Natural Killer (NK)/melanoma cell interaction induces NK-mediated release of chemotactic High Mobility Group Box-1 (HMGB1) capable of amplifying NK cell recruitment

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Abbreviations: 2-DE, two-dimensional gel electrophoresis; CXCL12, chemokine (C-X-C motif) ligand 12; CXCR4, chemokine (C-X-C motif) receptor 4; DAMP, Damage Associated Molecular Pattern; DC, Dendritic Cell; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FBS, Fetal Bovine Serum; FHIL2, Familial Hemophagocytic Lymphohistiocytosis 2; GAM, Goat Anti-Mouse; HMGB1, High Mobility Group Box-1; IFN, Interferon-; IL, Interleukin; KIR, Killer Ig-like Receptor; LPS, lipopolysaccharides; mAb, monoclonal antibody; NCRs, Natural Cytotoxicity Receptors; NK cells, Natural Killer cells; NMDA, N-Methyl-D-Aspartate; PHA, phytohemagglutinin; RAGE, Receptor for Advanced Glycation End products; SDS/PAGE, Sodium Dodecyl Sulfate/PolyAcrylamide Gel Electrophoresis; TIM-3, T-cell Immunoglobulin- and mucin-domain-containing Molecule-3; TLRs, Toll-like Receptors.

In this study we characterize a new mechanism by which Natural Killer (NK) cells may amplify their recruitment to tumors. We show that NK cells, upon interaction with melanoma cells, can release a chemotactic form of High Mobility Group Box-1 (HMGB1) protein capable of attracting additional activated NK cells. We first demonstrate that the engagement of different activating NK cell receptors, including those mainly involved in tumor cell recognition can induce the active release of HMGB1. Then we show that during NK-mediated tumor cell killing two HMGB1 forms are released, each displaying a specific electrophoretic mobility possibly corresponding to a different redox status. By the comparison of normal and perforin-defective NK cells (which are unable to kill target cells) we demonstrate that, in NK/melanoma cell co-cultures, NK cells specifically release an HMGB1 form that acts as chemoattractant, while dying tumor cells passively release a non-chemotactic HMGB1. Finally, we show that Receptor for Advanced Glycation End products is expressed by NK cells and mediates HMGB1-induced NK cell chemotaxis. Proteomic analysis of NK cells exposed to recombinant HMGB1 revealed that this molecule, besides inducing immediate chemotaxis, also promotes changes in the expression of proteins involved in the regulation of the cytoskeletal network. Importantly, these modifications could be associated with an increased motility of NK cells. Thus, our findings allow the definition of a previously unidentified mechanism used by NK cells to amplify their response to tumors, and provide additional clues for the emerging role of HMGB1 in immunomodulation and tumor immunity.

Introduction

Thanks to their innate capability of killing transformed cells, Natural Killer (NK) cells represent a potential powerful tool to fight cancer.1-4 Nevertheless, the insurgence and progression of malignancies can significantly influence the efficacy of such effector cells in controlling tumors either at a systemic or at a local level.5,6 In particular, at the tumor site, the local microenvironment may limit the availability of efficacious NK cells by suppressing their effector functions or by inhibiting their recruitment to the tumor...
nests. Recent studies have suggested how in different solid tumors the NK cell infiltrate could be often poor or represented by altered cells.

While several mechanisms of tumor-mediated suppression of NK cells have been described, there is still limited information on how NK cell migration is regulated within the tumor microenvironment. Depending on the type of stromal, immune, and cancer cells, different chemokines can be produced in the tumor microenvironment. Moreover, the tumor itself can also affect the chemokine receptor pattern of NK cells. Besides chemokines, additional factors may regulate immune cell migration within the tumor tissue. For example, High Mobility Group Box-1 (HMGB1) is endowed with chemotactic properties and can be released within the tumor. However, its effect on NK cells has not yet been investigated. This molecule, generally located in the nucleus as a transcription regulator, can be actively exported, upon appropriate stimuli, by different innate immune cells including monocytes, macrophages, and NK cells. In addition, it can be passively released by dying cells. Extracellular HMGB1 operates as Damage Associated Molecular Pattern (DAMP) molecule capable of promoting the local inflammatory response. It can specifically modulate cell proliferation, differentiation, migration, gene expression, and cytokine release, depending on its interaction with various surface receptors including Toll-like Receptor (TLR) 2 and 4, Receptor for Advanced Glycation End products (RAGE), and T-cell Immunoglobulin- and mucin-domain-containing Molecule-3 (TIM-3). The molecular bases for its pleiotropy have not yet been fully characterized, however, it has been recently demonstrated that certain functions of HMGB1 are related to the occurrence of reversible and/or irreversible changes in the redox status of its three Cysteine residues. Specifically, fully reduced HMGB1 (all-thiol-HMGB1) is endowed with chemotactic function, while the generation of the intramolecular disulfide bridge involving Cys23 and Cys45 in the A box (disulfide-HMGB1) switches this molecule from a cell chemoattractant to a cytokine-inducing factor. Finally, terminal and irreversible oxidation of all Cys residues, including Cys106 in the B box, results in an inactive or even tolerogenic protein.

