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Targeting of Human Dendritic Cells by Autologous NK Cells

Julia L. Wilson,* Lena C. Heffler,† Jehad Charo,‡ Annika Scheynius,† Maria-Teresa Bejarano,* and Hans-Gustaf Ljunggren‡*

NK cells have the capacity to spontaneously kill tumor cell lines, in particular cell lines of hemopoietic origin. In contrast, they do not generally kill nontransformed autologous cells. However, here we demonstrate that short-term activated polyclonal human NK cells, as well as human NK cell lines, efficiently lyse autologous dendritic cells (DC) derived from peripheral blood monocytes as well as Langerhans-like cells derived from CD34+ stem cells isolated from umbilical cord blood. Lysis of autologous DC by short-term activated NK cells and NK cell lines was dependent on granule exocytosis, since total abrogation of lysis was observed in the presence of EGTA. Induction of DC maturation by LPS, monocyte conditioned media (MCM), or stimulation through CD40 ligand (CD40L) rendered the DC less susceptible to lysis by NK cells. Infection of DC with influenza virus was likewise associated with a reduced susceptibility to lysis by NK cells. Thus, susceptibility to lysis by autologous NK cells is a particular property of immature DC. The present results are discussed in relation to the ability of DC to interact with NK cells and to the ability of NK cells to regulate development of specific immunity. The Journal of Immunology, 1999, 163: 6365–6370.

Natural killer cells are a population of lymphoid cells distinct from B and T lymphocytes. Human NK cells represent 5–15% of lymphocytes in peripheral blood. They are characterized phenotypically by a combination of positive and negative criteria. They lack expression of the TCR-CD3 complex and surface Iggs, but, in contrast to most other hemopoietic cells, express CD56. Like neutrophils and macrophages, NK cells also express CD16 (reviewed in Ref. 1). In recent years, insights into the molecular understanding of NK cell recognition of target cells have evolved. In particular, attention has been devoted to the identification and characterization of inhibitory receptors with specificity for MHC class I molecules (1–3).

NK cells were initially characterized by their ability to mediate spontaneous cytotoxicity against tumor cell lines, in particular tumors of hemopoietic origin (1). Currently, it is understood that they play a significant role in the host defense against certain microorganisms, in part through their ability to secrete cytokines such as IFN-γ, TNF-α, and GM-CSF (4, 5). They also take part in the development and regulation of adaptive immune responses. The mechanisms underlying the latter processes are less well understood. They most likely involve cytokines but may also involve direct interactions with APC such as dendritic cell (DC)3 (6, 7).

DC are specialized APC with a unique ability to prime naive lymphocytes (8, 9). They are distinguishable from other APC due to their typical morphology and constitutive expression of MHC I and II molecules. Immature DC have a high capacity to capture and process Ags at peripheral sites in the body, but a weak capacity to stimulate T cells. After Ag capture, DC migrate from the non-lymphoid tissue to the blood or afferent lymph for transport to regional lymph nodes or other peripheral lymphoid organs. During this time, the DC acquire a “mature” phenotype; they lose their ability to efficiently take up and process Ag and at the same time up-regulate the expression of MHC class I and II, adhesion and costimulatory molecules. These mature DC have a strong capacity to stimulate T cells and initiate immune responses (8, 9).

It was observed recently that murine bone marrow-derived DC could be killed efficiently by syngeneic NK cells in vitro (10). To study whether this effect is specific for the murine system or whether it is observed also with human cells, we have established cultures of human DC from peripheral blood CD14+ monocytes as well as from CD34+ hemopoietic stem cells from cord blood. Using these cells as targets, we demonstrate that freshly isolated human NK cells and human NK cell lines readily kill autologous DC and that this lysis is dependent on granule exocytosis. Susceptibility to lysis by NK cells appears to be a particular property of immature DC. These results are discussed in relation to the ability of DC to interact with NK cells, and the ability of NK cells to regulate development of adaptive immune responses.

