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Expanded Effector Memory T-lymphocytes in DBA/2 Mice Do Not Inhibit the Growth of SL2 Tumours

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Abstract. *Background: The aim of this study was to analyse changes in levels of memory T-lymphocytes during growth of SL2 tumours in DBA/2 mice and to evaluate whether these lymphocytes may have an inhibitory effect on tumour growth. Materials and Methods: Percentages of naïve ($CD8^+CD44^{low}CD62L^+$), central memory ($CD8^+CD44^{high}CD62L^+$) and effector memory ($CD8^+CD44^{high}CD62L^-$) lymphocytes in the $CD8^+$ subset in peripheral blood, spleen and lymph nodes of tumour-bearing and control mice were analysed by flow cytometry. Results: The percentage of effector memory lymphocytes in the $CD8^+$ subset increased during growth of tumours, whereas that of naïve $CD8^+$ lymphocytes decreased. No correlation between the levels of effector memory lymphocytes in peripheral blood and the mass of tumours was found. Conclusion: SL2 tumours induce expansion of effector memory lymphocytes in DBA/2 mice. However, expanded effector memory lymphocytes do not inhibit the growth of tumours.*

Recent evidence indicates that expansion of some T-lymphocyte subsets, including both $CD4^+$ and $CD8^+$ cells, may take place during tumour growth. Regarding $CD4^+$ lymphocytes, an increase in numbers of regulatory T-cells ($CD4^+CD25^+FOXP3^+$) in cancer patients is well-established. These cells contribute to the evasion of antitumour immune response (1, 2). To improve immunotherapy, it is thought that the number of regulatory T-cells and their functions should be inhibited in patients with advanced cancer (3).

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Regarding $CD8^+$ lymphocytes, increased numbers of $CD8^+CD57^+$ ($CD8^+CD28^-$) lymphocytes were found both in the tumour microenvironment and in the peripheral blood of patients with solid tumours and haemato-oncological diseases (4). Interestingly, these lymphocytes seem to play both positive and negative roles in various types of cancers (5). Similarly to $CD8^+CD57^+$ in humans, $CD8^+$ lymphocyte expansion in tumour-bearing mice can be demonstrated by an increase in numbers of $CD8^+CD44^{high}$ lymphocytes. It has been demonstrated that long-term tumour immunity after adoptive transfer of tumour antigen-reactive T-cell subpopulations is accompanied by an elevation in the number of $CD8^+CD44^{high}$ lymphocytes in spleen and lungs of mice (6).

We have reported that $CD8^+CD44^{high}$ lymphocyte levels in peripheral blood increase during growth of SL2 tumours in DBA/2 mice (7). However, these expanded $CD8^+CD44^{high}$ lymphocytes do not seem to have an antitumour effect, as tumours grow, and all tumour-bearing mice die during the second or third week after tumour implantation.

The $CD8^+CD44^{high}$ lymphocyte subset can be further analysed according to the expression of the cell-trafficking molecule CD62L (L-selectin). At least two memory T-cell subsets can be identified based on the CD62L expression: central memory T-cells (T_{CM}) are of $CD8^+CD44^{high}CD62L^+$ phenotype, whereas effector memory T-cells (T_{EM}) are of $CD8^+CD44^{high}CD62L^-$ phenotype (8, 9). T_{CM} lymphocytes have little or no effector function. These lymphocytes home to T-cell areas of secondary lymphoid organs and readily proliferate and differentiate to T_{EM} cells in response to antigenic stimulation. T_{EM} lymphocytes migrate to inflamed peripheral tissues and display immediate effector function (8, 10). Naïve $CD8^+$ (T_N) cells express the $CD8^+CD44^{low}CD62L^+$ phenotype (11).

In this study, we analysed changes in levels of T_N, T_{CM} and T_{EM} lymphocytes during growth of SL2 tumours in DBA/2 mice. We were also interested to investigate whether any of these lymphocyte subsets may have an inhibitory effect on the growth of tumours.

