ARTICLE

Intratumoral INF-γ triggers an antiviral state in GL261 tumor cells: a major hurdle to overcome for oncolytic vaccinia virus therapy of cancer

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Oncolytic vaccinia virus (VACV) therapy is an alternative treatment option for glioblastoma multiforme. Here, we used a comparison of different tumor locations and different immunologic and genetic backgrounds to determine the replication efficacy and oncolytic potential of the VACV LIVP 1.1.1, an attenuated wild-type isolate of the Lister strain, in murine GL261 glioma models. With this approach, we expected to identify microenvironmental factors, which may be decisive for failure or success of oncolytic VACV therapy. We found that GL261 glioma cells implanted subcutaneously or orthotopically into Balb/c athymic, C57BL/6 athymic, or C57BL/6 wild-type mice formed individual tumors that respond to oncolytic VACV therapy with different outcomes. Surprisingly, only Balb/c athymic mice with subcutaneous tumors supported viral replication. We identified intratumoral IFN-γ expression levels that upregulate MHCII expression on GL261 cells in C57BL/6 wild-type mice associated with a non-permissive status of the tumor cells. Moreover, this IFN-γ-induced tumor cell phenotype was reversible.

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INTRODUCTION

Glioblastoma multiforme (GBM) is one of the most malignant forms of brain cancer (WHO grade IV) and also the most frequent type of glioma in adults.1,2 The standard of care for GBM is surgical resection, followed by radiation and temozolomide chemotherapy.2 In spite of extensive research effort, the disease is still incurable and the prognosis is very poor with a median survival of less than 15 months.2 Difficulties associated with treatment of GBM are the highly aggressive and infiltrative nature of the tumor into the brain parenchyma. In addition, the histological heterogeneity of the tumor mass, the location of the neoplasm within the brain, the infiltration of the tumor with microglia/macrophages, and the function and morphology of the blood-brain barrier aggravate the therapy.2–4

There is a broad range of alternative treatment options presently studied in preclinical and also clinical trials for GBM.3,4 One of those is oncolytic virotherapy, defined as the use of replication-competent viruses that selectively infect, replicate in and destroy cancer cells while leaving healthy, nontransformed cells and tissues unharmed.5

Vaccinia virus (VACV) is a favorable candidate for oncolytic virotherapy due to its safety profile demonstrated during its use as a vaccine in the immunization against smallpox and as double-stranded DNA virus with the unique characteristic to replicate in the cytoplasm only, without integrating into the host genome.6,9 The efficient killing of tumor cells by recombinant VACVs or VACV wild-type isolates was demonstrated in different tumor xenograft models including a GBM model.10–13 There is a number of oncolytic viruses tested against malignant gliomas in phase 1 and phase 1/2 clinical trials, e.g., Herpes simplex virus1, adenovirus, reovirus, Newcastle disease virus, and measles virus.14,15 The application of those replication-competent viruses was generally safe, however, their antitumor effects observed in the preclinical studies need to be confirmed in human patients.14,15 One successful approach for oncolytic virotherapy may be the concept of personalized medicine, the screening of cancer patients for best treatment options and thereby maximizing therapeutic outcome.16,17 Therefore, the identification of biomarkers to locate patients that respond to a particular therapy is paramount.18,19

Cancer formation is associated with a close interaction of malignant tumor cells and the tumor microenvironment.19,20 The amount and composition of infiltrating immune cells varies in different cancer types and in individual patients and is investigated as biomarker in several studies, highlighting the link between the immune status of a tumor and the clinical outcome.19,20 In addition to cellular interactions, soluble factors released from cells in the micromilieu or from tumor cells control tumor-host interactions.19,21

Possible mechanisms resulting in a diverse immune cell infiltration and activation of a particular tumor microenvironment are reviewed by Ascierto et al.22 and include the genetic background

