Immunity, Vol. 19, 561–569, October, 2003, Copyright ©2003 by Cell Press

Natural Killer Cells Activated by MHC Class I^{Low} Targets Prime Dendritic Cells to Induce Protective CD8 T Cell Responses

Ralph Mocikat,[1,*] Heidi Braumüller,[2,3] Alain Gumy,[4] Oliver Egeter,[3] Heike Ziegler,[5] Uwe Reusch,[5] Anja Bubeck,[5] Jacques Louis,[4] Reinhard Mailhammer,[6] Gert Riethmüller,[7] Ulrich Koszinowski,[5] and Martin Röcken[2,3,*]

[1]GSF-Institut für Molekulare Immunologie
81377 Munich
Germany

[2]Department of Dermatology
Eberhard Karls University Tübingen
72076 Tübingen
Germany

[3]Department of Dermatology and Allergology
Ludwig-Maximilians-University Munich
80336 Munich
Germany

[4]WHO Immunology Research and Training Center
Institute of Biochemistry
University of Lausanne
Switzerland

[5]Max-von-Pettenkofer-Institut
Ludwig-Maximilians-Universität Munich
80337 Munich
Germany

[6]GSF-Institut für Klinische Molekularbiologie und Tumorgenetik
81377 Munich
Germany

[7]Institute of Immunology
Ludwig-Maximilians-University Munich
80337 Munich
Germany

Summary

Conserved molecular patterns derived from pathogenic microorganisms prime antigen-presenting dendritic cells (DC) to induce adaptive T cell responses. In contrast, virus-infected or tumor cells that express low levels of major histocompatibility complex (MHC) class I activate natural killer (NK) cells for direct killing. It is unknown whether NK cell recognition of MHC class I^{low} targets can also induce adaptive T cell responses. Here, we show that MHC class I^{low} targets initiate a cascade of immune responses, starting with the immediate activation of NK cells. The activated NK cells then prime DC to produce IL-12 and to induce highly protective CD8 T cell memory responses. Therefore, sensing of MHC class I^{low} targets by NK cells can link innate and adaptive immunity to induce protective T cell responses and may alarm the immune system during early infection with noncytopathic viruses.

*Correspondence: mocikat@gsf.de (R.M.), mrocken@med.uni-tuebingen.de (M.R.)

Introduction

Activation of dendritic cells (DC) by conserved pathogen-associated molecular patterns (PAMP) induces cytotoxic CD8$^+$ T cells (CTL) that recognize peptides presented on MHC class I molecules. These activated CTL can specifically recognize and eliminate pathogenic microbial agents (Kägi et al., 1996; Medzhitov and Janeway, 2002; Matzinger, 2002). Viruses and tumors try to evade adaptive immune responses by reducing self-MHC class I molecules on the cell surface (Xu et al., 2001; Alcami and Koszinowski, 2000). Even though loss of MHC protects against CTL, it remains unclear whether this really reflects an escape mechanism, since MHC class I also acts as a ligand for killer inhibitory receptors (KIR). Cells missing MHC class I molecules lose resistance to NK cells and become susceptible to NK cell-mediated lysis (Lanier, 1998; Long, 1999; Ravetch and Lanier, 2000; Moretta et al., 2001).

Experiments with NKG2D receptor ligand (NKG2DRL) expressing tumors unraveled that these NK cell-stimulating ligands cannot only activate NKG2DR-expressing NK cells but also CD8$^+$ T cells and can induce protective CTL responses (Diefenbach et al. 2001, Hayakawa et al., 2002). However, data performed with either CD70- or CD80-transfected tumors suggest a novel pathway for the induction of protective T cell responses that is both NK cell- and interferon-γ (IFN-γ) dependent (Kelly et al., 2002a, 2002b). This hypothesis is supported by in vitro data showing that activated NK cells can release inflammatory cytokines and stimulate DC through an IFN-γ-dependent signal cascade (Glas et al., 2000; Höglund et al., 1997; Brown et al., 2001; Kelly et al., 2002a, 2002b; Piccioli et al., 2002; Gerosa et al., 2002; Ferlazzo et al., 2002; Zitvogel, 2002). Thus, in vivo recognition of MHC class I^{low} targets by NK cells might not only result in direct killing of these target cells. Recognition of MHC class I^{low} targets may also activate NK cells and enable them to prime DC for IL-12 production. These IL-12-producing DC1 then acquire the capacity to induce protective CTL responses. Such interactions between innate and adaptive immunity may be crucial for the induction of specific CTL responses that follow infection with cytomegalovirus (CMV) and other noncytopathic viruses, which rapidly inhibit surface expression of MHC class I molecules on the infected cells (Biron et al., 1999).

To test this hypothesis and to study the in vivo role of CMV-induced MHC class I suppression independently from other viral evasion mechanisms, we transfected various tumor cells with mouse CMV (mCMV)-derived genes that inhibit MHC class I export without affecting the expression of costimulatory molecules. We show that MHC class I^{low} target cells can rapidly activate NK cells in vivo. These activated NK cells then initiated a cascade that started with the IFN-γ-dependent priming of DC toward an IL-12-producing DC1-phenotype, which, subsequently, induced strong, protective CD8 T cell responses.