The redox status and the function of extracellular HMGB1 may be influenced by different factors, including the mechanisms of release (active vs. passive) and/or the type of cell death (necrosis vs. apoptosis) leading to its passive diffusion outside the cell. In this context, the NK-tumor cell interaction may have functional consequences, as HMGB1 may be either actively exported by NK cells or passively released by killed tumor cells. That NK cells could actively release HMGB1, however, has been solely demonstrated in the context of the NK-Dendritic Cell (DC) cross-talk. In that case, HMGB1 release was promoted by DC-derived Interleukin (IL)-18 and NKp30 engagement, and resulted in the induction of DC maturation. No information is presently available on the possible release of HMGB1 during NK-tumor cell interaction and on its possible effects on the recruitment of effector cells to the tumor site.

In this study, we show that upon melanoma cell recognition, NK cells (but not tumor cells) can release a chemotactic form of HMGB1 capable of amplifying the antitumor response by attracting additional NK cells at the site of tumor-NK cell interaction.

**Results**

Several triggering receptors induce the active release of HMGB1 by NK cells

The major NK triggering receptors were analyzed for their ability to induce HMGB1 release. To this end, polyclonal NK cell lines were derived from different healthy individuals and stimulated with receptor-specific monoclonal antibodies (mAbs). The culture supernatants were then subjected to insoluble-heparin HMGB1 extraction followed by Western Blotting analysis. As shown in Fig. A, all of the Natural Cytotoxicity Receptors (NCRs; i.e. NKp30, NKp46, and NKp44) were able to induce an active HMGB1 release. In addition, the mAb-mediated stimulation of activating Killer Ig-like Receptors (KIRs) on KIR2DS1+ or KIR2DS2+ NK cell clones induced HMGB1 release at levels comparable to those induced by NKp30 (Fig. B). The phenotypic and functional features of activating-KIR+ NK cell clones are shown in Supplmentary Figure 1.

In order to assess whether HMGB1 release could be regulated independently from cytoxicity or cytokine secretion, NK cells were stimulated by mAb-mediated cross-linking of the receptors known to variably trigger NK cell function, including the NCRs, NK2D, DNAM-1 and 2B4. The supernatants were then analyzed for the content of HMGB1, Interferon-γ (IFN-γ), and Granzyme B (as a marker of cytotoxic degranulation). As shown in Figure 1C, all the receptors analyzed were able to induce HMGB1 release. NKp30, followed by NKp46 and NKp44, induced maximal effects, whereas NK2D, 2B4, and DNAM-1 were less efficient. Some receptors, however, showed a differential ability to induce the various functions (Fig. 1C). For example, NKp30 could equally trigger all of the three functions, while 2B4 induced HMGB1 release but was far less efficient in inducing Granzyme B and IFNγ release.

In conclusion, different activating receptors involved in NK cell-mediated recognition and killing of tumor cells can induce, at various extents, the active release of HMGB1.

Analysis of HMGB1 released following NK-melanoma cell interaction

We next assessed whether the direct interaction with melanoma cells could induce NK cells to release HMGB1. To this end, NK cells were co-incubated with the melanoma cell line FO-1 (which is typically targeted by NK cells) and the HMGB1 content was then quantified in the culture supernatants. As positive control, we measured the HMGB1 released by NK cells upon the direct mAb-mediated engagement of NKp30 (a crucial receptor for the recognition of FO-1). Moreover, as NK cells exert their cytotoxic function by the delivery of pro-apoptotic mediators (Granzyme B playing a major role), we also analyzed the supernatant from apoptotic FO-1 cells (i.e.,
undergoing apoptosis following γ-radiation – Fig. S2A). As shown in Figure 2A, we detected abundant HMGB1 in the NK-FO-1 co-culture supernatant, at levels higher than those released by the NKp30-stimulated NK cells or by radiation-induced apoptotic FO-1 cells. This could suggest that the co-culture supernatant contains HMGB1 derived both from active release by NK cells and passive diffusion from killed FO-1 cells. To further confirm this possibility and to try to dissect the HMGB1 of different origin, we performed co-culture experiments using NK cells from a patient affected by Familial Hemophagocytic Lymphohistiocytosis 2 (FHL2).32 This disease is characterized by mutations in the perforin gene (prf1) leading to a defective protein and poor or absent NK cell-mediated killing activity. At variance with healthy NK cells, FHL2-NK cells neither killed FO-1, nor induced their apoptosis (Fig. S2). Thus, supernatant from FHL2-NK-melanoma cell co-cultures should include HMGB1 derived from NK cells but not from tumor cells. As shown in Figure 2A, FHL2-NK cells were able to release HMGB1 following either NKp30 engagement or exposure to FO-1 cells, thus demonstrating that, indeed, NK cells actively release HMGB1 following interaction with tumor targets.

We next tried to assess whether different forms of HMGB1 could be released in the various (co-) cultures. It has been previously demonstrated that in non-reducing conditions the partially oxidized HMGB1 (i.e. the disulfide bond containing-molecule) displayed an increased electrophoretic mobility as compared to the fully reduced HMGB1 (i.e., the all-thiol-containing molecule).22 Thus, in our experiments, HMGB1 was extracted from culture supernatants and assessed in Western Blotting under non-reducing conditions. Our analysis indicated that HMGB1 from supernatant of NKp30-stimulated NK cells or apoptotic FO-1 cells mainly showed an electrophoretic mobility compatible with the “fully reduced” or the “partially oxidized” status of the molecule, respectively (see the respective upper and lower bands in Fig. 2B). Remarkably, in the supernatant from co-cultures both “bands” of HMGB1 were well represented (although the lower one was much more intense). These findings indicated...
that, following reciprocal interaction, NK and melanoma cells contribute to extracellular HMGB1 accumulation, each providing, chiefly, a specific molecular form. This hypothesis was definitively demonstrated by the evaluation of the supernatant from FHL2-NK-FO-1 co-cultures. In this case, FO-1 cells could not be killed by perforin-negative NK cells (see Fig. S2B) and the co-culture supernatant virtually contained the sole upper HMGB1 band. The same pattern was observed in Nkp30-stimulated FHL2-NK cells (Fig. 2B).