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of the host, genetic polymorphism of cytokine receptors, and the genetics of the tumor itself as potential factors. It is known, that one of the most important determinants in the regulation of immune responses in humans and mice is their genetic background.25,26 Two inbred mouse strains are used in this study which are defined as prototypical type 1 T helper (Th1) mouse strain in case of C57BL/6 and type 2 T helper (Th2) mouse strain in case of Balb/c mice.25-26 Adaptive immune response is divided into Th1/Th2 immune response based on a distinct cytokine secretion pattern: Classical Th1 cytokines are, e.g. interleukin 2 (IL-2), IL-12, interferon-γ (IFN-γ), and tumor necrosis factor alpha (TNF-α) whereas IL-4, 5, 6, 10 are Th2 cytokines.24 In addition to T-cell response, the innate immune cells, primarily macrophages, also show different characteristics in the two mouse strains used in response to pathogenic stimuli like lipopolysaccharide (LPS).23,25-27 It is well known, that the outcome of diseases in mice infected with several intracellular pathogens is dependent on the genetic background and the Th1/Th2 balance with IFN-γ as one major factor.24,26,28

In this study, we set out to identify microenvironmental differences or factors that decisively influence treatment outcome of oncolytic virotherapy and which may be relevant to affect therapeutic success in human patients. We investigated the oncolytic potential of the VACV LIVP 1.1.1, an attenuated wild-type isolate of the Lister strain, in murine GL261 glioma models in a comparative approach: Specifically, we used immunocompetent C57BL/6 wild-type (wt) mice and immunodeficient mouse strains of different genetic background (C57BL/6 athymic nude and Balb/c athymic nude mice) and also studied the effect of different tumor locations (subcutaneous and orthotopic).

RESULTS

Murine GL261 glioma cells were susceptible to VACV infection and oncolysis in cell culture

Infection of GL261 glioma cells with LIVP 1.1.1 (multiplicity of infection (MOI) 0.1) in cell culture revealed replication capacity of 612±244% within 24 hpi, and 6,272±3,821% at 72 hpi referred to the initial infection dose of the cells which was set 100% (Figure 1a). In addition, LIVP 1.1.1-mediated cell death could be demonstrated in a MTT-cell survival assay with only 20±11% surviving cells (MOI 1.0) or 55±12% (MOI 0.1) at 96 hpi (Figure 1b).

Differences in mouse strains and location of tumors influenced viral replication in GL261 glioma models

Using a comparative approach with different tumor locations, immunocompetent and immunodeficient mouse strains as well as different genetic backgrounds, we set out to analyze the replication efficacy of the VACV LIVP 1.1.1 upon intratumoral injection in murine GL261 glioma models.

Viral replication was an increased in viral titer compared to the initial injection dose (5×10⁶ pfu/mouse) delivered i.t. The results revealed viral replication exclusively in Balb/c athymic mice bearing subcutaneous tumors with 5×10⁶ ± 3×10⁵ pfu/g tissue 1 dpi and 8×10⁶ ± 2×10⁵ pfu/g tissue 7 dpi (Figure 1c). All other mouse models and tumor locations led to a decrease in the LIVP 1.1.1 titers during the observation time course. The replication capacity was 15-fold increased in subcutaneous tumors of Balb/c athymic mice at 7 dpi compared to a 28- and 8-fold decrease of the viral load in C57BL/6 wt mice and C57BL/6 athymic mice, respectively (Figure 1c). Replication was not detected either in Balb/c athymic mice (1×10⁶±8×10⁵ pfu/g tissue 7dpi) or in the C57BL/6 wt mice (6×10⁴±6×10⁴ pfu/g tissue 7dpi) with orthotopic brain tumors (Figure 1d). These findings coincide with a 4-fold decrease of the viral load in Balb/c athymic and 100-fold decrease in C57BL/6 wt mice at 7 dpi (Figure 1d).

Intratumoral LIVP 1.1.1 injection into subcutaneous GL261 tumors of Balb/c athymic mice significantly delayed tumor growth

Since major differences in virus replication in the subcutaneous tumors in different mouse strains were detected, we analyzed tumor growth kinetics in response to oncolytic virotherapy of these tumor models. In the LIVP 1.1.1 groups of all three models there was a slight tumor growth delay (Figure 1e–g) but only in Balb/c athymic mice a significant tumor growth delay from 4 dpi to the end of the study was detected (Figure 1e). All tumor models showed a very fast and aggressive tumor growth as mice reached a tumor size of 4,000 mm³ in 9–14 days.

