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ORIGINAL INVESTIGATION

Mechanism of tumor remission by cytomegalovirus in a murine lymphoma model: evidence for involvement of virally induced cellular interleukin-15

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Abstract A murine model of B and T cell lymphomas in recipients after hematoablative conditioning for hematopoietic cell transplantation (HCT) has previously revealed a tumor-repressive, metastasis-inhibiting function of murine cytomegalovirus (mCMV). More recently, this prediction from the experimental model was put on trial in several clinical studies that indeed gave evidence for a lower incidence of tumor relapse associated with early reactivation of latent human cytomegalovirus (hCMV) after allogeneic HCT in patients treated against different types of hematopoietic malignancies, including lymphoma and acute as well as chronic leukemias. Due to the limitations inherent to clinical studies, the tumor-repressive role of hCMV remained observational with no approach to clarify mechanisms. Although the tumor-repressive mechanisms of mCMV and hCMV may differ and depend on the type of tumor, experimental approaches in the murine model might give valuable hints for concepts to follow in clinical research. We have previously shown for the liver-adapted A20-derived B cell lymphoma E12E that mCMV does not infect the lymphoma cells for causing cell death by viral cytopathogenicity but triggers tumor-selective apoptosis at a tissue site of tumor metastasis distant from a local site of infection. This finding suggested involvement of a cytokine that triggers apoptosis, directly or indirectly. Here we used a series of differential high-density microarray analyses to identify cellular genes whose expression is specifically upregulated at the site of virus entry only by viruses capable of triggering lymphoma cell apoptosis. This strategy identified interleukin-15 (IL-15) as most promising candidate, eventually confirmed by lymphoma repression with recombinant IL-15.

Keywords Affymetrix probe array · Hematopoietic cell transplantation (HCT) · Infection-versus-leukemia/lymphoma effect (IvL) · Interleukin-15 (IL-15) · Lymphoma · Tumor remission

Introduction

Relapse of minimal residual leukemia (MRL), graft-versus-host disease, and infections in general, in particular reactivation of latent human cytomegalovirus (hCMV) in either the transplant or the recipient after myeloablative treatment, are major complications in the hematopoietic cell transplantation (HCT) therapy of hematopoietic malignancies (reviewed in [1–4]). Though all three complication entities are prone to potentiate post-transplant morbidity and mortality, a graft-versus-leukemia (GvL) reaction can counteract relapse of MRL [5] just as a graft-versus-infection (GvI) reaction can counteract post-transplant viral disease [6]. An additional donor lymphocyte infusion is a strategy for strengthening the beneficial effects of GvL [7, 8] and GvI reactions [9], including immunotherapy against hCMV [10–14]. Beneficial infection-versus-leukemia (IvL) effects that limit relapse of MRL after HCT are less frequently considered. Interestingly, however, early reports by Fujiwara and colleagues [15, 16] noted that reactivated

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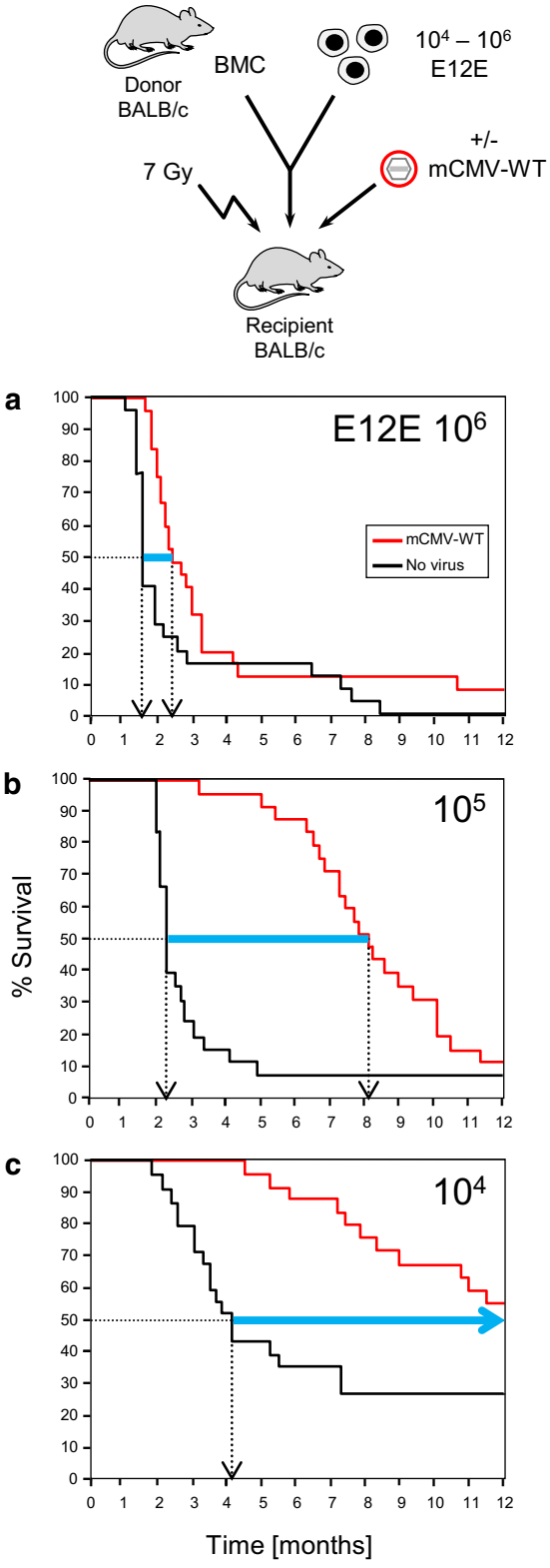


Fig. 1 Tumor dose-dependent survival benefit by mCMV infection after experimental HCT in the mouse model. (*Top*) Sketch of the experimental regimen. Step 1: Hematoablative conditioning of BALB/c HCT recipients by total-body γ-irradiation with a single dose of 7 Gy performed ~24 h prior to syngeneic HCT. Step 2: Intravenous infusion simultaneously of 10^7 femoral and tibial bone marrow cells from BALB/c donors for HCT and graded numbers of E12E lymphoma cells. Step 3: Intraplantar (*left hind footpad*) infection and mock infection of HCT recipients with 10^5 plaque-forming units (PFU) of mCMV-WT (strain Smith, ATCC VR1399) and solvent control (PBS), respectively. Kaplan–Meier plots show survival rates observed over a period of 12 months with 25 recipients per group dependent on a transferred initial lymphoma cell load of 10^6 (**a**), 10^5 (**b**), and 10^4 (**c**) E12E cells. Lethality of the lymphoma over time is shown with *black line*, reduced lethality in the presence of infection is shown with *gray* (in the online version: *red*) line. Survival benefit expressed as time of survival at survival rates of 50 % (*dotted lines* with *arrows* to the time axis) is highlighted by a *solid distance bar* (in the online version: *blue*). Note the general delay of mortality at all lymphoma cell doses and the curative effect of infection in ~60 % of HCT recipients receiving the moderate dose of 10^4 lymphoma cells, which is closer to the clinical correlate of minimal residual disease. Data are reproduced, with modification, from Ref. [18] with permission by the Journal of Virology, American Society for Microbiology (color figure online)

hCMV infection is not necessarily a poor predictor for the clinical course of adult T cell leukemia/lymphoma.

