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ORIGINAL ARTICLE

Loci controlling lymphocyte production of interferon γ after alloantigen stimulation in vitro and their co-localization with genes controlling lymphocyte infiltration of tumors and tumor susceptibility

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Abstract Low infiltration of lymphocytes into cancers is associated with poor prognosis, but the reasons why some patients exhibit a low and others a high infiltration of tumors are unknown. Previously we mapped four loci (*Lynf1–Lynf4*) controlling lymphocyte infiltration of mouse lung tumors. These loci do not encode any of the molecules that are involved in traffic of lymphocytes. Here we report a genetic relationship between these loci and the control of production of IFNγ in allogeneic mixed lymphocyte cultures (MLC). We found that IFNγ production by lymphocytes of O20/A mice is lower than by lymphocytes of OcB-9/Dem mice (both $H2^{pz}$) stimulated in MLC by irradiated splenocytes of C57BL/10SnPh ($H2^{b}$) or BALB/cHeA ($H2^{d}$) mice, or by ConA. IFNγ production in MLCs of individual (O20 × OcB-9)F_2 mice stimulated by irradiated C57BL/10 splenocytes and genotyped for microsatellite markers revealed four IFNγ-controlling loci (*Cypr4-Cypr7*), each of which is closely linked with one of the four *Lynf* loci and with a cluster of susceptibility genes for different tumors. This suggests that inherited differences in certain lymphocyte responses may modify their propensity to infiltrate tumors and their capacity to affect tumor growth.

Keywords Genetic control of interferon γ production · Gene mapping · Lymphocyte infiltration of tumors · Tumor susceptibility

Abbreviations

Cypr	Cytokine production
Lynf	Lymphocyte infiltration
MLC	Mixed lymphocyte culture
MLR	Mixed lymphocyte reaction
QTL	Quantitative trait locus

Introduction

Interaction between the immune system and cancer is one of the most important determinants of cancer growth, progression, and metastasis. The interconnected processes of innate and adaptive immunity co-determine the development of cancer from the very beginning, when acute inflammation tends to suppress and chronic inflammation to promote carcinogenesis [1]. The interaction of adaptive immunity with cancer is less clear. It is a powerful agent in rejection of virally induced tumors [2], but its effects on spontaneous and carcinogen-induced tumors are still subject of discussion [3, 4]. Recently, however, in a

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number of human cancers it has been observed that presence of infiltrating $CD3^+$ T lymphocytes is a positive prognostic factor [5], although in some studies their effect was mitigated by presence of T regulatory lymphocytes [6]. This indicated a positive role of $CD3^+$ T lymphocytes in suppression of cancer. However, it is not known why infiltrating T lymphocytes are abundant in cancers of some patients but scarce in others. Although in the past decade, a large progress has been made in unravelling the sequence of molecular steps that participate in the passage of lymphocytes from blood circulation into tumor, and more than 60 different molecules (or closely related members of the same family) that facilitate and mediate lymphocyte traffic were identified [7], differences in structure or expression of these molecules were not yet correlated with individual propensity to tumor infiltration.

To the contrary, a new insight into the regulation of lymphocyte infiltration in tumors has been provided by the observations that the presence of infiltrating lymphocytes in mouse lung tumors is pronounced in some strains but virtually absent in others [8] and that this difference is controlled by four chromosomal loci designated *Lynf1–Lynf4* (*Lynf* = Lymphocyte infiltration) that determine the presence of intra-tumoral infiltrating lymphocytes [9] in a cross between strains O20/A and OcB-9/Dem. Surprisingly, none of the four *Lynf* loci (*Lynf1*—chromosome 4; *Lynf2*—chromosome 8; *Lynf3* and *Lynf4*—chromosome 6) encoded any of set of >60 molecules that were reported to participate in migration of lymphocytes into tumors. Therefore, other processes than direct molecular interactions of lymphocyte and surrounding tissue may be the primary determinants of individual's propensity to lymphocyte infiltration of tumors.

Our previous data showed that genetic polymorphisms of the intensity of nonspecific inflammation-related activity of immunocytes are linked with cancer susceptibility genes. We defined previously two loci, *Marif1* controlling production of IL-12, TNFα and iNOS, and *Marif2* controlling production of TNFα and IL-12, by in vitro stimulated bone marrow-derived macrophages [10], and two loci, *Cinda3* and *Cinda5*, controlling in vitro proliferative response of lymphocytes to cytokines IL-2 and IL-4 [11]. *Marif1* and *Cinda3* are linked to each other and *Cinda3* is closely linked to *Lynf1* as well as the mouse lung cancer susceptibility loci *Sluc6/Sluc21* on chromosome 4. *Marif2* and *Cinda5* are linked to each other and to *Lynf2* as well as to the mouse lung cancer susceptibility locus *Sluc20* on chromosome 8. This suggests a possible functional connection between activation of immunocytes and capacity of tumor infiltration.

In the present study, we analysed the genetic control of production of another cytokine, IFNγ, during in vitro reaction to major histocompatibility complex (MHC) alloantigens. The differences in the MHC antigens induce the strongest responses [12]. But genetic disparity in minor alloantigens also modifies the alloresponse. Earlier studies found that some strong MLC-stimulations (*Mls*) antigens are coded by mouse *Mtv* (mammary tumor virus) [13]; later studies revealed a broad spectrum of additional minor human antigens [14]. Intensity of MLC-response to alloantigens is determined by two major factors—the genetic disparity between the responding and stimulating cells and the genetically defined intrinsic capacity of the responding cells to react to the stimulus [15–17].

