKG2DL surface expression on melanoma cells is modulated by IL-10

To study the cell surface expression profile of other NKG2DL on human melanoma cells, we selected three metastatic melanoma cell lines: FMS mel, BL mel and FM55 mel, as well as the ocular melanoma cell line OCM-1 mel. Cell surface expression of MICA, MICB, ULBP1, ULBP2 and ULBP3 was determined by flow cytometry. The surface expression of NKG2DL was different for the distinct cell lines studied: while FMS mel and BL mel lines presented MICA, MICB, ULBP2 and ULBP3 on the surface, OCM-1 mel displayed ULBP2, ULBP3 and barely expressed MICB (Figure 1a). None of the analysed NKG2DL molecules were found to be expressed by the FM55 mel cell line either on the cell surface or at the mRNA level (data not shown). ULBP1 was expressed by none of the cell lines (data not shown). MHC class I was detected in all cell lines, except FM55 mel (Figure 1a, lower row).

Next, we studied the effect of IL-10 on MICA expression on the cell surface of FMS melanoma cell line. Results obtained by dose–response and kinetic assays showed that IL-10, at 200 U ml^{-1}, induced the highest reduction of MICA cell surface expression after 48 h of culture (Figures 1b and c).

To determine the influence of IL-10 on NKG2DL surface expression patterns, melanoma cell lines were incubated with this cytokine, and expression of NKG2DL was analysed by flow cytometry (Figure 1a). We observed that recombinant IL-10 (rIL-10) decreased the expression of MICA on FMS mel cells, whereas MICB surface expression was increased in the presence of the recombinant cytokine (Figure 1a, upper row). BL mel cells showed a different expression response upon rIL-10 treatment, as this cytokine induced decreased surface expression levels of MICA and ULBP2 on these cells, whereas MICB showed no changes (Figure 1a, second row). On FMS mel and OCM-1 mel cells, ULBP2 expression was slightly reduced (Figure 1a, third row). Surface levels of MHC class I and ULBP3 did not change significantly in the presence of IL-10 in any of the studied cell lines. On synthesis, stimulus with rIL-10 induces a decrease in MICA and ULBP2 and an increase in MICB surface expression in melanoma cell lines.

NKG2DL surface expression is modulated on *IL-10*-gene-transduced melanoma cell lines

In order to study whether autocrine IL-10 also modulates the expression of MICA and MICB on melanoma cell lines, we used BLP, BLH and BLV melanoma cell lines transduced with the following vectors:[38] mock Hy-Tk retrovirus (BLP), human IL-10 Hy-Tk retrovirus (BLH) and the Epstein–Barr virus *BCRF-I* gene product, viral IL-10 Hy-Tk vector (BLV) (Figure 2a). Firstly, we evaluated the endogenous expression level of these cells *in vitro*. The cells were incubated for 48 h in culture medium, after which IL-10 secretion was quantified by enzyme-linked immunosorbent assay (ELISA). BLH and BLV secreted 0.5 and 0.9 pg ml^{-1} of IL-10 into the medium, respectively (Figure 2b). These levels were significantly higher than those observed in the transduction control (BLP) ($P<0.001$).

Next, we determined the influence of autocrine IL-10 on NKG2DL expression. BL melanoma cells were incubated for 12, 24 and 48 h in culture medium, and MICA and MICB surface expression was analysed by flow cytometry. After 48 h of culture, the expression of MICA on IL-10-secreting BLH and BLV cells was significantly reduced in comparison with the transduction control (BLP) ($P<0.05$ and <0.001, respectively) (Figures 2c and d). Although the *G* mean values in each histogram for MICB and particularly for MHC class I showed changes among the tested cell lines (Figure 2c), the mean fluorescence intensity variations of MICB and MHC class I expression levels were not statistically significant in the three independent experiments carried out (Figure 2d). In summary, our data show that MICA surface expression is decreased on *IL-10*-gene-transduced BL melanoma cells, which is independent of the cytokine origin (whether it is human or viral), and that this decrease is directly related to the amount of cytokine secreted by the transduced cell line. Using confocal microscopy, no differences could be detected in the intracellular distribution of MICA and MICB, neither on BLP, BLH, BLV cells nor on melanoma cell lines treated with exogenous rIL-10 (data not shown).

IL-10 downregulates MICA mRNA levels

The decrease of MICA expression might be the consequence of a reduction in its mRNA level. Therefore, we determined the influence of IL-10 on MICA mRNA levels on FMS cells treated with 100, 200 and 400 U ml^{-1} rIL-10 for 48 h using quantitative real-time polymerase chain reaction (qRT-PCR). MICA mRNA levels decreased significantly after cytokine treatment at 100 U ml^{-1}, as shown in Figure 3a. On the other hand, MICB mRNA expression levels increased on the FMS melanoma cell line treated with 200 and 400 U ml^{-1} of rIL-10 (Figure 3d).

We also determined the influence of transduced IL-10 on MICA and MICB mRNA levels on BL cell lines by semiquantitative reverse transcriptase (RT)-PCR and qRT-PCR. In both techniques, IL-10-secreting cells (BLH and BLV) presented a decrease in MICA mRNA levels in comparison with their transduction control (BLP) ($P<0.05$) (Figures 3b and c), whereas MICB-mRNA levels increased on the same cell lines (Figures 3e and f) ($P<0.05$). On synthesis, both endogenous expression and treatment with rIL-10 decrease MICA mRNA levels and increase MICB mRNA levels on melanoma cell lines.

Blocking the IL-10 receptor prevents reduction of MICA expression on the surface of IL-10-treated melanoma cells

To evaluate whether IL-10 has a direct effect on the surface expression of MICA on melanoma cells, we blocked the IL-10 receptor using an

