NK1.1$^+$ cells are important for the development of protective immunity against MHC I-deficient, HPV16-associated tumours

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Abstract. Loss or downregulation of MHC class I molecules on tumour cells is a common mechanism by which tumours can escape T-cell mediated immune responses. In this study, we examined the role of different immune cell lineages in the development of immunity against tumours of the same aetiology but with different MHC class I expression. *In vivo* depletion of CD8$^+$ cells, but not of CD4$^+$ or NK1.1$^+$ cells in the immunization period resulted in complete elimination of the protective effects of immunization with irradiated TC-1 cells (MHC class I-positive cell line) against the TC-1 tumour challenge. After immunization with irradiated TC-1/A9 or with MK16 tumour cells (MHC class I-deficient sublines) a remarkable dependence on the presence of NK1.1$^+$ cells was observed, while the tumour growth inhibition after CD4$^+$ or CD8$^+$ depletion was not efficient. Cytotoxic activity induced by TC-1 cell immunization was significantly abrogated in the CD8$^+$ and CD4$^+$ but not NK1.1$^+$ cell-depleted mice, as compared to the immunized only controls. After MK16 or TC-1/A9 cell immunization, NK1.1$^+$ but not CD8$^+$ and CD4$^+$ cell-depleted mice displayed significant reduction of specific cytotoxicity. Mice immunized with TC-1 cells showed similar percentage of IFNγ producing cells in CD8$^+$, CD4$^+$ and NK1.1$^+$ cell populations. On the other hand, the highest proportion of IFNγ producing cells after immunization with TC-1/A9 or MK16 cells was concentrated into the NK1.1-positive spleen cell population. Our data demonstrate that the development of immunity against MHC class I-deficient tumours is highly dependent on the activity NK1.1$^+$ cell population.

Introduction

Cell surface expression of MHC class I molecules on tumour cells is an important determinant in the interplay between tumour cells and the immune system. Absence or downregulation of the MHC class I molecules on tumour cells can abrogate tumour cell recognition by the cytotoxic T lymphocytes (CTLs); on the other hand, it can trigger the innate part of the immune system. Thus, the MHC class I levels on tumour cells participate in determining which part of the immune system (adaptive or innate) can interact with tumour cells and how these parts can co-operate in the immunity development (1-3). Among tumour escape routes, alteration of MHC class I cell surface expression is the mechanism used in a wide variety of human solid tumours, including cervical carcinoma (4-8).

Although MHC class I downregulation represents a common mechanism by which tumours can escape from the specific immunity, a number of studies have shown that the growth of MHC class I-deficient tumours can be inhibited by vaccines (1,3,7,9). The effective immunization protocols should respect the MHC class I status of target tumour cells. The mechanisms underlying the development of specific immunity against MHC class I-deficient tumours are not fully understood, they may be distinct from those mediating immunity against MHC class I positive tumours and, moreover, they may be specific for particular tumours according to the levels of residual MHC class I expression and its inducibility. The important role is attributed to the NK cells and interferon γ (IFNγ) production and their effects on the specific immune response activation. The role of indirectly activated CD8$^+$ and CD4$^+$ cells cannot be excluded either (10-13).

The data from our previous experiments have shown that immunization with MHC class I-deficient but not with MHC class I-positive tumour cells inhibited the growth of MHC class I-deficient, HPV16-associated murine tumours expressing E6 and E7 viral oncoproteins (14). *In vivo* depletion studies in the course of the tumour growth revealed that the effective immune responses against MHC class I-negative tumours in animals immunized with MHC class I-deficient tumour cells were dependent on natural killer cells. Using the same model, we have shown that induction of the specific immunity by immunization with a peptide comprising CTL and Th epitope but not solely a CTL epitope, can elicit protective immunity against MHC class I-deficient tumours (14,15).

In this study, the influence of CD8$^+$, CD4$^+$ and NK1.1$^+$ cell subpopulations on the development of protective immunity by prophylactic immunization with irradiated tumour cells in the model of MHC class I-deficient, HPV16-associated murine tumours was examined.

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Key words: MHC class I-deficient tumours, CD8$^+$, CD4$^+$, NK1.1$^+$ cell subpopulations, interferon γ