Responder cells react to stimulator cells by proliferation and by cytokine production. We have studied the genetic basis of these two responses, using the recombinant congenic (RC) strains, which were developed for analysis of multigenically controlled biological processes [18]. A series of RC strains is derived from two parental inbred strains: a "background" strain and a "donor" strain. Each of these RC strains contains a different subset of about 12.5% of genes from the parental donor strain and the remaining approximately 87.5% of background strain genes. The BALB/cHeA-c-STS/A (CcS) and O20/A-c-B10.O20/Dem (OcB) series of strains were used previously for analysis of alloantigen response. The strain distribution pattern of magnitude of proliferative response in MLR of individual RC strains to stimulator cells of four different strains was almost identical, indicating that differences in responsiveness, rather than the alloantigenic difference itself, determine the magnitude of the response, and that the responsiveness to different MHC alloantigens is largely controlled by the same genes [19, 20]. We have mapped previously two of these responsiveness genes, *Alan1* and *Alan2* (Alloantigen response 1, 2) located on chromosomes 17 and 4, respectively, that control differences in proliferative response to several alloantigens in CcS and OcB RC strains [16, 17]. We have also shown that in the individual RCS the levels of proliferation and IL-2 production in MLR are not correlated, and therefore these two responses are under a different genetic control [19].

In the present work we defined four loci, *Cypr4–Cypr7* (*Cypr* = *Cytokine production*), that control IFNγ production by responder cells in MLR. IFNγ plays important role in immunity against infection, and it has been associated with a number of autoinflammatory and autoimmune diseases (reviewed in [21]). IFNγ has also multiple and diverse essential functions in immune response to cancer [22–25]. For that reason, genetics of capacity of IFNγ production may be important for a variety of biological reactions. Here we show that control of IFNγ production is genetically linked to and possibly responsible for the control of intensity of lymphocyte infiltration in tumors and control of tumor growth and progression.

Materials and methods

Mice

Males and females of strains O20/A (abbrev. O20), OcB-9/Dem (abbrev. OcB-9) (both strains have the MHC haplotype $H2^{pz}$) and F_2 hybrids between O20 and OcB-9 came from P. Demant's breeding colony. The haplotype $H2^{pz}$ is described in [26]. The genetic composition of strain OcB-9 has been described in detail by Stassen and co-workers [27]. When used for these experiments strain OcB-9 passed more than 31 generations of brother–sister mating and was highly homozygous. C57BL/10SnPh (abbrev. B10) ($H2^{b}$) mice were from the Institute of Molecular Genetics (Prague). As the strains O20 and OcB-9 are MHC identical, all mixed lymphocyte reactions tested in this paper involved the same MHC incompatibility ($H2^{pz}$–$H2^{b}$).

Mixed lymphocyte culture

Spleen cells of O20, OcB-9, and of individual F_2 hybrid mice (6×10^5 cells per well) were mixed with 8×10^5 irradiated (3000 R) C57BL/10 stimulator cells in 800 µl in 24-well tissue culture plates in complete RPMI 1640 medium, respectively (Nunc, Roskilde, Denmark) [28]. Supernatants were removed after 96 h incubation period and stored at −70°C before use. Each day in a separate experiment 20 F_2 mice and appropriate controls (O20 and OcB-9 mice) were tested. In the experiments, comparing IFNγ, IL-2, and IL-4 production by stimulated lymphocytes of strains O20 and OcB-9, supernatants were removed also 48 and 72 h after stimulation.

Concanavalin A-stimulated cultures

Spleen cells (4×10^5 cells per well) were incubated in 800 µl in 24-well tissue culture plates in complete RPMI 1640 medium with 2.5 µg/ml ConA. Supernatants were collected after 24, 48, or 72 h of incubation and assayed for IFNγ presence.

Estimation of cytokine levels

Supernatants from MLR cultures were assayed for IFNγ, IL-2, and IL-4 presence using the primary and secondary mAbs (R46A2, XMG1.2; JES6-1A11, JES6-5H4; 11B11, BVD6-24G2) and standards (mIFNγ, mIL-2, and mIL-4) from BD Bioscience, San Jose, CA. Similarly, supernatants from ConA stimulated cultures were assayed for IFNγ. The ELISA was performed using the conditions recommended by BD Bioscience. In brief, a 96-well flat-bottom plate (Costar, Cambridge, MA) was coated with primary mAb at a concentration of 1 µg/ml in 0.1 M $NaHCO_3$ pH 8.2 and incubated overnight at 4°C. The plates were washed with PBS-Tween (Costar, Corning, NY) and incubated for 2 h with 200 µl of PBS 8% BSA. After washing, aliquots of supernatants were added to duplicate wells and incubated overnight at 4°C. Plates were washed again and biotin-labeled secondary mAb was added for 45 min. Plates were washed, followed by addition of horseradish peroxidase streptavidin (Vector Laboratories, Burlingame, CA). 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich, St. Louis, MO) and peroxide were used as chromogen. Color reaction was stopped by sodium dodecyl sulfate (SDS) in *N*,*N*-dimethylformamide and plates were read at OD 405 nm and the IFNγ levels were estimated by the ELISA reader Tecan and the curve fitter program KIM-E (Schoeller Pharma, Prague, Czech Republic) using least square nonlinear regression analysis. Sensitivity of detection of IFNγ, IL-2, and IL-4 levels in supernatants was 32, 4, and 15 pg/ml, respectively.