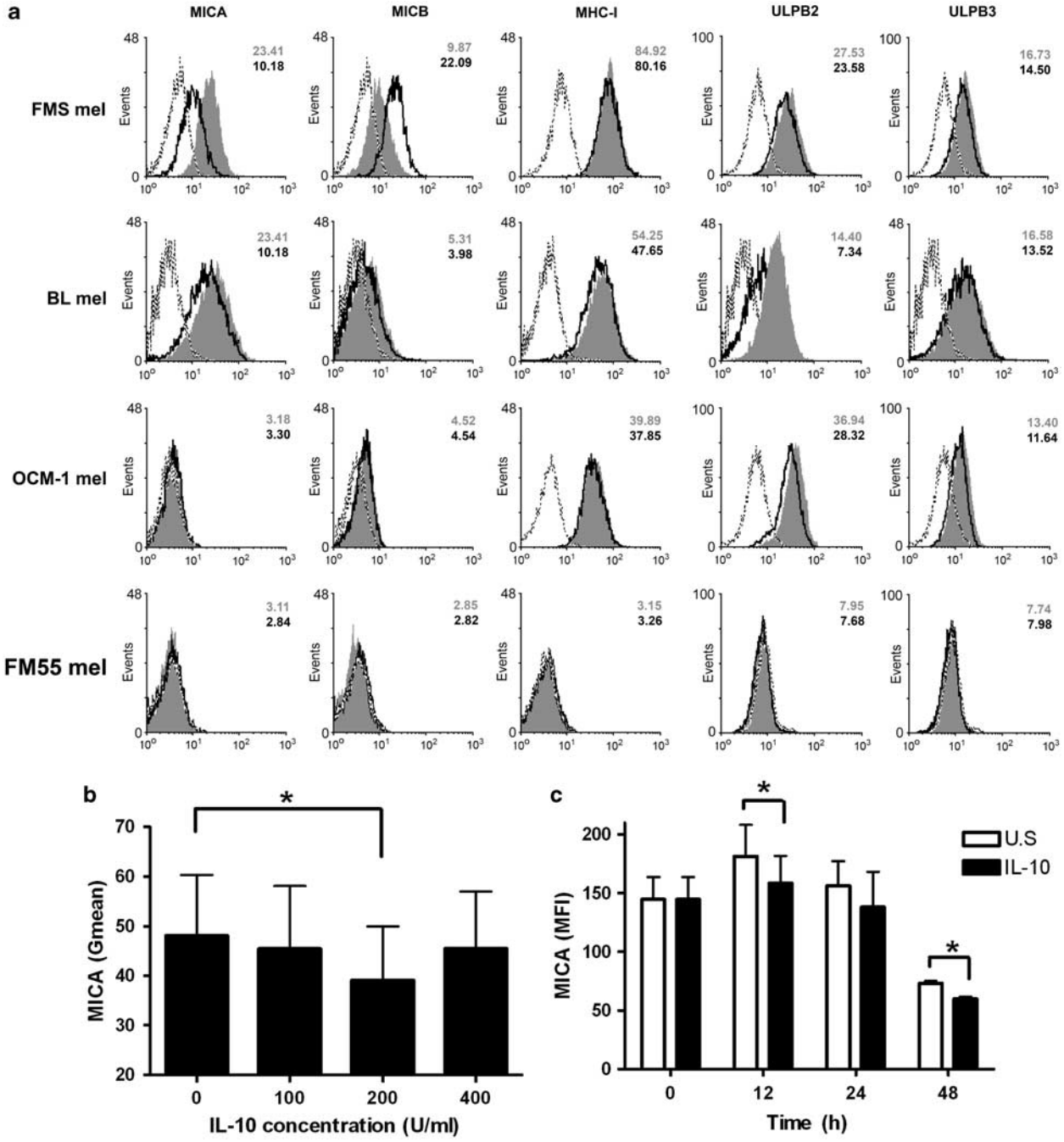

Figure 1 Modulation of NKG2D ligand surface expression upon IL-10 treatment. (**a**) The metastatic cutaneous melanoma cell lines FMS mel, BL mel and FM55 mel, and the ocular melanoma cell line OCM-1 mel were incubated for 48 h in the absence or presence of rIL-10 (200 U ml^{-1}). Surface expression of MICA, MICB, MHC class I, ULBP2 and ULBP3 molecules was determined by flow cytometry. Histograms show the expression of untreated (grey filled histograms) and IL-10-treated melanoma cells (thick lines). Dotted black histograms indicate control staining performed with IgG2b isotype. One representative out of three independent experiments is depicted. The *G* mean values of each determination for the untreated (grey number) and IL-10-treated cells (bold number) are also shown. (**b**) MICA expression levels on FMS cell surface under IL-10 treatment. Cells were incubated for 48 h in the absence or presence of 100, 200 or 400 U ml^{-1} rIL-10. MICA expression on the cell surface was then determined by flow cytometry. Statistically significant differences were detected between untreated and 200 U ml^{-1} rIL-10-treated cells. The results of five independent experiments are shown; $^{*}P<0.05$. (**c**) Kinetics of MICA expression levels on FMS cell surface under IL-10 treatment. Cells were incubated for 0, 12, 24 and 48 h in the absence or presence of 200 U ml^{-1} rIL-10. MICA expression on the cell surface was then determined by flow cytometry. Statistically significant differences were detected between untreated and rIL-10-treated cells at 12 and 48 h. The results of three independent experiments are shown; $^{*}P<0.05$.

inhibitor monoclonal antibody (mAb) (clone 3F9).[39] According to the literature,[40] we analysed phosphorylated STAT3 (p-STAT3) as a transduction signal to reveal the intracellular effect of rIL-10 on melanoma cells. Immunoblotting assays revealed that 200 U ml^{-1} of IL-10 was the maximum level of detectable p-STAT3 (Figure 4a). Next, to study the STAT3 signalling inhibitory effect of anti-IL-10 receptor