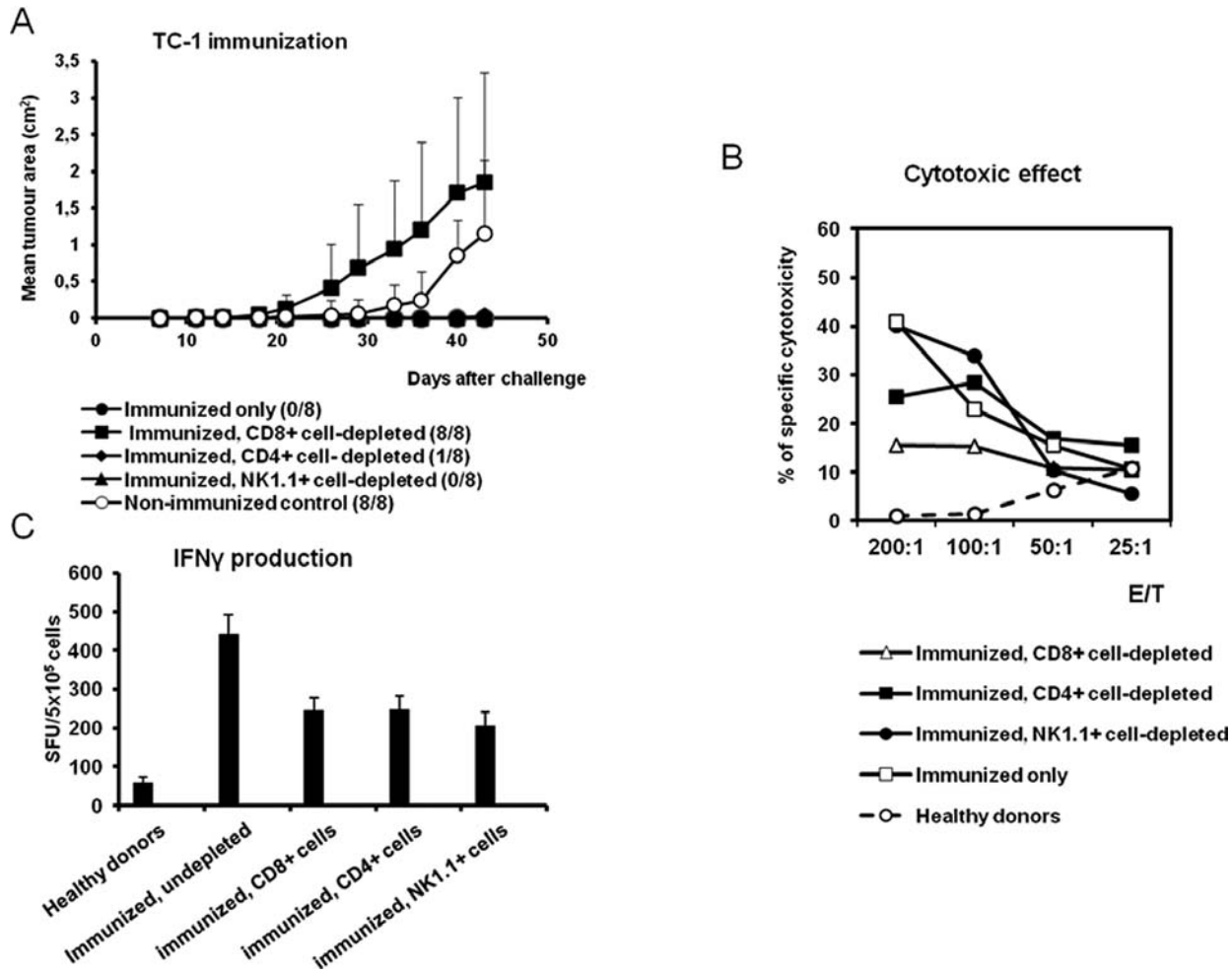

Figure 1. Immunization with TC-1 cells. (A) $CD8^+$, $CD4^+$ or $NK1.1^+$ cell-depleted and undepleted mice were immunized twice in a 3-week interval with irradiated tumour cells. Ten days after the second immunization, mice were challenged with the corresponding tumour cells. In parentheses: no. of mice with tumour/total no. of mice. Statistical significance (analysis of variance): Immunized only x non-immunized control, immunized + $CD8^+$ cell-depleted, $p<0.05$. Analysis of tumour takes: Immunized only, immunized + $CD4^+$ or $NK1.1^+$ cell-depleted x non-immunized control, immunized + $CD8^+$ cell-depleted, $p<0.001$ - χ^2 test. (B) ^{51}Cr microcytotoxicity test. CD8-depleted *in vivo* depleted mice exhibited decreased activity against TC-1 targets. (C) ELISPOT assay. Production of IFNγ is equally distributed among $CD8^+$, $CD4^+$ or $NK1.1^+$ subpopulations obtained after MACS separation of spleen cells of immunized, but undepleted mice ($p>0.05$, t-test).

Materials and methods

Mice. C57BL/6 (B6) male mice, 8-10 weeks old, were obtained from AnLab Co., Prague, Czech Republic. Experimental protocols were approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics, Prague.