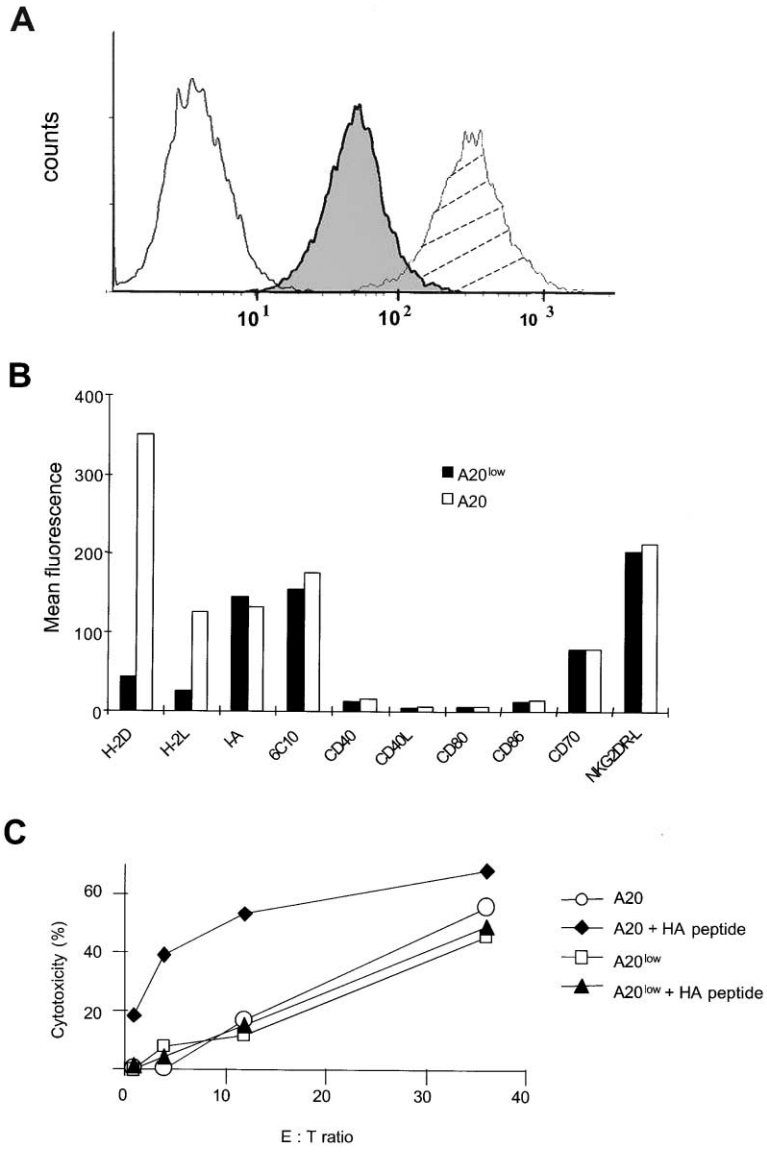

Figure 1. In Vitro Characterization of MHC Class I^{low} Tumor Cells

(A) MHC class I downregulation in m152-transfected A20 cells (gray area), as detected by FITC-labeled anti-H-$2D^d$ mAb HB87. The hatched area represents A20 wild-type tumor, the white area the isotype control. When compared to the parental cells, the remaining $H2D^d$ mean fluorescence intensity of the transfectants used in the experiments was 12% for A20-m152, 23% for A20-m06, and 19% for CT26-m06, respectively. Cells transfected with empty cassettes had normal MHC class I expression. Reduced MHC class I expression by CMV gene-transfected cells was verified prior to all experiments.

(B) Expression of H-2 and various other surface molecules by m152-transfected A20 cells (filled bars) or by A20 wild-type tumors (open bars). Isotype controls had a mean fluorescence <8. Representative results from at least two experiments.

(C) Specific lysis of m152-transfected $A20^{low}$ or A20 wild-type lymphoma by TCR-transgenic influenza hemaglutinin (HA)-specific CTL. Tumors were pulsed overnight with ^{3}H-thymidine and HA peptide, and specific lysis was determined using the JAM test as described in the Experimental Procedures. Experiments were performed in triplicates.

Results and Discussion

Inhibition of MHC Class I Expression by mCMV-Derived Genes Establishes Immunogenicity in Lymphoma or Carcinoma Cell Lines

Growth or rejection of tumors is a rigorous read-out system. Therefore, acceptance or rejection of tumor cells that differ only by the degree of MHC class I expression should provide important information on the effects of MHC class I suppression on the various cellular components of the innate and adaptive immune system in vivo. We transfected the A20 lymphoma and the CT26 carcinoma cell line with mCMV-derived genes, either m152 or m06, that prevent cell surface expression of MHC class I-peptide complexes in two different ways. m152 blocks the export of MHC class I complexes from the ER/*cis*-Golgi compartment (Krmpotic et al., 1999), while the m06 gene product targets MHC class I complexes to lysosomes (Ziegler et al., 1997). m152- and m06-transfected A20 cells expressed low levels of MHC class I (Figure 1A) but normal levels of MHC class II and of costimulatory molecules, including CD70, CD80, or NKG2DRL (Figure 1B). Similarly, MHC class I was selectively suppressed on the CT26 carcinoma. Cell lines transfected with empty vector cassettes had normal expression of MHC class I (not shown) and served as negative controls. Importantly, MHC class I^{low} mutants were not lysed by CTL (Figure 1C) and stimulated neither IFN-γ production nor proliferation by CTL (not shown). Therefore, transfection of A20 or CT26 cell lines prevented MHC class I expression without inducing costimulatory molecules that promote direct T cell activation by the MHC class I^{low} mutant tumor.

As predicted by the original studies of Kärre and coworkers (Kärre et al., 1986), reduced MHC class I expression protected against immune recognition in vitro only. In vivo, inhibition of MHC class I expression by either m152 or m06 strongly reduced or even abolished the capacity of A20 or CT26 tumor cells to grow in normal BALB/c mice (Figure 2A). Parental tumors or tumors transfected with empty vectors grew rapidly and caused death within 30 to 40 days (Figure 2A). Rejection of MHC

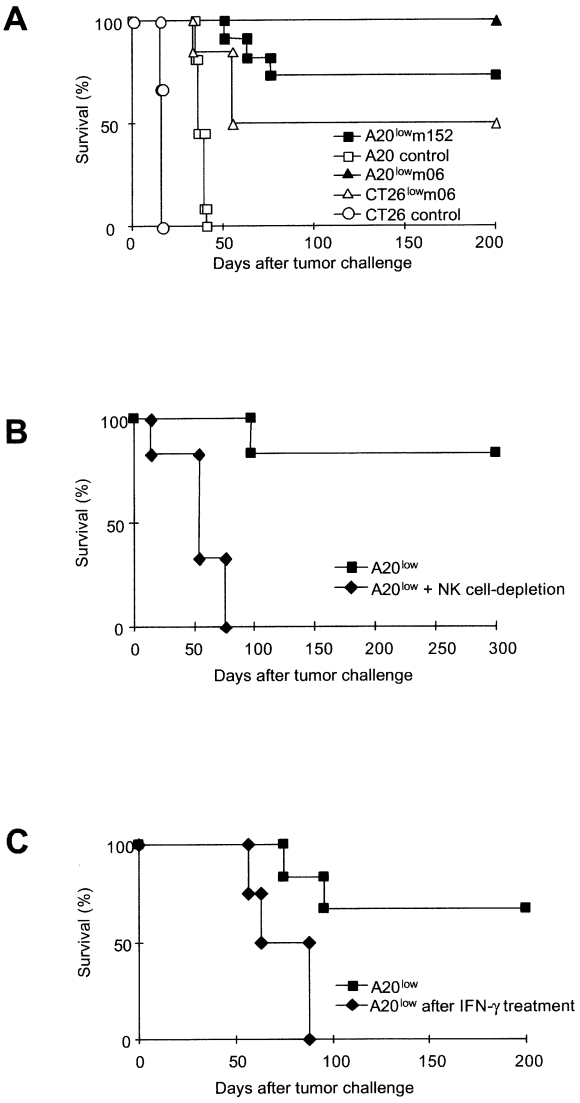

Figure 2. Rejection of MHC Class I^{low} Tumor Cells Requires NK Cells

(A) Survival of BALB/c mice after injection of 10^6 A20 or 10^6 CT26 cells. Injected tumor cells were either controls or cells transfected with either the m152 gene ($A20^{low}$-m152) or the m06 gene ($A20^{low}$-m06 or $CT26^{low}$-m06). Tumor cells transfected with empty vector cassettes showed identical growth kinetics as wild-type cells.