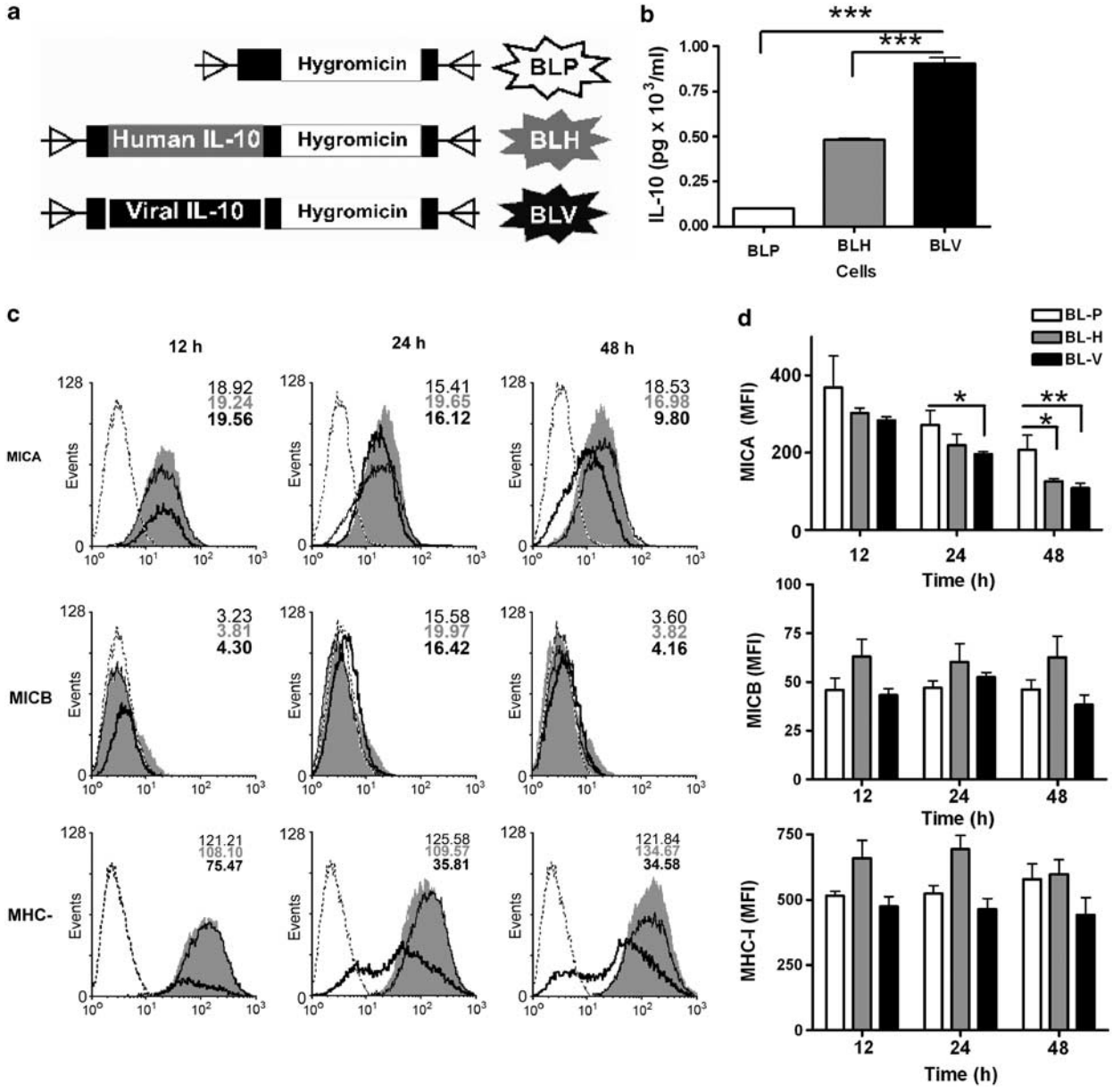

Figure 2 Melanoma cell lines transduced with the *IL-10* gene show differential surface expression of MICA and MICB. (**a**) Diagrams of BL melanoma cell lines transduced with retroviral constructs: mock Hy-TK vector (BLP), human *IL-10* gene (BLH) and viral *IL-10* gene (BLV). (**b**) Levels of IL-10 in the supernatant of BLH, BLV and BLP cell culture. Supernatants from cultures of 4×10^5 melanoma cells were harvested at 48 h, and quantification of IL-10 was performed by ELISA, as described in Materials and methods. Each graph represents one of three similar experiments. Error bars represent standard deviation of triplicate wells. (**c**) Surface expression of MICA, MICB and MHC class I molecules on IL-10-transduced BL melanoma cells. NKG2DL expression was determined at 12, 24 and 48 h of cell culture by flow cytometry. Histograms show the surface expression of these ligands on IL-10-transduced, BLV (thick lines) and BLH (thin lines) and empty vector-transduced BLP (grey filled histograms) melanoma cells. The *G* mean values of each determination for BLP (black numbers), BLH (grey numbers) and BLV (bold numbers) are also shown. One representative out of three independent experiments is depicted. (**d**) Mean fluorescence intensity (MFI) values for surface expression of MICA, MICB and MHC class I. For each MFI value, background MFI (staining with IgG2b isotype control) was subtracted. The results of three independent experiments is shown (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

antibody, we incubated FMS melanoma cells with 10 μg ml^{-1} of 3F9 antibody for 2 h previously to IL-10 stimulus. Interestingly, the activation effect of IL-10 on its receptor was reduced, as indicated by the detection of decreased levels of p-STAT3 (Figure 4b). In addition, anti-IL-10 receptor mAb also prevented the decrease of MICA on the cell surface of FMS cells treated with IL-10, as revealed by flow cytometry (Figure 4c), indicating that this cytokine directly controls the levels of MICA on melanoma cells.

IL-10 impairs NKG2D-mediated lysis of melanoma cells

Next, we addressed the impact of IL-10 on the cytotoxicity mediated by the NKG2D/NKG2DL system. For this purpose, we selected melanoma cell lines that presented differential expression of NKG2DL upon IL-10 treatment (Figures 1 and 2). First, we tested the capacity of these cells, which had not received previous treatment with IL-10, to challenge LAK cells at 20:1, 10:1 and 5:1 effector:target (E:T) cell ratios. Target cell lysis was then determined in^{51} Cr release

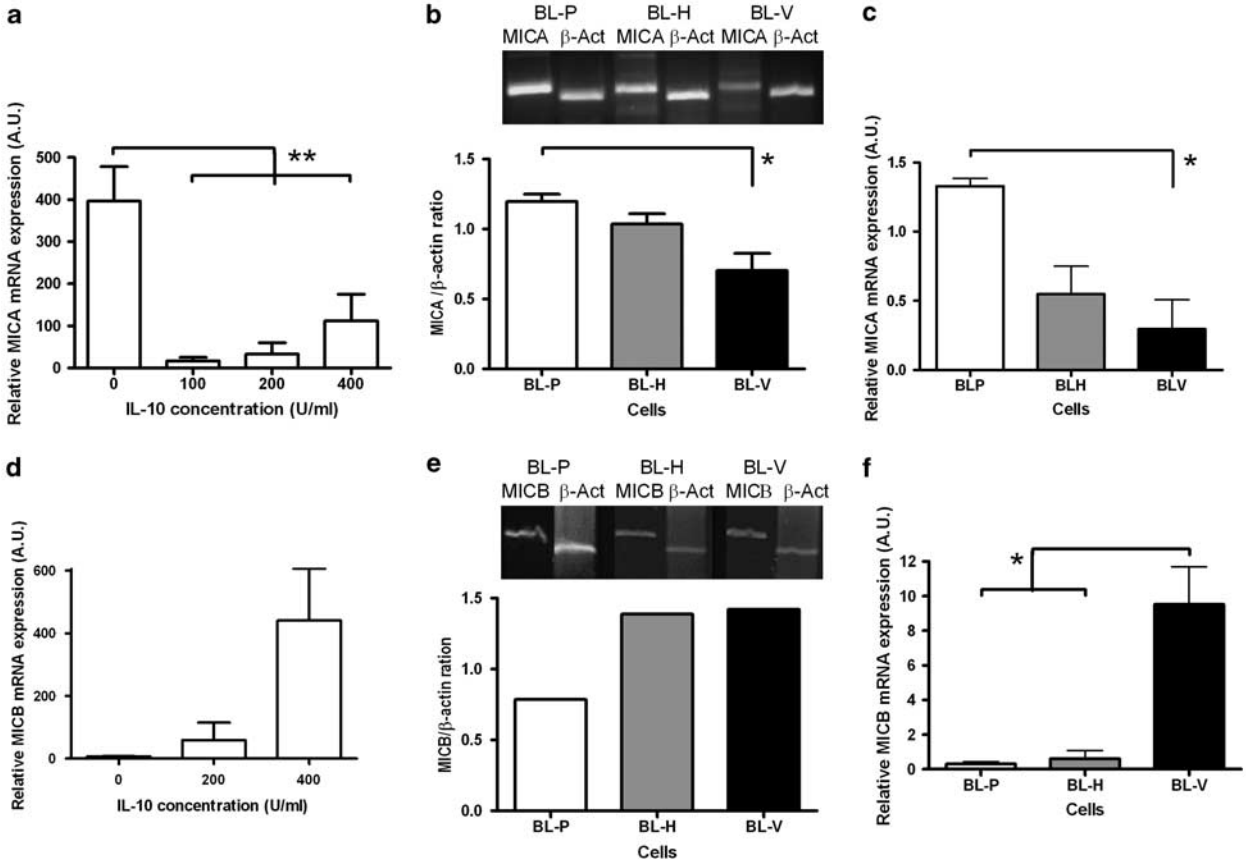

Figure 3 Relative changes in MICA and MICB mRNA expression levels in melanoma cell lines induced by IL-10. FMS cells were incubated with increased doses of human rIL-10 (0, 100, 200 and 400 U ml^{-1}) for 48 h, and evaluated for MICA (**a**) and MICB (**d**) mRNA levels by qRT-PCR, as described in Materials and methods. The graphs depict the results of arbitrary units (AU) in comparison with *GADPH* housekeeping gene and a reference control included in the experiment. (**b**) MICA and (**e**) MICB mRNA expression in IL-10-transduced (BLH and BLV) and mock Hy-TK vector empty-transduced (BLP) melanoma cell lines incubated for 48 h was determined by semiquantitative RT-PCR. Changes in ligand-specific mRNA levels, normalized to the internal β-actin mRNA levels, are indicated. Density analysis of three independent experiments for MICA and one assay for MICB are shown. qRT-PCR of MICA (**c**) and MICB (**f**) mRNA levels in the same melanoma cell lines. The graphs depict the results of arbitrary units in comparison with *GADPH* housekeeping gene and a reference control included in the experiment. Each graph represents one out of three similar experiments, except in (**e**), which represents one experiment. Error bars correspond to standard deviation of triplicates (*$P<0.05$; **$P<0.01$).

assays. At 20:1 E:T ratio, LAK cells effectively killed FMS mel (65% lysis) (Figure 5a), OCM-1 mel (45% lysis) (Figure 5b) and BL mel (30% lysis) cells (Figure 5c). In order to evaluate whether the lysis of melanoma cells was mediated by the NKG2D receptor, we incubated LAK cells with an mAb directed against NKG2D (clone 1D11), previous to cytotoxic assays. We observed that cytotoxicity towards these three melanoma cell lines was strongly inhibited by the anti-NKG2D mAb, as lysis of target cells in the presence of LAK cells treated with the blocking antibody was significantly lower than that of cells challenged with untreated effector cells (Figures 5a–c).