**Figure 2.** Analysis of HMGB1 released following NK-melanoma cell interaction. (A) Polyclonal NK cells from healthy donors (NK) or from a FHL2 patient (FHL2-NK) were cultured without stimuli, co-cultured with FO-1 cells, or stimulated with anti-Nkp30 specific mAb for 6 h. FO-1 cells were left untreated or exposed to γ-radiation to induce apoptosis and cultured for 24 h. Supernatants were analyzed by ELISA for their HMGB1 content. The data are means ± SEM of three independent experiments. (B) The supernatants derived from cells stimulated as in (A) were submitted to HMGB1 extraction and the eluted proteins were analyzed in non-reducing 15% SDS/PAGE followed by immunoblotting for HMGB1. The supernatant from anti-Nkp30 mAb-secreting hybridoma (SN αNkp30), or from FO-1 left untreated for 6 or 24 h were also analyzed to evaluate background.

**NK cells but not tumor cells release HMGB1 capable of inducing NK cell chemotaxis via RAGE**

The redox status of extracellular HMGB1 can influence its functional capabilities, including its chemotactic properties. The possible effect of HMGB1 on NK cell chemotaxis, however, is presently unknown. We performed a chemotaxis assay to assess whether the supernatants containing different HMGB1 forms could induce the NK cell migration (Fig. 3A). The supernatant from NK-FO-1 cell co-culture (containing both HMGB1 forms) strongly induced NK cell chemotaxis, at levels comparable to those obtained with IL-8 (used as positive control). Also the supernatants from FHL2-NK-FO-1 co-cultures and Nkp30-stimulated NK cells (both containing mainly the HMGB1 form with lower mobility – i.e. upper band) potently induced NK cell chemotaxis, whereas the supernatant from apoptotic FO-1 cells (essentially containing HMGB1 with higher mobility – i.e., lower band) had minimal effect. These results suggested that, during their reciprocal interaction, NK cells but not tumor cells could release a form of HMGB1 (possibly displaying lower mobility) capable of exerting chemotactant effects on NK cells (Fig. 3A). This hypothesis was definitively proved by the demonstration that an anti-HMGB1 blocking mAb could significantly reduce the effect of NK-FO-1 co-culture supernatant on NK cell chemotaxis (Fig. 3B). Remarkably, a similar inhibitory effect could also be induced by the specific blockade of RAGE, a HMGB1 receptor inducing cell migration (Fig. 3B). As controls, we showed that both anti-HMGB1 and anti-RAGE antibodies could inhibit chemotaxis induced by recombiant HMGB1, whereas they were ineffective on IL-8-induced NK chemotaxis (Fig. 3C).

To our knowledge, RAGE expression on NK cells has never been demonstrated. Thus, we assessed the expression of this receptor on polyclonal NK cell lines derived from two different healthy donors. As shown in Figure 3D and Supplemental Figure 3, mRNA coding for RAGE and RAGE protein were expressed in both donors’ polyclonal NK cell populations. In addition, FACS analysis confirmed RAGE expression at the NK cell surface (Fig. 3E).

In order to assess whether the above described phenomenon could be restricted to FO-1 cells or it could currently occur during NK-melanoma cell interaction, we analyzed a panel of eight melanoma-derived cell lines (including six primary cell lines – see Material and Methods). As shown in Figure 4, in all cases the supernatant from NK-melanoma co-cultures induced potent NK cell chemotaxis that could be significantly inhibited by anti-HMGB1 antibody. In keeping with these results, the Western Blot analysis of HMGB1 content revealed the presence of both upper and lower HMGB1 bands in all co-cultures (Fig. 5). Analysis of supernatants from apoptotic cells indicated that the amount of HMGB1 after irradiation greatly varied among the cell lines, suggesting that cell intrinsic factors could influence this process. However, the upper HMGB1 band was never detectable.

Since the major biological roles of NK cells consist in tumor cell killing and cytokine production (predominantly IFNγ), we evaluated whether HMGB1 could affect these functions. In particular, we analyzed the possible effect of HMGB1 released during NK-melanoma cell interaction or upon NK-receptor stimulation. To this end, the anti-HMGB1 antibody was used in proper functional assays. As shown in Supplemental Figures 4 and 5, neither NK cell cytotoxicity nor IFNγ production were altered by anti-HMGB1 antibody, thus ruling out a possible involvement of HMGB1 in the regulation of these specific functions.

**Effect of extracellular HMGB1 on NK cell proteome**

To get a broad picture of the possible effects of HMGB1 on NK cells, we carried out a proteomic study. In this experiment, a polyclonal NK cell line (deprived of IL-2 to reduce possible background) was cultured overnight in the absence or in the presence of recombiant HMGB1. Cell extracts derived from stimulated or unstimulated NK cells were then subjected to two-dimensional electrophoresis (2-DE). By this
approach, 430 protein spots were separated (Fig. S6). A comparative image analysis of the 2-DE map revealed 17 spots differentially expressed in control and HMGB1 treated cells ($p < 0.05$). As shown in Table 1, β-actin and several cytoskeletal or cytoskeleton-associated proteins were upregulated following cell treatment with HMGB1. Thus, for example: annexin A4 is positively involved in cell migration; moesin plays a non-redundant role in lymphocyte egress from lymphoid organs and undergoes dynamic regulation during cell shape changes and migration; Rho GDP-dissociation inhibitor 1 controls cell motility as a regulator of Rho GTPases; EFHD2 is a cytoskeleton associated adaptor and Ca$^{2+}$-binding protein involved in the modulation of cell migration and cytokine production; P64 CLCP cross-links the cell membrane and the cortical actin cytoskeleton promoting cell motility; protein disulfide isomerase is a chaperone protein that activates cell migration.