In support of this, and shortly after the observational reports from the Japanese group, we have established a murine model of experimental HCT and liver metastasis of transplanted B or T cell lymphomas [17–19]. In the specific example of the experimental model of the liver-adapted A20 B cell lymphoma-derived clonal variant E12E [17, 19], we previously documented a significant tumor remission and survival benefit associated with an acute murine CMV (mCMV) infection even at exceedingly high tumor cell doses, with ultimate survivors (>1 year) at lower initial tumor cell burden (Fig. 1; reproduced, with modification, from [18]). In immunocompetent mice, tumor lethality was completely prevented by the infection [17]. Notably, although CMVs are cytopathogenic upon infection of the specific tissue cell types in which CMV can replicate, for instance, in hepatocytes of the liver parenchyma [20–22], the lymphomas under study were not 'permissive' to infection, and hence, the mechanism of the IvL effect was not a trivial viral cytolysis, so-called oncolysis [23], of the lymphoma cells. The mechanism of tumor repression proved to be a tumor-selective caspase-dependent cell death/apoptosis triggered by mCMV infection even over a distance between a local tissue site of viral replication and the tissue site infiltrated by the tumor cells, the liver in the specific experimental models [18]. While virus replication was essential, a virus mutant unable to spread to the liver due to deletion of the anti-apoptotic viral gene M36 [24] induced the IvL effect on the E12E lymphoma in the liver in absence of liver infection [18], which calls for a viral initiation of long-distance, systemic signaling events, putatively involving cytokine(s).

More recently, encouraged by the murine model or not, the topic of a possible IvL effect of reactivated

hCMV in HCT, specifically HLA-matched minor histocompatibility-disparate allogeneic HCT, was taken up again in a number of observational clinical studies. As a bottom-line message, these studies confirmed a favorable effect of early CMV reactivation on the incidence of relapse in acute myeloid leukemia [25–27] and possibly chronic myeloid leukemia [28], in particular after myeloablative conditioning (MA) rather than after reduced-intensity conditioning (RIC) [27], which may relate to a higher load of reactivated virus under the stronger immunosuppression by MA compared to RIC. Mechanisms of the tumor repression were not addressed but were only speculated. Sadly, however, all these observational studies, and commentaries to them [29–31], ignored the findings from the experimental model, in which some of the speculated mechanisms had already been addressed experimentally.

For further exploring the mechanism, we expand here on the experimental model of a CMV IvL effect by showing that repression of a B cell lymphoma by mCMV infection of the immunocompromised host—even when no HCT is performed for reconstitution [18]—involves virally induced cellular interleukin (IL)-15. Local infection is shown here to jointly activate the transcription of cellular genes encoding IL-15 as well as the IL-15 receptor α-chain (IL-15Rα), which, as a complex, are capable of trans-signaling also to cells not expressing the complete IL-15R but expressing the signal-transducing IL-2Rβ chain, which is shared between IL-2R and IL-15R, and the IL-2R common γ chain (CD132), which both share with receptors for a panel of other interleukins, including IL-4, IL-7, IL-9, and IL-21 [32–34]. Complex formation with IL-15Rα is known to increase the affinity of IL-15 for the IL-2R β chain, and this allostery is considered to be required for IL-15 trans-signaling [34].

Notably, in the absence of infection, lymphoma remission is induced by recombinant IL-15 as efficient as with recombinant IL-15/IL-15Rα, indicating that direct signaling by IL-15 rather than trans-signaling is involved in tumor remission, either directly or indirectly. An indirect effect of IL-15, not targeting the tumor cells directly but involving an immune cell type as a potential anti-tumoral effector cell, is suggested by our finding that recombinant IL-15 fails to control lymphoma growth in the H-2d;BALB-RAG2γc ($RAG2^{-/-}/\gamma c^{-/-}$) mouse strain ([35, 36], backcrossed to BALB/c) that is combined deficient in recombinase activating gene 2 (RAG2) and the IL-2R common γ-chain (IL-2Rγ). As a consequence, these mice lack mature lymphocytes, including T and B lymphocytes, as well as invariant natural killer T cells due to an inability to initiate VDJ rearrangement [35], and they also lack NK cells due to the panel of deficient IL responses, including deficient IL-15 signaling.

Though CMV or spread-deficient CMV vectors are not to be seriously considered as therapeutics in HCT, learning concepts from CMV may help reduce relapse of MRL.

Candidate mining by a differential high-density microarray strategy

As CMV infections have a profound impact on the host cell transcriptome, up- and downregulating the expression of numerous genes (for hCMV, see the review [37]; for mCMV, see [38, 39]), a sophisticated screening strategy employing the mathematical 'set theory' was needed to have a chance for defining candidates in vivo that could mediate tumor remission. First, as we knew from our previous work that—also in absence of hematopoietic reconstitution by HCT—intraplantar infection of immunocompromised mice with wild-type mCMV, strain Smith (mCMV-WT), that is, infection of footpad tissue on the day after hematoablative treatment with 7 Gy of γ-irradiation, must induce a cytokine-'X' that operates over a distance [18], we focused on cellular genes that are specifically upregulated in vivo by the infection in footpad tissue. Though induction of apoptosis by downregulation of an inhibitor of apoptosis is a theoretical possibility, this idea was—in our view—too speculative for pursuing it with preference. Detailed previous time course analyses have shown that lymphoma cells infused intravenously after the γ-irradiation but shortly (~1 h) before intraplantar infection still successfully metastasize to liver parenchyma, where they grow out for 2 days with a doubling time of ~26 h to form small colonies of ~4 cells until they are driven into apoptosis, with total tumor cell numbers declining between day 2 and day 5 [18]. We therefore chose day 3 for the analysis of gene expression in the infected footpad. For this, total RNA was isolated from the hind limbs of mCMV-infected and control mice by using Lipid Tissue Mini Kit (Qiagen), quality-controlled rigorously, and subjected to a differential high-density microarray analysis with one-cycle target labeling based on Affymetrix Probe Array Type Mouse430A_2 with a total probe set number of 22,693 corresponding to ~14,000 genes.