Materials and Methods

mAbs and flow cytometry

FITC- and PE-labeled mAbs against CD3 (HIT3a), CD14 (M5E2), CD34 (8G12), CD56 (MY31), CD80 (L307.4), CD86 (2331), HLA-DR (L243), HLA-ABC (G46-2.6), and isotype-matched labeled controls were obtained from Becton Dickinson (Stockholm, Sweden). Anti-CD83 (HB15A) mAb was obtained from Immunotech (Kebolab, Stockholm, Sweden). Anti-CD40 (S2C6) mAb was kindly provided by Dr. S. Pauli (Stockholm University, Stockholm, Sweden). Anti-CD1a mAb (OKT6) was kindly provided by Dr. S. A. Porcelli (Brigham and Womens Hospital, Boston, MA). Abs were used to characterize cell surface phenotypes and purity by flow cytometry. For staining, cells were washed and resuspended in PBS supplemented with 1% heat-inactivated FCS and 0.01% NaN3. Abs were diluted in this buffer and used at a final concentration of between 2–20 μg/ml. Incubations with Abs were conducted for 30 min on ice. Following the final washing step, labeled cells were fixed with 1% formaldehyde solution (Sigma, Stockholm, Sweden), and 10,000 cells were analyzed by

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3 Abbreviations used in this paper: DC, dendritic cell; MCM, monocyte-conditioned media; EBV-BLCL, EBV-transformed B lymphoblastoid cell line; LAK, lympho-kine-activated killer cell; LGL, large granular lymphocyte; CD40L, CD40 ligand; rh, recombinant human.
flow cytometry on a FACSscan flow cytometer using Cellquest software (Becton Dickinson).

Cell lines
K562 (human erythroleukemia), t. cells, t. cells transfected with CD40L (kindly provided by Dr P. Garrone, Schering Plough, Dardilly, France), RPMI 8666 cells (kindly provided by Dr. G. Trinchieri, Philadelphia, PA), and EBV-transformed B lymphoblastoid cell lines (EBV-BLCL) were cultured in complete medium consisting of RPMI 1640 media (Life Technologies, Taby, Sweden) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies). To generate EBV-BLCL, 5 x 10^6 PBL, were added to 1 ml of culture supernatant from the EBV-secreting B95-8 cell line (ECACC, Wiltshire, U.K.) and incubated in a 37°C water bath for 2h before culturing in complete medium supplemented with 1 µg/ml cyclosporin A (Sandoz, Basel, Switzerland) to prevent outgrowth of EBV-specific T cells. After 2-3 wk of culture, lines were established and used.

Generation of DC cultures
Monocyte-derived (myeloid) DC were generated as described previously (11). Briefly, human PBMC were isolated from heparinized venous blood or cytophresis buffy coats from normal volunteer donors by Ficoll-Hypaque density centrifugation (Lymphoprep; Nycomed, Oslo, Norway). Isolated cells were resuspended at a concentration of 3.5 x 10^6 cells/ml in complete medium, and 1 ml was added to a 25-cm^2 flask and incubated in a 37°C water bath for 45 min at 37°C. Nonadherent cells were removed by gentle pipetting, and the plastic adherent cells were rinsed with 20 ml of complete media. The remaining adherent cells were cultured in complete media supplemented with 800 U/ml recombinant human (rh) IL-4 (Genzyme Corporation, Cambridge, MA) and 50 ng/ml rhGM-CSF (Leukomax; Sandoz). For induction of maturation, cultures of monocyte-derived DC were supplemented with 10 µg/ml LPS (Sigma) on day 6 of culture for 48 h before use. Additional cultures were matured using 25% v/v MCM, which was added to 1 ml of culture supernatant from the EBV-secreting B95-8 cell line (ECACC, Wiltshire, U.K.) and incubated in a 37°C water bath for 2h before culturing in complete medium supplemented with 1 µg/ml cyclosporin A (Sandoz, Basel, Switzerland) to prevent outgrowth of EBV-specific T cells. After 2-3 wk of culture, lines were established and used.