Genotyping of microsatellites by PCR

DNA was isolated from tails using a standard proteinase procedure. The strain OcB-9 differs from O20 by carrying B10.O20-derived segments at 10 chromosomes [27]. These differential segments were typed in the F_2 hybrid mice using 15 microsatellite markers: D2Mit7, D2Mit56; D4Mit54; D6Mit31, D6Mit52; D7Mit12, D7Nds2; D8Mit3, D8Mit65; D10Mit12; D11Mit15; D16Mit130; D18Mit17; D19Mit3, D19Mit63. These markers were selected because they cover optimally the chromosomal segments at which the OcB-9 strain has genetic material from the B10.O20 strain. They carry different alleles in the two parental strains, the O20 allele (designated *o*), and the B10.O20 allele (designated *b*). PCR genotyping was performed as described [29]. Briefly, amplification was performed with a MJ Research Thermal Cycler PTC 100 Model 96 (MJ Research, Watertown, MA) in a volume of 10 µl with 20 ng DNA, 0.23 µM of forward primer [γ-^{32}P]ATP end-labeled with T4 polynucleotide kinase (New England Biolabs, Schwalbach, Germany), 0.23 µM of reverse primer, 200 µM of each dNTP and 0.04 u Taq polymerase (GIBCO, Grand Island, NY) in a 96-well U-bottom microtiter plate (FALCON, Oxford, UK) with 1 cycle of 3 min at 94°C and 30 cycles of 30 s at 94°C, 1 min at 55°C and 1 min at 72°C and 1 cycle of 1 min at 55°C, 3 min at 72°C, and 1 min at 23°C. Each PCR product was mixed with 30 µl of loading buffer and electrophoresed in 6% acrylamide gels for 2–2.5 h at 50 W. Gels were wrapped in Saran Wrap (Dow Chemicals Co., Midland, MI) and exposed to X-ray film X-OMAT AR (Kodak, Weesp, The Netherlands) for 1.5–24 h at −70°C.

Statistical analysis

The role of genetic factors in IFNγ production in MLR response was examined by analysis of variance [ANOVA, PROC GLM statement of the SAS 8.2 for Windows (SAS Institute, Cary, NC)]. The effect of each marker, sex, and experiment on IFNγ level in supernatants was tested. Each individual marker and its interactions with other markers and sex or experiment were subjected to ANOVA. A backward elimination procedure [30] was followed wherein the interaction of marker (or interaction) bearing the highest P value (if $P > 0.05$) was eliminated first. The markers and interactions with P value smaller than 0.05 were pooled for the next round of ANOVA. The backward elimination procedure was repeated till the final set of significant markers and interactions was obtained. The P values ($Pc = \alpha_T$) were corrected according to Lander and Kruglyak [31] using the formula:

$$\alpha_T* \approx [C + 2\rho G h(T)]\alpha_T$$

with $G = 2$ M (the length of the segregating part of the genome: 12.5% of 16 M); $C = 10$ (number of chromosomes segregating in this cross); $\rho = 1.5$ for F_2 hybrids; and $h(T) =$ the observed statistics (F ratio).

IFNγ, IL-2, and IL-4 production in MLR by cells of responder strains O20 and OcB-9 was compared by Mann–Whitney U test (STATISTICA for Windows 5.0, StatSoft, Inc., Tulsa, OK).

Results

Strain differences in proliferation and production of cytokines after stimulation with alloantigen or ConA

The proliferation of lymphocytes of the strain OcB-9 in MLC is higher than that of the MHC-identical strain O20 (both $H2^{pz}$) after stimulation with lymphocytes of strains with C57BL/10 ($H2^b$), BALB/cHeA ($H2^d$), and CBA ($H2^k$) that carry unrelated MHC haplotypes (Fig. 1 and Ref. 17). This indicates that strain OcB-9 has a higher propensity to respond to a variety of alloantigens than O20. The production of IFNγ in MLC is also significantly higher by OcB-9 than by O20 responder cells when stimulated by C57BL/10 ($H2^b$) cells (Fig. 2a) and BALB/c ($H2^d$) cells, but it is low in both with weaker stimulators DBA ($H2^q$) and CBA ($H2^k$) (Fig. 2b). OcB-9 lymphocytes are also better producers of IFNγ than O20 lymphocytes after stimulation with ConA (Fig. 3). In MLC with C57BL/10 stimulator cells, the production of IL-2 (Fig. 4a) but not of IL-4 (Fig. 4b) is higher by OcB-9 than O20 responder cells.

