Suppression of Primary Allogenic Response by CD8+ Memory Cells

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Immune response to allogenic tumor cells is associated with the appearance of long-living CD8+ memory cells capable of rapid restimulation and lysis of tumor cells in case of repeated injection of these cells. In order to acquire the effector function, allorestricted memory cells need antigen restimulation for 2 days, which is a specific feature of central memory cell population. These cells can suppress proliferation of naive splenocytes *in vitro*. In mixed lymphocyte cultures containing memory cells, antigen stimulation induces more intensive IL-10 production and deeper suppression of IL-2 production in comparison with cultures containing naive cells. The conditions for activation of naive cells during secondary immune response are not optimal.

Key Words: *T lymphocyte; memory cell; suppression; alloantigen*

Maturation and selection of T cells in the thymus throughout the whole life span of the organism permits the appearance of naive cells with specificity coinciding with already existing memory T cells. Immunoregulatory interactions between these cell subpopulations are little studied. Functioning of the immune system is characterized by antagonistic interrelationships between responses to related antigenic epitopes (so-called "primary antigenic sin") [2]. This attracts special attention of immunologists as a mechanism allowing escape of rapidly mutating viruses, *e.g.* HIV, from the immune control [4,6]. For CD8+ T cell responses this mechanism is mediated by the production of IL-10, suppressing the generation of new effector CD8+ cells secreting γ-IFN [5].

Using mice with transgenic expression of green fluorescent protein (GFP), we studied the effect of allospecific memory T cells on the proliferation of naive T cells, traced the time course of appearance of cytolytic activity in them, and analyzed the spectrum of cytokines produced by memory T cells and naive cells during secondary immune response *in vitro*.

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MATERIALS AND METHODS

The study was carried out on C57BL/10 (H-2^b), B10.D2 (H-2^d), B10.D2(R101) ($K^dI^dD^b$) mice from Vivarium Breeding Department of N. N. Blokhin Cancer Research Center (Table 1) and transgenic C57BL/10-TgN(ACTbEGFP)1Osb (H-2^b) mice from Jackson Laboratory (Bar Harbor). In order to obtain B10.D2(R101) ($K^dI^dD^b$) mice expressing GFP, C57BL/6-TgN(ACTbEGFP)1Osb mice were mated with R101 mice, while F_1 hybrids were back-crossed with the same parental strain. In the second generation R101 homozygotes expressing the transgene were selected by survival after intraperitoneal injection of 2×10^7 EL4 (K^bD^b) thymoma cells. The presence of the transgene in animals was identified by green fluorescence of open body parts using a UV transilluminator.

P815 (K^dD^d) mastocytoma was obtained from N. N. Blokhin Cancer Research Center. Cell strains

TABLE 1. Mouse Strain Haplotypes Used in the Study

Strain	Expression of H-2 complex allele products				
	K	I-A	I-E	D	L
B10.D2(R101)	d	d	d	b	b
B10.D2	d	d	d	d	d
C57BL/10	b	b	b	b	b

were maintained by *in vitro* passages in RPMI-1640 (Sigma) with 10% FCS (Pan Eco), 20 mM HEPES (Sigma), 4 mM L-glutamine (Serva, 5×10^{-5} M 2-mercaptoethanol (Merck), and 0.016% gentamicin sulfate (Moskhimpreparaty).

C57BL/10 mice were intraperitoneally immunized with P815 (K^dD^d) mastocytoma cells (2×10^7). After 2 months splenocytes of immunized animals were used as the source of long-living memory cells.

Stimulator and responder lymphocytes were isolated from mouse spleen in a Potter homogenizer with a conical pestle by gentle squeezing from the organ stroma. Erythrocytes were removed by hypotonic shock and the resultant mononuclears were washed 3 times by centrifugation (200*g*, 5 min). Viable cells were counted in a mixture of trypan blue and eosin. Mouse splenocytes (prospective stimulators) were treated with mitomycin C (25 μg/ml, 37°C, 30 min). Acute heat shock was induced by splenocyte incubation at 45°C for 60 min.