Major differences in the tumor microenvironment were detectable between immunocompetent wild-type mice and immunodeficient athymic mice

Replication analysis affirmed that the mouse genetic background had a major impact on viral replication in the subcutaneous GL261 tumor models, as both C57BL/6 mouse models (athymic and wt) did not support viral replication, whereas the BALB/c background does. This led us to the question, which additional immunological factors in the tumor microenvironment of the subcutaneous tumor model of Balb/c athymic mice enabled virus replication on the one hand and prevented replication in mice with C57BL/6 background on the other side. We found that the virus titer differed significantly at 1 dpi (Figure 1c) implicating that the differences seem to exist in tumors before virus infection. Therefore, we performed a detailed characterization of tumor microenvironments of the subcutaneous tumor models already on d0.

In a first step, a subset of 59 biomarkers was tested in subcutaneous tumors of five mice of each mouse strain using a mouse immune related antigen profiling. Nineteen biomarkers that showed significant differences in the mouse strains tested are listed in Table 1. The significant differences of the proinflammatory signature were all detectable in the C57BL/6 wt mice compared to both athymic mouse strains (C57BL/6 and Balb/c) but not in the C57BL/6 athymic in comparison to Balb/c athymic mice. In most cases, highest biomarker concentrations were detectable in C57BL/6 wt mice followed by C57BL/6 athymic and Balb/c athymic mice.

The profiling revealed that there was a significant upregulation of proinflammatory cytokines such as interferon-γ-induced protein 10 (IP-10), IL-1α, IL-11 and macrophage inflammatory protein-1β (MIP-1β), and chemoattractants such as monocyte chemotactic protein (MCP)-1, MCP-3, MCP-5 recruiting mainly monocytes, macrophages, lymphocytes, eosinophils, MIP-2, and granulocyte chemoattractant protein-2 (GCP-2) attracting neutrophils and lymphokinin and IP-10 recruiting lymphocytes.39 Factors responsible for the proinflammatory signature (GCP-2, MIP-1β, MIP-2, MCP-3, and MCP-5) are mainly produced by macrophages. Another factor which was almost fivefold upregulated in the wild-type mice was vascular endothelial growth factor A (VEGF-A) as well as CD40.

In a second experiment, we performed an immune cell profiling of the subcutaneous GL261 tumors at d0 to determine, whether there were major qualitative and/or quantitative differences in the immune cell populations within these tumor models (Table 2). Single cell suspensions of the tumors were prepared and flow
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Cytometry analysis was performed. In the C57BL/6 wt mice besides CD3+CD4+ T-lymphocytes, immune cell subsets of the innate immune system such as CD49+ natural killer (NK) cells, CD11b+CD11c+ immature myeloid cells and cells of the monocyte/macrophage lineage (CD45+CD11b+, CD11b+/CD11c+, F4/80+/CD11c+) were significantly upregulated compared to the C57BL/6 athymic and Balb/c athymic mice (Table 2). Flow cytometry analysis of the blood in these mice (Table 2) showed that besides the expected CD3+/CD4+ and CD3+/CD8+ T-lymphocytes present exclusively in the wild-type mice, significantly more CD19+ B-lymphocytes, CD49+ NK cells, CD11b+/Ly6c+ dendritic cells, CD11b+/CD11c+ immature myeloid cells, and CD11b+/Gr1+ myeloid-derived suppressor cells (MDSCs) were detected in the C57BL/6 athymic mice compared to the Balb/c athymic mice. In case of CD49+ NK cells and CD11b+/CD11c+...
CD11c+ immature myeloid cells, the differences were also significant between C57BL/6 wt and C57BL/6 athymic mice.

This study highlighted that implantation of the same tumor cells in three different mouse strains resulted in a completely different tumor microenvironment with highest differences between immunodeficient and immunocompetent mice. It also demonstrated the influence of the adaptive immune system as main microenvironmental modulator. The comparison of these three mouse strains further revealed a tendency that the C57BL/6 athymic mice might be in an “intermediate state” between C57BL/6 wt and Balb/c athymic mice.