Additional upregulated proteins included molecules involved in cell survival or proliferation responses. Cofilin-1-like protein, also belonging to a family of essential actin regulators, was the only protein found to be downregulated in NK cells following treatment with extracellular HMGB1.

Changes of NK cell functional properties induced by extracellular HMGB1

The above described proteomic analysis suggested that NK cells could respond to extracellular HMGB1 by increasing the expression of proteins mostly involved in cell motility. In view of these data, we analyzed whether such proteomic profile changes could result in functional effects. To this end, a polyclonal NK cell line was stimulated as in the proteomic study (overnight culture without IL-2 in the absence or presence of HMGB1) and then analyzed in chemotaxis assays. As control, NK cells not exposed to IL-2 starvation were also analyzed. As shown in Figure 6, IL-2 deprivation reduced chemotactic response to both IL-8 and HMGB1. The stimulation with HMGB1 (overnight)
was ineffective for the recovery of chemotaxis but increased NK cell motility (i.e. migration in the absence of chemotactic stimuli) (see Materials and Methods). Thus, the long-term exposure to HMGB1 can increase the NK cell motility even if it may inhibit the NK cell ability to respond to chemotactic stimuli. However, it has to be noted that, while the increased motility is well documented by our data, the inhibition of chemotaxis should be considered with caution due to the high random migration background (i.e., the migration of cells in the absence of stimuli).

**Discussion**

In this study we identify an important role for HMGB1 in a new context: the NK-tumor cell interaction. We show that the engagement of major activating NK receptors or the interaction with melanoma target cells induces NK cells to release a form of HMGB1 capable of promoting NK cell chemotaxis. In addition, long-term exposure to HMGB1 enhances NK cell non-specific motility. These mechanisms may both amplify the recruitment of NK cells at the tumor site, and strengthen the ability of residing NK cells to move along and patrol the tumor tissue even in the absence of specific chemotactic stimuli.

Accumulating data suggest a multifaceted role for HMGB1 in tumor progression. Nuclear HMGB1 can be upregulated in certain tumor cells and may favor cell survival and proliferation by promoting the transcription of relevant genes. Extracellular HMGB1 may either elicit host antitumor responses, or favor the tumor by promoting neo-angiogenesis and contributing to the aberrant tumor-associated inflammation. In this context, the release of HMGB1 by tumor cells has been recently associated with the progression of the tumor. In particular, apoptotic tumor cells have been shown to passively release an oxidized form of HMGB1 capable of promoting immune tolerance. On the other hand, the disulfide-HMGB1 or all-thiol-HMGB1 can promote cytokine production or chemotaxis, respectively. The latter two forms could be distinguished on the basis of their electrophoretic mobility, while, to our knowledge, no information is presently available on the terminally oxidized HMGB1.

![Figure 4. Chemotactic effect of supernatants from NK cells co-cultured with different melanoma cell lines. The supernatants derived from the indicated melanoma cell lines cultured alone or with NK cells were assessed for their ability to induce NK cell chemotaxis. Supernatants from co-culture were assessed in the absence or in the presence of anti-HMGB1 antibody. The data are means ± SEM of three independent experiments and are calculated as percentage of the spontaneous migration (see Materials and Methods).](image-url)
In our experiments, HMGB1 derived from (co-)culture supernatants variably showed one or two bands whose position in the gel was compatible with the disulfide- and the all-thiol-HMGB1. By the use of “non-cytotoxic” NK cells (i.e. FHL2 NK cells)\(^3\) and the analysis of apoptotic melanoma cells, we could distinguish HMGB1 actively exported by NK cells (upper band, Fig. 2B) from that deriving from killed tumor cells, and could assign the chemotactic property uniquely to the NK-derived HMGB1. A predominant lower band possibly corresponding to the oxidized molecule (disulfide-HMGB1) was present in the supernatants derived from melanoma cells that were killed via either NK cells or pro-apoptotic stimuli (i.e., irradiation). This finding is in line with the concept that actually NK cells, through the release of Granzymes, kill the target by inducing apoptosis. As other pro-apoptotic stimuli, also NK-derived Granzymes may induce mitochondria to produce ROS favoring HMGB1 oxidation in dying tumor cells. In one out of nine analyzed melanoma cell lines (i.e. FO-1), a faint upper band is actually also detectable in supernatant from apoptotic (irradiated) melanoma cells in the absence of NK cells. This may indicate that HMGB1 oxidation during apoptosis is a dynamic process that, in some instances, may not target simultaneously the totality of the HMGB1 molecules. Alternatively, although showing the same mobility as that of NK cell-derived HMGB1, this upper HMGB1 band may represent (or include) a different molecular form. Anyhow, supernatant from apoptotic cells showed only minimal chemotactic properties, suggesting that the release of a specific form of HMGB1 by NK cells is required to trigger the recruitment of additional NK cells to the tumor site.

