# Adjuvant Cytokine Treatment of Minimal Residual Disease after Surgical Therapy in Mice Carrying HPV16-Associated Tumours: Cytolytic Activity of Spleen Cells from Tumour Regressors

( HPV16 / gene therapy / IL-2 / minimal residual tumour disease / cytotoxic effectors )

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**Abstract. It has been found previously that IL-2, IFNγ and GM-CSF were capable of reducing the recurrence rate of HPV 16-associated tumours in mice with SMRTD. We were interested whether the therapeutic effect of the surgery and adjuvant cytokine treatment was accompanied by cytolytic activity of spleen cells and whether the activity of the spleen cells was different in mice that had rejected tumour residua after surgery and adjuvant therapy with cytokines (tumour regressors) as compared to those that had not rejected the tumour residua (tumour progressors). We have examined the cytolytic activity of spleen cells from MHC class $I^+$ TC-1 tumour regressors and progressors after treatment of TC-1 SMRTD with GM-CSF, and the activity of spleen cells from MHC class $I^-$ MK16 tumour regressors and progressors after treatment of MK16 SMRTD with IL-2 and IFNγ. It has been found that irrespective of the tumour type and adjuvant treatment, the spleen cells from tumour regressors after surgery were regularly more cytolytic when allowed to react with target cells from HPV 16-associated tumours than the spleen cells from tumour progressors. No substantial differences between the cytolytic activity of spleen cells from the operated-only and operated plus cytokine (GM-CSF, IL-2, IFNγ) adjuvant treated groups were observed. The cytolytic activity of spleen cells from mice with SMRTD allowed to react with MHC class $I^+$ , MHC class $I^-$ , NK-sensitive and NK-resistant targets is compatible with the interpretation that in the mice with MHC class $I^+$ TC-1 tumours, primarily cytotoxic T lymphocytes (CTL) were efficient, whereas in the mice with MHC class $I^-$ MK16 tumours, both NK and non-lymphocytic effector cells were involved.**

In the majority of clinical tumour immunotherapy trials, complete and partial responses have been found to be rather rare, although simultaneous *in vitro* monitoring of the presence of the activated major component of tumour rejection reaction, $CD8^+$ CTLs, provided positive results. Monitoring of other tumour-reactive cytolytic effector cells, such as activated natural killer (NK) cells or non-lymphocytic cells, has usually not been performed (Offringa et al., 2000). To investigate the correlation of the results of immunotherapy and the presence of cytolytic effector cells in tumour-bearing individuals in a clinically relevant setting, an experimental model of human tumour papiloma virus (HPV 16)-induced murine tumours transplanted in syngeneic mice and treated by surgery plus adjuvant cytokine therapy was utilized (Lin et al., 1996; Bubeník et al., 1999; Šmahel et al., 2001). The mice were divided into two groups, those that were cured by the therapy (tumour regressors) and those that did not respond to the therapy (tumour progressors). Spleen cells from both groups and from the untreated controls were allowed to react *in vitro* with $^{51}Cr$-labelled, MHC class $I^+$ or MHC class $I^-$ HPV 16-induced tumour targets as well as with NK-sensitive and NK-resistant HPV $16^-$ targets to quantitatively examine the differences between the cytolytic activity of spleen cells from tumour regressors and tumour progressors and to characterize the effector cells with regard to their origin.

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Abbreviations: HPV – human papilloma virus, NK – natural killer, rGM-CSF – recombinant granulocyte-macrophage colony-stimulating factor, rIFNγ – recombinant interferon gamma, rIL-2 – recombinant interleukin 2, SMRTD – surgical minimal residual tumour disease.

## Material and Methods

### *Mice*

C57BL/6 (B6) males, 8–10 weeks old, were obtained from Anlab Co., Prague, Czech Republic.

### *Cell lines*

The murine malignant, non-metastasizing, MHC $I^+$ cell line TC-1, immunogenic in the syngeneic B6 mice and the murine malignant, spontaneously metastasizing, MHC class $I^-$ cell line MK16/1/IIIABC (MK16), moderately immunogenic in syngeneic B6 mice, were previously described in detail elsewhere (Lin et al., 1996, Šmahel et al., 2001). Both cell lines were obtained after *in vitro* co-transfection of murine B6 cells with HPV16 *E6/E7* and activated human Ha-*ras* (G12V) oncogene DNA. For some *in vitro* studies, YAC-1, Moloney virus-induced T-cell lymphoma of A/Sn origin sensitive to NK cell-mediated cytolysis (Wigzell and Ramstedt, 1986), and C1498, spontaneous murine leukaemia cell line of B6 origin relatively resistant to NK cell-mediated cytolysis (Goldie et al., 1953) were utilized.