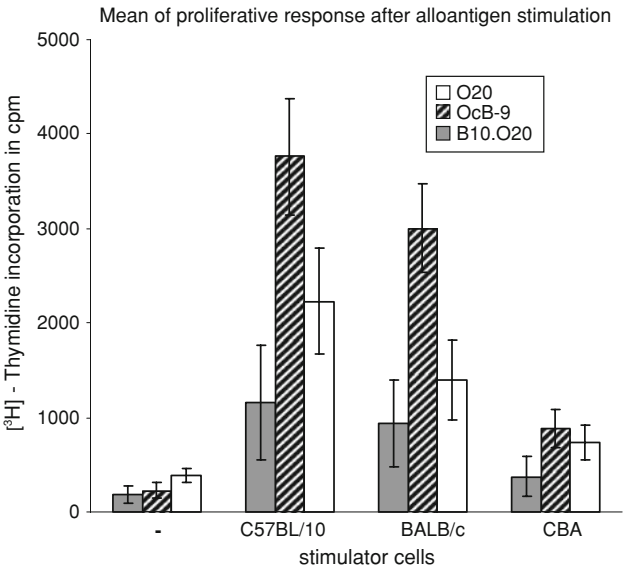

Fig. 1 The MLC reactivity of O20, B10.O20, and OcB-9 strains. Proliferative response of spleen cells in RPMI medium only and stimulated by C57BL/10Sn ($H2^b$) (B10), BALB/c ($H2^d$), and CBA/Ph ($H2^k$) alloantigens. Spleen cells (1.5×10^5 cells per well) were mixed with 2×10^5 irradiated (3000 R) B10, BALB/c or CBA/Ph stimulator cells in 200 µl in 96-well tissue culture plates. [3H]-thymidine (0.5 µCi/well) was added into the cultures for the last 6 h of the 96-h incubation period as described previously [17]. The data show the mean ± SE from three independent experiments. Reproduced with the kind permission from Ref. [17]

Detection of loci that control IFNγ level in MLC

Supernatants of the spleen cells of the strain OcB-9 contain higher concentration of IFNγ 96 h after stimulation with C57BL/10 cells than supernatants of spleen cells of strain O20 ($P < 0.014$) (Fig. 2a). We examined production of IFNγ in 240 F_2 hybrids between the strains O20 and OcB-9. The strains O20 and OcB-9 differ in their genetic material at 10 chromosomes [27]. These differential segments were typed in the F_2 hybrids mice using 15 microsatellite markers listed above.

Statistical analysis revealed four genetic loci that influence level of IFNγ in supernatants of (O20xOcB-9)F_2 splenocytes stimulated by irradiated C57BL/10 cells. Two of these loci have individual effects, but the other two operate in mutual nonadditive interaction. Two loci, *Cypr4* (Cytokine production 4) linked to D6Mit31 (corrected P value < 0.00717) and *Cypr5* linked to D6Mit52 (corrected P value < 0.000257) have effect on IFNγ production after alloantigen stimulation that is not influenced by interaction with other genes (main effects) (Table 1). These loci have an opposite effect on the studied trait. The homozygosity for the O20 allele of *Cypr4* (*oo*) determines about twofold higher level of IFNγ than the homozygosity of the B10.O20 allele (*bb*), whereas homozygosity for the O20 allele of