Con A-blasts served as targets for the cytotoxic test. To this end the cells isolated from the lymph nodes of C57BL/10-GFP and B10.D2(R101)-GFP mice by gentle squeezing from the stroma were precipitated by centrifugation (200*g*, 5 min) in 5 ml phosphate saline buffer, placed into flasks (3×10^7 cells in 15 ml medium per flask), and incubated with Con A in a final concentration of 5 μg/ml in a thermostat at 37°C, 5% CO_2, and 100% humidity for 48 h.

For mixed lymphocyte culture (MLC), 3×10^6 responder splenocytes were incubated with allogenic or syngeneic mitomycin C-treated stimulators (5×10^6 cells/well) in a 24-well flat-bottom plate. For selective activation of memory cells, the stimulators were exposed to acute heat shock [3,7] and added to wells (3×10^6/well). The cells were incubated in complete growth medium: RPMI-1640 with 5% human serum, 0.016% gentamicin sulfate, 4 mM L-glutamine, 20 mM HEPES, and 5×10^{-5} M 2-mercaptoethanol at 37°C, 5% CO_2, and absolute humidity for 72 h.

The targets were washed from Con A in 15 ml phosphate saline buffer. Activated memory cells from MLC were separated from dead cells by centrifugation in single-step Ficoll-Verografin density gradient (1.09) for 15 min. The cells were transferred to a 96-well flat-bottom plate in different memory/target cell ratios (1:1,1:5, 1:25, 1:50). The cells were precipitated by centrifugation (200*g*, 5 min) and incubated in a thermostat at 37°C, 5% CO_2, and 100% humidity for 4 h, and the count of remaining target cells expressing GFP was analyzed on a flow cytofluorometer. The test was carried out 24, 48, and 72 h after activation of memory cells in MLC.

The following antibodies were used for description of cell population composition by flow cytofluorometry: to CD4, phycoerythrin-labeled,

TABLE 2. Primer Sequences

Gene product	Primer sequence	Size of amplification product, bp
$TGF\beta_1$-AS	5'-ATCCACTTCCAACCCAGGTCCTT-3'	303
$TGF\beta_1$-S	5'-AGGAGACGGAATACAGGGCTTTCG-3'	303
IL-4-AS	5'-TGCATGATGCTCTTTAGGCTTTCC-3'	313
IL-4-S	5'-AGATCATCGGCATTTTGAACGAGGTC-3'	313
IL-2-AS	5'-TGATGAAATTCTCAGCATCTTCCA-3'	329
IL-2-S	5'-GACACTTGTGCTCCTTGTCAACAG-3'	329
IL-10-AS	5'-GTGGGTGCAGTTATTGTCTTCCCG-3'	200
IL-10-S	5'-GCCTTCAGTATAAAAGGGGGACC-3'	200
GAPDH-AS	5'-CTCAGTGTAGCCCAGGATGC-3'	528
GAPDH-S	5'-ACCACCATGGAGAAGGCTGG-3'	528