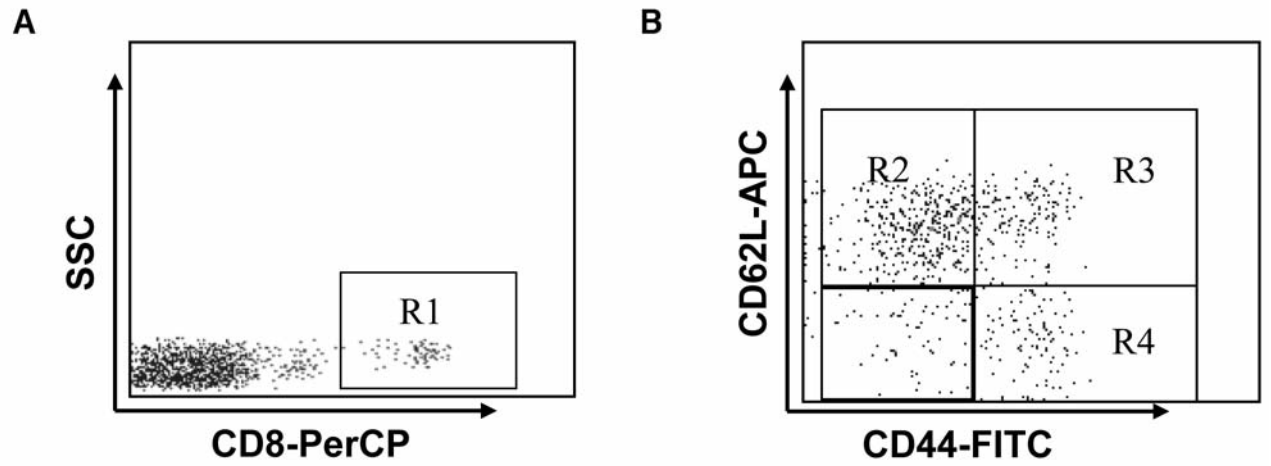

Figure 1. *Analysis of CD8, CD44 and CD62L antigens in peripheral blood lymphocytes in DBA/2 mice. A: Side scatter (SSC)/CD8 dot plot was used to gate CD8+ (R1) lymphocytes. B: Analysis of CD44 and CD62L antigens on gated CD8+ T-lymphocytes. $CD8^+CD44^{low}CD62L^+$ (R2) lymphocytes were identified as naïve (T_N) cells. $CD8^+CD44^{high}CD62L^+$ (R3) lymphocytes were identified as central memory (T_{CM}) cells. $CD8^+CD44^{high}CD62L^-$ (R4) cells were identified as effector memory (T_{EM}) lymphocytes.*

Materials and Methods

Mice and tumours. Male DBA/2 mice at the age of 8-12 weeks, weighing 20-26 g, were obtained from the local breeding facility at the State Research Institute Centre for Innovative Medicine, Vilnius, Lithuania.

SL2 lymphoma cells were maintained by weekly intraperitoneal passage in syngeneic DBA/2 mice. SL2 cells were collected from the peritoneal cavity, then washed and diluted in RPMI-1640 medium. Solid tumours were induced by subcutaneous injection of 10^7 SL2 cells in phosphate-buffered saline (PBS). The phenomenon of concomitant tumour immunity is observed in the DBA/2–SL2 model (12). This means that the immune reaction towards primary and secondary tumours may be different. Therefore, each mouse received two injections of SL2 tumour cells, one on the left (primary tumour) and one on the right side (secondary tumour) of the chest with an interval of two days.

Mice were sacrificed by cervical dislocation on day 9 of the experiment, tumours were removed and weighed (Denver Instrument GmbH, Göttingen, Germany). Experimental research on animals has been conducted according to recommendations of the Lithuanian Ethics Committee for the Laboratory Animal Use.

Preparation of lymphocytes for flow cytometric analysis. Peripheral blood was collected from the tail vein of individual mice on day 0 (prior to tumour implantation), on day 5 and on day 9 after implantation of the primary tumour. Heparin was used as an anti-coagulant. After sacrifice, spleens and lymph nodes were removed from tumour-bearing and naïve DBA/2 mice and lymphocyte suspensions were obtained by pressing spleens and lymph nodes through a nylon mesh. Contaminating red blood cells from spleen cell suspensions were removed by buffered ammonium chloride (5 min at room temperature). After centrifugation, the supernatant was decanted and the pellet resuspended in PBS.