In a triplicate array, referred to as Array 1a,b,c-WT (mCMV-WT vs. PBS), three independent microarray analyses were performed, each with RNA isolated from one mCMV-WT-infected hind limb compared to one mock-infected (phosphate-buffered saline, PBS) hind limb. It is important to note that mock infection covers all gene expression upregulated by other parameters of the protocol, including genes associated with DNA repair and with cell death in consequence of γ-irradiation. Based on mean values of induction, using Benjamini–Hochberg statistics, a total of 623 transcripts were accepted to be upregulated, which included genes not necessarily upregulated

in all three infected limbs. To test reproducibility of this result, a second infection experiment was performed under *bona fide* identical conditions, except that Array 2-WT (mCMV-WT vs. PBS) was performed with RNA pooled from three hind limbs. This resulted in only 446 upregulated transcripts. Of a union of 704 transcripts upregulated in either array (Array 1a,b,c-WT ∪ Array 2-WT), only 365 were shared between the two arrays (Fig. 2a, Venn diagram; intersection of sets Array 1a,b,c-WT ∩ Array 2-WT = 365). The imperfect match may indicate a high inter-experimental variance but may also be influenced by the lower stringency of defining upregulation based on Benjamini–Hochberg statistics in Array 1a,b,c-WT, as it is suggested by the high number of upregulated transcripts that were not confirmed by Array 2-WT, whereas most transcripts upregulated in Array 2-WT were included in Array 1a,b,c-WT. Notably, however, within the area of intersection, there was a good correlation in expression strengths between the two (actually the four) arrays (Fig. 3). As in our extensive experience from the previous publications, representing dozens of experiments [17–19], the tumor-repressive effect of mCMV absolutely never failed, we focused our attention on the consensus group of 365 upregulated transcripts, aware of a risk to miss a candidate. Still, however, this number was far too high to define candidate genes. So, in the next step, we performed Array 3-ΔIE1Ex4 with virus mCMV-ΔIE1Ex4 [40] not expressing the immediate early (IE) protein IE1, a known transactivator of cellular genes [41], including genes involved in nucleotide metabolism [42–44]. Importantly, this virus, though growth-attenuated [40, 41, 44], still induced tumor repression. In accordance with the idea, the number of upregulated transcripts was reduced to 324, yet with an overlap of 307 shared with the intersection of the preceding arrays (Fig. 2b, Venn diagram; intersection of Array 1a,b,c-WT ∩ Array 2-WT ∩ Array 3-ΔIE1Ex4 = 307), still too many.

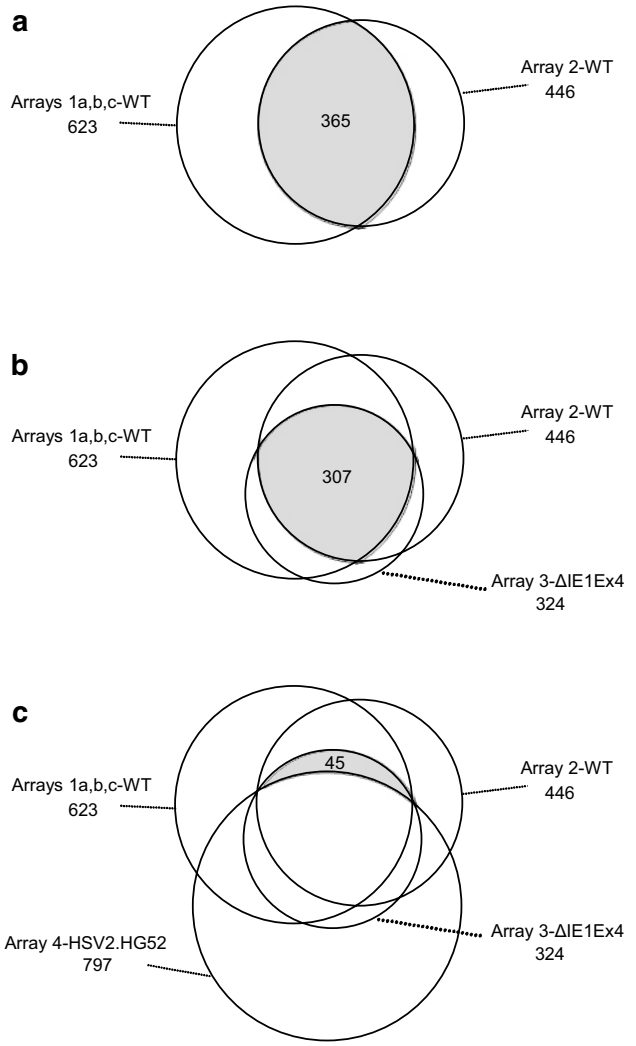


Fig. 2 Results of differential high-density microarray analyses identifying cellular transcripts upregulated reproducibly and selectively after infection with tumor-repressive viruses. Shown are Venn diagrams, based on the 'set theory': **a** intersection of Array 1a,b,c-WT ∩ Array 2-WT, identifying 365 transcripts reproducibly upregulated in footpad tissue by infection with mCMV-WT; **b** intersection of Array 1a,b,c-WT ∩ Array 2-WT ∩ Array 3-ΔIE1Ex4, identifying 307 transcripts upregulated in footpad tissue by infection with mCMV-WT as well as after infection with a mutant virus that lacks expression of the transactivator protein IE1; **c** set difference of Array 1a,b,c-WT ∩ Array 2-WT ∩ Array 3-ΔIE1Ex4∖Array 4-HSV2.HG52, identifying 45 transcripts upregulated in footpad tissue by infection with mCMV-WT and mutant virus mCMV-ΔIE1Ex4 but not with HSV2 strain HG52, a virus that fails to induce tumor repression [18]. Relevant set intersection and set difference areas are highlighted by shading. See the main body of the text for more detailed explanation