(B) Survival of BALB/c mice or NK cell-depleted BALB/c mice after injection of $A20^{low}$ cells. NK cells were depleted using rabbit anti-asialo GM1 antibody.

(C) Survival of BALB/c mice after injection of m152-transfected A20 cells, following the restoration of MHC class I expression by preincubation with 670 U/ml IFN-γ.

class I^{low} tumors was strictly dependent on NK cells (Figure 2B) and, as predicted (Höglund et al., 1997), most mice survived even when given 10-fold higher numbers of MHC class I^{low} tumor cells (not shown). Enhancement of MHC class I expression on m152-transfected tumors by IFN-γ pretreatment prior to injection restored their capacity to grow in immunocompetent mice (Figure 2C). Since under most conditions IFN-γ rather enhances the immunogenicity of tumors (Ikeda et al., 2002), this result supports the concept that the suppression of MHC class I was directly responsible for the NK-dependent rejection of the m152-transfected tumors.

NK Cells Activated by MHC Class I^{low} Tumors Prime DC to Produce IL-12 and to Stimulate CD8 T Cells In Vivo

In vitro activation of NK cells by MHC class I^{low} targets enables them to interact with DC (Gerosa et al., 2002; Zitvogel, 2002). To test in vivo whether NK cells only kill the MHC class I^{low} tumor cells or whether this NK-tumor cell interaction results in a more complex interaction that also involves other immune cells, such as DC or T cells, we isolated lymphoid cells various times after intravenous injection of MHC class I^{low} A20 lymphomas from the lung, the site of the first tumor cell passage, and the spleen. Already 4 hr after injection of MHC class I^{low} A20 lymphomas, IFN-γ, IL-12p40, and IL-12p35 mRNA were strongly expressed in the lung and in the spleen. In order to determine the precise cellular origin of the IFN-γ or IL-12 mRNA, we prepared ≥99% pure populations of either NK, DC, or non-NK cells by fluorescence-activated cell sorting, using multicolor staining, as described (Biedermann et al., 2001). At 4 hr, IFN-γ mRNA and protein was exclusively expressed by NK cells (Figure 3A and insert). No IFN-γ mRNA was detectable in the remaining pool of non-NK cells of either spleen or lung (Figure 3A and data not shown). In sharp contrast, IL-12p40 and IL-12p35 mRNA appeared only in highly purified DC (Figure 3B and data not shown). Virtually no or only very little IFN-γ mRNA, IL-12 mRNA, or IFN-γ protein appeared in either population after injection of the parental A20 lymphoma (Figures 3A and 3B and data not shown). After 1 day, IFN-γ or IL-12 mRNA expression had declined. But on day four, IFN-γ mRNA was again strongly expressed in both organs (Figure 3C). However, at this time IFN-γ mRNA was expressed by the $CD3^+CD8^+$ T cell population (Figure 3C). IFN-γ mRNA was also translated, as we found IFN-γ protein on the surface of freshly isolated $CD3^+$ T cells (Figure 3C, insert). Moreover, CD8 T cells from the spleen produced large amounts of IFN-γ either spontaneously (not shown) or after in vitro stimulation (not shown) when isolated and cultured for 2 days in vitro. In agreement with the mRNA data, only T cells derived from mice primed with MHC class I^{low} A20 lymphomas produced IFN-γ. These data are best conceivable with the concept that MHC class I^{low} A20 lymphoma activated NK cells and these activated NK cells then primed the local DC to stimulate CD8 T cells. To directly test this hypothesis, we deleted NK cells prior to the injection of MHC class I^{low} A20 lymphomas and analyzed again IFN-γ and IL-12 mRNA in these three populations. NK depletion entirely abrogated the early IFN-γ peak by NK cells (Figure 4A, upper two lanes), IL-12 mRNA expression by DC (Figure 4A, lower two lanes), and subsequently the IFN-γ mRNA expression by CD8 T cells (Figure 4B). Thus, deletion of NK cells did not only abolish the resistance of mice against MHC class I^{low} tumors (Figure 2B), it also prevented activation of either DC or T cells. Together, these data exclude that either DC or T cells recognized the MHC class I^{low} A20 lymphoma and strongly suggest a cascade, where NK cells first primed DC to become an IL-12-producing DC1. These DC1 then stimulated CD8