HMGB1, associated with different molecules (i.e., CXCL12, LPS, Pam\(_3\)Csk\(_4\), nucleosomes, nucleic acids, IL-1\(\beta\)) or even alone, can activate a variegated group of receptors, including chemokine (C-X-C motif) receptor 4 (CXCR4), TLR2, 3, 4, 9, TIM-3, IL-1R and RAGE\(^1\) leading to different functional effects such as cytokine production, chemotaxis, or even the delivery of negative regulatory signals.\(^2\) In particular, RAGE and CXCR4, which can recognize chemokine (C-X-C motif) ligand 12 (CXCL12)-HMGB1 complex, have been shown to promote chemotaxis in different cell types.\(^2\) Our data demonstrate that NK cells express RAGE and use this receptor for the chemotactic response to HMGB1. An involvement of the HMGB1-CXCL12-CXCR4 axis cannot be ruled out in our experiments, but its effect may be limited, since chemotaxis assays were performed on activated NK cells, which generally poorly express CXCR4 (Fig. S7). Moreover, recombinant HMGB1 could induce NK chemotaxis at levels comparable to those induced by the supernatant of NKp30-stimulated NK cells (Fig. 3A, B), thus suggesting that the engagement of RAGE by HMGB1 alone can play a major role for the supernatant-induced chemotaxis. Supernatants derived from NK+FO-1 or FHL2-NK+FO-1 co-cultures (characterized by the presence or the absence of tumor-derived HMGB1, respectively) didn’t greatly differ for their chemotactic properties, thus suggesting that tumor-derived HMGB1 doesn’t apparently compete for the binding to RAGE.

Figure 5. Analysis of HMGB1 released following co-cultures of NK cells with different melanoma cell lines. Polyclonal NK cells from a healthy donor were co-cultured with the indicated melanoma cells (+NK). Alternatively, melanoma cells were left untreated (ctrl) or exposed to \(\gamma\)-irradiation to induce apoptosis (Apopt). Supernatants were submitted to HMGB1 extraction and the eluted proteins were analyzed in non-reducing 15% SDS/PAGE followed by immunoblotting for HMGB1. A representative blot of two for each melanoma cell line is shown.

Our experiments suggest that HMGB1 may not influence major NK cell effector functions, such as cytolytic activity or cytokine release (Figs. S4 and S5). However, new receptors capable of recognizing HMGB1, including TIM-3\(^4\) or the N-Methyl-D-Aspartate-receptor\(^4\) (NMDA-R), have been recently demonstrated to be expressed on NK cells.\(^4\) Thus, at this stage, it cannot be ruled out that HMGB1 could modulate different aspects of the NK cell biology through these receptors. We have specifically assessed the potential role of TIM-3 and NMDA-R in the regulation of NK cell chemotaxis by using a specific anti-TIM-3 mAb and the NMDA-R antagonist MK-801. As shown in Fig. S9, neither anti-TIM-3 mAb nor MK-801 significantly modified the NK cell chemotactic response to HMGB1 or co-culture supernatant, suggesting that these two receptors should not play an important role in this phenomenon.
| ID  | Protein                                                                 | gi NCBI accession number | Biological process*                                                                 | N matched peptides | Sequence coverage (%) | Mr (Da)/pI theoretical | Fold⁴/p-value | Ref. |
|-----|-------------------------------------------------------------------------|--------------------------|-------------------------------------------------------------------------------------|--------------------|-----------------------|------------------------|--------------|------|
| 799 | Moesin                                                                  | 4505257                  | Regulation of lymphocyte migration                                                  | 19                 | 39                    | 67776/6.08             | 1.26/0.04    | (51) |
| 833 | Transketolase                                                           | 37267                    | Energy reserve metabolic process                                                    | 7                  | 18                    | 67751/7.90             | 1.14/0.04    | (52) |
| 952 | Protein disulfide-isomerase precursor                                   | 20070125                 | Cell redox homeostasis                                                              | 23                 | 50                    | 57081/4.76             | 1.26/0.04    | (53) |
| 1117| Poly(rC)-binding protein                                                | 14141166                 | Innate immune response                                                              | 3                  | 11                    | 38197/6.33             | 1.30/0.01    | (54) |
| 1319| Annexin A5                                                             | 4502107                  | Signal transduction                                                                | 2                  | 68                    | 35914/4.94             | 1.43/0.01    | (55) |
| 1328| Annexin A4                                                             | 1703319                  | Signal transduction                                                                | 9                  | 35                    | 35860/5.84             | 1.51/0.01    | (56) |
| 1332| SAP domain-containing ribonucleoprotein                                | 32129199                 | Regulation of translation                                                           | 3                  | 13                    | 23656/6.10             | 1.32/0.04    | (57) |
| 1342| P64 CLCP                                                               | 895845                   | Signal transduction                                                                | 2                  | 13                    | 23528/5.12             | 1.34/0.04    | (58,59) |
| 1352| EF–hand domain-containing protein D2                                    | 20149675                 | Calcium ion binding                                                                | 6                  | 24                    | 26680/5.15             | 1.28/0.03    | (60) |
| 1404| Prohibitin                                                              | 4505773                  | Negative regulation of cell proliferation                                           | 4                  | 15                    | 29786/5.57             | 1.50/0.01    | (61) |
| 1461| Rho GDP-dissociation inhibitor 1 isoform α                              | 4757768                  | Cellular response to interleukin-6                                                 | 5                  | 42                    | 23193/5.02             | 1.60/0.05    | (62) |
| 1504| Glutathione S-transferase                                               | 2204207                  | Cellular response to lipopolysaccharide                                             | 14                 | 70                    | 23367/5.43             | 1.30/0.05    | (63) |
| 1675| Superoxide dismutase [Cu-Zn]                                           | 4507149                  | Positive regulation of cytokine production                                          | 4                  | 37                    | 15926/5.70             | 1.53/0.01    | (64) |
| 1709| Translation initiation factor eEF5A                                     | 183448388                | Positive regulation of cell proliferation                                          | 4                  | 47                    | 15140/5.80             | 1.31/0.03    | (65) |
| 1730| Cofilin-1-like                                                         | 5031635                  | Actin cytoskeleton organization                                                      | 11                 | 69                    | 18360/8.26             | 0.40/0.002   | (66) |
| 1825| Histone H2A                                                            | 4504239                  | Nucleosome assembly                                                                 | 2                  | 21                    | 13952/10.90            | 1.31/0.02    | (67) |
| 1902| Actin                                                                  | 4501885                  | Cellular component movement                                                         | 13                 | 46                    | 41579/5.29             | 1.23/0.02    | |  