Generation of large granular lymphocytes (LGL), polyclonal NK cell populations, and NK cell lines
For preparation of LGL, mononuclear cells were isolated by Ficoll-Hypaque density centrifugation (see above). PBMC were depleted of monocytes by plastic adherence for 1 h at 37°C; then the flask was turned over and incubated for one further hour. The nonadherent cells were collected and resuspended in 2 ml of warm (37°C) complete media, and the cells were loaded onto a nylon wool column (Polysciences, Warrington, PA). Columns were incubated for 1h at 37°C before 50 ml of warm media was run through the column. Collected cells were pelleted by centrifugation and then resuspended in 4 ml of a 30% Percoll solution (Sigma) in PBS with 10% FCS. This was overlaid on a 40% Percoll solution and centrifuged for 30 min at 2000 rpm. Cells at the LGL interface were harvested and washed three times with PBS before being resuspended in complete media and used immediately as effector cells. For preparation of lymphokine activated killer (LAK) cells, mononuclear cells were isolated as above, and PBMC were depleted of monocytes by plastic adherence for 45 min at 37°C. The recovered cells were resuspended in complete medium supplemented either with 1000 U/ml rhIL-2 or 2000 U/ml rhIFN-α (Peprotech, London, U.K.) and cultured at 37°C for 48 h. NK cell lines were generated by resuspending nonadherent cells in IMDM medium (Life Technologies) supplemented with 5% heat-inactivated FCS, 5% heat-inactivated AB blood, 5% human serum, and 10% leukocyte-conditioned medium. (16). Cells (2.5 x 10^6 cells/well) were cultured in 24-well plates at 37°C, in the presence of 0.5 x 10^5 irradiated (5000 rad) RPMI 8666 cells. Following 7 days of culture, cells were collected and enriched for NK cells by incubating for 45 min at 4°C with saturating amounts of anti-CD3 (OKT3), anti-CD4 (OKT4), and anti-CD8 (OKT8) hybridoma supernatants. Cells were washed twice with PBS before resuspending in rabbit complement (Pel-Freez, Brown Deer, WI) and incubated at 37°C for 1 h. After washing, 1 x 10^8 recovered cells were further expanded in 24-well plates in complete medium supplemented with 200 U/ml rIL-2, in the presence of 1 x 10^6 RPMI 8666 cells to generate NK cell lines. Typical NK cell lines were >99% positive for the human NK cell marker CD56 and negative for CD3 and other T cell markers.

NK cell-mediated cytotoxicity
Cytotoxicity of cells was measured in a standard 4-h ^51Cr-release assay using Na^2CrO_4-labeled cells in triplicate at various E:T ratios. To assess extracellular Ca^{2+} requirements, cytotoxicity assays were performed in media supplemented with 8 mM EGTA and 6 mM Mg^{2+} (EGTA/Mg^{2+}). The percentage specific ^51Cr release was calculated according to the formula: % release = ([experimental release - spontaneous release]/(maximal release - spontaneous release)) x 100. The spontaneous release was usually less than 20% of the maximum release.

Statistical analysis
Statistical analysis was performed using the Student two sample t test, where p < 0.05 is significant.

Results
Characterization of monocyte-derived DC
In a typical experiment, after 7 days of culture with GM-CSF and IL-4, peripheral blood-derived leukocytes appeared as loosely adherent clumps with typical dendritic morphology with 80–95% purity as assessed by flow cytometry (data not shown). The cell surface phenotype of such cells exhibited a classic “immature” DC phenotype (Table I). The cells expressed MHC class I and class II as well as CD40, but low levels of CD80, CD86, CD1a, and CD83. Expression of CD14 was negligible. Following maturation by culturing the cells with MCM, LPS, or culture on CD40L-expressing L cells, cell surface Ag expression was modulated. Expression of CD14 was negligible. Following maturation by culturing the cells with MCM, LPS, or culture on CD40L-expressing L cells, cell surface Ag expression was modulated. Expression of MHC class I and class II molecules, CD1a, and the costimulatory molecules CD40, CD80, and CD86 was increased. Expression of the known DC maturation marker CD83 was also up-regulated (Table I). The cells expressed MHC class I and class II as well as CD40, but low levels of CD80, CD86, CD1a, and CD83. Expression of CD14 was negligible. Following maturation by culturing the cells with MCM, LPS, or culture on CD40L-expressing L cells, cell surface Ag expression was modulated. Expression of MHC class I and class II molecules, CD1a, and the costimulatory molecules CD40, CD80, and CD86 was increased. Expression of the known DC maturation marker CD83 was also up-regulated (Table I).