As rIL-10 decreases the expression of MICA on melanoma cells (Figure 1), the recognition of this ligand on target cells, as well as the activation of effector cells through NKG2D would be compromised. Therefore, we decided to determine the effect of rIL-10 in the cytotoxic capacity of LAK cells. We incubated FMS mel, OCM-1 mel and BL mel cells with 200 U ml^{-1} of rIL-10 for 72 h previous to cytotoxic assays. When treated with IL-10, FMS cells showed a decrease in the susceptibility to killing by LAK cells obtained from three out of five healthy donors (Figure 5a). However, this effect was not observed for OCM-1 (Figure 5b) or BL cells (Figure 5c). IL-10-transduced melanoma cells (BLH and BLV) were also used to stimulate LAK cells, and lower lysis susceptibility was observed in cell lines that overexpress IL-10 (Figure 5d). Thus, these results suggest that IL-10 exerts inhibitory effects on cytotoxicity mediated by the NKG2D receptor.

To isolate the effect of MICA and MICB on the cytotoxicity mediated by effector cells, we incubated BLP, BLH and BLV melanoma cell lines with a cocktail of 10 μg ml^{-1} mAbs directed to MICA (clone 159227), MICB (clone 236511) and MICA/B (clone D7), and incubated them with an enriched culture of NK cells at 20:1 E:T ratio. As shown in Figures 5e and f, the percentage of each transduced melanoma cell lysis did not change significantly when the MICA/B antibody cocktail was used, as compared with lysis of untreated melanoma cells and lysis of target cells in the presence of effectors treated with anti-NKG2D blocking antibody. The positive control for cell lysis, the K562 cell line, was used in all experiments, with over 50% cell lysis (data not shown). NK cells without previous activation with IL-2 did not lyse either IL-10-treated or -untreated melanoma cells lines (data not shown). In addition, we did not find differences in the spontaneous release of chromium by melanoma cells untreated or treated with rIL-10 when incubated with NK cells that had not received IL-2 stimulus (data not shown).

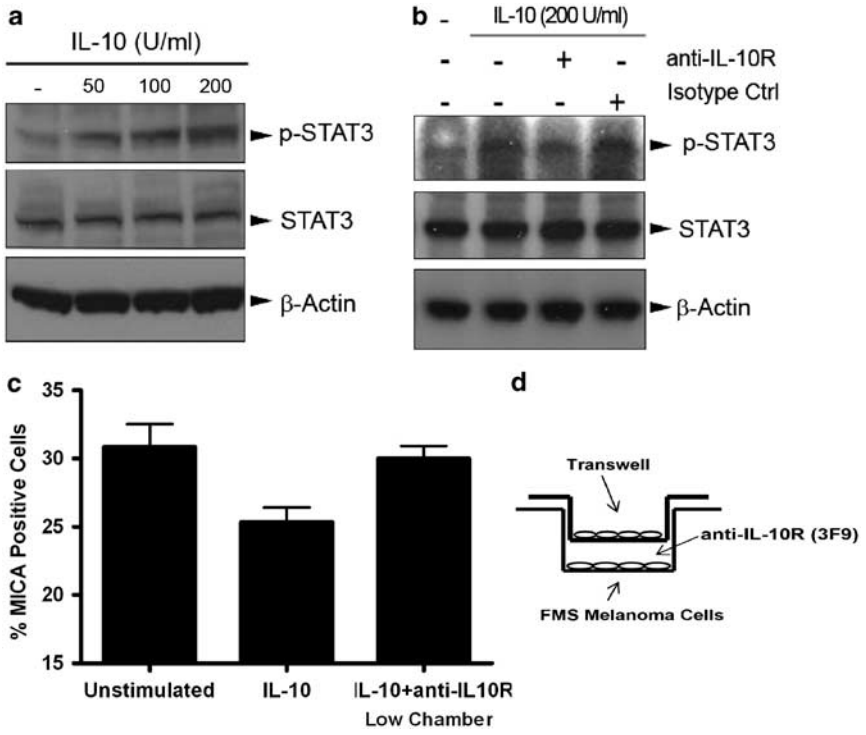

Figure 4 Blocking the IL-10 receptor prevents the reduction of MICA levels on melanoma cell surface. (**a**) Induction of p-STAT3 by IL-10 on melanoma cell line. The FMS melanoma cell line was incubated with increased doses of human rIL-10 (0, 50, 100 and 200 U ml^{-1}) for 30 min, and evaluated for p-STAT3, total STAT3 and β-actin (loading control) by immunoblotting. A representative experiment, out of three independent assays, is shown. (**b**) An anti-human IL-10 receptor mAb prevents the activation of p-STAT3 on FMS cell line. Cells were incubated for 2 h in the presence of 10 μg ml^{-1} of rat anti-human antibody (clone 3F9) and stimulated with 200 U ml^{-1} rIL-10. Cell extracts were analysed for p-STAT3, total STAT3 and β-actin. A representative, out of three independent experiments, is shown. (**c**) Percentage of MICA-positive cells was determined by flow cytometry after 48 h of FMS cell culture in the presence or absence of rIL-10 and/or blocking IL-10 R antibody. (**d**) Schematic diagram of a Transwell assay, which shows the position where the cells were seeded into the chambers.

NK cell activation is reduced upon challenge with IL-10-treated melanoma cells

CD107a is a marker of intracytoplasmic cytolytic granules that indicate effector cell degranulation.[41–43] Given the strong cytotoxic capacity of NK cells, we decided to assess the expression of CD107a on these cells to evaluate the effect of IL-10 as a potential inhibitor of NK cell activation. For this purpose, a CD107a degranulation assay was carried out, in which enriched cultures of NK cells from peripheral blood mononuclear cells (PBMCs) of healthy donors, other than those cells used for the cytotoxic assays (Figures 5a–d), were challenged with IL-10-treated FMS mel cells as effectors. Following a 4-h incubation period in the presence of anti-CD107a mAb and monensin, NK cells were stained for CD3 and CD56. Within the CD3$^-$/CD56$^+$ population of cells, we observed that CD107a expression was lower in NK cells stimulated with IL-10-treated FMS mel cells when compared with melanoma cells that received no IL-10 treatment, as indicated by the percentage of CD107a$^+$ NK cells under both stimulatory conditions (8.1 and 14.6% of NK cells, respectively) (Figure 5g) ($P<0.01$). CD107a was expressed by 21.5% of NK cells following co-incubation with K562 cells, whereas baseline activity (NK cells cultured alone) induced CD107a expression in only 4.6% of NK cells. Taken together, our data suggest that IL-10 impairs the functional activation of NK cells upon contact with melanoma target cells.