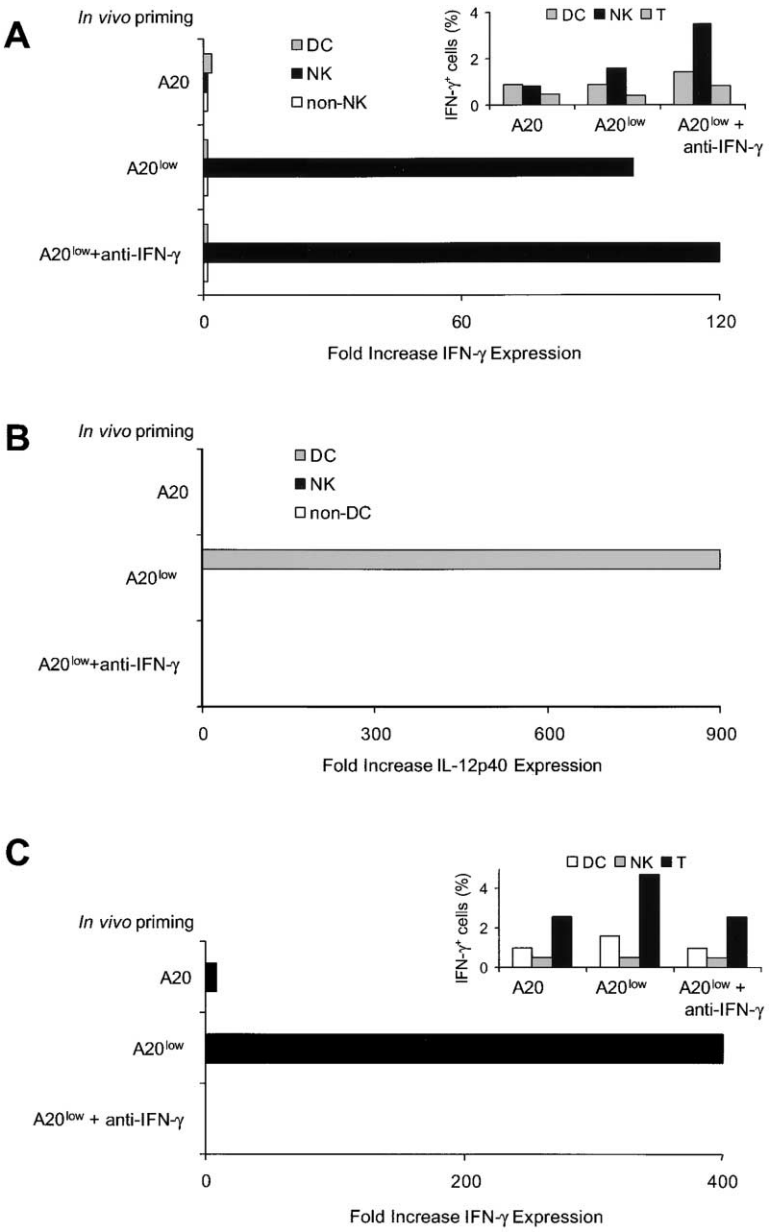

Figure 3. MHC Class I^{low} Tumors Activate NK Cells to Prime DC for IL-12 Production and for the Induction of $CD8^+$ T Cell Responses

(A) IFN-γ mRNA expression by sorted NK cells, DC, and non-NK cells from spleens 4 hr after injection of either A20 or $A20^{low}$ lymphoma cells. Some of the mice had received anti-IFN-γ mAb 4 hr before tumor challenge. The insert shows IFN-γ^+ DC, NK, or T cells in the spleen detected by an IFN-γ capture assay and counterstained for CD11c, DX5, and CD3 expression. The high amounts of IFN-γ detected in mice treated with $A20^{low}$ and anti-IFN-γ are reproducible and are due to the enrichment of IFN-γ on the cell surface after anti-IFN-γ mAb treatment.

(B) IL-12p40 mRNA expression by sorted $CD11c^+$ DC or non-DC from spleens 4 hr after intravenous injection of either A20 or $A20^{low}$ lymphoma cells. Some of the mice had received anti-IFN-γ antibody 4 hr before tumor challenge.

(C) IFN-γ mRNA expression in $CD3^+CD8^+$ T cells 4 days after injection of either A20 or $A20^{low}$ lymphoma cells. Some of the mice received anti-IFN-γ mAb 4 days before injection of $A20^{low}$ lymphoma cells. The insert shows percentages of IFN-γ^+ DC, NK, or T cells in the spleen detected by an IFN-γ capture assay and counterstained for CD11c, DX5, and CD3 expression. All panels show one out of two similar experiments.

T cells. Yet the data cannot exclude that NK cells directly stimulated CD8 T cells through DC-independent mechanisms. As in vitro data revealed that priming of DC by activated NK cells is IFN-γ dependent (Gerosa et al., 2002; Zitvogel, 2002), we neutralized IFN-γ during the injection of MHC class I^{low} A20 lymphoma. Neutralizing IFN-γ had no effect on IFN-γ mRNA or protein expression by NK cells (Figure 3A and insert), but entirely abrogated activation of both DC (Figure 3B) and T cells (Figure 3C and insert), strongly supporting the concept that the DC-T cell interactions induced the IFN-γ-producing CD8 T cells and not direct NK-T cell interactions.

To directly test the functional relevance of this postulated NK-DC1-T cell axis, we blocked, further downstream, the action of DC-derived IL-12 with mAb as described (Biedermann et al. 2001). Indeed, anti-IL-12 entirely prevented the activation of IFN-γ-producing T cells that were specifically induced by the MHC class I^{low} A20 lymphoma (Figure 4C). To test whether this consecutive activation of first NK cells and then DC1 was really T cell independent and to exclude any direct effects of $A20^{low}$ lymphoma on T cells, we injected $A20^{low}$ cells into mice with severe combined immune deficiency (SCID). Only $A20^{low}$ clearly induced detectable IFN-γ production by NK cells (about 5%), while the effect of A20 lymphoma cells was much weaker (data not shown). In consequence, $A20^{low}$ cells induced a strong and IFN-γ-dependent increase in IL-12-producing DC1, while A20 lymphoma cells had only a weak effect (Figure 5A).

MHC Class I^{low} Tumors Induce Protective CD8 T Cell Responses

Kärre et al. found that NK cells can directly reject MHC class I-deficient tumors (Kärre et al., 1986). The data