LAK cells mediate lysis of allogeneic and autologous monocyte-derived DC
Peripheral blood-derived LAK cells, representing short-term activated polyclonal NK cells, do not efficiently kill EBV-BLCL. In
contrast, day 7 monocyte-derived DC were readily killed by allogeneic rhIL-2 or rhIFN-α-activated LAK cells (Fig. 1). It was important to verify that this lysis was not simply a result of NK cell activation due to allogeneic stimulation; thus, LAK cell cultures and DC were generated from the same donors. It was found that autologous DC were highly susceptible even to autologous LAK cells (Fig. 2). In this context, it is important to note that activation of the NK cells was necessary to obtain efficient lysis of DC. Using LGL as effector cells, in the absence of stimulation, only low levels of lysis of autologous DC was observed (Fig. 3). Cell to cell contact appeared to be required for NK cell-mediated lysis of DC since supernatant from the NK cell cultures had no cytolytic activity (data not shown).

**NK cell lines mediate lysis of autologous monocyte-derived DC**

LAK cell cultures could be a potential source of T cells activated by IL-2. Thus, it was of importance to determine whether the population of cells responsible for DC lysis resided within the NK cell subset. For this purpose, five independent CD3⁺/CD56⁺ NK cell lines and corresponding autologous monocyte-derived DC were generated. Although the CD3⁻/CD56⁺ NK cell lines spared the autologous EBV-BLCL, they specifically lysed the DC targets and the NK-sensitive K562 cell line (Fig. 4).

**Mature monocyte-derived DC are less susceptible to lysis than immature DC**

Several studies have shown that DC can be further induced to mature by treatment with different stimuli, such as MCM (12) and LPS (17), or through CD40L stimulation (8). To address the effect of DC maturation with respect to their susceptibility to lysis by autologous NK cell lines, monocyte-derived DC cultures were induced to mature before being used as targets. DC treatment with LPS or MCM or stimulation through CD40L resulted in mature populations of cells (Table I) that were less susceptible to NK lysis than corresponding untreated day 8 DC (Fig. 5).

**Influenza virus infection of monocyte-derived DC**

It has recently been demonstrated that DC infected with influenza virus are potent APC for the initiation of specific antiviral CTL responses (18, 19). This function suggests that infected DC may display a mature Ag presenting rather than an Ag uptake phenotype. To study how influenza virus infection affects susceptibility to NK cells, immature day 7 monocyte-derived DC were infected with the A/Japan/305/57 influenza virus strain. Productive virus infection was verified by expression of hemagglutinin on the cell surface (data not shown). Cell viability was not affected by influenza virus infection as determined by trypan blue exclusion. In line with observations above on the reduced NK cell susceptibility of mature DC, these virally infected cells were rendered less sensitive

### Table I. Flow cytometric analysis of monocyte-derived DC populations

<table>
<thead>
<tr>
<th>Cell Surface Expression</th>
<th>Immature DC</th>
<th>DC Stimulated with MCM</th>
<th>LPS</th>
<th>CD40L</th>
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<td>CD86</td>
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<td>112</td>
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</table>

*Mean fluorescence intensity. Representative data from one out of five independent experiments.

**FIGURE 1.** Monocyte-derived DC are susceptible to allogeneic LAK cell-mediated lysis. LAK effectors were stimulated with rhIFN-α (squares) or rhIL-2 (circles). Target cells were day 7 DC (closed symbols) or EBV-BLCL (open symbols). Data represent one of four independent experiments.

**FIGURE 2.** Monocyte-derived DC are susceptible to autologous LAK cell-mediated lysis. LAK effector cells were activated with rhIL-2 and used against K562 targets (■) and day 7 monocyte-derived DC (○). Data represent one of five independent experiments.