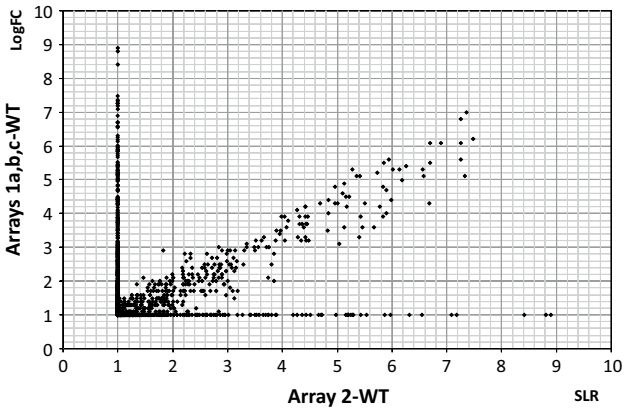


Fig. 3 Correlation of expression induction for candidate genes. The plot quantifies the induction of the 365 cellular transcripts from the intersection of Array 1a,b,c-WT ∩ Array 2-WT (recall Fig. 2a), that is, the transcripts whose expression was found to be reproducibly upregulated in footpad tissue infected with mCMV-WT. SLR: signal $\log_2$ ratio. LogFC: log fold change. An SLR of 1 is equivalent to a fold change of 2, an SLR of >1 indicates a significant differential expression

The breakthrough came with the idea of discarding transcripts that are upregulated also by infection with a distantly related α-herpesvirus HSV2, strain HG52, known not to induce E12E lymphoma repression [18]. This strategy should exclude genes commonly upregulated by the innate antiviral defense or inflammatory responses. Array 4-HSV2.HG52 detected 797 upregulated transcripts, that is, even more than in any of the mCMV arrays tested, and most transcripts upregulated during mCMV infection were included. This left us with a manageable number of just 45 transcripts upregulated reproducibly and selectively by tumor-repressing strains of mCMV (Fig. 2c, Venn diagram; set difference Array 1a,b,c-WT ∩ Array 2-WT ∩ Array 3-ΔIE1Ex4∖Array 4-HSV2.HG52 = 45). These 45 transcripts actually represent only 33 annotated genes (Table 1), of which the genes coding for IL-15 and the IL15R α chain, capable of direct signaling and trans-signaling, shine out as most promising candidates for signaling over a distance. It is important to consider that these 33 genes are not necessarily all upregulated in *cis*, that is, in infected footpad tissue cells, but could also be upregulated in *trans*, that is, in uninfected cells stimulated by recognition of signatures on infected cells, by 'factors' (cytokines, chemokines) secreted by infected cells, or by components released as a result of cell death.

With this focused set of candidate genes, we went back to the first group of arrays, Array 1a-WT, 1b-WT, and 1c-WT, to look at the inter-individual variance in the induction by mCMV-WT infection, and found a remarkable reproducibility for all candidate genes including those coding for IL-15 and IL-15Rα (Fig. 4). In accordance with the published observation of missing tumor repression by UV-inactivated virus mCMV-WT^{UV} [17, 18], an array designated Array 5-WT^{UV} did not detect an upregulated expression of any of the 33 candidate genes, including the genes coding for IL-15 and IL-15Rα (negative data not shown). As the viral genome does not contain homologies to the sequence of the cellular IL-15 gene, the infection apparently upregulated the expression of cellular IL-15.

Table 1 List of cellular genes whose expression is upregulated reproducibly and selectively by mCMV in footpad tissue

No.	Probe set ID	Gene	Protein
1	1452013_at	Atp10a	ATPase, class V, type 10A
2	1449473_s_at	Cd40	CD40 antigen
3	1423393_at	Clic4	Chloride intracellular channel 4 (mitochondrial)
4	1419026_at	Daxx	Fas death domain-associated protein
5	1424375_s_at	Gimap4	GTPase, IMAP family member 4
6	1418219_at	Il15	Interleukin-15
7	1422397_a_at	Il15ra	Interleukin-15 receptor, alpha chain
8	1448436_a_at	Irf1	Interferon regulatory factor 1
9	1416714_at	Irf8	Interferon regulatory factor 8
10	1431591_s_at	Isg15	ISG15 ubiquitin-like modifier
11	1437226_x_at	Marcksl1	MARCKS-like 1
12	1419254_at	Mthfd2	Methylenetetrahydrofolate dehydrogenase (NAD + dependent), methenyltetrahydrofolate cyclohydrolase
13	1448618_at	Mvp	Major vault protein
14	1422512_a_at	Ogfr	Opioid growth factor receptor
15	1426519_at	P4ha1	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide
16	1452178_at	Plec1	Plectin 1/Plec1 protein
17	1417056_at	Psme1	Proteasome (prosome, macropain) 28 subunit, alpha
18	1417189_at	Psme2	Proteasome (prosome, macropain) 28 subunit, beta
19	1451321_a_at	Rbm43	RNA-binding motif protein 43
20	1452289_a_at	Rnf135	Ring finger protein 135
21	1423986_a_at	Scotin	Scotin gene
22	1450027_at	Sdc3	Syndecan 3
23	1418206_at	Sdf2l1	Stromal cell-derived factor 2-like 1
24	1431055_a_at	Snx10	Sorting nexin 10
25	1450378_at	Tapbp	TAP binding protein
26	1451544_at	Tapbpl	TAP binding protein-like
27	1420635_a_at	Tcirg1	T cell, immune regulator 1, ATPase, H + transporting, lysosomal V0 protein A3
28	1455900_x_at	Tgm2	Transglutaminase 2, C polypeptide
29	1428346_at	Trafd1	TRAF-type zinc finger domain containing 1
30	1418077_at	Trim21	Tripartite motif protein 21
31	1425974_a_at	Trim25	Tripartite motif protein 25
32	1434813_x_at	Wars	Tryptophanyl-tRNA synthetase
33	1427874_at	Zfp313	Zinc finger protein 313

Recombinant IL-15 mediates lymphoma repression

It is particularly intriguing that mCMV infection jointly upregulates transcripts for IL-15 and its receptor, a finding that strengthened the hypothesis that the tumor-repressive effect of mCMV may involve IL-15 signaling. As it is appreciated that IL-15 is induced by mCMV infection of conventional dendritic cells (cDC) in the spleen, as evidenced from neutralization of its function in NK cell proliferation [45], but that it is difficult to detect in vivo in host tissues or serum (reviewed in [46]), we followed the straightforward approach to test directly whether recombinant IL-15 alone or in complex with IL-15Rα can mediate lymphoma repression in the absence of infection (Fig. 5). We first reproduced

the original observation of tumor repression by mCMV infection and found almost complete prevention of liver metastases when infection preceded the infusion of the lymphoma cells by 1 day (Fig. 5a, panel A vs. B). Note that previous work has shown that infection between day −6 and day 0 relative to lymphoma is comparably efficient in repressing lymphoma growth [18]. Importantly, in the absence of infection, intravenous infusion of recombinant murine IL-15 (rmIL-15) also significantly reduced tumor growth (Fig. 5a; panel A vs. C, $P = 0.014$). The immunohistological (IHC) detection of tumor noduli in liver tissue sections gives a visual impression of the reduction in tumor burden by rmIL-15 (Fig. 5b). Some residual tumor, reduced in absolute tumor cell numbers and tumor colony size, should not be overinterpreted, because systemic single or even repeated administration of an arbitrary dose of cytokine may not fully reflect the effects of a more continuous supply or of cell-to-cell supply of the cytokine in vivo. In a qualitative interpretation, however, we can conclude that soluble IL-15 indeed mediates lymphoma repression. Combining rmIL-15 with IL-15Rα also revealed a significant tumor-repressive effect (Fig. 5a; panel A vs. D, $P = 0.009$), but no improvement compared to rmIL-15 alone (Fig. 5a; panel C vs. D, $P = 0.836$). Taken together, tumor growth retardation in the two cytokine therapy groups combined was highly significant (Fig. 5a; panel A vs. panels C plus D, $P = 0.006$).