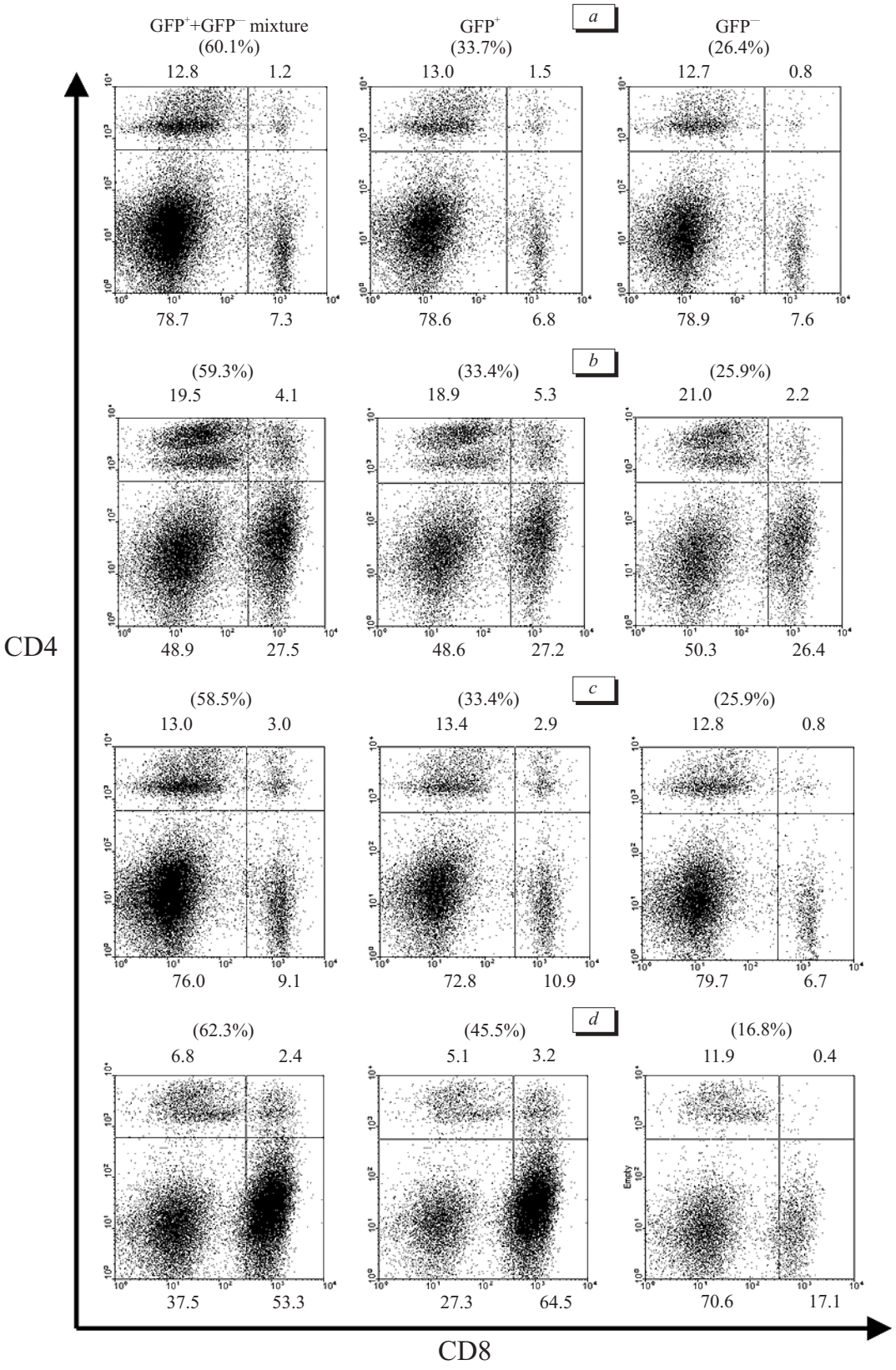

Fig. 1. Cytofluorometric analysis of C57BL/10 splenocyte proliferation in a mixture with intact (*a*, *b*) or immune (*c*, *d*) splenocytes of GFP mice during response in MLC to syngeneic (C57BL/10 — *a*, *c*) and allogenic (B10.D2 — *b*, *d*) stimulators treated with mitomycin C. Figures in parentheses: percentage of viable cells of respective fractions (in cell mixture and separately in GFP^+ and GFP^- fractions) in the total number of events analyzed on a flow cytofluorometer.

and allophycocyanin-labeled (Pharmingen). Dead cells were excluded from the analysis by propidium iodide staining. The samples were analyzed on a FacsCalibur flow cytofluorometer. The results were analyzed using WinMDI 2.8 software.

For evaluation of cytokine expression, total RNA was extracted from MLC using Tri Reagent (Sigma). The resultant RNA was dissolved in deionized water. For more precise evaluation of the ratio of mRNA for some cytokines the samples with initial cDNA concentration were scanned and the respective cDNA quantities were taken for PCR. The structure of primers and conditions of reverse transcription PCR (RT PCR) were described previously [9] (Table 2). After PCR the reaction mixture was applied onto 2% agarose gel containing ethidium bromide and separated by electrophoresis in Tris-borate buffer at 8 V/cm for 45 min. The gels were photographed and analyzed using UV transilluminator fitted with a CD camera.

The arithmetic mean and mean error for a sample were calculated for each experimental group. The results were statistically processed using Student's *t* test.