Cell lines. HPV16-associated, non-metastasizing, MHC class I-positive cell line TC-1, moderately immunogenic in syngeneic B6 mice was obtained by *in vitro* co-transfection of murine lung C57BL/6 cells with HPV16 E6/E7 and activated human *H-ras* (G12V) oncogenes (16). TC-1/A9 tumour cell line, deficient in MHC class I molecules, represents tumour cell derivative which escaped from the selection pressure mediated by the specific immune response (17). Another E6/E7-expressing MHC class I-deficient, spontaneously metastasizing cell line MK16/1/IIIABC (MK16), moderately immunogenic in syngeneic mice, was developed by *in vitro* co-transfection of murine C57BL/6 kidney cells with a mixture of plasmids carrying activated *H-ras* oncogene (plasmid pEJ6.6), HPV16 E6/E7 genes (plasmid p16HHMo) and neomycin resistance gene (plasmid pAG60) (18,19). These cell lines were maintained in RPMI-1640 medium supplemented with 10% foetal calf serum, 2 mM L-glutamine and antibiotics (complete medium) and were cultured at 37˚C in a humidified atmosphere with 5% CO_2.

In vivo immunization-challenge protocol. Mice were immunized twice with 10^7 irradiated tumour cells (days -30 and -10, subcutaneous injection, irradiation dose 150 Gy) and challenged on day 0. The number of tumour-bearing mice was recorded bi-weekly, and the tumour size was expressed as the mean tumour area (cm^2). For *in vitro* analyses, spleen cells from three immunized or naive (control) mice were collected and treated with ammonium chloride-potassium lysing buffer (1 min) to deplete erythrocytes.

In vivo depletion studies. *In vivo* depletion of $NK1.1^+$, $CD4^+$ and $CD8^+$ cells was performed using monoclonal antibodies PK 136 (anti-$NK1.1^+$), GK 1.5 (anti-$CD4^+$), and 2.43

Table I. IFNγ-production (pg/ml) by different cell subsets after immunization.

Spleen cells	Cells used for immunization		
	TC-1	TC-1/A9	MK16
Undepleted *in vivo*, unseparated *in vitro*	1045±405	516±352	520±266
Undepleted *in vivo*, separated *in vitro*			
$CD8^+$	1236±139	727±230	415±312
$CD4^+$	710±268	498±185	574±235
$NK1.1^+$	60±14[a]	1398±616[c]	2265±1080[c]
Depleted *in vivo*, unseparated *in vitro*			
$CD8^-$	503±406[c]	718±426	1060±200
$CD4^-$	560±344[c]	751±611	1220±636
$NK1.1^-$	1401±870	228±119[c]	176±39[b]
Non-immunized controls			
Tumour bearing mice	250±115	152±131	358±210
Healthy donors		33±41	

T-test: [a]$p<0.001$, [b]$p<0.01$, [c]$p<0.05$ as compared to the corresponding 'Undepleted *in vivo*, unseparated *in vitro*' groups (ELISA assay).

(anti-$CD8^+$), respectively, for 4 weeks, starting day -37 (14,20-25). To deplete the effector cells, 0.1 mg of the antibody was injected i.p. into mice; during the first week injections were given 3 times and in the following two weeks, mice received injections once a week. Depletion was verified by the staining of spleen cells with labelled antibodies and FACS analysis.

Preparation of spleen cells. After the immunization period, spleen cells from immunized or naive (control) mice were collected, suspended, treated with ammonium chloride-potassium lysing buffer (1 min) to deplete erythrocytes and used for *in vitro* determination.

Flow cytometry. To determine CD8, CD4, NK1.1 and MHC class I cell surface expression, the PE-labelled rat anti-mouse CD8a (53-6.7), CD4: PE labelled rat anti-mouse CD4, clone GK1.5, PE-labelled anti-mouse NK-1.1 (NKR-P1B and NKR-P1C) PE-labelled mouse anti-mouse H-2Db (KH95) + PE-labelled mouse anti-mouse H-2Kb (AF6-88.5) were used. As isotype controls, PE-labelled antibodies of irrelevant specificity were utilized. All products were purchased from Pharmingen, San Diego, CA. Flow cytometry was performed using an LSR II flow cytometer (BD Biosciences, San Jose, CA).

Magnetic separation (MACS). For MACS separation, cells were labelled with MACS anti $CD8^+$, $CD4^+$ or $NK1.1^+$ MicroBeads Antibodies according to the manufacturer's instructions. Positive cells were separated in the AutoMACS Pro cell separator (Miltenyi Biotec, GnbH, Bergisch-Gladbach, Germany). The purity of separed cells ranged between 85-95%.