In conclusion, IL-15 mediates tumor repression by a mechanism that does not appear to depend on trans-signaling to IL15Rα-negative cells based on complex formation with IL-15Rα.

Fig. 5 Repression of E12E lymphoma growth in the liver by IL-15.▶ In slight modification of the experimental protocol outlined in Fig. 1, BALB/c mice were 7-Gy γ-irradiated followed by intraplantar mock infection (group A, PBS) or infection with 10^5 PFU of mCMV-WT (group B) one day before intravenous application of 2×10^6 E12E lymphoma cells (day 0), with no HCT being performed. In groups C and D, the mice were left uninfected but, instead, received three doses of intravenous recombinant murine IL-15 (3 × 2 μg rmIL-15, specific activity of 10^4 units/mg; cat. no. 34-8151, eBioscience) or three doses of a mixture of rmIL-15 and recombinant murine IL-15R α chain (3 × 2 μg rmIL-15 plus 13 μg rmIL-15Rα Fc chimera; acc. no. Q60819, RDSYSTEMS) according to the protocol in [47]), respectively, on consecutive days 1, 2, and 3. Quantitation of tumor growth in the liver was performed for day 8, based on IHC staining of lymphoma cells in 2-μm liver tissue sections as described in greater detail previously [17, 18]. **a** Tumor-size diagrams for individual mice of groups A–D, representing the numbers of tumor noduli of defined size intervals present in representative 100 mm^2 of liver tissue section area. Size classes *1–6* are defined as <0.05 (*1*), 0.05–0.1 (*2*), 0.1–0.2 (*3*), 0.2–0.4 (*4*), 0.4–0.8 (*5*), and 0.8–1.6 (*6*) mm^2 (see also [18]). Values indicate the percentages of liver tissue section area covered by tumor. Asterisks indicate individuals for which representative images are shown in, **b** comparing tumor burden in group A and reduction of tumor burden by rmIL-15 in group C. *Black* IHC staining of CD45R (B220) antigen visualizes E12E lymphoma noduli. The *bar marker* represents 100 μm. For comparisons of interest, *P* values provided in the main body of the text were calculated based on the % tumor-covered areas by using Student's *t* test (unpaired, two sided) with Welch's correction to account for a potentially unequal variance

Recombinant IL-15 operates indirectly for mediating lymphoma repression

It was an obvious question if IL-15 acts as an effector molecule that targets the lymphoma cells directly or if it is part of a signal chain involving other cytokines and/or the activation of innate immune cells present in the host even after the

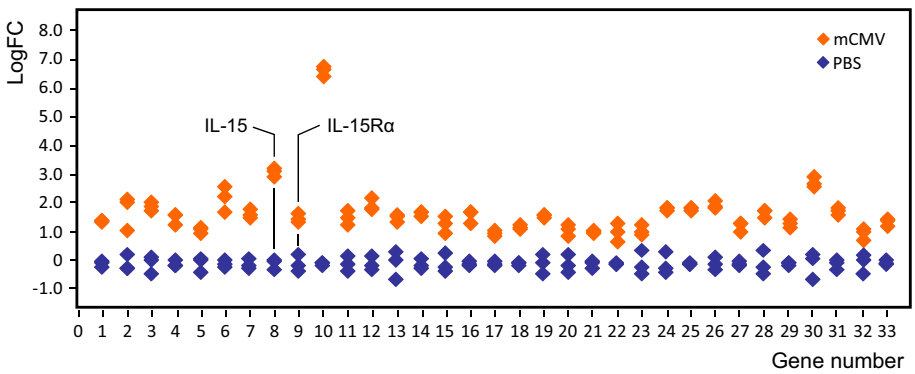
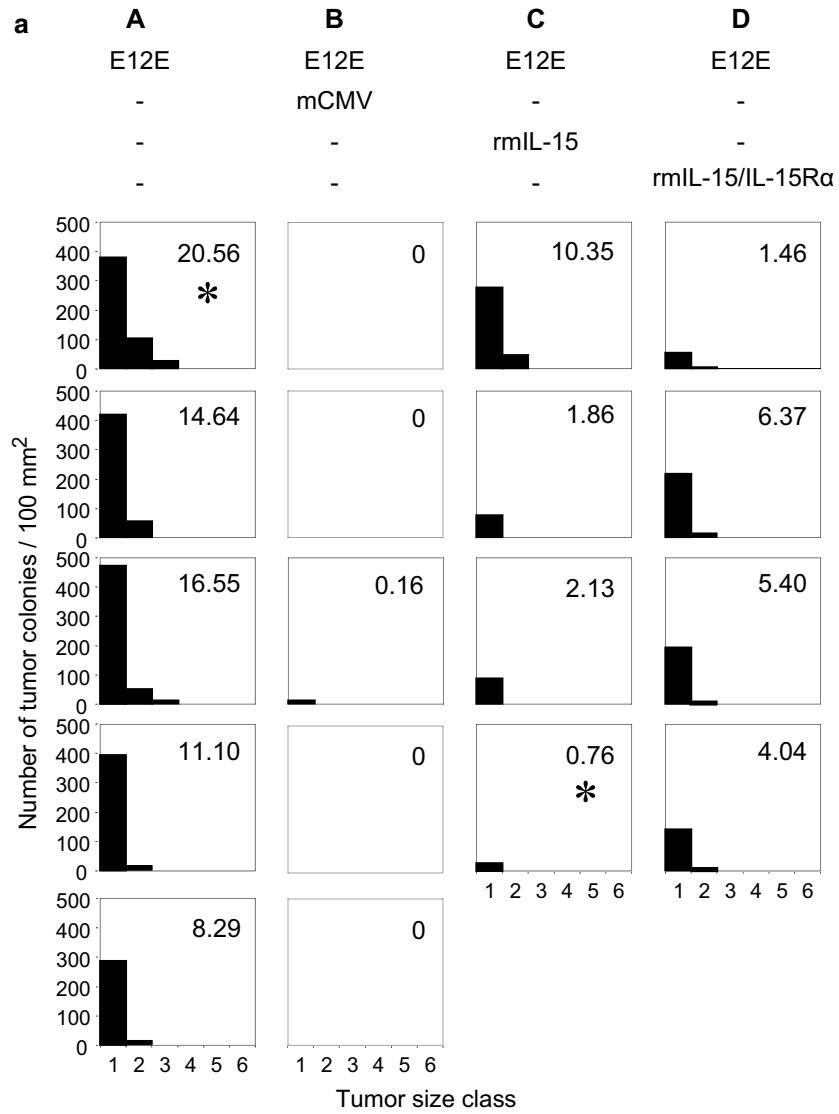