Phagocytic macrophages were excluded as factor responsible for viral clearance in C57BL/6 wt mice.

After characterization of the subcutaneous GL261 tumor model in different mouse strains, we tried to elucidate whether therapeutic efficiency in the “non-responder” C57BL/6 wt mice can be improved. As the proinflammatory signature was mainly produced by macrophages (Table 1) which were significantly recruited to subcutaneous GL261 tumors in C57BL/6 wt mice in comparison to athymic mice, we depleted macrophages in C57BL/6 wt mice with clodronate liposomes prior to virus infection. The depletion efficiency was confirmed by flow cytometry analysis with different macrophage marker combinations 1 dpi (Figure 2a–d). Standard viral plaque assay revealed that the virus titer in both groups (PBS and clodronate liposomes) did not differ significantly either on day 1 or on day 7 post infection (Figure 2e). Therefore, macrophages were excluded as factor responsible for the pronounced reduction of viral particles in this model detected already 1 dpi.

Uptregulation of MHCII observed on nonmonocytic cells from subcutaneous GL261 tumors in C57BL/6 wt mice at the day of infection

The immunohistochemical analysis of subcutaneous GL261 tumors sections of C57BL/6 wt and athymic as well as Balb/c athymic mice at d0 revealed a significant upregulation of MHCII in the C57BL/6 wt mice (Figure 3a). The upregulation of MHCII in C57BL/6 athymic mice compared to Balb/c athymic mice was not significant. Additionally, amounts of CD68+ immune cells were not significantly different in the three mouse strains (Figure 3b). Surprisingly, the expression of the marker MHCII did not colocalize with the immune cell marker CD68 (Figure 3d,e), implicating the existence of a MHCII+ non-macrophage population. The expression of MHCII was distributed homogeneously throughout the tumor center and at the tumor rim.

Table 1: Mouse immune-related protein antigen profiling of C57BL/6 wt, C57BL/6 athymic, and Balb/c athymic mice in GL261 tumors at the day of infection (d0)*

| Biomarker                                           | Abbr.          | Unit       | C57BL/6 wt | C57BL/6 athymic | Balb/c athymic | P value       |
|-----------------------------------------------------|----------------|------------|------------|-----------------|----------------|---------------|
| Granulocyte chemotactic protein-2                   | GCP-2          | ng/ml      | 0.2 ± 0.03 | 0.1 ± 0.02      | 0.1 ± 0.01     | 0.005         |
| Interferon-γ-induced protein 10                     | IP-10          | pg/ml      | 2940.0 ± 602.2 | 921.0 ± 1123.9 | 423.0 ± 154.2  | 0.003         |
| Interleukin-1α                                       | IL-1α          | pg/ml      | 1035.5+/−123.5 | 572.5 ± 158.6  | 438.8 ± 218.3  | 0.002         |
| Interleukin-11                                       | IL-11          | pg/ml      | 101.3 ± 17.9   | 70.8 ± 6.2      | n.d.           | —             |
| Leukemia inhibitory factor                          | LIF            | pg/ml      | 775.0 ± 112.9   | 533.8 ± 167.1  | 452.3 ± 71.5   | 0.006         |
| Lymphotactin                                         |                |            | 879.5 ± 201.4   | 516.2 ± 267.6  | 188.0 ± 32.0   | 0.006         |
| Macrophage inflammatory protein-1β                  | MIP-1β         | pg/ml      | 1457.5 ± 281.2  | 294.2 ± 164.5  | 171.0 ± 15.9   | 0.003         |
| Macrophage inflammatory protein-2                   | MIP-2          | pg/ml      | 34.0 ± 6.6      | 17.6 ± 4.9      | 15.3 ± 2.8     | 0.006         |
| Monocyte chemotactic protein 1                       | MCP-1          | pg/ml      | 436.8 ± 122.1   | 160.3 ± 51.3   | 115.8 ± 44.0   | 0.010         |
| Monocyte chemotactic protein 3                       | MCP-3          | pg/ml      | 320.5 ± 120.9   | 143.4 ± 64.2   | 96.8 ± 25.5    | 0.032         |
| Monocyte chemotactic protein-5                       | MCP-5          | pg/ml      | 184.5 ± 38.9    | 94.8 ± 38.2    | 87.6 ± 33.0    | 0.007         |
| T-cell-specific protein RANTES                       | RANTES         | pg/ml      | 0.3 ± 0.1      | 0.1 ± 0.1      | 0.03 ± 0.01    | 0.017         |
| Vascular cell adhesion molecule-1                    | VCAM-1         | ng/ml      | 47.0 ± 7.5      | 27.0 ± 4.0     | 26.6 ± 3.9    | 0.007         |
| Vascular endothelial growth factor A                 | VEGF-A         | pg/ml      | 9105.0 ± 2539.6 | 1684.2 ± 829.3 | 2230.0 ± 600.3 | 0.011         |
| von Willebrand factor                                | vWF            | ng/ml      | 12.0 ± 2.2      | 8.6 ± 1.5      | 8.3 ± 2.3     | 0.045         |
| Stem cell factor                                     | SCF            | pg/ml      | 1380.0 ± 175.7  | 791.6 ± 237.3  | 542.8 ± 195.9  | 0.0003        |
| CD40                                                | CD40           | pg/ml      | 724.8 ± 361.3   | 138.0 ± 51.7   | 154.6 ± 29.9   | 0.051         |
| Tissue inhibitor of metalloproteinases 1             | TIMP-1         | ng/ml      | 10.1 ± 1.3      | 9.5 ± 1.9      | 5.9 ± 1.6     | 0.003         |
| Matrix metalloproteinase-9                          | MMP-9          | ng/ml      | 20.8 ± 5.9      | 11.0 ± 4.1     | 6.7 ± 1.7     | 0.014         |