ELISPOT assay. To determine the amount of IFNγ-secreting cells, an ELISPOT kit for detection of murine IFNγ (BD PharMingen, San Diego, CA) was used. Splenocytes were cultured for 48 h and then placed to the wells of ELISPOT plates (concentration $5x10^5$ cells/well) for 24 h. The plates were then processed according to the manufacturer's instructions (BD PharMingen). Coloured spots were counted with CTL Analyzer LLC (CTL, Cleveland, OH) and analysed using the ImmunoSpot Image Analyzer software.

Cytometric bead array (CBA) analysis. Frozen samples of supernatants obtained after 48-h cultivation of spleen lymphocytes ($2x10^6$/ml) were thawed and tested in the CBA assay kit (BD Biosciences, San Diego, CA) allowing simultaneous detection and quantification (pg/ml) of soluble murine IL-2, IL-4, IL-5, IFNγ and TNFα in a single sample, according to the manufacturer's instructions.

ELISA assay. For ELISA assay, supernatants obtained after 48 h of cultivation of splenocytes ($2x10^6$/ml) were collected and frozen. The thawed supernatants were used in the ELISA assay kit (BD Biosciences) according to the manufacturer's instructions.

^{51}Cr microcytotoxicity assay. The cytolytic activity of immune effector cells was tested using the ^{51}Cr release assay, as described previously (22-27). ^{51}Cr-labelled tumour targets were mixed with the effector cells in the target-to-effector cell ratios 200:1, 100:1 50:1 25:1 in complete RPMI medium supplemented with mercaptoethanol ($2x10^{-5}$ M). For cytotoxic tests, erythrocyte-depleted, nylon wool non-adherent cell fractions were used. The mixtures were incubated in triplicate in 96-well round-bottom microtiter plates (Nunc, A/S, Roskilde, Denmark). Percent of specific ^{51}Cr release was expressed: [(cpm experimental release - cpm control release)/(cpm maximum release - cpm control release)] x100.

Statistical analyses. For statistical analyses of *in vivo* experiments, analysis of variance from the NCSS, Number Cruncher Statistical System (Kaysville, UT) statistical package was used. For comparison of tumour takes in experimental and control groups, the χ^2 test was utilized. For statistical analyses of *in vitro* studies, the Student's t-test was used.

Results

Immunization

Immunization of undepleted mice. Significant inhibition of the tumour growth in immunized mice compared to the controls was recorded in mice immunized with the irradiated TC-1 cells and challenged with the TC-1 cells, in mice immunized with the TC-1/A9 cells and challenged with TC-1/A9 cells, and also in mice immunized with the irradiated MK16 cells and challenged with the MK16 cells (Figs. 1A, 2A and 3A).

Immunization of depleted mice. *In vivo* removal of $CD8^+$ cells in the TC-1 cell-immunized mice within the immunization