Flow cytometry. Twenty μl of whole blood or 6 μl of cell suspension from spleens and lymph nodes were incubated for 30 min on ice in the dark with 0.2 μl of anti-CD8-peridinin chlorophyll protein (PerCP), 0.2 μl of anti-CD44-fluorescein isothiocyanate (FITC) (PharMingen, San Diego, USA) and 0.2 μl of anti-CD62L-allophycocyanin (APC) (Miltenyi Biotec GmbH, Teterow, Germany) monoclonal antibodies. All stainings were performed in the presence of purified anti-CD16/32 (PharMingen), at saturation to block nonspecific staining *via* FcRII/III. After incubation of blood samples, red blood cells were lysed by FACS Lysing Solution (Becton Dickinson, San Jose, CA, USA). The samples were analysed on a FACScalibur flow cytometer (Becton Dickinson) with a laser tuned at 488 nm. Data were acquired and analysed with the CellQuest software (Becton Dickinson). List mode files were collected for 10,000 cells from each sample. T_N T_{CM} and T_{EM} lymphocytes were identified as shown in Figure 1.

Statistics. Comparisons between the percentages of T_N, T_{CM} and T_{EM} lymphocytes in the $CD8^+$ subset, at different time points of the experiment, were made using the non-parametric sign test for dependent samples. Comparisons between the variables of tumour-bearing and control mice were made using the non-parametric Mann–Whitney *U*-test. Correlations between the percentage of T_{EM} lymphocytes in the $CD8^+$ subset in peripheral blood and tumour mass were evaluated by Spearman's rank correlation test. The level of significance was set at p=0.05.

Results

Changes in peripheral blood levels of T_N, T_{CM} and T_{EM} lymphocytes during tumour growth. Changes in the percentages of T_N, T_{CM} and T_{EM} lymphocytes in the $CD8^+$ subset during growth of SL2 tumours in DBA/2 mice are shown in Figure 2. The percentage of T_N lymphocytes in the

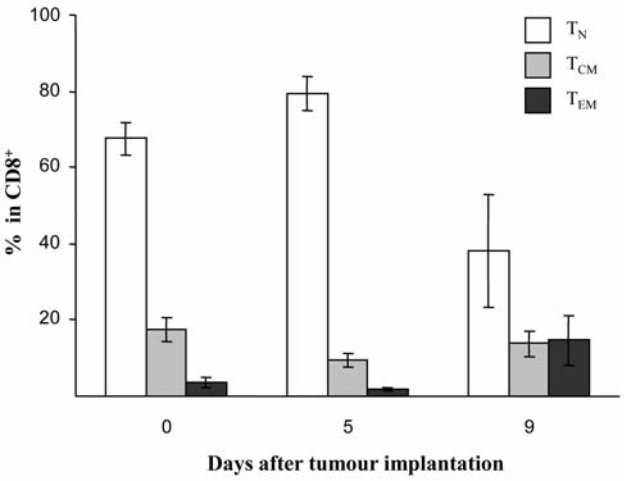

Figure 2. *Changes in naïve (T_N), central memory (T_{CM}) and effector memory (T_{EM}) lymphocyte levels in peripheral blood of DBA/2 mice during growth of SL2 tumours. Peripheral blood was collected from the tail vein of 9 mice on days 0, 5 and 9. Lymphocytes were analysed by flow cytometry. The increase in the percentage of T_{EM} lymphocytes in the CD8+ subset on day 9 is statistically significant compared to day 0 and day 5 (p=0.004). The decrease in the percentage of T_N lymphocytes in the CD8+ subset on day 9 is statistically significant compared to day 0 and day 5 (p=0.004). The percentage of T_{CM} lymphocytes in the CD8+ subset decreases from day 0 to day 5, then slightly increases at day 9 (p=0.008), but does not reach the level of day 0 (p=0.046). Values are given as means±SD.*