*Shown are the mean values and standard deviation (n = 5; C57BL/6 and Balb/c athymic and n = 4 C57BL/6 wt). Differences between the mouse strains were analyzed using two-sided t-test with unequal variances, *P < 0.05 (yellow), **P < 0.01 (orange), ***P < 0.001 (dark orange). Proinflammatory cytokines and chemoattractants are marked in green; modulators of tissue homeostasis are marked in blue.
CD68− cells could be detected (Figure 3f).

Further analysis revealed that intratumoral LIVP 1.1.1 administration had no impact on MHCII expression in the different mouse models 1 dpi with no difference between the LIVP 1.1.1 and PBS groups (Figure 3g). The expression of MHCII positive cells was homogenously scattered throughout tumor center and rim in the C57BL/6 wt mice. The MHCII pattern 1 dpi with strongest MHCII expression in subcutaneous tumors in C57BL/6 wt, followed by C57BL/6 athymic and at least in Balb/c athymic mice was also present 7 dpi (data not shown).

Analysis of orthotopic tumors of C57BL/6 wt mice revealed that 1 and 7 dpi in VACV- and PBS-injected tumors a large proportion of tumor cells expressed MHCII (Figure 3h–j). Tumor cells could be distinguished well from Iba-1-positive microglial cells and from astrocytes (data not shown).

Taken together, these data implicated that expression of MHCII on GL261 tumor cells was not limited to subcutaneous tumors but also occurred in the orthotopic natural location of the tumor within the brains of these mice. Further, it was not a consequence of virus infection but of the tumor microenvironment itself.

Diminished viral replication and MHCII upregulation on GL261 tumor cells in cell cultures upon pretreatment with IFN-γ. In our biomarker profiling, we detected factors such as IP-10, MCP-1 or MIP-1β which were differentially expressed with highest

(quantification not shown). An upregulation of MHCII on CD68− cells was observed in C57BL/6 athymic mice with patchy distribution, but not in Balb/c athymic mice (Figure 3c,d). Further, the morphology of the MHCII+/CD68− cells was distinct from immune cell populations and resembled that of the GL261 glioma cells. The upregulation of MHCII on the surface of non-immune cells/nonantigen-presenting cells is described especially in the context of malignant gliomas.30 Indeed, the expression of MHCII in our experiments was observed on tumor cells in cell cultures upon pretreatment with IFN-γ.