DISCUSSION

In this study, we showed that, in melanoma cells, IL-10 regulates the expression of NKG2DL and participates in the regulation of the functional activity of NK cells. There are several described mechanisms involved in the regulation of NKG2DL expression in tumour cells, such as enzymatic release of the molecules from the cell surface,[15–18] retention in the vesicular system[22] and regulation via microRNA.[44–46] Also, it has been described that MICA, and less frequent ULBP2, are downregulated in the majority of the analysed melanoma cell lines under IFN-γ treatment.[23,24] Another cytokine involved in the regulation of NKG2DL surface expression is TGF-β. It has been shown that this cytokine downregulates the transcription of human *MICA*, *ULBP2* and *ULBP4* genes.[25]

Here we provide evidence that IL-10, a pleiotropic cytokine secreted by several tumours, affects the expression of NKG2D ligands in melanoma cell lines. IL-10 was able to decrease MICA and ULBP2 surface expression in all cell lines that expressed them (Figure 1), as observed for other cytokines.[23–25] Nevertheless, IL-10 did not significantly influence the surface levels of ULBP-1 and ULBP3. We also observed an inverse association in the BL melanoma cell lines transduced with human *IL-10* (BLH) and viral *IL-10* (BLV) genes: the highest expression of IL-10 correlated to the lowest expression of MICA on the cell surface of these cells (Figure 2). In the IL-10-transduced melanoma cell model and exogenous IL-10-treated FMS mel cell line, we also detected a reduction of MICA expression on the cell surface over culture time (Figures 1c and 2d). In addition, our analyses revealed a decrease in MICA mRNA levels in FMS melanoma cell line treated with IL-10 (Figure 3a), an effect that could partially explain the reduction of MICA on the cell surface, suggesting transcriptional regulatory mechanisms. Moreover, using semiquantitative

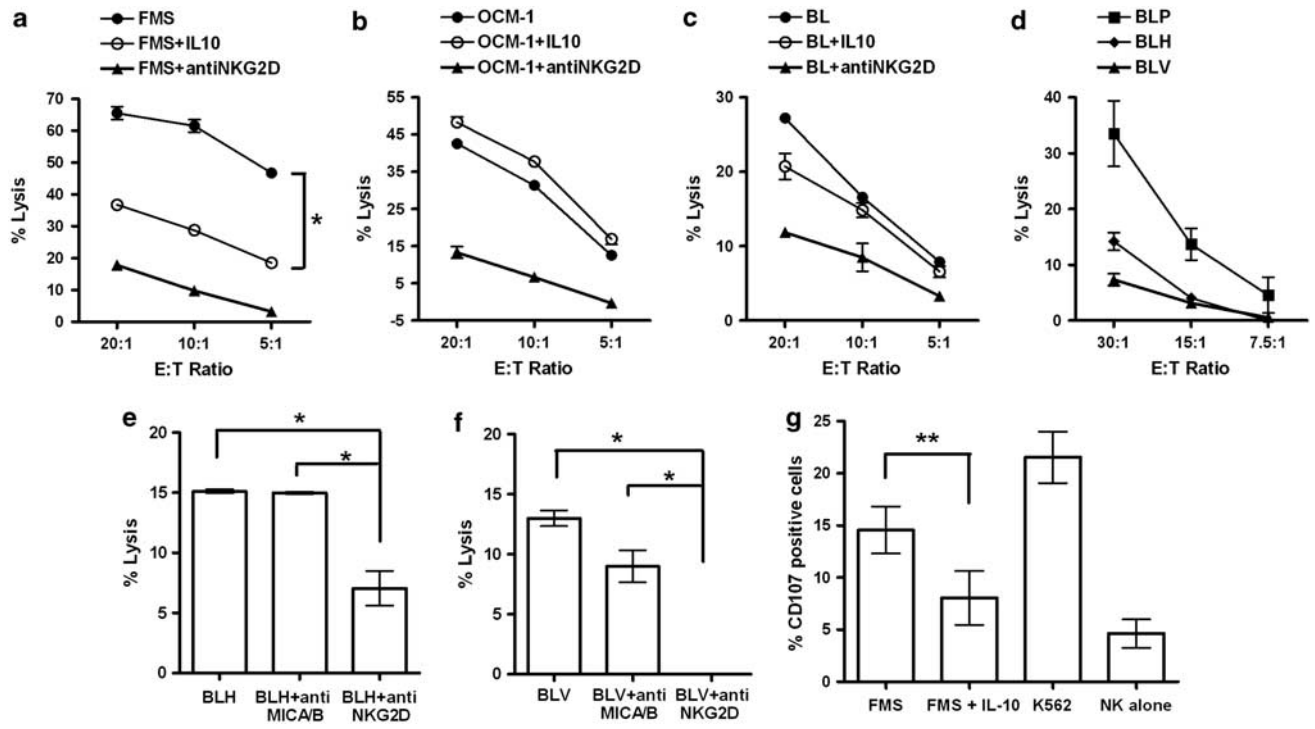

Figure 5 IL-10 modifies the sensitivity of melanoma cells for NKG2D-dependent LAK cell cytotoxicity. (**a**) FMS, (**b**) OCM-1 and (**c**) BL melanoma cell lines were incubated for 72 h in the absence (black circles) or presence of 200 U ml^{-1} rIL-10 (white circles) and analysed in a standard ^{51}Cr-release assay using LAK as effectors cells, as described in Materials and methods. To determine the impact of NKG2D-mediated signals on tumour cell killing, the LAK cells were incubated with an anti-NKG2D blocking antibody (clone 1D11) (5 μg ml^{-1}) for 30 min before co-incubation with ^{51}Cr-labelled melanoma cells (black triangles). Lysis values are given as mean of triplicate wells at 20:1, 10:1 and 5:1 E:T ratios. One representative out of three independent experiments is shown for the FMS mel cells, whereas one out of five and one out of three independent assays are shown for the OCM-1 mel and BL mel cell lines, respectively. (**d**) IL-10-transduced melanoma cell lines show lower susceptibility to NK cell lysis. BLP (squares), BLH (rhombi) and BLV (triangles) cell lines (**d**) were incubated for 48 h and analysed in a ^{51}Cr-release assay using enriched NK as effectors cells. One representative out of three independent experiments is shown. (**e**) BLH and (**f**) BLV cell lines were incubated with a cocktail of 10 μg ml^{-1} mAb against MICA, MICB and MICA/B before cytotoxic ^{51}Cr-release assay in a 20:1 E:T ratio using enriched NK cells incubated with anti-NKG2D mAb where it is shown. Error bars represent standard deviation of triplicate wells (*$P<0.05$). (**g**) Enriched NK cells isolated from PBMC from two healthy donors were activated with IL-2 (150 U ml^{-1}) for 12 h and incubated with FMS target cells, as described in Materials and methods, at 20:1 E:T ratio for 4 h in the presence of fluorescein isothiocyanate-conjugated anti-human CD107a and monensin. K562 cells were used as a positive control of NK cell activation. CD3$^-$/CD56$^+$ were analysed for CD107a expression. Results indicate the mean percentage ± standard deviation of reactive NK cells detected in two independent experiments, in triplicate wells; **$P<0.01$.

RT-PCR, we did not observe the expression of endogenous TGF-β in FMS melanoma cell line in the presence or absence of IL-10 (data not shown). We also performed a Transwell assay, which showed that the decrease of MICA on the cell surface could be attributable to the effect of IL-10 alone (Figure 4c). Our results showed different surface expression patterns for NKG2DL in each melanoma cell line analysed (Figure 1a). Interestingly, in FMS cells, we detected that MICB is increased on the cell surface (Figure 1a), as well as its mRNA levels (Figure 3d) upon IL-10 treatment. This phenomenon correlates with mRNA levels of MICB in BL cell lines transduced with *IL-10* genes (Figures 3e and f), but the cell surface expression of MICB on the IL-10-transduced melanoma model did not show any clear tendencies. Previous studies hypothesized that NKG2DL have different expression pathways.[23–25] *MICA* and *MICB* genes are highly polymorphic,[47] and share many sequences of response elements.[47,48] Here we observed that MICA and ULBP2 show similar behaviour under the same stimulus (IL-10), unlike what was seen for MICB (Figure 1a). However, this phenomenon, for the moment, is cell line specific.