RESULTS

The effect of memory cells on naive cell proliferation in immune response to alloantigen *in vitro* in MLC was evaluated using a mixture of responder splenocytes from intact wild type C57BL/10 mice and splenocytes from intact or immunized transgenic C57BL/10-TgN(ACTbEGFP)1Osb mice expressing GFP. Mytomicin C-treated allogenic splenocytes of B10.D2 (H-2^d) mice served as stimulators (Fig. 1). On day 3 the cells were removed from the culture and stained with monoclonal fluorescent antibodies to CD4 and CD8 molecules and the blast population was analyzed on a flow cytofluorometer. The proliferation of naive $CD8^+$ (GFP^-) cells was partially suppressed and that of naive $CD4^+$ (GFP^-) cells was completely suppressed in the presence of memory cells (GFP^+) (Fig. 1). Similar results were obtained in another system, with memory cells specific to H-$2K^b$ molecule obtained by immunization of B10.D2(R101)($K^dI^dD^b$) mice with EL4 (K^bD^b) thymoma cells.

We hypothesized that memory cells suppress proliferation of naive cells via cytokines, such as $TGF\beta_1$ and IL-10 (a signal blocking T lymphocyte proliferation is mediated by these cytokines). For induction of primary and secondary immune response splenocytes of intact or immune B10.D2(R101) mice were activated in MLC. On day 3 mRNA was isolated from the cells, the expression of $TGF\beta_1$, IL-10, IL-4, and IL-2 cytokines in primary and secondary immune response was analyzed by RT-PCR. The concentration of TGFβ mRNA in cell cultures derived from intact and immune animals was similar (Fig. 2). The concentration of IL-10 mRNA in selective activation of memory T cells was 4-fold higher than the concentration during primary re-

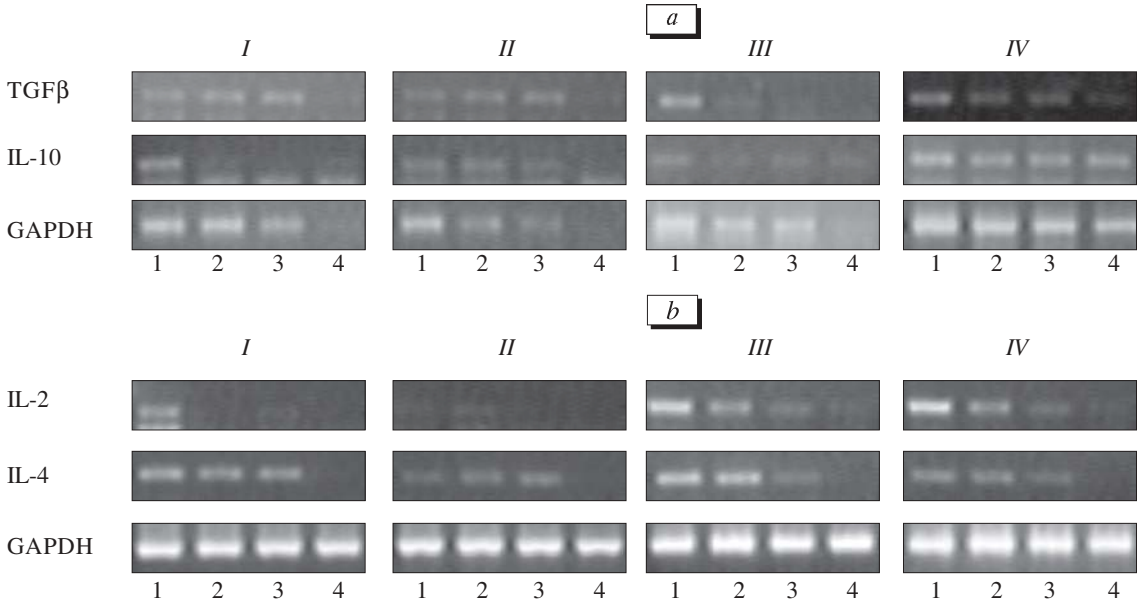

Fig. 2. Comparison of cytokine gene mRNA expression. Results of electrophoresis of amplified cDNA products at different dilutions. *a*) cDNA concentrations (c — initial concentrations): 1) $c/4^3$; 2) $c/4^4$; 3) $c/4^5$; 4) $c/4^6$; *b*) concentrations: 1) 4c, 2) c, 3) c/4; 4) c/16. Identity control: expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene in corresponding dilutions. *I*: mitomycin C, immune; *II*: thermal shock, immune; *III*: mitomycin C, intact; *IV*: thermal shock, intact.