### Table 2 Immune cell profiling and comparison of single cell suspensions isolated from subcutaneous GL261 tumors and blood of C57BL/6 wt, C57BL/6 athymic, and Balb/c athymic mice on d0

| Tumor Marker | C57BL/6 wt (%) | C57BL/6 athymic (%) | Balb/c athymic (%) | C57BL/6 wt: C57BL/6 athymic | C57BL/6 wt: Balb/c athymic | C57BL/6 athymic: Balb/c athymic |
|--------------|---------------|---------------------|-------------------|-----------------------------|---------------------------|-------------------------------|
| Lymphocytes  | CD3+/CD4+     | 2.4 ± 0.7           | < 1               | 0.030                       | 0.039                     | –                             |
|              | CD3+/CD8+     | < 1                 | < 1               | –                           | –                         | –                             |
|              | CD19+         | 2.4 ± 2.0           | 1.1 ± 0.1         | 2.5 ± 0.7                   | 0.26                      | 0.71                          |
| NK cells     | CD49b+        | 5.5 ± 0.3           | 1.9 ± 0.0         | 4.1 ± 0.6                   | 0.001                     | 0.04                          |
|              | –             |                     |                   |                             |                           | 0.01                          |
| Dendritic cells | CD11b+/Ly6c+   | 5.7 ± 1.7           | 1.9 ± 0.8         | < 1                         | 0.16                      | 0.08                          |
| Immature myeloid cells | CD11b+/CD11c+ | 4.7 ± 0.9           | 3.3 ± 1.1         | 1.5 ± 0.4                   | 0.04                      | 0.03                          |
| MDSC         | CD11b+/Gr1+   | < 1                 | 1.7 ± 0.5         | < 1                         | 0.03                      | –                             |
| Neutrophils  | CD11b+/Ly6G+  | < 1                 | < 1               | < 1                         | –                         | –                             |
| Monocytes    | CD45+/CD11b+  | 8.8 ± 1.1           | 3.5 ± 0.1         | 2.3 ± 0.5                   | 0.01                      | 0.004                         |
| Macrophages/ | CD11b+/MHCII+ | 6.78 ± 0.0          | 1.9 ± 0.4         | < 1                         | 0.0002                    | 0.0004                        |
| Macrophages  | CD68+/MHCII+  | 3.5 ± 2.0           | 1.6 ± 0.8         | 1.0 ± 0.3                   | 0.22                      | 0.16                          |
| Macrophages  | F4/80+/MHCII+ | 2.5 ± 0.5           | < 1               | < 1                         | 0.02                      | 0.01                          |
| Blood        | CD3+/CD4+     | 16.9 ± 3.0          | < 1               | < 1                         | 0.01                      | 0.01                          |
| Lymphocytes  | CD3+/CD8+     | 16.9 ± 2.0          | < 1               | < 1                         | 0.006                     | 0.006                         |
| CD19+        | 52.2 ± 5.0    | 61.2 ± 1.9          | 44.6 ± 3.1        | 0.10                        | –                         | 0.02                          |
| NK cells     | CD49+         | 10.0 ± 2.4          | 29.1 ± 3.0        | 13.1 ± 0.7                  | 0.001                     | 0.03                          |
| Dendritic cells | CD11b+/Ly6c+ | 10.2 ± 4.5          | 15.4 ± 1.7        | 8.7 ± 2.3                   | 0.05                      | 0.68                          |
| Immature myeloid cells | CD11b+/CD11c+ | 5.6 ± 1.1          | 19.3 ± 2.2        | 4.6 ± 0.8                   | 0.002                     | 0.29                          |
| MDSC         | CD11b+/Gr1+   | 5.5 ± 3.6           | 12.7 ± 2.1        | 6.4 ± 2.4                   | 0.05                      | 0.72                          |
| Neutrophils  | CD11b+/Ly6G+  | 6.2 ± 3.6           | 13.0 ± 1.8        | 8.2 ± 2.9                   | 0.07                      | 0.51                          |
| Monocytes    | CD11b+/MHCII+ | 4.2 ± 1.3           | 5.6 ± 1.6         | 7.0 ± 0.8                   | 0.29                      | 0.06                          |
| Macrophages  | F4/80+/MHCII+ | 1.0 ± 0.2           | 1.5 ± 0.3         | 3.3 ± 0.2                   | 0.04                      | 0.002                         |