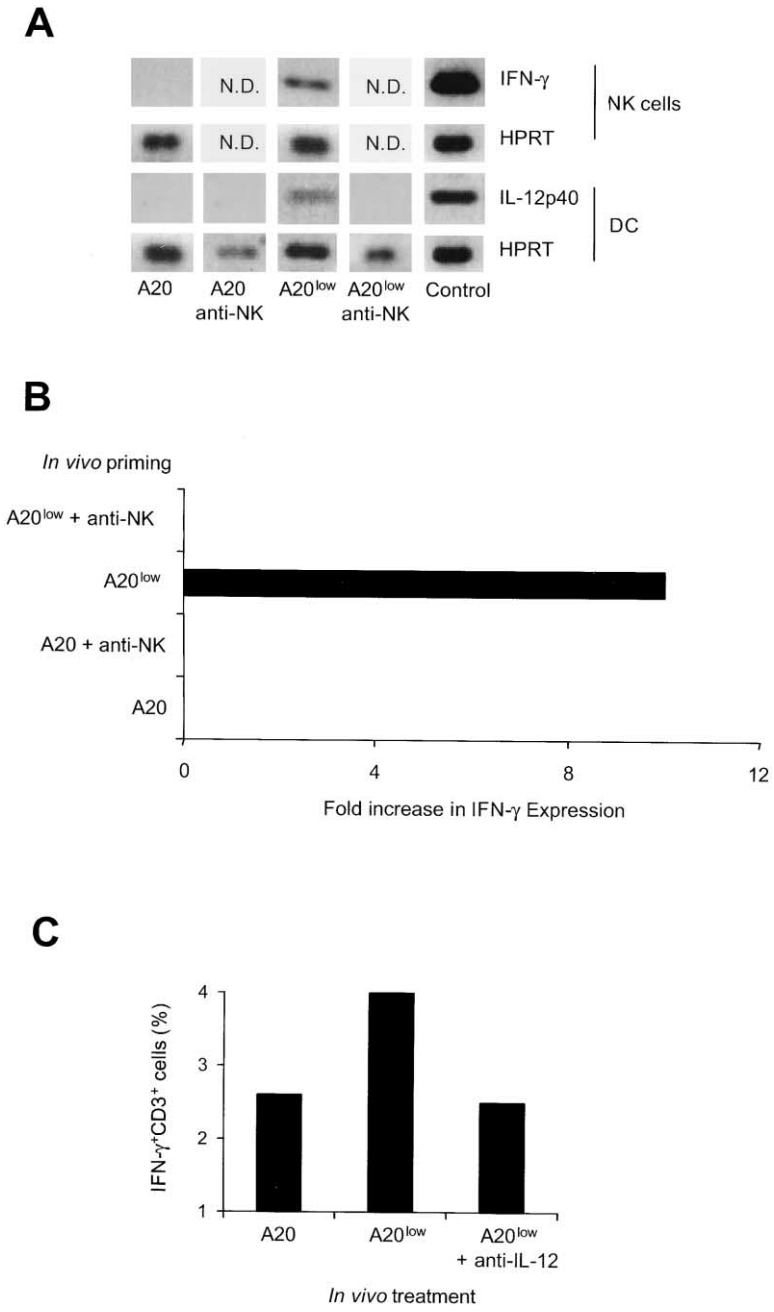

Figure 4. T Cell Priming Is Dependent on NK Cells and IL-12

(A) IFN-γ and HPRT expression by NK cells (upper two lanes) or IL-12p40 and HPRT expression by DC (lower two lanes). Mice received either A20 lymphoma or A20^{low} lymphoma cells. In half of the mice, NK cells were depleted 3 days before by rabbit anti-asialo GM1 antibody. Four hours after intravenous tumor cell injection, the indicated cell population was positively sorted and mRNA was amplified and determined as described (Biedermann et al., 2001). N.D., not detectable because of efficient NK cell depletion (confirmed by FACS analysis).

(B) Four days after injection of either A20 or A20^{low} lymphoma cells, CD3^{+}CD8^{+} T cells were isolated and IFN-γ mRNA expression was determined by quantitative RT-PCR. In half of the animals, NK cells were depleted.

(C) Percentages of IFN-γ^{+}CD3^{+} spleen cells as determined by an IFN-γ capture assay after treating mice either with A20, A20^{low}, or A20^{low} + anti-IL-12 mAb.

above showed that injection of MHC class I^{low} A20 lymphomas did not only activate NK cells but also primed DC to stimulate CD8 T cells. To test the biological relevance of this T cell stimulation in vivo, we next studied MHC class I^{low} A20 lymphomas in nude/nude mice. Surprisingly, nude/nude mice were entirely unable to control MHC class I^{low} A20 lymphomas. As MHC class I^{low} A20 lymphomas grew in nude/nude mice as rapidly as wild-type tumors (Figure 5B), the data showed (1) that the MHC class I^{low} tumors tested had no growth defect and (2) that resting NK cells alone were not capable of controlling these MHC class I^{low} tumors. NK cells can be further activated and their functioning can be enhanced by IL-2, IL-12, or immunostimulatory DNA motifs, such as CpG-DNA 1668 (Biron et al., 1999; Dokun et al., 2001). To determine whether in vivo activated NK cells are more efficient in rejecting MHC class I^{low} tumors, SCID mice received CpG-DNA 1668 prior to injection of either MHC class I^{low} or wild-type tumors. Pretreatment with CpG-DNA 1668 resulted in solid protection, but only against MHC class I^{low} tumor cells, whereas parental tumors grew normally (Figure 5C). Therefore, only activated NK cells deleted MHC class I^{low} tumors efficiently in the absence of T cells.

The failure of nude/nude or unprimed SCID mice to control MHC class I^{low} tumor cells demonstrated that resting NK cells alone could not achieve tumor cell elimination and suggested that T cells contributed to the

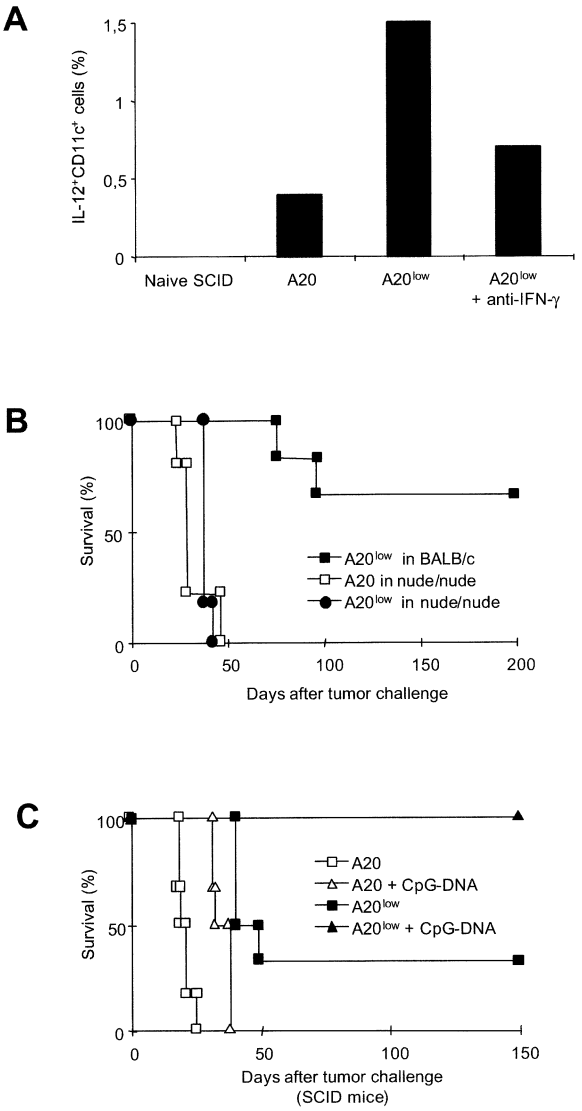

Figure 5. DC-Activation by MHC Class I^{low} Tumors Is T Cell Independent, but Rejection of MHC Class I^{low} Tumor Cells Is T Cell Dependent

(A) Percentages of IL-12-producing DC as determined by intracellular FACS staining of spleen cells from SCID mice after treatment with either A20, A20^{low}, or A20^{low} + anti-IFN-γ mAb.