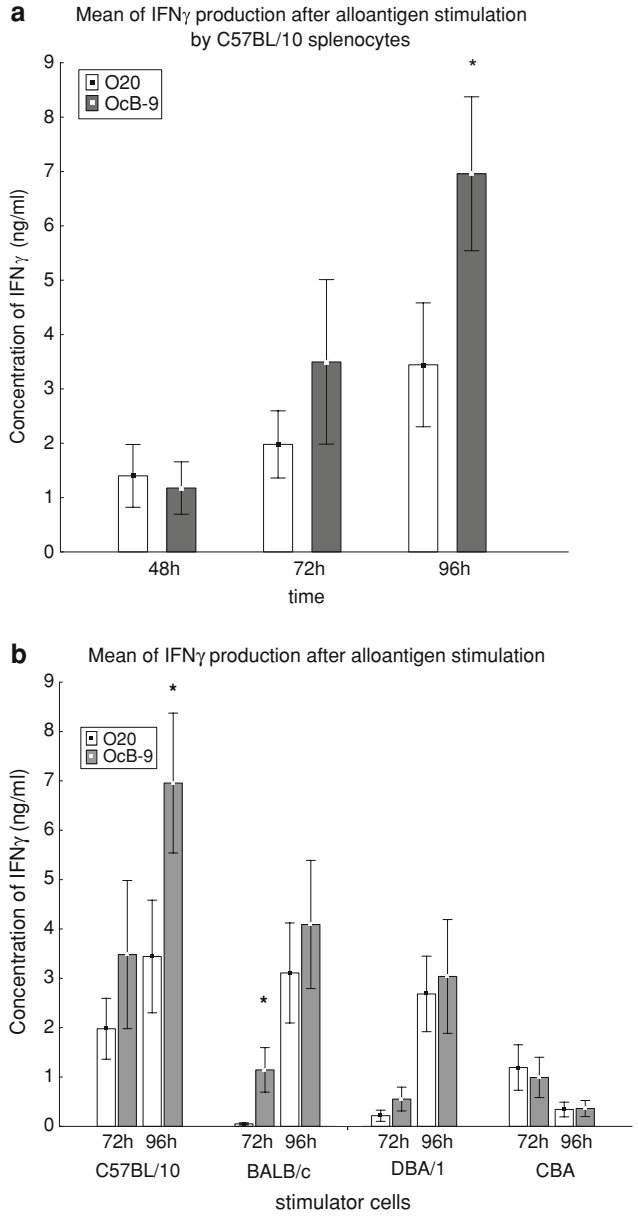

Fig. 2 **a** Comparison of concentration of IFNγ in supernatants of the spleen cells of the strains OcB-9 and O20 after alloantigen stimulation. Spleen cells (6×10^5 cells per well) were mixed with 8×10^5 irradiated (3000 R) C57BL/10 stimulator cells in 800 µl in 24-well tissue culture plates. Cell supernatants were analyzed 48, 72, and 96 h after stimulation. Data summarize the result of 13 independent experiments. Both female and male mice were used in our analysis, but no influence of sex on strain difference was observed. The columns show the means ± SE of IFNγ concentration in ng/ml. *Filled square*: OcB-9, *unfilled square*: O20. **b** Comparison of concentration of IFNγ in supernatants of the spleen cells of the strains OcB-9 and O20 after stimulation by C57BL/10Sn ($H2^b$), BALB/c ($H2^d$), CBA ($H2^k$), and DBA/1 ($H2^q$) alloantigens. Spleen cells (6×10^5 cells per well) were mixed with 8×10^5 irradiated (3000 R) C57BL/10Sn, BALB/c, CBA, or DBA/1 stimulator cells in 800 µl in 24-well tissue culture plates. Cell supernatants were analysed 72 and 96 h after stimulation. Data summarize the result of 13 independent experiments

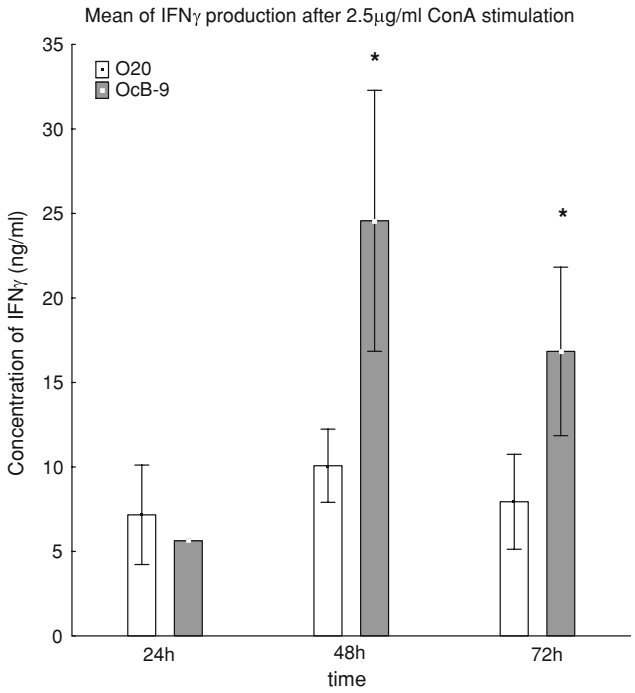

Fig. 3 Comparison of concentration of IFNγ in supernatants of the spleen cells of the strains OcB-9 and O20 after stimulation with 2.5 µg/ml ConA. To test IFNγ concentration, 800 µl of spleen cells (4×10^5 cells per well) were incubated in 24-well tissue culture plates in complete RPMI 1640 medium with 2.5 µg/ml ConA. After 24, 48, or 72 h of incubation supernatants were collected and assayed for IFNγ presence. The columns show the means ± SE of IFNγ concentration in ng/ml. Data summarize the result of four independent experiments. *Square*: OcB-9, *unfilled square*: O20

Cypr5 is associated with about two-and-half fold decrease level of this cytokine than the homozygosity of the B10.O20 allele. Influence of the experimental group was evident ($P < 1.75 \times 10^{-24}$), but no significant interaction between experimental group and marker was observed.

Cypr6 influences IFNγ level in interaction with *Cypr7* (corrected $P < 0.00111$). F_2 mice with homozygous B10.O20 (*bb*) alleles at *Cypr6* and O20 (*oo*) alleles at *Cypr7* or homozygous for B10.O20 allele at *Cypr7* and homozygous for O20 alleles in *Cypr6* produce approximately twice higher IFNγ level in supernatants in alloantigen-stimulated spleen cells in comparison with other combinations of *Cypr6* and *Cypr7* O20 and B10.O20 alleles (Table 2). The levels of IFNγ seem to be negatively correlated with the intensity of lymphocyte infiltration, both in the strains and in comparable genotypes. The production of IFNγ is higher in the strain OcB-9 than O20, while in O20 mice the numbers of intratumoral lymphocytes are higher than in OcB-9 [8]. A similar negative correlation is seen also at locus level: IFNγ levels at the three *Cypr4* genotypes are negatively correlated with lymphocyte infiltration scores at the corresponding