FMS mel cells showed the highest variations in NKG2DL expression with IL-10 treatment (Figure 1a). In addition, an impact on susceptibility to NK cell lysis could be observed under treatment of these cells with this cytokine (Figure 5), as in ^{51}Cr release assays, the cytotoxicity exerted by LAK cells derived from three out of five healthy donors was reduced to almost 50% on IL-10-treated cells, probably due to a decrease in MICA and ULBP2 levels on the cell surface after IL-10 stimulus. It is known that MICA and ULBP2 behave in a similar way under the influence of TGF-β and IFN-γ.[23–25] Although IL-10 induced an increase in MICB expression on the cell surface, this stimulus was not sufficient to modulate cytotoxicity mediated by this ligand. This may be owing to the fact that MICB is normally briefly exposed on the cell membrane, unless some trafficking mechanisms are altered.[49] IL-10 treatment of the OCM-1 cell line, which does not express MICA, induced a slight decrease in ULBP2 expression (Figure 1a). This variation showed no difference between IL-10-treated and -untreated melanoma cells on five independent LAK-mediated lysis assays (Figure 5b). However, blocking of the NKG2D receptor reduced cell lysis on all melanoma cell lines tested (Figures 5a–f), implying that the cytotoxicity is dependent on NKG2DL. Hence, we speculate that this cell line expresses other NKG2DL (ULBP4–6),[8] which were not tested in this study and that may mediate cell lysis. Our results showed a slight reduction of MHC class I cell surface expression on melanoma cells treated with IL-10 (Figure 1a), a phenomenon also described by other authors,[28,50] who showed that human leukocyte antigen-A2 decreases in melanoma cell lines treated with IL-10. We consider that

this reduction is not sufficient to modify the inhibitory effect of these molecules on the cytotoxic potential of NK cells (Figure 5). In addition, the pattern of cytotoxic response observed when FMS mel cells (treated or untreated with IL-10) were used as target cells for primary NK cells from two unresponsive healthy donors (data not shown) is similar to that observed for IL-10-treated OCM-1 cells used to challenge NK cells from the five studied donors (Figure 5c). This response correlates with what has been observed by Schwinn *et al.*,[24] who showed that primary NK cells from different donors present a different lysis pattern when co-incubated with melanoma cell lines treated with IFN-γ. These authors speculate that target cells treated with IFN-γ might upregulate the surface expression of ligands that bind to stimulatory NK cell receptors other than NKG2D, such as DNA accessory molecule-1 and natural cytotoxic receptors (NCRs). According to the authors, this upregulation could, therefore, mask the impact of IFN-γ on the expression of NKG2D ligands on melanoma cells, which could reflect in variability on NK cell lysis capacity, depending on the donor. In our study, the effect of IL-10 on the expression of NKG2D ligands could also have been masked by signals received through other NK cell receptors,[51] resulting in a different response in target cell lysis by different NK cell donors. This response could be a result of the individual genetic background of each donor, NKG2D polymorphisms[52,53] or to the variation on MICA expression, as OCM-1 cells do not express this ligand (Figure 1). These are interesting topics to evaluate, as they could be related to susceptibility to cancer.

CD107a is a marker of NK cell activation and function.[54] In our study, the CD107a degranulation assay correlated with the[51] Cr release assay, as FMS cells treated with IL-10 were less susceptible to LAK cell lysis (Figure 5a), and induced lower CD107a cell-surface mobilization on NK cells (Figure 5g). These results do reflect the capacity of freshly isolated IL-2-activated NK cells from PBMC from healthy donors to be activated by target cells, as K562 cells, a highly undifferentiated human erythroleukaemic cell line that does not express MHC class I molecules, significantly induced upregulation of CD107a on the surface of the same NK cells (Figure 5g). However, the cytolytic potential of NK cells stimulated with melanoma cells could not be compared with IFN-γ secretion, as no statistically significant differences among target cells have been detected for the production of this intracellular cytokine (data not shown). The expression of CD107a can be detected in both IFN-γ-secreting and -non-secreting NK cells.[54] Therefore, it is possible that the NK cells selected for our experiments might not have been able to express this cytokine. On the other hand, the method applied by us to evaluate IFN-γ secretion might have presented some limitations. Fauriat *et al.*[55] have recently described that CD107a expression can be detected after 4 h of target and effector cells co-incubation, whereas a 6-h co-incubation time is ideal for the detection of IFN-γ expression, which differs from the 4-h period used by us and other authors.[56,57] Other studies have shown differences between NK cells from various donors in ^{51}Cr release cytotoxicity assays[24] and CD107a degranulation assays, and we hypothesized that this phenomenon explains the variability among our assays.

Therefore, in the present work, cell killing is clearly triggered by the activating mechanisms of cytotoxicity through the NKG2D/NKG2DL system, as the anti-NKG2D blocking antibody inhibited cell lysis (Figure 5). The effect of each NKG2D ligand could not be shown reliably because the monoclonal antibodies used to inhibit MICA and MICB molecules were not as effective as the blocking anti-NKG2D mAb (Figures 5e and f). The inhibition of cytotoxicity observed when the NKG2D blocking mAb was used reflects the importance of this system on lysis and subsequent elimination of tumour cells.[13] Other authors have also described the role of other activation receptors on NK cells, such as the NCRs and the DNA accessory molecule-1 on the recognition and elimination of melanoma cells.[58] These authors highlight the effect of both receptors on the elimination of malignant melanoma cell lines over the activity of the NKG2D receptor. However, these authors used cell lines that express low amounts of NKG2D ligands or that do not express these ligands at all, whereas the melanoma cell lines studied in the present work constitutively present NKG2DL on the cell surface, which shows that the NKG2D/NKG2DL system does have an important role in tumour elimination. According to previous studies,[59] expression of NCR, DNA accessory molecule-1 and NKG2D ligands on myeloma cell lines is temporarily different; moreover, expression of ligands to NCR and NKG2D receptors present an inverse correlation on the cell surface, showing the complexity in the relation between tumours and NK cells. In addition, the activation of the NCR and NKG2D receptors is synergic,[60] such as in the case of 2B4 molecule and intercellular adhesion molecule-1.[55] Therefore, it would be interesting to evaluate, in future investigations, the expression of these receptors ligands on the cell surface of the melanoma cell lines used in this study in order to describe the behaviour and function of these ligands in the presence of IL-10.

NK receptor ligands on melanoma cells that are modulated by IL-10 have not been described previously. The untransduced melanoma cell lines used in this study were previously screened by ELISA after 48 h of cell culture, in order to evaluate the endogenous secretion of IL-10. Marginal levels of this cytokine were detected (data not shown). After 4 h of co-culture of target melanoma cells with cytotoxic effector cells, the presence of soluble IL-10 in the medium would be negligible, and would probably not affect the lysis mediated by these effector cells.

The role of IL-10 in the regulation of MHC class I has been described, affecting antigen-specific CD8 lymphocyte cytotoxicity.[28] We presented here evidence that IL-10 participates in the modulation of cytotoxicity mediated by the NKG2D/NKG2DL system, expanding the knowledge about the effect of the microenvironment in the capacity of malignant cells to evade the immune response. In this sense, besides the immunosuppressive activity of IL-10, it possibly acts as a negative surface regulator of NKG2DL, such as IFN-γ and TGF-β,[23–25] as it has been shown that TGF-β is capable of decreasing the expression of NKG2DL on tumour cells *in vivo*.[61,62] Therefore, this phenomenon may be added to other post-translational mechanisms of immune evasion, such as endocytosis or shedding of NKG2DL from the membrane.[49,63] We have also observed in this study that viral IL-10 could modulate MICA cell surface expression as efficiently as human IL-10 (Figures 2, 3 and 5), showing a novel function of the *BCRF-1* gene, as described by others.[64,65]

STAT3 is constitutively activated in several human cancers, including melanoma,[66] and p-STAT3 is currently a target in antitumour research. We showed the basal level of p-STAT3 in a melanoma cell line used in this study (Figures 4a and b). The treatment with IL-10 was able to increase the activation state of this pathway, a phenomenon reversed by the use of an anti-IL-10 receptor blocking antibody. In some tumours, STAT3 can induce the activation of metalloproteinases, which can modulate the cell surface expression of MICA.[15] An important role of matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, in tumour development has been well established.[67] A study using a mouse melanoma model has shown that *MMP-2* is a direct target gene of STAT3, and that melanoma metastatic ability is STAT3 dependent.[68] Therefore, we hypothesize that IL-10 affects the surface level of MICA in melanoma cell lines by the STAT3–MMP pathway. However, this speculation requires further investigation.