**FIGURE 3.** LGL lyse autologous monocyte-derived DC less efficiently than IL-2-activated LAK cells. Target cells were K562 (squares) or day 7 monocyte-derived DC (circles). Effectors were autologous IL-2-activated LAK cells (closed symbols) or LGL (open symbols). Data represent one of two independent experiments.
to autologous NK cells than similar uninfected DC. A reduction in killing was observed after infection and culture for only 2 h (Fig. 6A). Twenty-four hours after infection, the DC were almost completely resistant to autologous NK cell-mediated lysis (Fig. 6B).

LAK cell and NK cell line-mediated lysis of autologous monocyte-derived DC is dependent on granule exocytosis

Calcium ions are necessary for degranulation and perforin polymerization (20). To investigate the calcium dependence of NK cell-mediated killing of DC, EGTA/Mg$^{2+}$, a calcium chelator, was used. LAK or NK cell line-mediated lysis of autologous DC was almost entirely dependent on the presence of calcium ions, since all $^{51}$Cr release was abrogated in the presence of EGTA/Mg$^{2+}$ (Fig. 7). This dependence on extracellular Ca$^{2+}$ suggests that LAK cells and NK cell lines lyse autologous DC using perforin and granzyme action. Although our data indicate that perforin-mediated killing is the predominant cytolytic mechanism of DC killing, we cannot exclude an additional role for the Fas-mediated cytolytic pathway. Using flow cytometry, we detected the expression of CD95 (Fas) on the DC cell surface. This was not altered by DC maturation, and Fas-Ligand expression was detected on NK cell lines (data not shown).

LAK cell and NK cell line-mediated lysis of Langerhans-like cells

To investigate whether NK cell susceptibility was restricted to peripheral blood-derived DC, an alternative source of DC was utilized, namely CD34$^+$ umbilical cord blood-derived Langerhans-like cells. The DC cultured from CD34$^+$ stem cells from umbilical cord blood were found to have a phenotype similar to that described previously (14, 21, 22). These DC were for susceptibility to lysis using allogeneic LAK cells or allogeneic NK cell lines as effectors. Like the monocyte-derived DC, these Langerhans-like cells were demonstrated to be highly susceptible to lysis (Fig. 8).

Discussion

In the present study we demonstrate that short-term activated polyclonal human NK cells and human NK cell lines readily kill autologous monocyte-derived DC. Induction of DC maturation by LPS or MCM or stimulation through CD40L rendered the DC less susceptible to lysis by autologous NK cells. Infection of DC with influenza virus was also associated with a reduced susceptibility to
lysis by autologous NK cells. This phenomenon was not restricted to peripheral blood monocyte-derived DC, since we also demonstrate that Langerhans-like cells generated from cord blood CD34+ stem cells are also targeted by LAK cells and NK cell lines. The susceptibility to killing is not a property of any type of target cell since autologous EBV-BLCL and other targets tested, including freshly isolated monocytes, were resistant to NK cell lysis. Thus, the NK cell culture conditions or other factors have not generated killer cells, which, per se, have the capacity to lyse any target cell. Taken together, these lines of evidence indicate that human monocyte-derived DC and Langerhans-like cells are highly susceptible to lysis by autologous NK cells and, moreover, that this may be a particular property of immature DC.

Cytotoxic T lymphocytes and NK cells kill targets by two mechanisms, namely the perforin and Fas-Fas ligand (FasL) pathways. The relative contribution of granule exocytosis was studied using the calcium chelator EGTA. Degranulation and perforin polymerization are entirely dependent on the presence of extracellular calcium (20, 23). EGTA totally abrogated LAK and NK cell mediated lysis of dendritic cell targets, confirming that, in this case, cytotoxicity is almost entirely granule dependent. This is in line with previous experiments where it was found that perforin−/− mice could not kill autologous DC (10). This differs slightly from studies using the YT leukemic cell line and its derivatives, since it has been suggested that YT cells may use both perforin- and Fas-mediated pathways (23, 24). We do not formally exclude a Fas-mediated component of DC killing by NK cells since it is well documented that NK cells express Fas Ligand (25) and DC express CD95. Furthermore, it has been suggested that there may be preferential activation of either the Fas or perforin mechanisms, depending on the type of target cell (20).