*Shown are mean and standard deviation of three mice per group in percentages (%). Differences in marker expression between mouse strains were analyzed using two-sided t-test with unequal variances. *P < 0.05 (yellow), **P < 0.01 (orange), ***P < 0.001 (dark orange). Highlighted in red is the mouse strain showing the highest percentage of a particular marker combination. 10,000 events/sample and staining were measured. Percentages below 1% are described as < 1.
LIVP 1.1.1 but the virus titers were more than 100-fold lower than stimulated (Figure 4b).

Seventy-two hours post stimulation, proliferation of IFN-γ+ cells revealed that cells preincubated with IFN-γ compared to cells (w/o) or IL-4 preincubation. Virus-mediated toxicity (MOI 1 and MOI 0.1) was not express MHCII constitutively. We set out to analyze whether this phenotypic change of GL261 tumor cells may have an impact on viral replication or virus-mediated toxicity.

For this, GL261 cells in cell cultures were either stimulated with rm-IFN-γ (10, 30, 60, 100) ng/ml or with rm-IL-4 (10 ng/ml) which are cytokines for induction of M1/M2 phenotypes in macrophages. Indeed, stimulation of GL261 cells with 10 ng/ml IFN-γ for 24 hours resulted in an increased amount of MHCII+ GL261 cells from 0% to 30% ± 3% which increased further to 79% ± 2% at 72 hours post stimulation. Mock (w/o) or IL-4 stimulated cells did not show MHCII expression on their surface (Figure 4a). Expression of MHCII increased over time from 24 to 72 hours after stimulation with IFN-γ and dependent on the dose at various concentrations (10, 30, 60, and 100) ng/ml (Figure 4a). Flow cytometry analysis using propidium iodide (PI) as a marker for cell death revealed that IFN-γ stimulation at a concentration of 10 ng/ml had no effect on cell viability compared to GL261 cells without stimulation (w/o) or stimulated with 10 ng/ml IL-4 at 24 and 72 hours post stimulation (Figure 4b). Seventy-two hours post stimulation, proliferation of IFN-γ stimulated cells was significantly reduced compared to cells (w/o) or IL-4 stimulated (Figure 4b).

Replication analysis in differentially stimulated GL261 tumor cells revealed that cells preincubated with IFN-γ were infected with LIVP 1.1.1 but the virus titers were more than 100-fold lower than cells infected after preincubated with IL-4 or (w/o) (Figure 4c). The detected virus titers (24–72 hpi) of IFN-γ preincubated cells remained below the initial infection dose, implicating that no viral replication occurred in these cells. No difference was detectable between w/o or IL-4 preincubation. Virus-mediated toxicity (MOI 1 and MOI 0.1) was significantly reduced in IFN-γ preincubated cells compared to cells without preincubation with IFN-γ. Seventy-two hours after infection with MOI 0.1 only 21 ± 14% cells survived in the unstimulated samples compared to 94 ± 18% in the IFN-γ stimulated samples (Figure 4d). In summary, these results in cell culture revealed the capacity to upregulate MHCII in the murine glioma cell line GL261 by INF-γ stimulation and highlighted an antiviral state of the MHCII+ GL261 tumor cells.

Impact of endogenous IFN-γ levels in C57BL/6 wt mice on viral infection

To affirm the findings that IFN-γ is responsible for the upregulation of MHCII on the surface of the tumor cells and the reduced viral replication in the C57BL/6 models in vivo, we compared viral replication in C57BL/6 wt mice with that of C57BL/6 IFN-γ knockout (KO) mice. Flow cytometry analyses revealed a significantly lower percentage of MHCII+/CD11b- or MHCII+/F4/80- tumor cells in subcutaneous GL261 tumors of C57BL/6 IFN-γ KO mice compared to C57BL/6 wt mice (Figure 4e,f). Furthermore, the replication capacity of LIVP 1.1.1 was significantly increased in C57BL/6 IFN-γ KO compared to the C57BL/6 wt mice (Figure 4g). These findings confirmed the role of endogenous IFN-γ levels in C57BL/6 wt mice as candidate factor responsible for diminished VACV replication and as factor in charge for upregulation of MHCII on GL261 tumor cells in this particular mouse model.