(B) Survival of BALB/c nude/nude mice injected with either A20 wild-type cells or m152-transfected A20 MHC class I^{low} cells.

(C) Survival of SCID mice after injection of either 10^6 m152-transfected A20 MHC class I^{low} cells or 10^6 A20 wild-type cells. Half of the SCID mice had received 10 nMol CpG-DNA 1668 oligonucleotide on days –1 and 1 of the tumor cell transfer.

control of MHC class I^{low} tumors in normal BALB/c mice. If T cells were involved in the rejection of MHC class I^{low} tumors, MHC class I^{low} A20 lymphomas should establish long-lasting protective immunity against the parental tumors in BALB/c mice, but not in T cell-deficient SCID mice. To test this, we injected MHC class I^{low} A20 lymphoma cells either into BALB/c mice or into SCID mice pretreated with CpG-DNA 1668. Three months later, we challenged the surviving mice with a lethal dose of parental A20 lymphoma. T cell-bearing BALB/c mice were protected while all SCID mice succumbed to the second tumor challenge (Figure 6A). Notably, these experiments also showed that only BALB/c mice "primed" with 10^6 MHC I^{low} tumors developed protective immunity, while mice that received only 10^5 MHC class I^{low} A20 cells during the initial priming remained fully susceptible to the wild-type tumor (Figure 6A). Therefore, recognition of 10^6 MHC class I^{low} A20 lymphoma cells by NK cells was required to generate the innate signals that primed for protective immunity. As 10^6 tumor cells provide a stronger stimulus for NK cells than 10^5 tumor cells, it is most likely that the 10-fold higher number of MHC class I^{low} A20 lymphoma cells also provided a stronger alarm signal for DC.

To determine the relative contribution of CD4 and CD8 cells to the T cell-mediated protection induced by MHC class I^{low} tumor cells, we depleted either CD4$^+$ or CD8$^+$ T cells with mAb. Each treatment deleted more than 99% of the targeted population (not shown). Injection of control mAb or depletion of CD4$^+$ cells did not affect the resistance against MHC class I^{low} tumors (Figure 6B). In sharp contrast, depletion of CD8$^+$ entirely abrogated protection against the MHC class I^{low} tumor cells, as did depletion of NK cells with either anti-asialo GM1 antibody or anti-IL-2Rβ chain mAb (Figures 2B and 6B).

In vitro, MHC class I^{low} A20 cells induced neither CTL-mediated killing nor T cell stimulation (Figure 1C and data not shown). As the rapid stimulation of CD8$^+$ T cells was obviously essential for the rejection of MHC class I^{low} A20 cells in vivo, we tried to develop CD8$^+$ T cell lines, in order to obtain functional proof for the in vivo stimulation of T cells. Indeed, we easily derived IFN-γ-producing, A20-specific T cell lines from mice injected with MHC class I^{low} A20 cells (Figure 7A), but only rarely from mice that had received the parental A20 lymphoma or from A20^{low}-injected mice that were depleted of NK cells. To test the in vivo relevance of this fast generation of A20-specific CTL responses by MHC class I^{low} tumors, we injected MHC class I^{low} A20 lymphoma cells together with a lethal dose of A20 wild-type tumor. Simultaneous injection of parental A20 lymphomas and MHC class I^{low} A20 lymphomas protected 25% of BALB/c mice against the otherwise lethal wild-type tumor load (not shown), directly showing that recognition of MHC class I^{low} A20 lymphomas by NK cells rapidly generated A20-specific CTL responses that even protected mice against a simultaneous challenge with the lethal wild-type A20 lymphoma.

Following injection of MHC class I^{low} A20 lymphoma, NK cells linked innate and adaptive immunity and established long-term protection against the parental tumor in normal BALB/c but not in SCID mice. To test whether this protection was based on T cell memory or other mechanisms, such as priming for antibody-dependent cytotoxicity, we primed mice with MHC class I^{low} A20 lymphomas and deleted T cells after 3 months, directly prior to a second tumor challenge. As shown above, inoculation with MHC class I^{low} A20 tumors established protective tumor immunity in BALB/c mice (Figure 6A). However, when T cells were deleted directly prior to the challenge with A20 lymphoma, all mice died within 30 days (Figure 7B). Thus, activation of NK cells with MHC class I^{low} A20 lymphomas initiated a cascade that started with the priming of DC to become an IL-12-producing DC1 phenotype and ultimately resulted in a CD8-dependent T cell memory response that even protected against the parental A20 lymphoma.

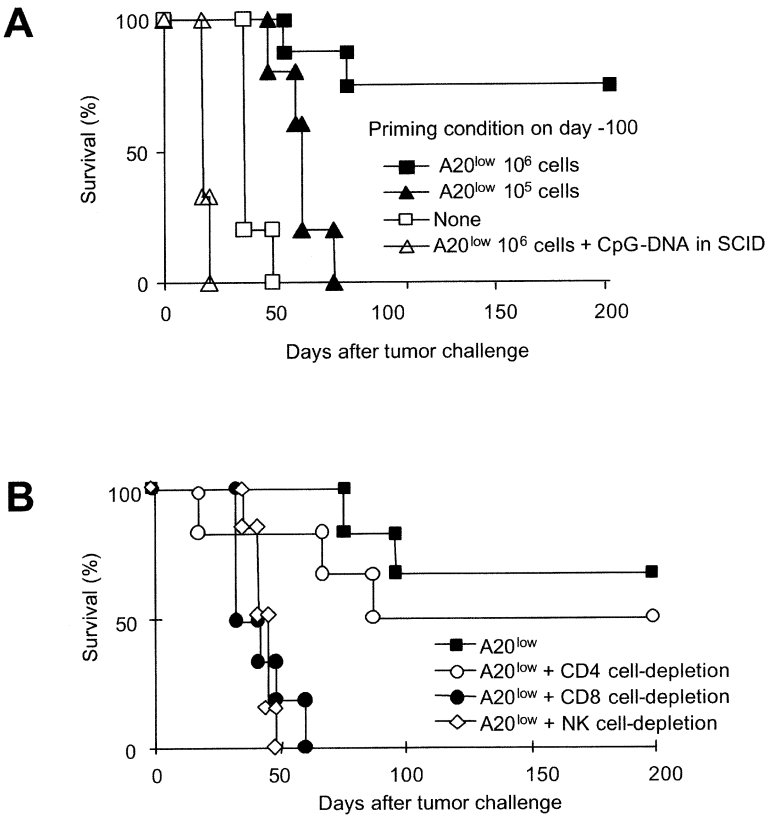

Figure 6. NK Cell-Induced Activation of DC Establishes Long-Lasting Memory

(A) Long-term memory induced by NK cell-mediated rejection of $A20^{low}$ variants. BALB/c mice were primed with either 10^5 or 10^6 of MHC class I^{low} A20 cells or remained unprimed. A fourth group of SCID mice was primed with 10 nMol CpG-DNA and10^6 MHC class I^{low} A20 cells. On day +100, mice received 10^6 A20 wild-type tumor cells and survival was monitored.