The HPV16 E6/E7-associated tumour cell lines were maintained in RPMI 1640 medium supplemented with 10% of foetal calf serum (Biomedia, Brno, Czech Republic), L-glutamin and antibiotics. For YAC-1 cells, C1498 cells and spleen cells, the medium was supplemented with $10^{-5}$ M mercaptoethanol and the cells were cultivated in a humified atmosphere with 5% $CO_2$. For induction of MHC class I molecules on the MHC class $I^-$ MK16 cells, the cells were cultivated for 48 h in the RPMI 1640 medium in the presence of 1 µg/ml IFNγ (R&D Systems, Minneapolis, MN) and designated as MK16-IFNγ (Mikyšková et al., 2003a).

### *Therapy of mice prior to in vitro analysis*

For therapy, B6 mice were inoculated s.c. with 5 x $10^5$ MK16 or 2 x $10^5$ TC-1 cells. After 30 days, when the transplanted tumours reached approximately 8–12 mm in diameter, the tumours were excised. The hypothetical microscopic tumour residua after surgery were designated as surgical minimal residual tumour disease (SMRTD, Vlk et al., 1998). Mice were then injected twice a day, on days 3–7 and 10–14 after the operation, with 5 x $10^4$ i.u. of human rIL-2 (Chiron, Emerville, CA), 50 ng of murine rGM-CSF (R&D Systems), or with 0.5 µg of murine IFNγ (R&D Systems) (Bubeník et al., 2003; Mikyšková et al., 2003a,b).

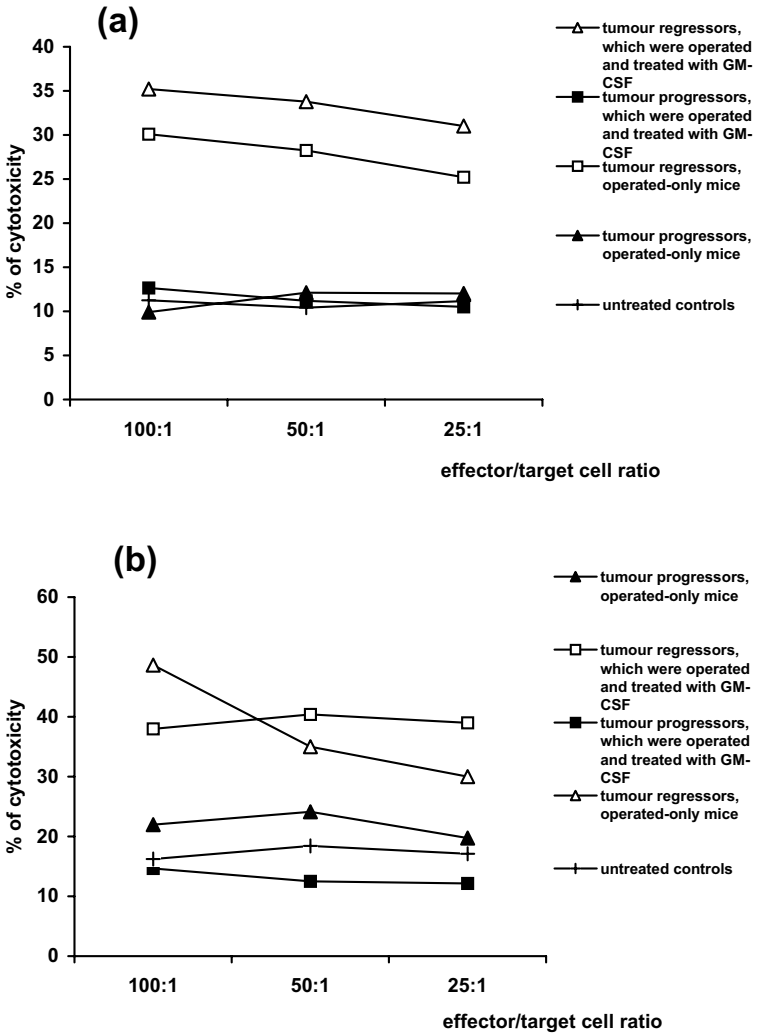


*Fig. 1.* Cytolytic activity of spleen cells from mice carrying TC-1 tumour residua which were injected with GM-CSF and allowed to react with: (a) TC-1 cells, (b) MK16 cells cultivated in IFNγ