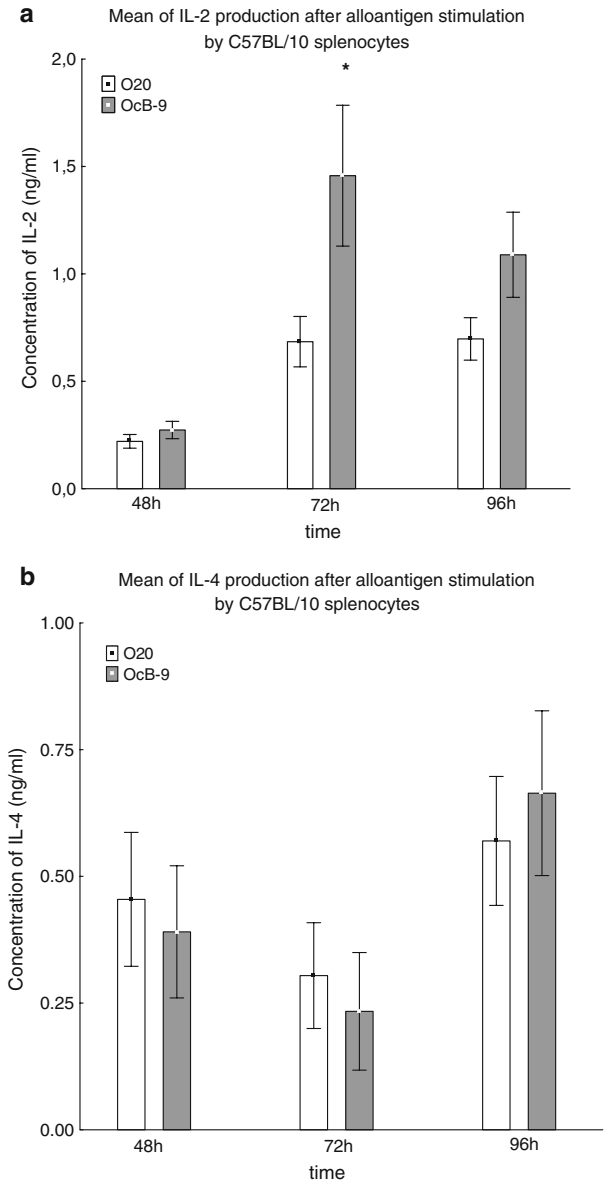

Fig. 4 **a** Comparison of concentration of IL-2 in supernatants of the spleen cells of the strains OcB-9 and O20 after alloantigen stimulation. Spleen cells (6×10^5 cells per well) were mixed with 8×10^5 irradiated (3000 R) C57BL/10 stimulator cells in 800 µl in 24-well tissue culture plates. Cell supernatants were analysed 48, 72, and 96 h after stimulation. Data summarize the result of 13 independent experiments. Both female and male mice were used in our analysis, but no influence of sex on strain difference was observed. The columns show the means ± SE of IL-2 concentration in ng/ml. *Filled square*: OcB-9, *unfilled square*: O20. **b** Comparison of concentration of IL-4 in supernatants of the spleen cells of the strains OcB-9 and O20 after alloantigen stimulation. Spleen cells (6×10^5 cells per well) were mixed with 8×10^5 irradiated (3000 R) C57BL/10 stimulator cells in 800 µl in 24-well tissue culture plates. Cell supernatants were analysed 48, 72, and 96 h after stimulation. Data summarize the result of 13 independent experiments. Both female and male mice were used in our analysis, but no influence of sex on strain difference was observed. The columns show the means ± SE of IL-4 concentration in ng/ml. *Filled square*: OcB-9, *unfilled square*: O20

genotypes [9] of the co-localizing *Lynf4* locus: *oo*: IFNγ—7.74 ng/ml, infiltration score (IS)—0.04; *ob*: IFNγ—5.77 ng/ml, (IS)—0.39; *bb*: IFNγ—3.78 ng/ml, (IS)—0.34. Allelic correlations within the other *Cypr-Lynf* could not be assessed, because these loci were defined by inter-locus interactions.

Discussion

In the present study, we demonstrate genetic linkage between in vitro production of IFNγ and in vivo lymphocyte infiltration of tumors. Previously, we have shown that responder's genotype strongly influences various in vitro responses of lymphocytes to stimulation by allogeneic cells [16, 17], anti-CD3 antibodies [28], mitogens [32], and cytokines [11, 28]. Importantly, in MLC these differences in response were obvious in spite of identical MHC incompatibility. We have shown that these differences are controlled by several loci with quantitative additive effects, and that different components of a response, for example proliferation or production of different cytokines, are controlled by different genes. We observed that two genetic loci that control activation of macrophages [10] and lymphocytes [11] co-localize with the genes that control susceptibility to lung tumors. This suggested that the loci regulating activity of lymphocytes and macrophages in vitro could have also in vivo effect on tumor growth. The genetic analysis of production of IFNγ by responder lymphocytes in allogeneic mixed lymphocyte cultures demonstrated four genetic loci with significant influence. We analyzed the difference in the phenotype of interferon production between the strain O20 and the RC strain OcB-9 that is in about 87.5% genes identical to O20, but received a random subset of about 12.5% of genes from the strain B10.O20 [18, 27]. The genetic material obtained from B10.O20 is almost entirely contained in several contiguous chromosomal segments, mostly 10–20 cM long. They were described previously [27, 33] and are covered by 15 polymorphic microsatellite markers listed in "Materials and methods" section. Thus, any genetic differences between these two strains must be controlled by a gene or genes located in one of these B10.O20-derived segments [34]. It should be pointed out that the strains O20 and OcB-9 have identical MHC haplotype ($H2^{pz}$), so they respond to the same MHC incompatibility ($H2^b$) and any difference in the response must be host-related. The stimulation by non-MHC alloantigens is not a likely cause of the observed differences in production of IFNγ between O20 and OcB-9, because the two strains have the same genotype at the strong stimulatory locus *Mtv13* (*Mls1*) [27] and no other stimulators in MLC were described in the vicinity of the *Cypr* loci mapped here.