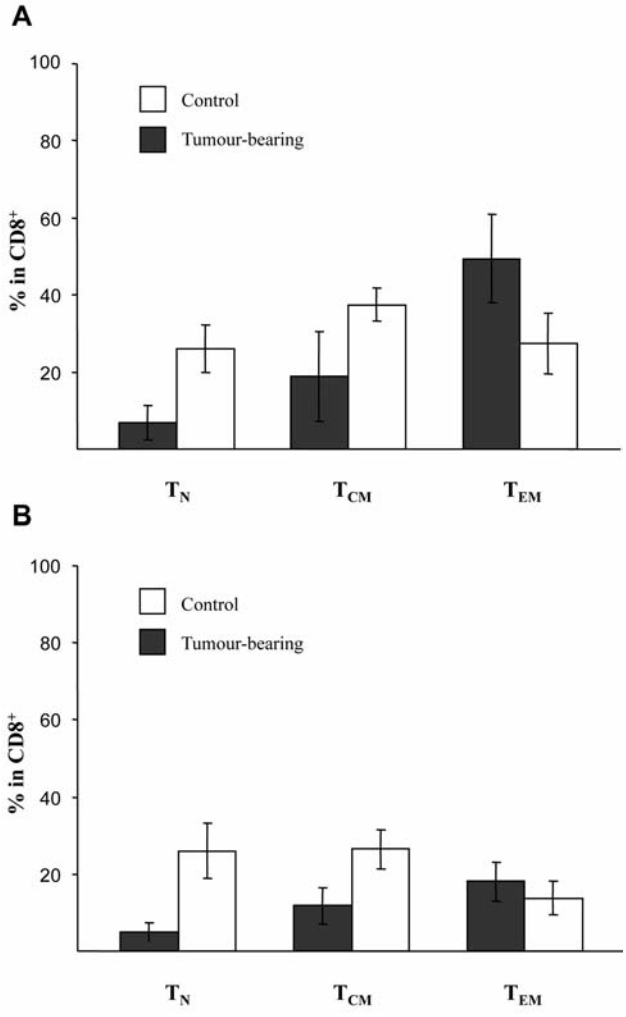

Figure 3. *Percentages of naïve (T_N), central memory (T_{CM}) and effector memory (T_{EM}) lymphocytes in the CD8+ subset in spleen (A) and lymph nodes (B) of tumour-bearing and control mice. Control mice (n=5) were killed on day 0 and tumour-bearing mice (n=10) were killed on day 9 of the experiment. Spleen and lymph nodes were removed and cell suspensions were prepared for flow cytometric analysis. The percentage of T_{CM} lymphocytes in the CD8+ subset decreased in the spleen (p=0.005) and in lymph nodes (p=0.003) of tumour-bearing mice compared to control mice. The percentage of T_N cells in the CD8+ subset is significantly lower in the spleen and in lymph nodes of tumour-bearing mice compared to control mice (p=0.002). The percentage of T_{EM} lymphocytes in the CD8+ subset was significantly higher in the spleen of tumour-bearing mice compared to control mice (p=0.002). Values are given as means±SD.*

$CD8^+$ subset decreased, whereas that of T_{EM} lymphocytes was higher on day 9 compared to day 0 and day 5. The percentage of T_{CM} lymphocytes decreased from day 0 to day 5, then slightly increased at day 9, but did not reach the level of day 0.

Changes in levels of T_N, T_{CM} and T_{EM} lymphocytes in spleen and lymph nodes of tumour-bearing mice. T_{CM} lymphocytes are known to proliferate in secondary lymphoid organs. Therefore, we wanted to determine whether the tumour-bearing state is associated with an increase in T_{CM} lymphocyte levels in spleen and lymph nodes. The percentages of T_N, T_{CM} and T_{EM} lymphocytes in spleen and in lymph nodes of tumour-bearing and control mice are shown in Figure 3. Figure 3 shows that the percentage of T_{CM} lymphocytes in spleen and in lymph nodes of tumour-bearing mice was lower than that in control mice, as was the percentage of T_N lymphocytes. However, the percentage of T_{EM} lymphocytes in the $CD8^+$ subset was increased in the spleen of tumour-bearing mice, whereas changes of T_{EM} lymphocytes in lymph nodes were not significant.

Relationship between the level of T_{EM} lymphocytes in peripheral blood and tumour mass. To determine whether expansion of T_{EM} lymphocytes may inhibit the growth of tumours, correlation between the percentage of T_{EM} cells in the $CD8^+$ subset and tumour mass in individual mice was analysed. As can be seen in Figure 4, no significant correlation between the level of T_{EM} lymphocytes in peripheral blood and the mass of primary or secondary tumour was found.