Fig. 4 Low interindividual variance in the expression of candidate genes. For the 33 genes whose expression was found to be upregulated reproducibly and selectively by infection with tumor repression-inducing viruses (listed in Table 1, derived from the set difference of 45 upregulated transcripts identified by Array 1a,b,c-WT ∩ Array 2-WT ∩ Array 3-ΔIE1Ex4∖Array 4-HSV2.HG52), the intensity of upregulation expressed as LogFC is depicted for the triplicate arrays Array 1a-WT, Array 1b-WT, and Array 1c-WT. *Black diamond symbols* (*blue* in the online version) and *gray diamond symbols* (*orange red* in the online version) represent the triplicate data for uninfected (PBS control) and mCMV-WT-infected footpad tissue, respectively. Data for the genes encoding IL-15 and IL-15 receptor alpha chain (IL-15Rα) are marked (color figure online)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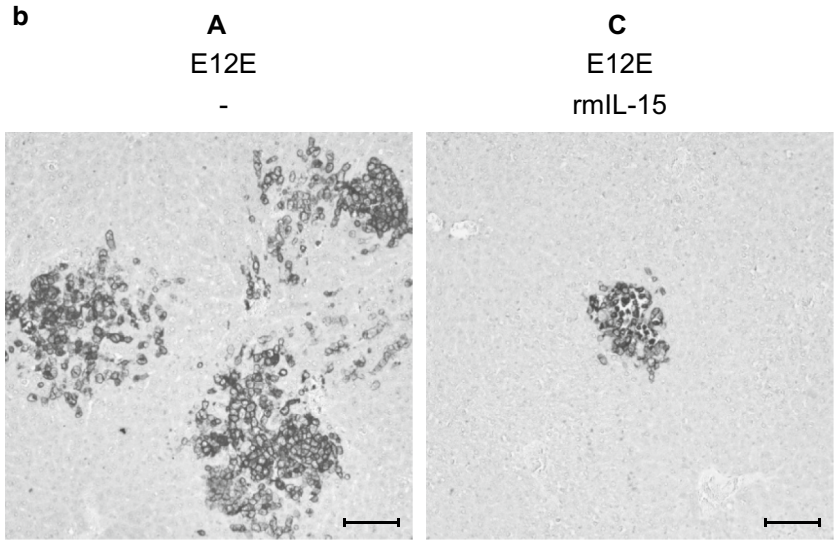
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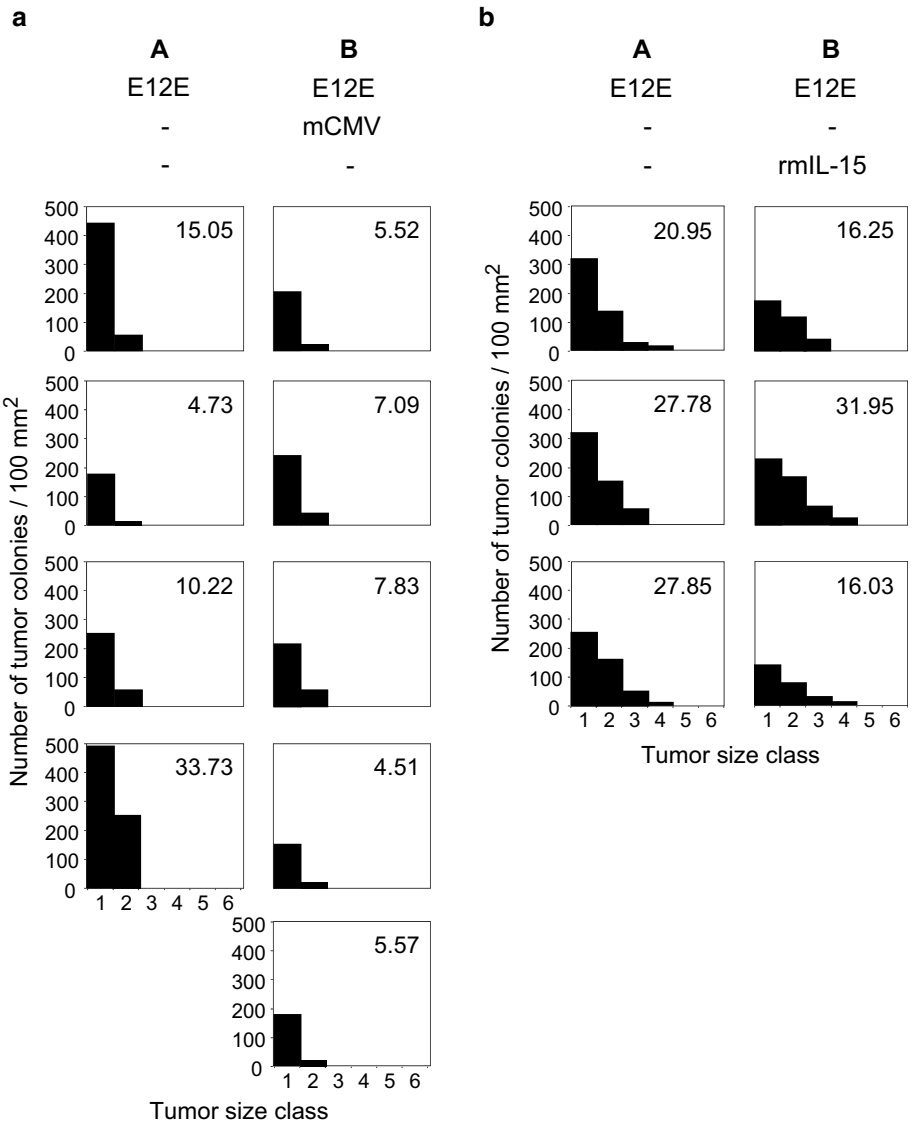