Table 1 Effects of genotype at the loci *Cypr4* and *Cypr5* on the concentration of IFNγ in supernatants of spleen cells of individual F_2 hybrids between OcB-9 and O20 stimulated by irradiated B10 splenocytes

Marker/locus	Genotype			*P* value	Corrected *P*
	oo	*ob*	*bb*		
D6Mit31 (*Cypr4*)	7.74 ± 0.60 ($n = 64$)	5.77 ± 0.47 ($n = 105$)	3.78 ± 0.69 ($n = 63$)	0.000100	0.00717
D6Mit52 (*Cypr5*)	3.66 ± 0.74 ($n = 55$)	5.09 ± 0.47 ($n = 121$)	9.43 ± 0.66 ($n = 63$)	0.00000296	0.000257

The phenotypic values given for individual genotypes indicate mean and standard errors of the concentration of IFNγ and (in *brackets*) the number of mice (for computations the measured values of IFNγ in supernatants (ng/ml) were normalized by natural logarithm of (value + 0.5) to the power of 0.005)

Table 2 Effects of genotype at the loci *Cypr6* and *Cypr7* on the concentration of IFNγ in supernatants of spleen cells of F_2 hybrids between OcB-9 and O20 stimulated by irradiated B10 splenocytes

	D8Mit3 *(Cypr7)*		
	oo	*ob*	*bb*
D4Mit54 (*Cypr6*)			
oo	5.57 ± 1.89 ($n = 15$)	3.13 ± 0.93 ($n = 29$)	10.64 ± 1.57 ($n = 10$)
ob	5.16 ± 0.83 ($n = 31$)	5.66 ± 0.55 ($n = 58$)	4.67 ± 0.69 ($n = 33$)
bb	9.06 ± 1.38 ($n = 10$)	4.77 ± 0.77 ($n = 28$)	4.61 ± 0.85 ($n = 11$)

The phenotypic values given for individual genotypes indicate mean and standard errors of the concentration of IFNγ and (in *brackets*) the number of mice. (B10.O20 allele *b*, O20 allele *o*), *P* value = nominal *P* value, corrected *P* = *P* value corrected for total genome screen)
P value = 0.000189, corrected *P* value = 0.00111

However, the higher production of IFNγ by OcB-9 cells is not likely a mere consequence of a higher proliferative response. We measured proliferation in parallel MLCs of individual F_2 hybrid animals that were tested for IFNγ production. We found in that population only one locus controlling the proliferation, *Alan2* [17]. It is linked to a marker that is different from but relatively close (6 cM) to the marker identifying *Cypr6*, so the identity of the two loci is neither indicated nor ruled out. The other three loci (*Cypr4*, *5*, and *7*) were not linked to proliferative activity in MLR. This prevailing genetic independence of proliferation and IFNγ production in MLC is similar to the extensively documented distinct genetic control of lymphocyte proliferation and IL-2 production in MLC [19].

An unexpected feature of our data is the complete co-localization of the four newly detected loci *Cypr* controlling production of IFNγ in mixed lymphocyte culture with the four *Lynf* loci that control infiltration of lymphocytes into tumors: *Cypr4–Lynf4*; *Cypr5–Lynf3*; *Cypr6–Lynf1*; *Cypr7–Lynf2*. This general *Cypr-Lynf* co-localization contrasts sharply with a complete lack of co-localization of *Lynf* loci with any genes encoding the more than 60 molecules that were either reported to participate in traffic of lymphocytes from the circulation to tumors or belong to the same family [9]. This suggests that, contrary to the prevailing emphasis in the literature, the principal control of capacity of lymphocytes to infiltrate tumors might reside in the regulation of the processes of lymphocyte activation.

In addition, the four *Cypr* loci described here co-localize with or are linked to a number of loci that control susceptibility to or patterns of progression of a variety of tumors that were independently described by different groups (Table 3). *Cypr4* is linked to four such loci, *Mmom1*, *Ots1*, *Pas1c*, *Skts11*, *Cypr5* to one locus, *Ltsd8*, *Cypr6* to 15 loci (10 of them within 5 cM) *Gct1, Ltsd4, Naad5, Nbl1, Pcts, Sluc6, Sluc21, Ssic1, Stmn1, Tlsr3*, and *Cypr7* to 7 loci *Dlc1, Ltq, Ltsd3, Mfhas1, Sluc20, Scc8, Poats1*.

IFNγ has an important role in host defenses, in both innate and acquired immunity. IFNγ is produced by many different cell types, such as by T cells, NK cells, NKT cells [35] upon their stimulation by antigens or mitogens. Macrophages and other accessory cells, such as dendritic cells, besides having a boosting effect on IFNγ production by resting T cells [36] can themselves also produce large amounts of IFNγ [37]. The interaction of T cells with antigen-presenting cells results in the production of IL-1, which then activates the T cells to produce IL-2, which promotes the IFNγ production [38]. All IFNγ inducers activate T cells either in a polyclonal (mitogen or antibodies) or a clonally restricted (antigen-specific) manner.

IFNγ (class II interferon) mediates broad immune responses to pathogens. It binds to IFNGR1/IFNGR2

Table 3 Co-localization of the four *Cypr* loci with cancer susceptibility loci/genes