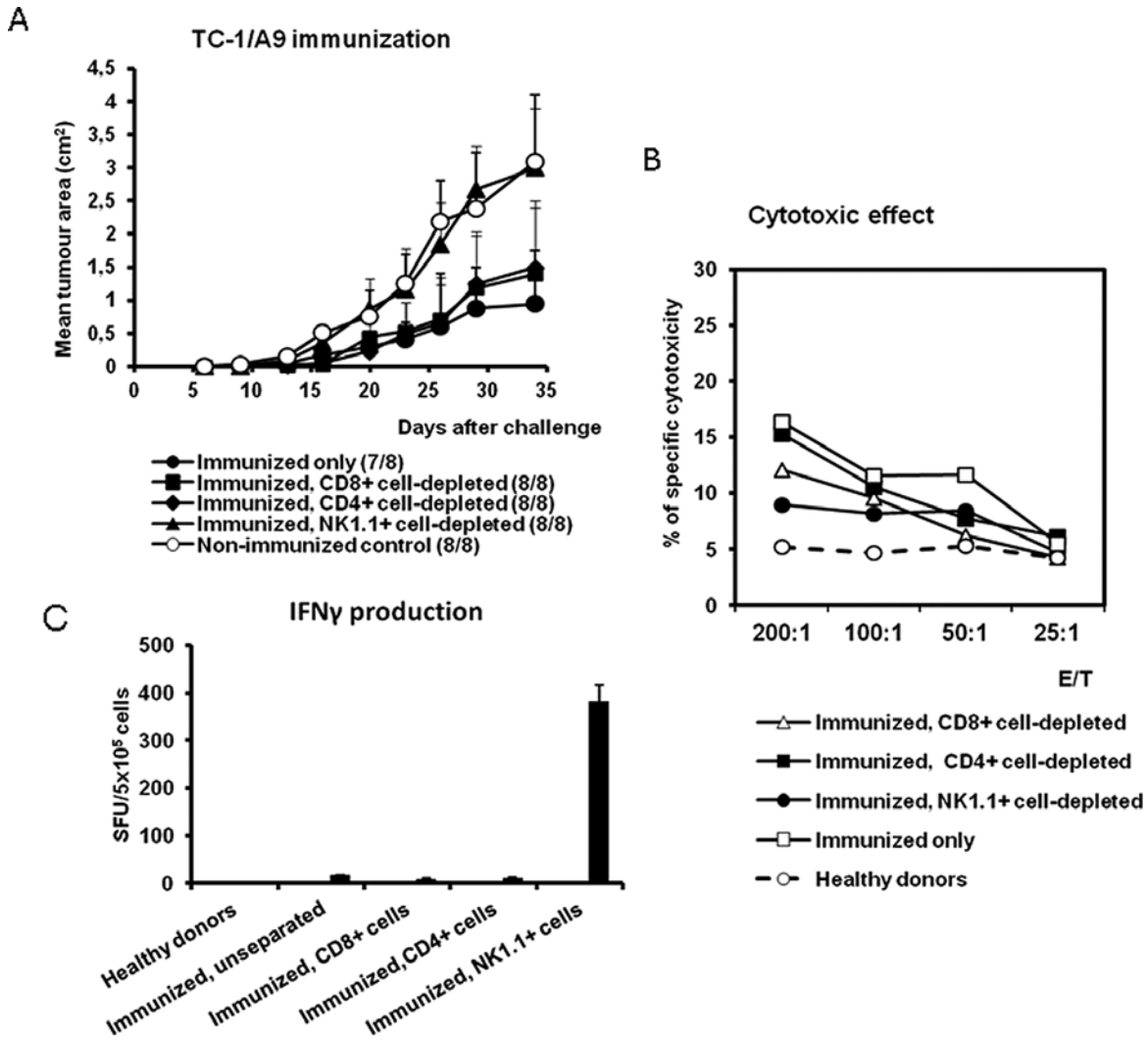

Figure 2. Immunization with TC-1/A9 cells. (A) CD8+, CD4+ or NK1.1+ cells depleted and undepleted (immunized only) mice were immunized twice in a 3-week interval with irradiated tumour cells. Ten days after second immunization, mice were challenged with the corresponding tumour cells. In parentheses: no. of mice with tumour/total no. of mice. Statistical significance (analysis of variance): Immunized only x non-immunized control, immunized + NK1.1+ cell-depleted, $p<0.05$. (B) ^{51}Cr microcytotoxicity test. NK1.1+ *in vivo* depleted mice exhibited decreased activity against TC-1/A9 targets ($p<0.05$). (C) ELISPOT assay. Production of IFNγ is concentrated into NK1.1+ subpulation. The subpopulation were obtained after MACS separation of spleen cells of immunized, but undepleted mice ($p<0.05$, as compared with other groups, t-test).

period resulted in the complete elimination of the protective effect. CD4+ or NK1.1+ depletion was without any effect on the tumour growth inhibition in immunized animals (Fig. 1A). After prophylactic immunization with irradiated MHC class I-deficient TC-1/A9 or MK16 tumour cells the anti-tumour effect of immunization exhibited remarkable dependence on the presence of NK1.1+ cells. The removal of the CD8+ or CD4+ cells was without significant influence on the protective level of prophylactic vaccination (Figs. 2A and 3A).

In vitro monitoring of the immune response

Effect of cytotoxic lymphocytes. The analyses of the spleen effector cells showed that the cytotoxic activity was abrogated in cells isolated from the TC-1 cell-immunized, CD8+ and CD4+ but not of the NK1.1+ cell-depleted mice, as compared to the cells from undepleted immunized mice (Fig. 1B). The analyses of the spleen cells from MK16 or TC-1/A9 immunized mice documented the decrease of activity in effectors from the NK1.1+ cell-depleted mice (Figs. 2B and 3B). The decrease of the cytotoxic activity of the spleen cells from CD8+ and CD4+ cells-depleted mice was not significant.

Production of IFNγ. After immunization with irradiated TC-1 cells, immunized mice showed similar percentage of IFNγ producing cells measured by the ELISPOT assay in CD8+, CD4+ and NK1.1+ cell populations, obtained after MACS separation of spleen cells. On the other hand, after the immunization with irradiated TC-1/A9 or MK16 cells, IFNγ-producing cells were concentrated into NK1.1-positive spleen cell populations (Figs. 1C, 2C and 3C). In addition, the ELISPOT data were completed by the IFNγ analysis detected with ELISA assay in cell supernatants from 48 h cultivated spleen cell cultures. These analyses also showed that the level of IFNγ-production in distinct cell subpopulation was dependent on the MHC class I expression on cells used for