### *$^{51}$Cr microcytotoxicity assay*

For the microcytotoxicity assay, mice were sacrificed 40 days after surgery and pooled suspensions of cells from three spleens from tumour progressors, regressors and untreated healthy controls were prepared. After lysis of the erythrocytes with Tris-$NH_4Cl$ buffer, spleen cells were passed through a nylon wool column. The effluent fraction was designated as non-adherent effector cells. These cells were cultivated for three days in complete RPMI medium supplemented with recombinant IL-2 (20 i.u./ml, Proleukin, Cetus, Emeryville, CA) and recombinant IL-7 (5 ng/ml, R&D Systems). After cultivation, the cells were collected and used for the cytotoxicity assay in various target-to-effector cell ratios (1 : 25, 1 : 50, 1 : 100). The mixtures of the effector cells with the $^{51}$Cr-labelled tumour targets were incubated for 18 h in triplicate in microtitre plates. Percent specific $^{51}$Cr release was expressed according to the formula: [cpm experimental release –

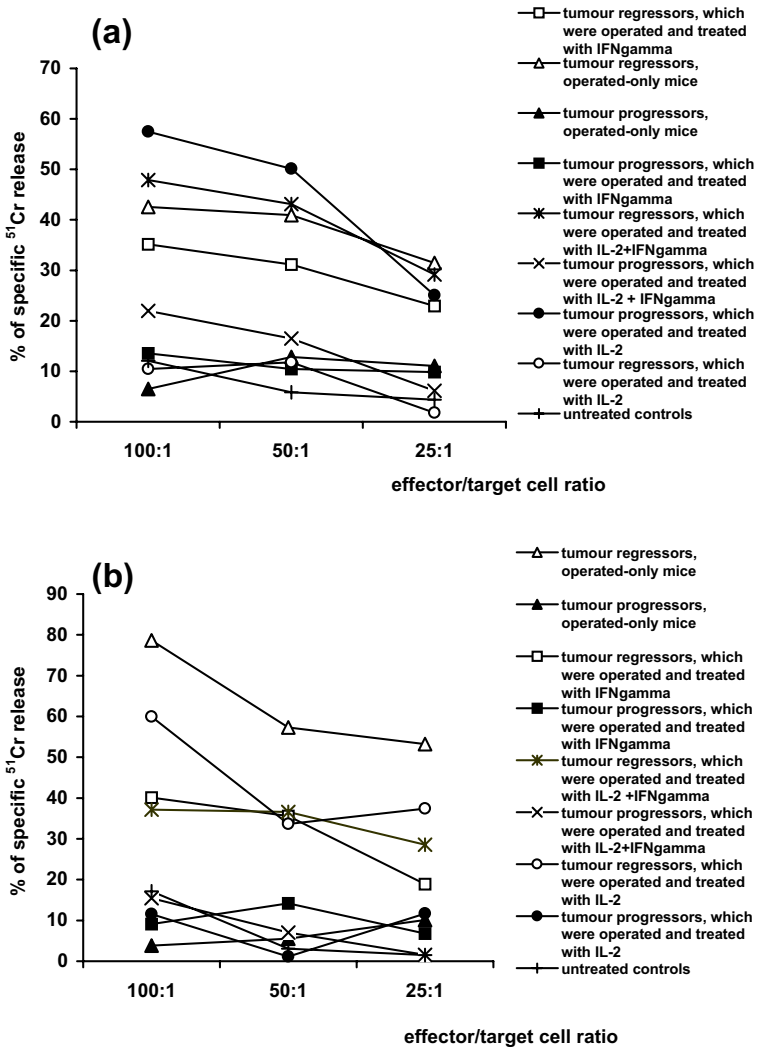


*Fig. 2.* Cytolytic activity of spleen cells from mice carrying MK16 tumour residua which were injected with IL-2/IFNγ and allowed to react with: (a) TC-1 cells, (b) MK16 cells cultivated in IFNγ