It is probable that the DC cell surface expression of several different molecules contributes to the NK cell-sensitive phenotype of these cells. Alterations in the expression of some of these molecules during DC maturation may contribute to the more NK cell resistant phenotype observed. Expression of HLA-A, -B, and -C are increased upon monocyte-derived DC maturation and also when these cells are infected with influenza virus. Since NK cells express inhibitory receptors with specificity for MHC class I, it is not unlikely that the increased levels of MHC class I expressed by mature DC may in part account for the reduction of NK cell susceptibility (3, 26). The role of costimulatory molecules in NK cell killing has recently been highlighted in a number of publications (10, 27–31). Briefly, in murine tumor models it has been demonstrated that the introduction of CD80 (B7-1), CD86 (B7-2), and CD40 is associated with enhanced susceptibility to NK cell lysis (10, 29–31). In the human system, similar results have been observed in different models involving CD80- or CD40-expressing cells (32, 33). Given these results, the present observations were somewhat surprising. The expression of the costimulatory molecules CD80, CD86, and CD40 and other adhesion molecules increase upon DC maturation; in fact, one of the hallmarks of mature DC is the high expression of costimulatory molecules (8). If these are the only molecules that control NK cell susceptibility, one might expect that mature DC would be even more NK cell sensitive than immature DC, although the opposite pattern was observed. In relation to these results, it is of interest to note that EBV-BLCL have extremely high expression of all costimulatory molecules yet are among the most resistant NK cell target documented. Although the results generated with tumor cell targets transfected with costimulatory molecules suggest that these molecules can trigger NK cells, they do not necessarily imply that these molecules alone are responsible for the NK cell-sensitive phenotype of DC. Most likely, modulation of other molecules may counteract the (possible) triggering effect. In line with a possible triggering role for costimulatory molecules on DC, Carbone and colleagues observed recently that NK cell recognition of DC was partially blocked by incubating targets with mAbs against CD40, suggesting that this molecule may be one of several ligands involved in NK cell lysis of DC (E. Carbone, manuscript in preparation). On the other hand, recent studies by Chambers have demonstrated that murine DC from CD80, CD86, or CD80/CD86 and CD40 deficient mice are still susceptible to lysis by autologous murine NK cells at levels similar to wild type DC (B. J. Chambers, personal communication). Taken together, several different cell surface molecules, costimulatory and MHC class I molecules most likely contribute to render DC, and in particular immature DC, highly susceptible to lysis to autologous NK cells. Further studies should focus on identifying key molecules in these interactions.

One implication of the present observations is that there is a molecular match between receptors and ligands on DC and NK cells. This suggests that, under certain conditions, DC and NK cells may be able to communicate with each other, either to stimulate or prevent immune responses. Indeed, indirect evidence for a functional role of DC-NK cell interactions can be found in the literature. In short, NK cells have in different models been demonstrated to affect the outcome of adaptive immune responses (6, 7, 34), and, in certain in vitro experimental models, a direct role for DC-NK cell interactions has been suggested (35–38). Hypothetically, NK cells could, under certain circumstances, have a role in activating DC cells. Such a role has recently been described for CD4+ T cells in their interactions with DC (39–41). Likewise, DC may, under certain circumstances, play a role in the activation of NK cells. Such dialogue may occur either directly via cell surface receptor-ligand interactions or via cytokines.

In conclusion, the present studies demonstrate that human DC can be targeted by NK cells. The present results should stimulate studies aimed at identifying molecules involved in the interactions between DC and NK cells and the physiological relevance of such interactions between DC and NK cells in vivo. A particular subject of interest is the NK cell-mediated control of development of adaptive immune responses and the role of DC in this event. In this respect, it is of interest to note that a recent study in a murine model suggested that cell-to-cell contact between DC and resting NK cells resulted in a substantial increase in both NK cell cytolytic activity and IFN-γ production (42).
Acknowledgments

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