Finally, we provide here new contributions to the understanding of the behaviour of the NKG2D system and its ligands, which may be relevant to the knowledge of the mechanisms related to immunosurveillance and to the discovery of new strategies for cancer treatment.

METHODS

Cell lines

BL mel and FMS mel melanoma cell lines were derived from metastatic lesions of patients treated at Radiumhemmet, Karolinska Hospital (Solna, Sweden). The FM55 mel melanoma cell line was obtained from the Danish Cancer Society by the courtesy of Dr J Zeuthen. The OCM-1 mel is an ocular melanoma line kindly provided by Dr M Jager (University of Leiden, Leiden, the Netherlands). The retroviral-transduced BL melanoma cell lines BLP, BLH and BLV were generated in a previous work using the construct Hy-TK-human *IL-10* gene retrovirus, Hy-TK-viral *IL-10* gene retrovirus and Hy-TK-mock retrovirus, respectively.[38] The transduced cells were selected and maintained with 300 and 20 $\mu g\,ml^{-1}$ of hygromycin B, respectively (Invitrogen Life Technologies, Gaithersburg, MD, USA). All melanoma cell lines and human K562 erythroleukaemia cells (ATCC, Pennsylvania, PA, USA) were maintained in RPMI 1640 culture medium (Hyclone, Logan, UT, USA) supplemented with 5% foetal calf serum (FCS), 1 mM glutamine and 100 $mg\,ml^{-1}$ penicillin–streptomycin (Hyclone).

Enriched NK and LAK cell culture

PBMCs were isolated from whole blood or buffy coats from healthy donors (Blood Bank, Hospital Clínico, Universidad de Chile, Santiago, Metropolitana, Chile) by separation with Ficoll–Hypaque (GE Healthcare AB, Stockholm, Sweden). The enrichment of NK cells was obtained by negative selection using NK Cell Isolation Kit and MS or LS columns (all from Miltenyi Biotec, Cologne, Germany) from 50×10^6 PBMC, following the manufacturer's instructions. The eluted-enriched NK suspension was activated with 150 $U\,ml^{-1}$ of rIL-2 (Tecnofarma, Santiago, Chile) for 12 h. The analysis of NK cell surface phenotype using phycoerythrin (PE)-conjugated anti-CD14 (BD Biosciences, San Jose, CA, USA), allophycocyanin-conjugated anti-CD3 (eBiosciences, San Diego, CA, USA) and PE-conjugated anti-CD56 (eBiosciences) or fluorescein isothiocyanate-conjugated anti-CD56 (BD Biosciences) was performed by flow cytometry (FACScanto; BD Biosciences, Heidelberg, Germany). For LAK assay, 1.5×10^6 per ml PBMCs were cultured in RPMI medium and 10% FCS with 300 $U\,ml^{-1}$ of rIL-2 for at least 5 days.

Treatment of tumour cells with IL-10

Melanoma cells were harvested using phosphate-buffered saline (PBS)-EDTA 2 mM solution and seeded for 24 h before IL-10 treatment. Cells (3×10^5) in 1.5 ml per well were incubated in RPMI 1640 medium and 3% FCS in a six-well plate (Falcon BD, San Jose, CA, USA) in the absence or presence of 100, 200 or 400 U of recombinant human IL-10 (rIL-10) (R&D Systems, Minneapolis, MN, USA) for 12, 24 and 48 h. The adherent cells were recovered with PBS-EDTA 2 mM solution, and prepared for staining.

To isolate the IL-10 effect, a Transwell assay was performed in a 24-well plate (Corning, Boston, MA, USA), in which FMS melanoma cells (1×10^5 cells) were seeded and pre-incubated for 2 h in the lower chamber with an IL-10 receptor-neutralizing antibody (clone 3F9) (Biolegend, San Diego, CA, USA). Later, cells were washed three times with PBS and the same amount of unblocked melanoma cells were seeded in the upper chamber. RPMI supplemented with 1% FCS and 200 $U\,ml^{-1}$ of rIL-10 was added, and the cells were maintained for 48 h at 37 °C. Cells from the lower chamber were harvested, and flow cytometry analysis was performed.

Flow cytometry analysis

After cell washing, melanoma cell lines (2×10^5 cells in PBS-1% FCS), either untreated or stimulated with rIL-10, were incubated with 40 $\mu g\,ml^{-1}$ of anti-MICA mAb (clone 159227), anti-MICB mAb (clone 236511), anti-ULPB1 mAb (clone 170818), anti-ULBP2 mAb (clone 165903) or anti-ULPB3 mAb (clone 166510) (all anti-human molecules from R&D Systems). Anti-human leukocyte antigen-A, -B and -C mAb (clone w6/32), mouse immunoglobulin G (IgG)2b and mouse IgG2a (isotype controls) were purchased from eBiosciences. Cells were incubated for 30 min on ice, washed three times with PBS-1% FCS and incubated with 20 $\mu g\,ml^{-1}$ of secondary fluorescein isothiocyanate -conjugated goat anti-mouse antibody (KPL, Gaithersburg, MD, USA) for 30 min on ice. For IL-10 receptor neutralization assays, a PE-conjugated anti-human MICA mAb was used (R&D Systems). After staining, cells were washed three times with PBS and fixed with 1% paraformaldehyde in PBS containing 0.1% FCS. Flow cytometry was performed using a FACScanto flow cytometer, acquired by the CellQuest program (BD Biosciences) and analysed using the WinMDI version 2.9 software (J Trotter, Scripps Research Institute, La Jolla, CA, USA). Averages of 1×10^4 events were collected and analysed per sample.

Semiquantitative RT-PCR

For semiquantitative RT-PCR, 3×10^5 cells in 2.5 ml per well of each melanoma cell line were cultured in a six-well plate (Falcon BD) for 48 h and lysated with Chomczynski reagent (Winkler, Santiago, Chile). Total RNA (free of genomic DNA) was isolated using the standard protocol of RNA extraction.[69] The integrity of total RNA was confirmed by 1.5% agarose gel electrophoresis in MOPS buffer, pH 7.0, using RNA ladder (Fermentas, Glen Burnie, MD, USA). The reverse transcriptase reaction was performed for 1 h at 37 °C using 100 pmol of polythymidine oligo (Alpha DNA, Montreal, QC, Canada), 2 mM dNTPs (Bioaxis, Santiago, Chile) and 200 U Moloney murine leukaemia virus reverse transcriptase (Promega, Madison, WI, USA). Specific amplification of MICA cDNA (forward primer, 5′-GGGACAGTGGGCAGA-3′; reverse primer, 5′-TTCCAGGGATAGAAG-3′), MICB cDNA (forward primer, 5′-AGGGAC AGTGGGCAG-3′; reverse primer, 5′-GGGGCACTGTTCTCC-3′) and β-actin cDNA (forward primer, 5′-GCCAACCGCGAGAAGATGACC-3′; reverse primer, 5′-CTCCTTAATGTCACGCACGATTTC-3′) was carried out by PCR using *Taq* polymerase (Stratagene, Cedar Creek, TX, USA). The cycling conditions were as follows: 2 min at 94 °C, followed by 28 (MICA) or 30 (MICB) cycles, 30 s at 94 °C, 30 s at 56 °C, 45 s at 72 °C and a final stage of 10 min at 70 °C. Samples were analysed in triplicates. PCR products were run on 2% agarose gels, stained with ethidium bromide and visualized by a ultraviolet transilluminator. The intensity of the bands was calculated using Scion Image Software (Amherst, MA, USA). The ratio of MICA and MICB mRNA expression levels was obtained by the quotient with internal β-actin mRNA levels. All oligonucleotides were synthesized by IDT (Coralville, IA, USA).