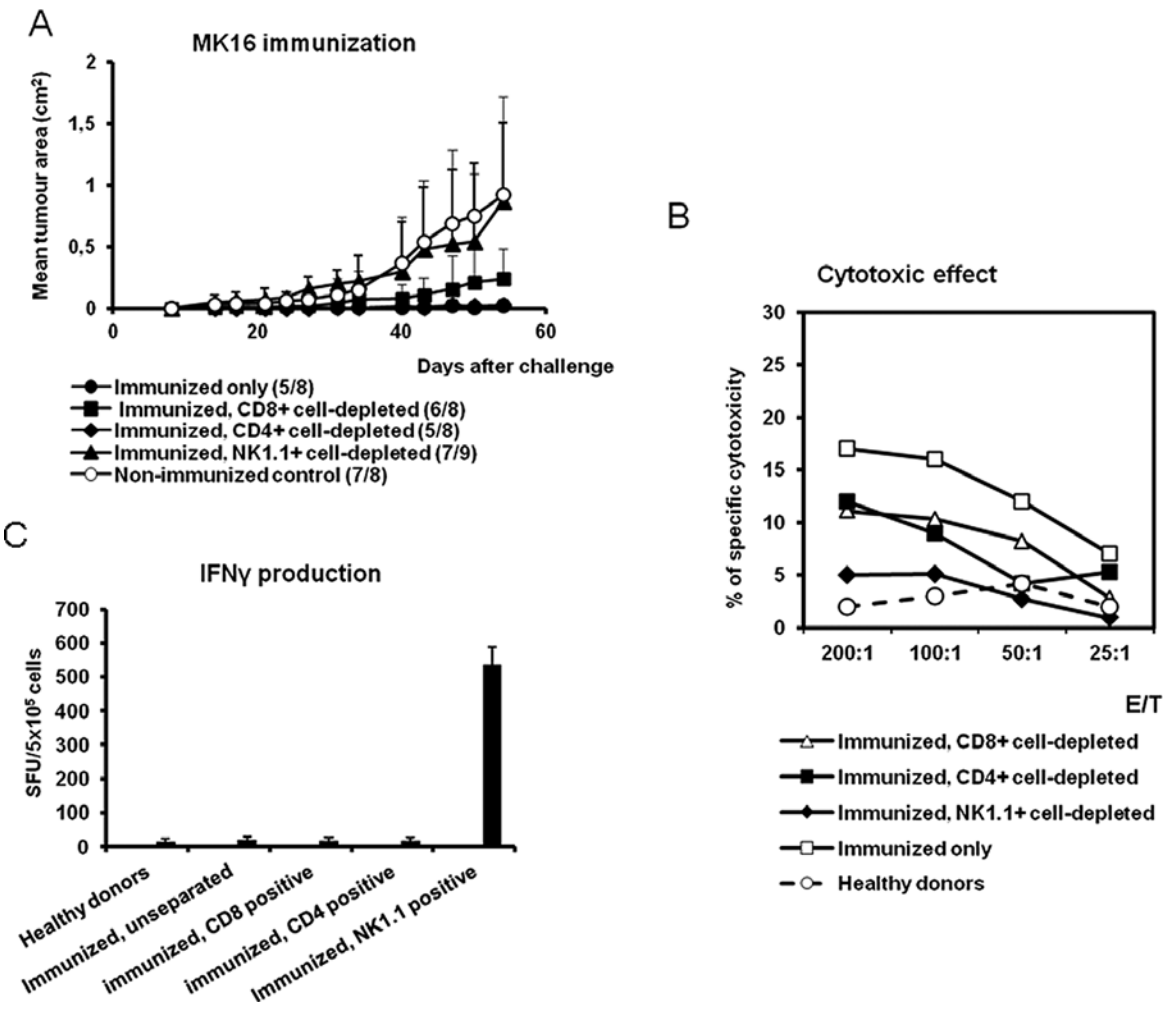

Figure 3. Immunization with MK16 cells. (A) CD8+, CD4+ or NK1.1+ cells depleted and undepleted (immunized only) mice were immunized twice in a 3-week interval with irradiated tumour cells Ten days after second immunization, mice were challenged with the corresponding tumour cells. In parentheses: No. of mice with tumour/total no. of mice. Statistical significance (analysis of variance): Immunized only x, non-immunized control, immunized + NK1.1+ cell-depleted $p<0.05$. (B) ^{51}Cr microcytotoxicity test. NK1.1 *in vivo* depleted mice exibited decreased activity against MK16 targets ($p<0.05$). (C) ELISPOT assay test: Production of IFNγ is concentrated into NK1.1+ subpulation. The subpopulation were obtained after MACS separation of spleen cells of immunized, but undepleted mice $p<0.05$, as compared with other groups, t-test.

immunization. The maximum production was concentrated into the CD8+ spleen cell subpopulation in the TC-1 cell-immunized mice. Further, the IFNγ production was decreased in the CD8+ and CD4+ cell-depleted but not in the NK1.1+ cell-depleted mice. In the TC-1/A9 or MK16 immunized mice, maximum production was concentrated into the NK1.1+ spleen cell subpopulation. IFNγ production was abrogated in the NK1.1+ spleen cell-depleted, but not in the CD4+ or CD8+ cell-depleted mice, as compared to the immunized only controls (Table I).