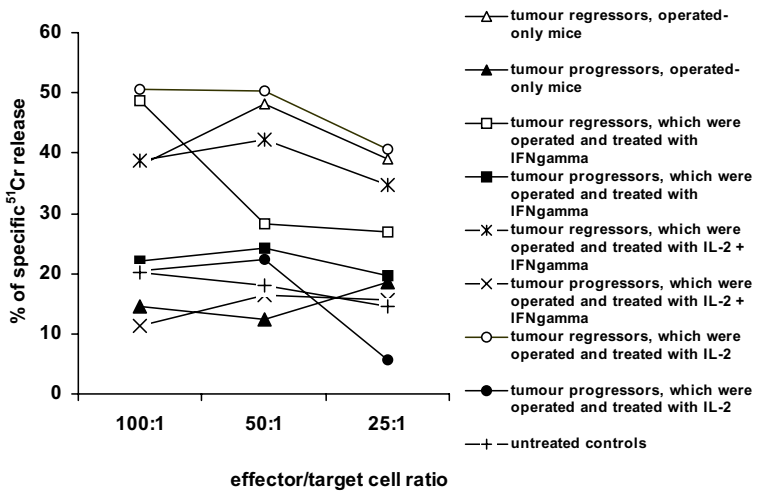


*Fig. 3.* Cytolytic activity of spleen cells from mice carrying MK16 tumour residua which were injected with IL-2/IFNγ and allowed to react with MK16 targets

cpm control release / cpm maximum release – cpm control release] x 100 (Bubeník et al., 1994).

## Results

Spleen cells from mice carrying tumour residua after surgery and adjuvant therapy of SMRTD with cytokines were examined *in vitro* in the $^{51}$Cr microcytotoxicity assay, using MHC class I$^+$ (TC-1, MK16-IFNγ) and MHC class I$^-$ (MK16) target cells, as well as NK-sensitive (YAC-1) and NK-resistant (C1498) targets. The spleen cells from mice that had rejected tumour residua after therapy (tumour regressors) and from mice that did not reject tumour residua (tumour progressors) were tested separately.

It has been found that spleen cells from mice carrying TC-1 (MHC class I$^+$) tumour residua after surgery were more cytolytic for MHC class I$^+$ target cells (TC-1 cells or MK16 cells cultivated in the presence of IFNγ) when the spleen cells were derived from tumour regressors (27–36% or 30–48% specific $^{51}$Cr release) than when they were derived from tumour progressors (10–15% or 12–23% specific $^{51}$Cr release) (Fig. 1a, b). Similarly, spleen cells from mice carrying MK16 (MHC class I$^-$) tumour residua after surgery were more cytolytic for MHC class I$^+$ target cells (TC-1 cells or MK16 cells cultivated in the presence of IFNγ) when the effector cells were derived from tumour regressors (28–58% specific $^{51}$Cr release) than when they were derived from tumour progressors (2–22% specific $^{51}$Cr release) (Fig. 2a, b). When the spleen cells from mice carrying MK16 (MHC class I$^-$) tumour residua after surgery were allowed to react with MHC class I$^-$ (MK16) target cells (Fig. 3), again the spleen cells from tumour regressors were more cytotoxic (27–51% specific $^{51}$Cr release) than those from tumour progressors (5–24% specific $^{51}$Cr release).

Since the MHC class I$^+$ (TC-1) tumour cells can be expected to activate MHC class I-restricted cytolytic T lymphocytes but not NK cells, whereas the MHC class I$^-$ (MK16) tumour cells can be expected to activate MHC class I-unrestricted effector cells, we have attempted to characterize the effector cells in spleen of the MK16 SMRTD-suffering mice more in detail, using NK cell-sensitive (YAC-1) and NK

cell-resistant (C1498) target cells. It has been found that the effector cells from MK16 tumour regressors were more cytolytic (30–79% specific $^{51}Cr$ release) than those derived from tumour progressors (2–23% specific $^{51}Cr$ release) when allowed to react with NK cell-sensitive (YAC-1) targets (Fig. 4a). When the effector cells from mice carrying MK16 (MHC class $I^-$) tumour residua were allowed to react with NK cell-resistant (C1498) targets (Fig. 4b), it has been found that the effector cells from tumour regressors were more cytotoxic (13–49% specific $^{51}Cr$ release) than those from tumour progressors (1–15% specific $^{51}Cr$ release).