Fig. 6 Lymphoma repression requires a component that is absent in H-2d;BALB-RAG2γc mice. **a** Absence of E12E lymphoma repression by mCMV infection of H-2d;BALB-RAG2γc mice. The experimental regimen was as described for Fig. 5 groups A and B, including preconditioning by 7 Gy of total-body γ-irradiation on day −1, to allow direct comparison with the situation in WT BALB/c mice. **b** Absence of E12E lymphoma repression by IL-15 in uninfected H-2d;BALB-RAG2γc mice. The experimental regimen was essentially as described for Fig. 5 groups A and C, except that the dose of rmIL-15 was raised to 3 × 3 μg and that tumor burden was quantitated on day 12. For the explanation of the diagrams and for statistical evaluation, see the legend of Fig. 5

myeloablative treatment, a 7-Gy total-body γ-irradiation in the specific case, which definitively wiped out adaptive immunity, at least for the timescale of the tumor repression assay. First evidence against direct pro-apoptotic signaling of IL-15 to the E12E lymphoma cells was provided by an unaltered growth in cell culture in the presence of graded doses of rmIL-15 (negative data not shown), like it was shown previously that mCMV virions do not inhibit growth of the E12E lymphoma in cell culture [17, 18]. Notably, our previous cell culture studies have also excluded tumor necrosis factor (TNF)-α as a pro-apoptotic ligand targeting E12E lymphoma cells [17].

With all this in mind, we tested whether the repressive effect on the E12E lymphoma in vivo is operative in H-2d;BALB-RAG2γc mice that show a combined immunodeficiency by being devoid of all those IL-signaling pathways that use the IL-2R common γ-chain CD132 (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21 signaling) and lack essentially all lymphocyte subpopulations. As shown in Fig. 6 in two independent experiments, neither mCMV infection (Fig. 6a; panel A vs. B, $P = 0.218$) nor even increased doses of rmIL-15 (Fig. 6b; panel A vs. B, $P = 0.548$) directly effected or induced tumor repression in these mice. This finding confirmed that IL-15 does not target E12E lymphoma cells directly for a pro-apoptotic signaling and indicated that an IL-signaling pathway and/or a lymphocyte subpopulation missing in H-2d;BALB-RAG2γc mice must be involved in the tumor-repressive mechanism of mCMV to which IL-15 contributes.

Lymphoma repression by mCMV infection does not involve residual CD8 T cells

Notably, previous work by Epardaud et al. [47] has shown in two murine tumor models, namely transplantable B16

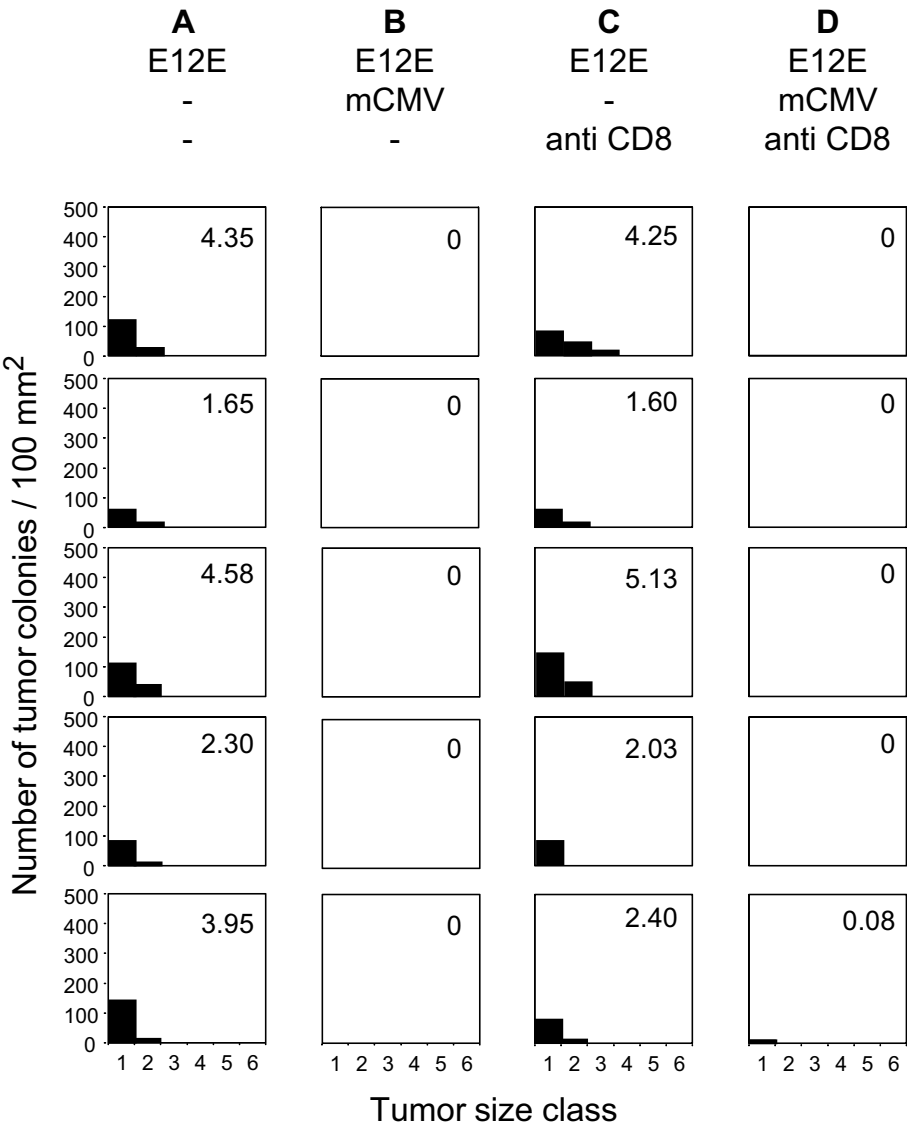

Fig. 7 Lymphoma repression does not depend on the presence of CD8 T cells. Experiments were performed with BALB/c mice according to the protocol described for Fig. 5 group A and group B, except that tumor burden was quantitated on day 7. In groups C and D, CD8 T cells in the recipients were depleted by intravenous administration of 100 µl (1.19 mg) of anti-CD8 monoclonal antibody YTS 169.4.2 ([48], purified and kindly provided by S. Jonjic, University of Rijeka, Croatia) on day −2, that is, on the day before preconditioning by 7 Gy of total-body γ-irradiation followed by infection (both on day −1) and transfer of E12E lymphoma cells (day 0). The antibody was given prior to the hematoablative treatment to make sure that antibody-dependent cellular cytotoxicity can still operate to deplete the CD8 T cells according to established protocols. For the explanation of the diagrams and for statistical evaluation, see the legend of Fig. 5