*Biological process is defined according to Gene Ontology (GO);

⁴Average ratio of the protein abundance in HMGB1-treated vs. control cells.
HMGB1 can play a positive role in the NK cell trafficking both by stimulating the rapid recruitment of these cells through a RAGE-mediated chemotaxis, and by inducing changes in the proteome network with upregulation of several cytoskeletal or cytoskeleton-associated proteins that favor a high motile cell behavior. The only cytoskeleton-associated protein downregulated by HMGB1 was cofilin 1-like protein. Reduced cofilin expression by antisense oligonucleotides has been previously reported to enhance superoxide production in mouse macrophages. In NK cells the HMGB1 induced downregulation of cofilin 1-like protein was accompanied by an upregulation of the Cu-Zn-superoxide dismutase. Hence, a further functional effect of HMGB1 in NK cells could consist in the activation of cell survival and defense programs against pro-oxidant conditions. This speculative hypothesis is consistent with the observed upregulation of several proteins involved in cell repair and in the intracellular redox homeostasis (Table 1).

The data presented in this study indicate that the active release of HMGB1 may represent a key step for the recruitment and the motility of activated NK cells within the tumor site. A similar mechanism may be important also for the NK-mediated response to viruses. We have analyzed two EBV-infected B cell lines, including one derived from a patient (CoP). In this case, how-ever, supernatant from EBV-infected cells (either alone or in coculture with NK cells) do not show chemotactic properties (Fig. S10).

The relative poor NK cell infiltration of certain solid tumors suggests that the recruitment of NK cells via HMGB1 may be counteracted by (still unknown) tumor escape strategies aimed at inhibiting NK cells in their ability to export or respond to HMGB1. The study of patients’ NK cells, coming from both peripheral blood and the tumor site, may help to shed light on this point. In addition, the evaluation of the elements that, in the tumor microenvironment, can modulate NK cell functions may give hints on the possible involved mechanisms.

A large array of activating receptors, including activating KIRs, are able to trigger active HMGB1 release. The meaning of activating KIRs in the biology of NK cells has not yet been fully elucidated; however, several hints suggest an important role in different pathological and physiological conditions, including the control of viruses, autoimmune diseases, and reproduction. In addition, the potential beneficial role of the activating KIRs in the context of Haematopoietic Stem Cell Transplantations (HSCT) has also been recently highlighted for the cure of leukemia. Thus, the active release of functional HMGB1 by NK cells may be considered, in the future, in the ever growing number of functional contexts in which NK cells play an important role.

Materials and Methods

Antibodies
Anti-RAGE antibody for FACS analysis (ab37647) and anti-HMGB1 antibody (ab18256) were from Abcam plc; anti-human RAGE antibody (AF1145) and peroxidase-conjugated anti-goat antibody (HAFO17) were from R&D Systems. Anti-β-actin antibody (sc-69879) was from Santa Cruz Biotechnology. Peroxidase-conjugated anti-rabbit (#7074) and anti-mouse (#7076) antibodies were from Cell Signaling Technology. Alexa Fluor 488 chicken anti-rabbit was from Life Technologies. PE-conjugated isotype-specific goat anti-mouse (GAM) antibodies were from Southern Biotechnology Associated (1090-09 and 1070-09).

The following anti-NK receptor mAbs, produced in our lab, were used to stimulate NK cells to release cytokines, Granzyme B or HMGB1: AZ20 (IgG1, anti-NKp30), BAB281 (IgG1, anti-NKp46), Z231 (IgG1, anti-NKp44), BAT221 (IgG1, anti-NKp44), GN18 (IgG3, anti-DNAM-1), PP35 (IgG1, anti-2B4), 11PB6 (IgG1, anti-KIR2DL1/S1), GL183 (IgG1, anti-KIR2DL2/L3/S2).

Generation of polyclonal or clonal NK cell lines
NK cells from healthy donors or FHL2 patient were purified from peripheral blood using the RosetteSep™ NK Cell Enrichment Cocktail (StemCell Technologies, 15025). Those populations displaying more than 95% of CD56+ CD3– CD14– NK cells were selected. Polyclonal or clonal NK cell lines were obtained by culturing purified NK cells at appropriate dilutions on irradiated feeder cells in the presence of 100 U/mL rhIL-2 (Proleukin, Novartis) and 1,5 ng/mL phytohemagglutinin (PHA; Gibco Ltd, 10576-015) in round-bottomed 96-well microtiter plates. After 3/4 weeks of culture the expanded NK cells were used for the NK cell stimulation experiments.