(B) Rejection of $A20^{low}$ lymphoma cells in BALB/c mice requires NK and $CD8^+$ T cells. BALB/c mice were depleted of the indicated cell populations with mAb (CD8 cells with RmCD8-2, CD4 cells with RmCD4-2, NK cells with anti-IL-2 receptor β chain TM-β1) 3 days prior to the injection of 10^6 $A20^{low}$ lymphoma cells.

Concluding Remarks

It is currently assumed that recognition of either PAMPs or "missing self" results in two distinct immune responses against microbial pathogens. PAMPs prime DC and possibly other antigen-presenting cells to express cytokines and costimulatory molecules that induce adaptive immune responses (Kägi et al., 1996; Medzhitov and Janeway, 2002; Matzinger, 2002). In contrast, NK cells directly lyse cells infected with viruses that inhibit MHC class I expression during primary infection (Medzhitov and Janeway, 2002; Matzinger, 2002; Lanier, 1998; Long, 1999; Ravetch and Lanier, 2000; Moretta et al., 2001). More recent data suggest that, in addition, NKG2DR-NKG2DRL interactions are capable to directly stimulate naive CD8 T cells and to induce T cell memory (Diefenbach et al. 2001; Hayakawa et al., 2002).

The data reported here show a novel pathway leading to adaptive immune responses. Activation of NK cells is dependent on activating receptors that are normally functionally silenced by MHC-recognizing KIR (Lanier, 1998; Long, 1999; Ravetch and Lanier, 2000; Moretta et al., 2001). Here, we demonstrate that activation of NK cells by missing self can functionally link innate with adaptive immune responses, through NK cell-dependent priming of DC. Sudden appearance of MHC class I^{low} tumor cells resulted in rapid activation of IFN-γ-producing NK cells. Activation of NK cells was needed to prime DC for IL-12 production through IFN-γ-dependent signals. Others have recently shown in vitro that NK cells and DC can directly interact (Glas et al., 2000, Gerosa et al., 2002). As anti-IFN-γ-mAb prevented DC1 priming also in vivo, it is likely that such NK cell-DC interactions occurred also in response to MHC class I^{low} cells, even though we did not address this point. These DC1 cells had the capacity to recruit and activate naive T cells through IL-12-dependent signals and to induce protective CTL responses in vivo. Even though activation of NK cells and induction of DC1 was entirely T cell independent, we cannot exclude that IFN-γ-producing $CD8^+$ T cells appearing around day 4 further enhanced the establishment of protective immunity. This cross-priming, that follows the classical T cell activation pathway, is fundamentally different from tumor immunity established by stimulation through ligands of NKG2DR, where NKG2DR-NKG2DRL interactions directly stimulate CD8 T cells (Diefenbach et al. 2001; Hayakawa et al., 2002; Zitvogel, 2002). Importantly, NK cells induced CTL only when activated by large numbers of MHC class I^{low} cells. This threshold may explain why noncytopathic viruses that rapidly inhibit self-MHC expression in large numbers of cells during primary infection induce strong CTL responses, whereas tumors that slowly and progressively lose self-MHC remain largely ignored by the adaptive immune system. Thus, sensing of missing self by NK cells or immune activation by PAMP are two alternative, innate pathways leading to the initiation of adaptive immune responses.

Experimental Procedures

Generation and In Vitro Characterization of MHC Class I^{low} Tumor Cells

The B cell lymphoma cell line A20 and the carcinoma cell line CT26 and their variants were maintained in RPMI 1640 medium supplemented with 5% FCS, 2 mM glutamine, nonessential amino acids, antibiotics, and 50 μM 2-mercaptoethanol. For gene transfer, cell

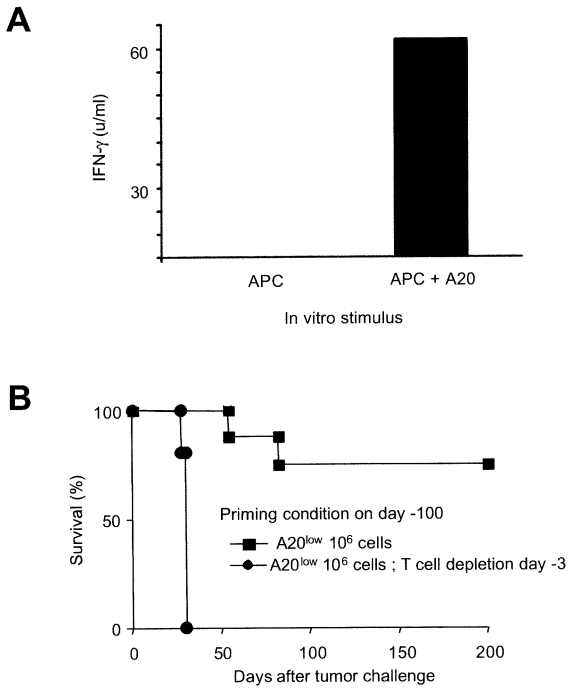

Figure 7. Rejection of MHC Class I^{low} A20 Lymphoma Cells Establishes T Cell Memory Responses

(A) Four days after injection of A20^{low} lymphoma, A20-specific T cell lines were generated. After 10 days, these T cell lines were stimulated with either APC alone or with APC and irradiated A20 cells. No T cell line could be established from NK cell-depleted mice. IFN-γ production was determined in the supernatant by ELISA. Representative data from three independent experiments which yielded similar results.