melanoma and spontaneous pancreatic tumor, that systemically administered IL-15/IL-15Rα complexes can mediate regression even of established solid tumors by releasing tumor-resident CD8 T cells from functional suppression by the tumor microenvironment. We therefore tested whether the IvL effect induced by mCMV, supposedly through the concerted induction of IL-15 and its receptor IL-15Rα, involves tumor-reactive, though unprimed, CD8 T cells that might have resisted the immunoablative 7-Gy γ-irradiation (Fig. 7). An involvement of CD8 T cells also would have been compatible with the absence of lymphoma repression in H-2d;BALB-RAG2γc mice (see above), in which CD8 T cells and IL-2/T cell growth factor signaling are missing. In this experiment, again, mCMV infection strongly repressed E12E lymphoma growth in the liver, resulting in complete remission in five out of five mice tested individually (Fig. 7; panel A vs. B). While a depleting anti-CD8 antibody [48] had, by itself, no effect on the lymphoma (Fig. 7; panel A vs. C, $P = 0.762$), depletion of CD8 T cells did not abrogate the lymphoma-repressive IvL effect of mCMV infection (Fig. 7, panel B vs. D). This finding excludes a critical contribution of CD8 T cells to lymphoma repression in the experimental setting under study.

Conclusion and outlook

The here presented data have identified virally induced cellular IL-15 as one link in a still largely unknown chain of events that eventually lead to lymphoma repression by CMV in the murine model. De-repression of tumor-reactive CD8 T cells by IL-15/IL-15Rα, as proposed for the solid tumor models discussed above [47], is unlikely to apply to our lymphoma metastasis models, as the tumor

Table 2 Current state of knowledge from the murine model of the IvL effect of CMV

Characteristics of mechanism	Evidence	References
Susceptibility of T and B cell lymphomas	A20-derived B cell lymphomas, including E12E	[17]
	T cell lymphomas Esb-MP and ESb-L-CI	[18]
Transient susceptibility	IvL only until a 32-cell stage of tumor noduli	[18]
Virus specificity	No IvL with HSV-1 strains Len, JES, KOS, and 1716 or HSV-2 strains ER-, HG52, and Bry	[18]
Requirement for virus replication in the host	No IvL with mCMV-WT^{UV}	[17, 18]
	mCMV-ΔMIEenh, and hCMV Towne	[18]
No cytopathogenic lymphoma cell infection	Absence of IE1 protein in lymphoma cells	[17]
No requirement for infection of tumor stroma	IvL operative also in uninfected tissue	[18]
	No IvL in infected tissue beyond a 32-cell stage of tumor noduli	[17, 18]
No requirement for virus spread to the tumor site	IvL with spread-deficient mCMV-ΔM36	[18]
Lymphoma cell death by apoptosis	Active caspase 3 in dying lymphoma cells in vivo	[18]
Apoptosis is tumor-selective	Only lymphoma cells stain for active caspase 3	[18]
Death receptor Fas (CD95) is not involved	No apoptosis of Fas$^+$ E12E cells with anti-Fas Ab	[18, 19]
TNFα[a] is not involved	No effect of rmTNFα; IvL despite anti-TNFα	[17]
No requirement for radiosensitive immune cells	IvL also after 7-Gy γ-irradiation with no HCT	[18]
No involvement of natural killer (NK) cells	No IvL by NK cell transfer	[18]
	IvL despite NK cell depletion	[19]
No involvement of residual CD8 T cells	IvL despite CD8 T cell depletion	[this report]
IL-15 is involved but is not the effector cytokine	Effect of rmIL-15, but not in RAG2γc mice	[this report]
Requirement for a RAG2 and/or common IL-2R γ chain signaling-dependent component	No IvL in infected RAG2γc mice	[this report]

[a] Tumor necrosis factor alpha

repression here operates after an immunodepleting 7-Gy γ-irradiation, as shown previously [18], but also after an additional depletion of CD8 T cells in the recipients (recall Fig. 7). Table 2 summarizes all the information that we currently have about lymphoma repression by CMV in the murine model.

If all predictions from the model hold true for the reported clinical cases of a lower incidence of leukemia relapse in HCT recipients who experienced an early reactivation of hCMV (see the "Introduction") is an open question, but the murine model sets the scene for a better mechanistic understanding of the clinical observations in that it offers a testable prediction. As we have described and discussed previously, however, the timing of host infection is critical [18, 19]; specifically, apoptosis of tumor cells that had just recently metastasized to the liver was inducible only until young tumor colonies/noduli had reached a ~32-cell stage. After that time, the colonies became resistant. This resistance was not genetically or epigenetically fixed, as tumor cells recovered from advanced tumor noduli that had escaped apoptosis in an infected first recipient proved to be susceptible after serial transfer [18]. In HCT patients latently infected with hCMV, the time of virus reactivation relative to MRL relapse cannot be predicted or controlled, so that it is a matter of chance if the viral signal meets the tumor cells in a susceptible stage. This may be one explanation for the rather modest reduction of relapse risk associated with hCMV reactivation in clinical studies [26] performed with cohorts of patients under conditions that are unavoidably not standardized in terms of preceding leukemia therapy, myeloablative conditioning, HCT donor and recipient genetic differences in unmatched loci, virus strains carried by the recipients, time of MRL relapse, time of virus reactivation, viral load after reactivation, and antiviral therapy—to name just some more obvious variables.

Clearly, this is not the end of the road, as our data have revealed IL-15 is not the terminal effector molecule that directly triggers the selective tumor cell apoptosis. Based on the missing lymphoma-repressive effect of mCMV in the H-2d;BALB-RAG2γc mouse model, current and future work aims at identifying the final effector mechanism in the chain of events that are initiated by mCMV infection.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard All procedures performed in studies involving animals were approved by the ethics committee of the Landesuntersuchungsamt Rheinland-Pfalz, permission no. 1.5 177-07-04/051-56 in accordance with German Federal Law §8 Abs. 1 TierSchG (animal protection law). BALB/c mice and H-2d;BALB-RAG2γc mice were bred and housed under specified pathogen-free conditions at the Central Laboratory Animal Facility (CLAF) at the University Medical Center of the Johannes Gutenberg-University, Mainz, Germany.

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