Melanoma cells
Melanoma cell lines MeCoP, MeDeBO, MePA, MeTA MeBO and MeOV were derived from metastatic melanoma.

Figure 6. Functional changes in motility and chemotactic properties of NK cells following long-term exposure to recombinant HMGB1. Polyclonal NK cell lines were exposed overnight to 0.5 μg/mL recombinant HMGB1 (−IL-2/+HMGB1 o/n) or to vehicle (−IL-2 o/n) in the absence of IL-2; or cultured in the presence of IL-2 (+IL-2 o/n). NK cells were then washed, seeded in the upper compartment of Transwell chambers, and assessed in chemotaxis assays for their ability to cross the membrane spontaneously or in response to HMGB1 or IL-8 (both at 100 ng/mL) placed in the lower compartment. Values indicate the number of migrated cells either in the absence or in the presence of chemotactic stimuli. The data are means ±SEM of three independent experiments.
samples, as previously described, obtained from IRCCS AOU S. Martino-IST – Genova. All the lesions were histologically confirmed to be cutaneous malignant melanomas. FO-1 cell line was kindly provided by S. Ferrone (New York Medical College). MEWO and C32 cell lines were purchased from European Collection of Cell Cultures. Cells were cultured in RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS) and antibiotic mixture (0.05 mg/mL penicillin, 0.05 mg/mL streptomycin). All cell lines were periodically tested and authenticated by morphologic check and flow cytometry for the expression of informative markers including Mel-CAM/CD146 and GD2.

**NK cell stimulation, NK-melanoma cell (co-)culture and generation of apoptotic melanoma cells**

NK cells were cultured in 24-well flat bottom plates (5 × 10⁵ cells/well) in the presence of the indicated melanoma cell lines (ratio 1:1), or PHA, or in wells pre-coated with GAM IgG (MP cells/well) in the presence of the indicated melanoma cell lines samples, as previously described, obtained from IRCCS AOU S. Martino-IST – Genova. All the lesions were histologically confirmed to be cutaneous malignant melanomas. FO-1 cell line was kindly provided by S. Ferrone (New York Medical College). MEWO and C32 cell lines were purchased from European Collection of Cell Cultures. Cells were cultured in RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS) and antibiotic mixture (0.05 mg/mL penicillin, 0.05 mg/mL streptomycin). All cell lines were periodically tested and authenticated by morphologic check and flow cytometry for the expression of informative markers including Mel-CAM/CD146 and GD2.

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**Extracellular HMGB1 extraction**

The supernatant from co-cultures or stimulated cells were incubated with 70 µL Heparin Sepharose CL 6B (GE Healthcare, 17-0467-01) pre-equilibrated in 50 mM sodium borate buffer (pH 8.5) containing 0.25 M NaCl. Heparin-bound proteins were washed four times in 50 mM sodium borate buffer (pH 8.5) containing 0.15 M NaCl. Samples were washed four times in 50 mM sodium borate buffer (pH 8.5) containing 0.15 M NaCl. Heparin-bound proteins were eluted by adding Laemmli sample buffer with or without 2% β-mercaptoethanol. Heparin-Sepharose-extracted supernatants were boiled and submitted to 15% SDS/PAGE followed by immunoblotting.

**Chemotaxis assay and evaluation of NK cell motility**

Polyclonal NK cell lines, previously expanded in rhIL-2 (100 U/mL), were seeded at 2 × 10⁶/mL in the upper compartment of Transwell® chambers (3 µm pore size; Corning Costar, 3415). Where indicated, recombinant HMGB1, IL-8 (MACS Miltenyi Biotec, 130-093-942), both dissolved in RPMI 1640 10% FBS, or the conditioned media were placed in the lower compartment. Cells were allowed to migrate for 2 h at 37°C in a 5% CO₂ atmosphere in a humidified incubator. After incubation, cells migrated in the lower compartment were collected and counted by using Burker chamber following Trypan blue dye exclusion staining method and then confirmed by using the MACSQuant Analyzer (Miltenyi Biotec). The specific chemotactic response of NK cells (Figs. 3 and 4) was assessed as percentage of spontaneous migration and was calculated as follows: (number of migrated cells in the presence of chemotactic stimulus / number of migrated cells in the absence of stimulus) × 100.

**Granzyme B activity assay**

Granzyme B activity was monitored fluorometrically (λex 405 nm; λem 535 nm) using the fluorogenic substrate N-Acetyl-Ile-Glu-Pro-Asp 7-amido-4-trifluoro-methylcoumarin (Ac-IEPD-AFC; Sigma-Aldrich, A6345). Aliquots of culture medium (10 µL) were added to 90 µL of 100 mM Hepes (pH 7.5) containing 20% glycerol, 5 mM DTT, 0.5 mM EDTA, and 400 µM Ac-IEPD-AFC in 96-well black microplate. After fluorescence intensity recording at 0 h, the microplate was covered with adhesive foil and dark incubated at 37°C for 24 h. The increase of fluorescence was determined as the difference of values recorded at 0 and 24 h. The standard curve was generated by using different amounts of free AFC (Sigma-Aldrich, 248924), and the value of fluorescent units per nanomole was extrapolated. The fluorescence intensity was measured using the top reading mode in the