The differences between the cytolytic activity of the spleen cells derived from tumour regressors and tumour progressors were observed in the operated-only mice as well as in mice treated by the operation plus adjuvant cytokine therapy. No substantial differences between the cytolytic activity of spleen cells from the operated-only and operated plus cytokine (GM-CSF, IL-2, IFNγ, IL-2 + IFNγ) adjuvant-treated groups were observed. The results suggest that the regression of both, MHC class $I^+$ and MHC class $I^-$ tumours after surgery is accompanied by the increase in the cytolytic activity of spleen cells in the operated mice. In tumour progressors, the cytolytic activity of spleen cells was either negligible or substantially lower than that observed in tumour regressors. From the spectrum of target cells sensitive to the cytolytic activity of the effector cells derived from mice carrying MK16 (MHC class $I^-$) tumour residua after surgery it can be concluded that the MHC class I-unrestricted lymphocytes as well as non-lymphocytic effector cells participate in the cytolytic effect (Fig. 4).

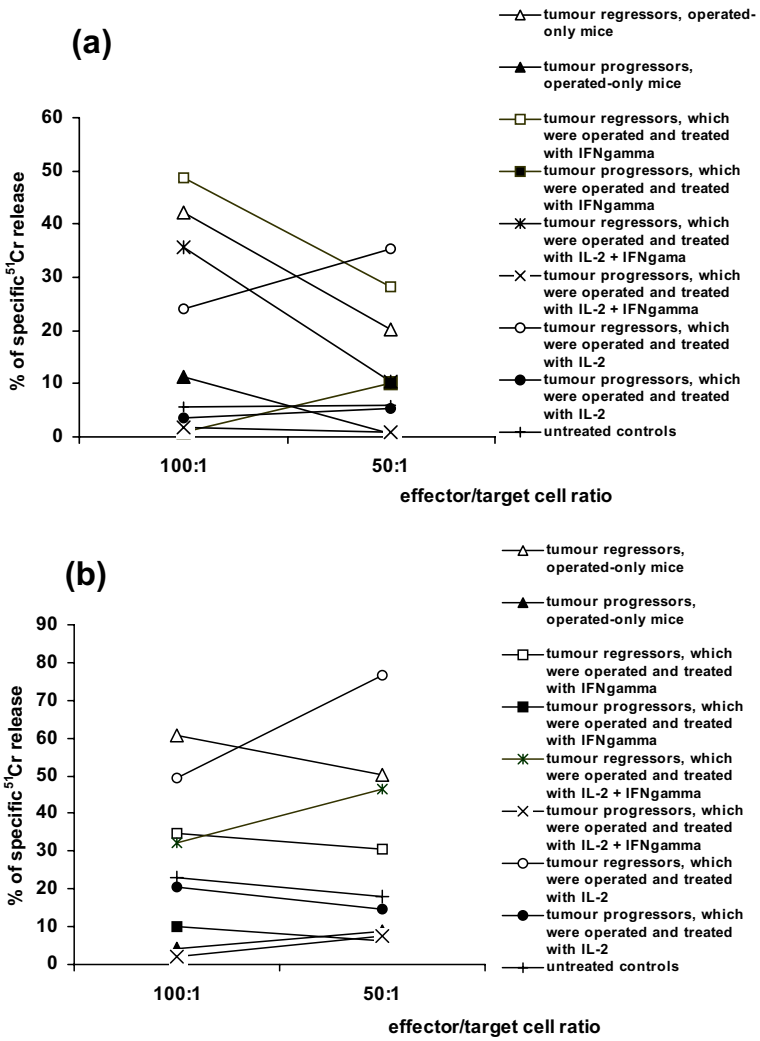


*Fig. 4.* Cytolytic activity of spleen cells from mice carrying MK16 tumour residua which were injected with IL-2/IFNγ and allowed to react with: (a) YAC-1 cells, (b) C1498 cells

## Discussion

Comparison of the cytolytic activity of spleen cells derived from TC-1 and MK16 tumour progressors and tumour regressors revealed that in both tumour systems the activity of the effector cells from tumour regressors was substantially higher than the activity of the spleen cells from tumour progressors. Therefore, it can be concluded that in the HPV 16-associated tumours utilized, a positive correlation has been found between the cure of SMRTD and the presence of the cytolytic effectors.