Th1/Th2 polarization. The expression of the MHC class I molecules on the cells used for prophylactic immunization led to the increased production of IFNγ (Fig. 4), but, in general, was without any effect on other Th1 (IL-2, TNFα) and Th2 (IL-4, IL-5) cytokine production measured by the CBA analysis. Prophylactic immunization led to the increased production of IL-2, IFNγ and TNFα (Th1 cytokines) and IL-5 (Th2 cytokines) in the comparison with the healthy donors. However, the increased production of IL-4 (Th2) cytokine was not observed (Fig. 4).

Discussion

In this study, we demonstrated that prophylactic vaccinations with the HPV16-associated, MHC class I-positive or MHC class I-deficient tumour cells induced effective immunity against the respective tumours by different mechanisms, corresponding to their MHC class I expression status. Further, we have shown that IFNγ was produced mainly by NK1.1+ cells upon vaccination with MHC class I-deficient tumour cells while its production was distributed into CD4+, CD8+, as well as into NK1.1+ cellular lineages in the MHC class I positive TC-1 tumour cell-immunized mice.

In our previous studies, it was found that immunization with cellular vaccines based on MHC class I-deficient tumour cells was effective against MHC class I-deficient tumours, as well as against their 'parental' MHC class I-positive tumours, due to the antigen cross-presentation (14). However, only immunization with MHC class I-deficient but not with MHC class I-positive tumour cells inhibited the growth of the MHC class I-deficient tumours. *In vivo* depletion studies during the