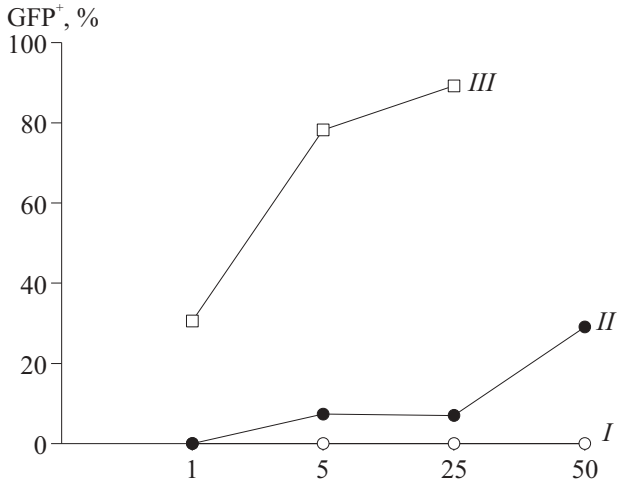

Fig. 3. Relationship between cytotoxic activity of memory T cells and duration of stimulation. Activated memory T cells were incubated with Con A-blasts in ratios: 1:1 (1), 5:1 (5), 21:1 (25), and 50:1 (50) for 4 h. The percentage of killed Con A-blasts was evaluated on a flow cytofluorometer. Abscissa: effector/target ratio; ordinate: percentage of GFP^+ cells killed by activated memory T cells. *I*: 24 h; *II*: 48 h; *III*: 72 h.

sponse to mitomycin C-treated allogenic stimulators. No clear-cut differences in the IL-4 mRNA concentrations were detected. The most significant differences were observed in expression of IL-2, the main growth factor for T cells: the concentration of IL-2 mRNA during secondary immune response was 12-fold lower than during primary response.

Hence, cooperation of suppressor cytokines and the absence of the main growth factor can be responsible for suppressed proliferation of naive cells.

Memory cells can be divided into effector and central by their functional characteristics. They differ by the time course of cytolytic response: for acquisition of cytolytic activity central memory cells need 24-h restimulation, while effector memory cells possess cytolytic activity *ex vivo* [7,8]. In our system central memory cells were activated, because they exhibited no cytolytic activity *ex vivo* and it was detected only 48 h after restimulation with heated allogenic cells (Fig. 3).

Thus, central memory cells are involved in suppression of the primary immune response. Expression of suppressor cytokines IL-4 and TGFβ was observed during primary and secondary immune responses, but the concentration of IL-10 increased significantly after memory cell activation. Moreover, the expression of IL-2, the main growth factor for naive T cells, was virtually absent during memory cells response, indicating that the proliferation of $CD8^+$ memory cells did not depend on IL-2 and that there existed a special mechanism for naive cell response suppression by these cells.

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REFERENCES

1. J. L. Casanova, P. Romero, C. Widmann, *et al.*, *J. Exp. Med*, **174**, 1371-1383 (1991).
2. L. R. Haaheim, *Dev. Biol.* (Basel), **115**, 49-53 (2003).
3. D. B. Kazanskii, V. N. Petrishchev, A. A. Shtil', *et al.*, *Bioorg. Khim.*, **25**, No. 2, 117-128 (1999).
4. P. Klenerman and R. M. Zinkernagel, *Nature*, **394**, No. 6692, 482-485 (1998).
5. X. S. Liu, Y. Xu, L. Hardy, *et al.*, *J. Immunol.*, **171**, No. 9, 4765-4772 (2003).
6. J. Mongkolsapaya, W. Dejnirattisai, X. N. Xu, *et al.*, *Nat. Med.*, **9**, No. 7, 921-927 (2003).
7. E. L. Pobezinskaya, L. A. Pobezinskii, D. B. Kazanskii, *et al.*, *Bull. Exp. Biol. Med.*, **137**, No. 5, 493-498 (2004).
8. F. Salusto, D. Lenig, A. Lanzavecchia, *et al.*, *Nature*, **401**, 708-712 (1999).
9. C. Tanchot, A. Lamonnier, B. Perarnau, *et al.*, *Science*, **276**, 2057-2062 (1997).