Quantitative reverse transcriptase-polymerase chain reaction

For quantification of MICA and MICB transcripts by qRT-PCR, Invisorb Spin Cell RNA Mini Kit (Invitek, Berlin-Buch, Denmark) and Brilliant SYBR Green qRT-PCR, AffinityScript Two-Step Master Mix (Stratagene) were used, in accordance with the instructions of the manufacturer using the Stratagene MX3000P real-time system. The specific primers used were as follows: MICA forward, 5′-CTGGCTGGCATCTTCCCTTTT-3′, MICA reverse, 5′-CTCCTGG TGCTGTTGTCTTC-3′; MICB forward, 5′-TCTCACCAGCACTTTCCCTC T-3′, MICB reverse, 5′-TCTTCCACAGCCCTTCGTATTT-3′; and GADPH forward, 5′-GTCAGCCGCATCTTCTTTTG-3′, and GADPH reverse, 5′-CATC GCCCCACTTGATTTTG-3′. All oligonucletotides were synthesized using Alpha DNA (Montreal, Quebec, Canada). The cycling conditions were one cycle of 10 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 56 °C, 30 s at 72 °C and 7 s at 78 °C, followed by a dissociation curve period of 60 s at 95 °C, 30 s at 55 °C and 30 s at 95 °C. Changes in MICA and MICB expression levels normalized to the internal GADPH mRNA levels and arbitrary values were used for comparison with a reference control. Real-time PCR data acquisitions and the analysis of the results were performed using the Stratagene Mxpro-Mx3000P software.

Enzyme-linked immunosorbent assay

Melanoma cells (4×10^5) were cultured in 0.5 ml RPMI supplemented with 10% FCS for 48 h in a 24-well plate (Falcon BD). The supernatants from triplicate wells were collected and analysed to quantify human IL-10 using the OptEIA Kit (BD Biosciences), following the manufacturer's instructions. Horseradish peroxidase activity was assessed by the addition of 2–2′-azino-di-(3-ethyl-benzthiazoline sulphonic acid) with H_2O_2 (Sigma-Aldrich, St Louis, MO, USA). Optical density was read at 405 nm in an automated ELISA reader (Bio-Rad, Hercules, CA, USA).

Analysis of STAT3 activity in melanoma cells

FMS melanoma cells were plated in six-well culture plates (8×10^5 cells per well) in 1 ml of complete medium containing 3% FCS. Cells were cultured for 2 h at 37 °C. rIL-10 (Prospec, Rehovot, Israel) was added for 30 min, after which cells were suspended at 4 °C in ice-cold radioimmunoprecipitation buffer plus a protease and phosphatase inhibitor cocktail (Sigma-Aldrich, Munich, Germany). Equal amounts of protein were resuspended in Laemmli 4× sample buffer; extracts were separated through a gradient of 4–12% sodium dodecyl sulphate-polyacrylamide gels (Invitrogen Life Technologies), transferred to nitrocellulose membranes (Bio-Rad) and incubated with mouse phosphor-specific (Tyr705) STAT3 mAb (diluted 1:1000) and rabbit STAT3 mAb (Millipore, Billerica, MA, USA). In addition, anti-β-actin mAb (Zymed, Carlsbad, CA, USA) was used as charge control. Enhanced chemiluminescence western blotting detection reagents (Amersham, Buckinghamshire, UK) were used.

Chromium (^{51}Cr)-release cytotoxicity assay

The ^{51}Cr-release assay was performed as described previously.[70] Briefly, 1×10^6 IL-10-treated and -untreated melanoma cell lines and K562 cells were labelled with 5–10 μCi of ^{51}Cr radionuclide chromium chloride in 0.5 M HCl (Perkin-Elmer, Waltham, MA, USA) for 1 h at 37 °C. After four abundant washes with RPMI media, target cells were co-incubated at different ratios with NK or LAK cells. After 4 h, 75 μl of the culture supernatants were collected and analysed for the presence of radioactivity by gamma counting (Packard Cobe Spectra Gamma Counter, Stamford, CT, USA). To determine the spontaneous release of ^{51}Cr from target cells (counts per min (c.p.m.) spontaneous), supernatants from wells containing only target cells (treated or untreated with rIL-10) were used. To determine the maximal release of ^{51}Cr from target cells (c.p.m. maximal), 1% of Tween 20 was added to the wells. Cytotoxic activity was calculated as the percentage of specific ^{51}Cr release using the following equation: % lysis=((c.p.m. experimental−c.p.m. spontaneous)/(c.p.m. maximal−c.p.m. spontaneous))×100. Experiments were performed in triplicate wells. For blocking experiments, anti-NKG2D mAb (clone 1D11) (eBiosciences) was added at 10 μg ml^{-1} to the NK/LAK cells during 30 min, and cells were then washed twice with PBS before co-incubation with target cells. As a control, effector cells were treated with an isotype IgG2b antibody (eBiosciences) under the same conditions. For treatment with IL-10, target cells were incubated for 72 h with 200 U ml^{-1} rIL-10 (R&D Systems), and then cells were washed in PBS and the adherent cells were recovered with PBS-EDTA 2 mM. Cell pellets were labelled with ^{51}Cr and tested for NK cell lysis susceptibility in a ^{51}Cr release assay, as described previously. The basal activity of NK cells that had not been previously activated with IL-2 and which were co-incubated with melanoma and K562 cells was also analysed.

CD107a mobilization assay

FMS cells, either untreated or treated with rIL-10 (200 U ml^{-1}) for 72 h, were used as target cells. An enriched culture of IL-2-stimulated NK cells was used as effector cells. To detect spontaneous degranulation, a negative control sample without target cells was included. The K562 cell line was used as a positive control for NK cell activation. An E:T ratio of 20:1 (1×10^5 effector cells:5×10^3 target cells in a total volume of 200 μl) was used. Fluorescein isothiocyanate - conjugated anti-human CD107a mAb (BD Biosciences) was added in each well (4 μl per well) before incubation. Monensin (BD Biosciences), at a final concentration of 2 mM, was also added before incubation to prevent the acidification of endocytic vesicles, avoiding the degradation of reinternalized CD107a proteins from the surface and allowing for the visualization of this marker following stimulation.[54] Effectors and targets cells were then co-incubated at 37 °C for 4 h. Cells were washed with PBS-1% FCS and stained for 30 min with a mixture of allophycocyanin-conjugated anti-human CD3 (eBiosciences) and PE-conjugated anti-human CD56 (eBiosciences). Thereafter, cells were fixed and permeabilized with BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences), according to the manufacturer's directions. Alternatively, intracellular IFN-γ was stained with PE-Cy7-conjugated anti-human IFN-γ mAb (BD Biosciences) for an additional 30 min. Surface expression of CD107a and intracellular detection of IFN-γ on CD3$^-$CD56$^+$ NK cells were analysed by flow cytometry. For each sample, a total of 10 000 events were acquired and analysed using the FlowJo software (version 7.6) (Tree Star Inc., Ashland, OR, USA).

Statistical analysis

Differences between paired data were calculated by the Student's *t*-test. Two-way analysis of variance with Bonferroni post-test was used in kinematic/IL-10-transduced cell line assays by flow cytometry and the CD107 mobility assay. For the analyses of ^{51}Cr release cytotoxic assays and the experiments involving endogenous secretion of IL-10-gene-transduced melanoma cells, one-way analysis of variance followed by Tukey's multiple comparison test was used. mRNA experiments were analysed with one-way analysis of variance with Bonferroni's multiple comparison test. All data were analysed with the GraphPad Prism software (version 4) (La Jolla, CA, USA); $P<0.05$ was considered statistically significant.

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