Locus with border markers	Marker	cM	Locus/Gene	cM	Reference
O20 (*o*)	D6Mit29	36.50	*Lynf4* (Lymphocyte infiltration 4)	35.15	[9]
B10 (*b*)	D6Mit31	38.50	*Skts11* (skin tumor susceptibility 11)	36.50	[42]
***Cypr4* (*b*)**	D6Mit31	38.50	*Pas1c* (pulmonary adenoma susceptibility 1c)	37.00	[43]
B10 (*b*)	D6Mit327	46.50	*Mmom3* (mammary modifier of *Min3*)	38.50	[44]
O20 (*o*)	D6Mit10	48.70	*Ots1* (ovarian teratoma susceptibility 1)	40.00	[45]
O20 (*o*)	D6Mit334	60.75	*Ltsd8* (lung tumor shape-determining 8)	61.20	[46]
B10 (*b*)	D6Mit256	60.95	*Lynf3* (Lymphocyte infiltration 3)	61.20	[9]
***Cypr5* (*b*)**	D6Mit52	61.40			
B10 (*b*)	D6Mit370	62.67			
O20 (*o*)	D6Mit13	63.60			
O20 (*o*)	D4Mit37	56.50	*Lci* (liver cell immortalization)	57.50	[47]
B10 (*b*)	D4Mit11	57.40	*Naad4* (Neu associated allelic deletion 4)	57.40	[48]
			Ril3 (radiation-induced leukemia sensitivity 3)	60.00	[49]
			Sluc21 (susceptibility to lung cancer 21)	63.00	[30]
			Tlsr3 (thymic lymphoma suppressor region 3)	63.10	[50]
			Stmn1 (stathmin 1)	65.70	[51]
***Cypr6* (*b*)**	D4Mit54	66.00	*Naad5* (Neu associated allelic deletion 5)	66.00	[52]
			Ltsd4 (lung tumor shape-determining 4)	67.00	[46]
			Sluc6 (susceptibility to lung cancer 6)	67.00	[53]
			Lynf1 (Lymphocyte infiltration 1)	67.00	[9]
			Ssic1 (susceptibility to small intestinal cancer 1)	67.00	[54]
			Nbl1 (neuroblastoma, suppression of tumorigenicity 1)	70.00	[55]
			Pcts (plasmacytoma susceptibility)	70.00	[56]
			Gct1 (granulosa cell tumorigenesis 1)	71.00	[57]
B10 (*b*)	D4Mit342	77.50	*Pctr2* (plasmacytoma resistance 2)	73.50	[58]
O20 (*o*)	D4Mit59	78.90	*Tlsr2* (thymic lymphoma suppressor region 2)	76.60	[50]
O20 (*o*)	Markers start at 1 cM		*Scc8* (colon tumor susceptibility 8)	4.0	[59]
			Poats1 (post-ovarectomy adrenal tumor susceptibility 1)	8.0	[60]
			Ltsd3 (lung tumor shape-determining 3)	10.00	[46]
B10 (*b*)	D8Mit155	1.00	*Sluc20* (susceptibility to lung cancer 20)	10.00	[30]
***Cypr7* (*b*)**	D8Mit3	10.00	*Lynf2* (lymphocyte infiltration 2)	10.00	[9]
			Mfhas1 (malignant fibrous histiocytoma amplified sequence 1)	20.00	[61]
B10 (*b*)	D8Mit98	28.00	*Dlc1* (deleted in liver cancer 1)	21.00	[62]
O20 (*o*)	D8Mit66	28.00	*Ltq* (lung tumor QTL)	30.00	[63]

receptor complex and uses JAK1/JAK2/STAT1 signaling pathway (reviewed in [39]). IFNγ modulates the expression of major histocompatibility antigens on many different accessory cells, and thus stimulates interaction of these cells with T cells. Enhanced expression of class II antigens after IFNγ treatment also takes place on cells of T and B lineage [40]. In the nonlymphoid organs, increased MHC antigens are found mainly on capillary endothelial cells, on renal tubular cells and on bronchiolar epithelial cells.

The role IFNγ in suppression of tumor growth has been known for long time and the extensive literature cannot be reviewed here. Availability of genetically modified mice allowed to demonstrate also the role of IFNγ in various aspects of anti-tumor immune responses [22–24, 41]. In some tests, it has been shown that this role may differ in different mouse strains [22]. The present data show not only a linkage between the four *Cypr* loci that control production of IFNγ, but also an apparently negative correlation between the IFNγ levels and intensity of infiltration, not only in the tested strains, but also when comparing different alleles. However, the statistical significance of this relationship is not clear. Moreover, we are comparing results of a short-term assay (production of IFNγ in MLR) and a condition of long duration (intensity of lymphocyte

infiltration in tumors). Before the responsible *Cypr-Lynf* genes are identified, it is difficult to assess, which of the multiple effects of IFNγ on lymphocytes, other inflammatory cells, vasculature, and tumor cells modifies the level of infiltration. *Cypr-Lynf* congenic strains, currently under production, will create possibilities for more detailed studies of function of these genes.

The data presented here may be a suitable model for analysis of the potent modifying effects of infiltrating lymphocytes in human cancer. Although the mechanisms of the suppressive effects of lymphocyte infiltration on growth of human cancers are not known, it is interesting that there is an extensive genetic association between the four *Cypr-Lynf* loci and more than 20 loci controlling susceptibility to and progression of a variety of mouse tumors (Table 3). Each of the four *Cypr/Lynf* loci is linked to a number of susceptibility loci to various tumors including lung, skin, ovary, liver, mammary gland, lymphomas, leukemia, plasmacytoma, and histiocytoma. This multiplicity of tumor types affected by these loci/genes is compatible with a genetic effect that is not organ specific but possibly systemic, like control of effective immunosurveillance.

In conclusion, our data provide a novel link between genetic polymorphisms of regulation of lymphocyte activation and genetic variation in immunoregulation of tumor growth. Analysis of the genes involved and their human homologues may contribute to the understanding of individual predisposition to a high or low anti-tumor activity of lymphocytes and thus improve the decisions about personalized selection of appropriate therapies.

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