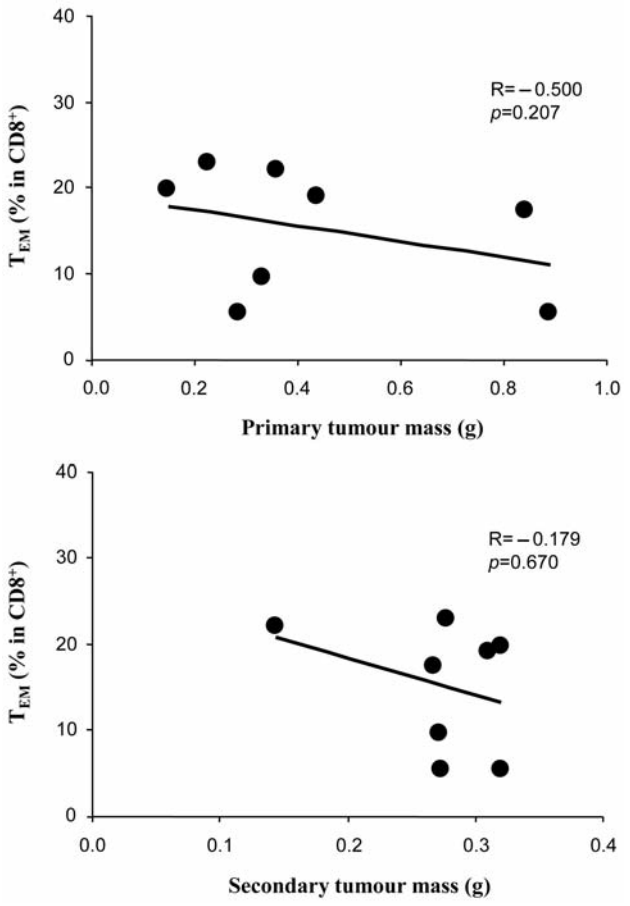

Figure 4. *Analysis of correlation between the percentage of peripheral blood T_{EM} lymphocytes in the CD8+ subset and the mass of primary or secondary tumour. Peripheral blood was collected from tumour-bearing mice (n=8) on day 9. The mice were killed and tumours were weighed.*

Thus, growth of SL2 tumours induced expansion of T_{EM} lymphocytes in peripheral blood and in the spleen of DBA/2 mice. However, these lymphocytes did not inhibit the growth of tumours.

Discussion

Our results show that the percentage of T_{EM} lymphocytes in the $CD8^+$ subset increases during growth of SL2 tumours in DBA/2 mice, whereas the percentage of T_N lymphocytes decreases. This suggests that tumours induce the development of T_N lymphocytes into T_{EM} cells. This type of change in $CD8^+$ lymphocytes is observed both in peripheral blood and in secondary lymphoid organs of mice. T_{EM} lymphocytes are known to exert immediate effector functions (13), mediated by the pre-expression of granzyme B and perforin within intracellular cytotoxic granules (8). However, lack or correlation between the levels of T_{EM} lymphocytes in peripheral blood and tumour mass in our experiments indicates that T_{EM} cells do not play a critical role in the inhibition of growth of SL2 tumours. Thus, cytotoxic function *in vitro* may not be important for the *in vivo* antitumour activity of T-lymphocytes. This finding is in agreement with the findings of other authors who did not find a correlation between cytotoxic T-cell function *in vitro* and the effect of cancer immunotherapy *in vivo* (14, 15).

Several previous studies using the adoptive transfer of T-lymphocytes have demonstrated that T_{CM} cells are more effective at eliminating established tumours than are T_{EM} cells (16), despite the fact that T_{CM} cells lack immediate effector function (13). The major difference between T_{CM} and T_{EM} lymphocytes seems to be the ability of T_{CM}, but not of T_{EM} lymphocytes, to rapidly proliferate and expand after re-encountering antigens (17). These data suggest that the proliferative potential of T-lymphocytes is more important than cytotoxicity for an effective antitumour response. Interestingly, the proliferative activity of intratumoural $CD8^+$ T-lymphocytes was found to be important as a prognostic factor in human renal cell carcinoma (18).

The available data indicate that $CD8^+$ T-lymphocytes follow a progressive pathway of differentiation from T_N cells into T_{CM} and T_{EM} cells (13). However, in our study, we did not observe an increase in T_{CM} lymphocyte levels. Thus, it can be hypothesised that progressive growth of SL2 tumours in DBA/2 mice is associated with the failure to generate T_{CM} lymphocytes.

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