**Total membrane proteins preparation**

Jurkat or NK cells (1.7 × 10⁶) were washed twice with PBS and lysed in 50 mM sodium borate buffer (pH 7.5) containing 1 mM EDTA (ethylenediaminetetraacetic acid), 10 µg/mL aprotinin, 100 µg/mL leupeptin, and 2 mM Pefabloc SC (500 µL) by three freeze-thaw cycles. The particulate fraction was collected by centrifugation at 100,000 × g for 20 min at 4°C, washed in 50 mM sodium borate buffer (pH 7.5) containing 0.1 mM EDTA, and solubilized in Laemmli sample buffer without β-mercaptoethanol and bromophenol blue (150 µL). Total proteins were quantified by Lowry method and, after addition of β-mercaptoethanol and bromophenol blue, the samples were boiled and submitted to 10% SDS/PAGE followed by immunoblotting.

**Recombinant HMGB1**

Eukaryotic recombinant HMGB1 protein was produced by using the baculovirus system, and was purified as previously described. HMGB1 was stored in 50 mM sodium borate buffer (pH 8.5) containing 0.4 M NaCl and 2 mM dithiothreitol (DTT).

**ELISA**

Extracellular HMGB1 concentration in the culture media (10 µL) was measured using the HMGB1 ELISA Kit II (IBL International Gmbh, ST51011) according to the manufacturer’s protocol. The range of quantification for the assay was 2.5–80 ng/mL. Each sample was run in duplicate.

**Extracellular IFNγ concentration in the culture media**

(50 µL) was measured (following proper dilution of the supernatants) using the IFNγ Human ELISA Kit (Life Technologies, KHC4021) according to the manufacturer’s protocol. The range of quantification for the assay was 0–1 ng/mL. Each sample was run in duplicate.
fluorescence multilabel reader LB 940 Mithras (Berthold Italia). Granzyme B activity was calculated as follows:

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\text{Unit of activity (nmol/mL/min)} = (F/\text{min})/(F/nmol) 	imes 100
\]

**Immunoblotting**

Proteins were separated by SDS/PAGE and transferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked in 5% nonfat dry milk, 0.1% Tween 20 and incubated for 16 h at 4°C with a primary antibody: anti-human RAGE (1:2,000), anti-HMGB1 (1:2,000), anti-β-actin (1:1,000). Peroxidase-conjugated secondary antibodies (1 h at 20°C) were anti-goat (1:2,000), anti-rabbit (1:5,000), and anti-mouse (1:5,000), respectively. Immunoreactive signals were developed using ECL Select™ Western Blotting Detection Kit (GE Healthcare, RPN2235), acquired and quantified using Chemi Doc XRS equipped with the Quantity One Image Software (Bio-Rad). In order to validate the identification of the two HMGB1 bands, detectable in non-reducing conditions, the nitrocellulose membrane was stripped and re-probed with an irrelevant antibody (see Fig. S8).

**FACS analysis**

For cytofluorimetric analysis, cells were stained with appropriately unlabeled Abs followed by appropriate second reagent and then analyzed by FACS (FACSCalibur, Becton Dickinson) using the FlowJo X software.

**Proteomic analysis and protein identification**

NK cells (2 × 10^7) were cultured without IL-2 in the absence or in the presence of 0.5 μg/mL recombinant HMGB1. After 16 h, cells were washed twice with PBS and lysed in 2 mL of 7 M urea, 2 M thiourea, 5% w/v CHAPS, 50 mM DTT, 5% Triton X-100, 5% ITPG Buffer (GE Healthcare, 17-6000-87), DNase (1 U/μL) and RNase (1 mg/mL). After centrifugation at 16,000 x g at 4°C for 5 min, equal amounts of soluble proteins (400 μg) from each NK cell subset were separated by 2-DE according to Spertino et al. using immobilized strip gels pH 3–10 linear, 13 cm (GE Healthcare, 17-6001-14). The Coomassie-stained gels were scanned using a densitometer GS-710 (Bio-Rad Laboratories). The gel images were recorded and computationally analyzed using Same Spot software (Progenesis), three biological replicates were used for comparative analysis. Spots of interest were manually excised from 2-DE gels and trypsin digested. MS/MS analysis of peptides mixture for protein identification was performed using a QSTAR XL hybrid quadrupole–TOF instrument (Applied Biosystems) coupled with LC Packings Ultimate 3000 nano-flow LC system (Dionex), as previously described.

**Statistical analysis**

Data are presented as mean ± SEM. Significance of the difference was analyzed by ANOVA followed by post-hoc Fisher’s test using the Prism 6.0 software package (GraphPad Software), with statistical significance taken at p < 0.05. For proteomics, statistical analysis was performed with StatView 4.5 (Abacus Concepts), spot optical densities were compared by ANOVA and p ≤ 0.05 was adopted as significance cut-off.

**Ethical statements**

The study protocol was approved by the internal review board (IRB) (Comitato Etico – IST, approval n. OMA09.001) and the Regional Ethic Committee (Comitato Etico Regionale, P.R. 023REG2013) for the study on the NK/melanoma cell interaction; and the IRB of Gaslini Institute (Comitato Etico – Istituto Gaslini – Genova, AIEOP HLH 2004) for the use of NK cells from the FHL patient. Biological samples from both healthy donors and patients were collected after obtaining informed consent according to the Declaration of Helsinki.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Supplemental Material**

Supplemental data for this article can be accessed on the publisher’s website.

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