Surprisingly, the cytolytic activity of the effector cells from mice with MHC class $I^-$ MK16 SMRTD was similar to that observed in spleens of mice with MHC class $I^+$ TC-1 SMRTD. Since in the mice carrying MHC class $I^-$ tumours the primary effector cell mechanisms mediated by $CD8^+$ CTLs are lacking, other effector cells have to be involved in tumour rejection.

It is generally accepted that a low MHC class I level favours NK cells as effectors, whereas a high level of MHC class I molecules on the surface of tumour cells favours T cells as effectors. One can envision that T cells can participate even in the therapeutic effects of some immunomodulatory cytokines used for the treatment of MHC class $I^-$ tumours. Interferon γ has been described to upregulate MHC class I molecule expression on MHC class $I^-$ tumours treated by peritumoral administration of IFNγ (Mikyšková et al., 2003a).

Also, IL-2 can enhance IFNγ production by both NK and T cells and the produced IFNγ can repair some defects in the MHC class I antigen presentation pathway and upregulate MHC class I expression on the tumour cell surface (Mikyšková et al., 2001; Indrová et al., 2002; Mikyšková et al., 2003a). The upregulation of the MHC class I expression due to the direct effects of

IFNγ or due to the indirect effects of IL-2 can then induce a sensitivity of the MHC class $I^-$ tumour cells to MHC class I-restricted immunity *in vivo*.

Immune therapy with cytokines or tumour vaccines carrying inserted cytokine genes was previously found to inhibit growth of both MHC class $I^+$ or MHC class $I^-$ HPV 16-associated tumours transplanted in syngeneic mice (Bubeník et al., 1999; Indrová et al., 2001, 2002, 2003; Bubeník et al., 2003; Jinoch et al., 2003). Interleukin-2, IFNγ and GM-CSF were found to be efficient (for a review, see Bubeník 2003) .

Therefore, we have examined and compared the cytolytic activity of the spleen cells from the operated-only mice and from mice treated by surgery and adjuvant GM-CSF, IL-2 and IFNγ therapy. Surprisingly, no substantial differences between the cytolytic activity of spleen cells from the operated-only and operated plus cytokine-treated mice were observed. These results indicate that in addition to spleen, other effector cell sources, such as tumour microenviroment or circulating leukocytes have to be examined.

Due to the redundancy of direct priming of $CD8^+$ CTLs and tumour antigen cross-priming through dendritic cells, even MHC class $I^-$ tumour cells can induce MHC class I-restricted CTLs. However, the MHC class $I^-$ tumour cells will not bind the induced CTLs and will not be destroyed by them, unless MHC class I molecule expression is upregulated on the tumour targets (Indrová et al., 2002).

Despite the MHC class I molecule deficiences and the resulting resistance of the MHC class $I^-$ tumours to the $CD8^+$ CTLs, in the HPV 16-related and some other experimental tumour systems the tumour hosts were found to be capable of being immunized against MHC class $I^-$ tumours (for review, see Bubeník, 2002). Various mechanisms were reported to operate in these MHC class $I^-$ tumour systems, such as NK cells, NK-mediated antibody-dependent cellular cytotoxicity, LAK cells, $CD4^+$ cytotoxic T cells and non-lymphocytic cytolytic effector cells.

The spectrum of the cytotoxic activities of spleen cells from mice with MK16 SMRTD allowed to react with MHC class $I^+$ , MHC class $I^-$ , NK-sensitive (Wigzell and Ramstedt, 1986) and NK-resistant (Goldie et al,. 1953; Bubeník et al, 1992; LaBelle and Truitt, 2002) targets is compatible with the interpretation that both, NK and non-lymphocytic effector cells can serve as redundant effector mechanisms supplementing the function of the lacking $CD8^+$ CTLs. However, from our results we cannot conclude whether and which non-lymphocytic effector cells also participate in the cytolysis exerted by spleen cells from mice with TC-1 SMRTD. Experiments designed to solve this question and using both, depletion of various effector cell lineages and subsets as well as purification of lymphocytic and non-lymphocytic effector cell lineages are at present in progress to elucidate this question.

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