(B) Long-term memory induced by NK cell-mediated rejection of A20^{low} variants depends on T cells. BALB/c mice were primed with 10^6 of MHC class I^{low} A20 cells and, on day 100, re-challenged with 10^6 A20 wild-type tumor cells. In some of the mice, total T cells were depleted 3 days prior to the second challenge with mAb MmT1. The data are part of the experiment shown in Figure 6A.

lines were electroporated with the mCMV-derived genes m152 or m06 (Krmpotic et al., 1999; Ziegler et al., 1997) or with empty vector cassettes using a Genepulser apparatus (Biorad, München, Germany). Transfectants were selected for resistance against geneticine in 96-well plates and resistant clones were tested for H-2D^d expression using the FITC-labeled mAb HB87 (ATCC). MHC class I^{low} cells were subcloned by FACS sorting. mAb specific for H-2L, I-A, CD80, CD86, CD40, and CD40L were purchased from Pharmingen (San Diego, CA). The 6C10 mAb specifically recognizes the A20 Ig Id (Selmayr et al., 1999). The PE-labeled NKG2D tetramer recognizes the NKG2D receptor ligand (Knabel et al., 2002).

Animals and In Vivo Experiments

Female BALB/c and BALB/c nude/nude mice purchased from Bommice (Ry, Denmark) and SCID mice grown in our animal facility were housed under specific pathogen-free conditions. If not otherwise indicated, groups of six mice were injected intravenously with 10^5-10^6 MHC class I^{low} or wild-type tumor cells. Mice were euthanized when showing tumor growth. Depletion of pan-T cells or CD4 or CD8 subpopulations was performed with mAb MmT1, RmCD8-2, or RmCD4-2, respectively, as described (Mocikat et al., 1997). For NK cell depletion, mice were intraperitoneally injected with 1 mg of IL-2 receptor β chain-specific mAb TM-β1 (Tanaka et al., 1993) 4 days before and 0.1 mg 2 and 14 days after A20^{low} challenge. Due to their lower expression density of IL-2 receptor β chain, T cells were not eliminated by this mAb (not shown). Alternately, rabbit anti-asialo GM1 antibody (Wako Chemicals, Neuß, Germany) was injected intravenously at days −3, 4, and 11. To stimulate NK cells in vivo, mice received 10 nMol CpG-oligonucleotide 1668 (ref. in Egeter et al., 2000) at days −1 and 1. All animal experiments were reproduced one to four times. Significance was tested using the logrank test; all results shown were between $p < 0.05$ and $p < 0.005$. Typical results are shown in the figures.

Generation of T Cell Lines

CD8$^+$ T cells were prepared from spleens of transgenic mice bearing a T cell receptor specific for influenza hemagglutinin (HA) by magnetic cell separation (Miltenyi Biotec, Heidelberg, Germany) using a one-step positive selection with anti-CD8-coated microbeads (Miltenyi Biotec). CD8 positive cells were then cultured with syngeneic APC and 7.5 μM HA peptide (SFERFEIFPK, Biotrend, Köln, Germany) and IL-2. After 10 days, cells were used for cytotoxicity experiments.

Ex Vivo T Cell Analysis

Four days after in vivo priming of mice with either A20 lymphoma or A20^{low} lymphoma, T cells were isolated from spleens by negative selection over Biotex T cell columns (TEBU, Frankfurt, Germany) to at least 95% purity. Isolated T cells were stimulated in vitro with irradiated A20 tumor cells and syngeneic APC. IFN-γ was determined in the 24 hr culture supernatant. Part of the cells was further expanded for 10 days with IL-2 (Chiron, Ratingen, Germany) and again restimulated with syngeneic APC in the presence or absence of tumor cells.

Cytotoxicity Assay

Specific killing of A20 or A20^{low} cells was determined using the JAM test (Matzinger, 1991). In brief, A20 or A20^{low} cells were loaded with ^{3}H-thymidine and pulsed with HA peptide overnight. ^{3}H-thymidine-labeled A20 or A20^{low} cells were incubated in 96-well plates (Greiner, Frickenhausen, Germany) with in vitro-generated HA-specific CTL at the indicated effector:target ratio. After 4 hr, cells were harvested on glass fiber filters and ^{3}H-thymidine containing cells were determined using an automatic filter counting system (TRACE96, Inotech, Dittikon, Switzerland). The percentage of specific lysis was calculated as described (Kirberg et al., 1994).

Detection of Cytokines

IFN-γ was quantified in cell culture supernatants by an ELISA using R4-6A2 as a capture and XMG-1.2 as a secondary mAb. All mAb were from BD Pharmingen (Heidelberg, Germany) and recombinant standards from R&D Systems (Wiesbaden, Germany). To quantify IFN-γ-expressing cells in vivo, spleen cells were isolated and subjected to an IFN-γ capture assay using the Miltenyi kit (Miltenyi, Bergisch-Gladbach, Germany). The cells were counterstained by mAb against CD11c, CD3, or DX5 and analyzed by FACS. IL-12-expressing cells were detected by intracellular FACS analyses. Splenocytes were incubated for 10 hr in the presence of 1 μg/ml Brefeldin A (Sigma, München, Germany). After staining with FITC-conjugated anti-CD11c mAb, cells were fixed and permeabilized using the Fix and Perm Kit (Caltag, Burlingame, USA). IL-12 was detected by incubation with the rat anti-IL-12 mAb C17.8 followed by phycoerythrine-labeled anti-rat IgG.

Determination of Cytokine mRNA

Either 10^6 A20 or 10^6 A20^{low} cells were intravenously injected into BALB/c mice that had received PBS or 1 mg of anti-IFN-γ intraperitoneally 4 hr before tumor cell injection. 4 hr after tumor cell injection, spleen cells were sorted by flow cytometry into NK cells (DX5$^+$CD3$^-$), DC (CD11c$^+$CD19$^-$F4/80$^-$) and the corresponding NK- and DC-depleted subpopulations. CD8$^+$ T cells were sorted from spleens and lungs 4 days after tumor injection using anti-CD3 and anti-CD8 antibody. After mRNA extraction, the relative levels of IFN-γ and IL-12p40 mRNA were determined in the different subpopulations by semiquantitative RT-PCR (Biedermann et al., 2001). Results were expressed as the increase of the specific mRNA as compared to noninjected control mice.

Acknowledgments

We are grateful to D. Schendel and C. Falk for helpful discussions and critically reviewing the manuscript. The gift of NKG2D tetramer by D. Busch is gratefully acknowledged. We thank H. Jennen, J. Jasny, B. Konkol, G. Alvarez, and C. Reitmeier for expert technical assistance, J. Mysliwietz for help with FACS sorting, and B. Frankenberger for RNA preparations. The work was supported by Wilhelm-Sander-Stiftung (97.041.2), Deutsche Forschungsgemeinschaft (RO 764/8-1), and SFB 455, 456, Deutsche Krebshilfe (10-1917), and the Swiss National Science Foundation.

Received: April 23, 2003
Revised: August 15, 2003
Accepted: August 20, 2003
Published: October 14, 2003

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