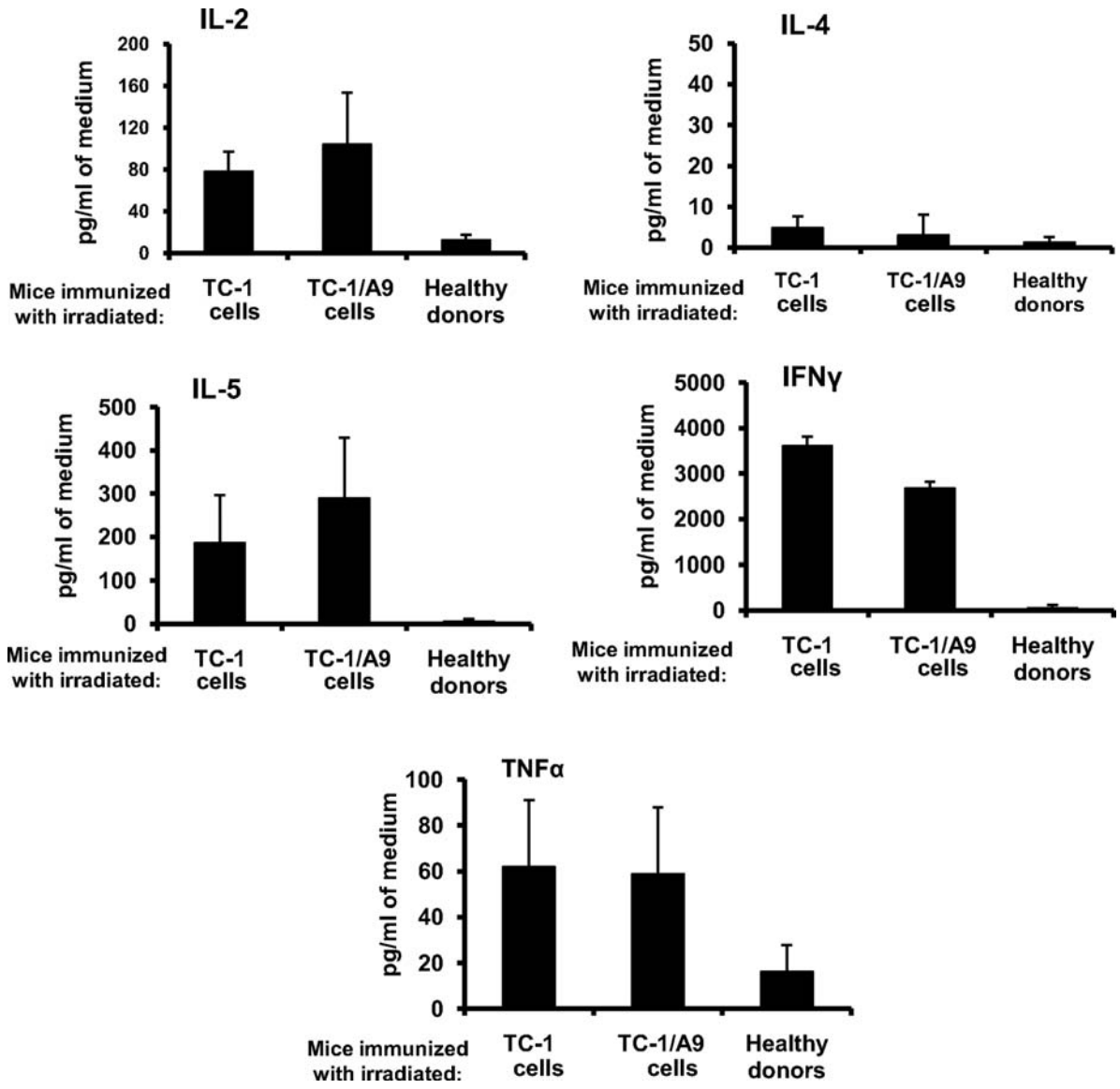

Figure 4. The level of expression of MHC class I molecules on tumour cells used for protective immunization had no effect on the Th1/Th2 polarization (CBA test). $p<0.001$ (IL-2, IL-5, IFNγ), $p<0.05$ (INFγ), compared to the healthy donors. IFNγ production. Mice immunized with irradiated TC-1 cells x mice immunized with irradiated TC-1/A9 cells, $p<0.05$ (t-test). Similar results were obtained after immunization with MK16 cells (data not shown).

tumour growth revealed that the tumour growth in animals immunized with MHC class I-deficient tumour cells was strongly dependent on natural killer cells.

Moreover, we have shown, using the same model, that immunization with peptides, peptide-pulsed DC or MHC class I-deficient tumour cells can elicit specific immunity against these tumours, but the MHC class I expression status has to be considered in optimization of the vaccination strategy (15). The peptide vaccine based on the peptide $E7_{49-57}$ harbouring solely the CTL epitope with CpG oligodeoxynucleotide as an adjuvant was effective only against MHC class I-positive but not -deficient tumours, while the peptide $E7_{44-62}$, harbouring CTL and Th epitopes, was also effective against MHC class I-deficient tumours. Unlike in the peptide immunization setting, treatment with dendritic cells pulsed with a 'short' peptide resulted in the MHC class I-deficient tumour growth inhibition, albeit weaker as compared to the immunization with the longer peptide.

Using the model of the TC-1 tumours Símová *et al* (28) found that depletion of NK1.1+ cells by repeated i.p. injections of anti-NK1.1 antibody substantially accelerated growth of MHC class I+ tumours, when starting before tumour transplantation or on the day of transplantation. These results indicate that NK1.1 cells play an important inhibitory role also during the early phase of the growth of MHC class I+ tumours as has been documented also in other models (13).

In this communication, our previously obtained results (14,15) were completed with new data on the impact of *in vivo* depletion of CD8+, CD4+ or NK1.1+ cells within the immunization period. This study revealed that the development of protective immunity against MHC class I-expressing and -deficient tumours, elicited by immunization with tumour cell-based vaccines, is mediated by different mechanisms, based on the CD8+ cells for the MHC class I-expressing tumours, and mainly on the NK1.1+ cells in case of the MHC class I-deficient tumours. The data suggest that the NK1.1+

cells are crucial for the development of specific immunity against MHC class I-deficient tumours elicited by tumour cell-based vaccines, but not against their MHC class I-positive counterparts.

Vaccination with irradiated tumour cells enhanced the production of IFNγ, regardless of the MHC class I expression on the cellular vaccines. The IFNγ, which has been suggested to be crucial for effective immune response against MHC class I-deficient tumours (10) may help to overcome the MHC class I downregulation, e.g. by partially restoring the MHC class I expression and by starting closer cooperation between the T cells and innate immune cells (2,29). Mice immunized with irradiated TC-1 cells showed similar percentage of IFNγ producing cells in $CD8^+$, $CD4^+$ and $NK1.1^+$ cell populations. On the other hand, the highest proportion of IFNγ-producing cells after the prophylactic immunization with irradiated TC-1/A9 or MK16 cells (MHC class I-deficient tumour cells) was concentrated into NK1.1-positive spleen cell population. These results are in accordance with those from the *in vivo* depletion experiments demonstrating that different subsets of lymphocytes are important for the anti-tumour effect against TC-1-based MHC class I-positive and -deficient tumours.

Collectively, our results indicate an important role of $NK1.1^+$ cells and IFNγ production by these cells in the induction of the protective immunity against MHC I-deficient HPV16-associated tumours. $NK1.1^+$ cells seem to be an important part of the complex mechanism in development of specific immunity against MHC class I-deficient tumours that includes the cooperation of various cell subsets.

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