IFN-γ-induced antiviral state in GL261 glioma cells is reversible

We showed that GL261 tumor cells which are initially in a MHCII-VACV-permissive status were modified or imprinted into
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a MHCII⁺-VACV non-permissive status after implantation into C57BL/6 wt mice. We wanted to elucidate whether this modification of the GL261 tumor cells in C57BL/6 wt mice was a reversible or a permanent effect and therefore, a potential target for improving oncolytic virotherapy in tumors with MHCII signatures.

Hence, single cell suspensions of subcutaneous GL261 tumors from C57BL/6 wt mice were prepared. As controls, GL261 tumors of Balb/c athymic mice were used since the status of those tumor cells was still MHCII⁻-VACV-permissive (Figure 5f). By flow cytometry analysis, we detected a reduction of the number
INF-γ impairs oncolytic VACV therapy of GL261 tumors

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Figure 4 Interferon-γ-induced MHCII-upregulation in vitro and in vivo on GL261 glioma cells and correlated with impaired virus replication and reduced virus-mediated cytotoxicity. MHCII expression on GL261 cells without stimulation (w/o) or after stimulation with 10 ng/ml IL-4 or 10, 30, 60, 100 ng/ml IFN-γ was analyzed by flow cytometry after 24 and 72 hours (a). Experiment was performed in triplicates and repeated in an independent experiment. Statistical significance was tested using two-sided t-test with unequal variances at each time point (24 and 72 hours) between samples stimulated with 10 ng/ml IFN-γ and 30 ng/ml, 60 ng/ml, or 100 ng/ml IFN-γ, respectively indicated by horizontal asterisk (*P < 0.05, **P < 0.01, ***P < 0.001). In addition, statistical significance was compared for each concentration (10 ng/ml, 30 ng/ml, 60 ng/ml, or 100 ng/ml IFN-γ) between different time points (24 and 72 hours), indicated by vertical asterisk (*P < 0.05, **P < 0.01, ***P < 0.001). (b) Propidium iodide (PI) staining and thus measuring of cell death of GL261 cells w/o, or stimulated with 10 ng/ml IL-4 or IFN-γ for 24 and 72 hours was determined by flow cytometry. The bar charts represent the mean value and standard deviation of dead cells in percentage. Cell proliferation was determined by measuring the total events per µl until a total of 10,000 “gated” events were reached. Cell debris was excluded from the measurement. (c) GL261 cells w/o, or stimulated with IL-4 (10 ng/ml) or IFN-γ (10 ng/ml) for 24 hours were infected with LIVP 1.1.1 at a MOI of 0.1 and viral titers were determined 24, 48, and 72 hpi. The chart shows the mean of triplicate samples and standard deviation. For MTT-assay, GL261 cells were infected with LIVP 1.1.1 (MOI 0.1 and 1.0) (d). Experiment was performed in triplicates in two independent experiments. Statistical significance was tested using two-sided t-test with unequal variances, *P < 0.05, **P < 0.01, ***P < 0.001. Flow cytometry analysis of single cell suspensions isolated from s.c. GL261 tumors of C57BL/6 wt and C57BL/6 IFN-γ KO mice on d0 (e,f). Shown are mean and standard deviation of four mice per group in percentages (%). Experiment was performed twice in independent set ups. Differences between the mouse strains were tested using two-sided t-test with unequal variances. Replication capacity (%) of s.c. GL261 tumors of C57BL/6 wt (n = 14) and C57BL/6 IFN-γ KO mice (n = 13) 1 dpi (g). Viral titers were determined by plaque assay. The experiment was performed twice in two independent set ups. For the final calculations, the experiments were taken together. Statistical power analysis of the experiment was performed.
of CD11b/MHCII+ tumor cells in the single cell suspensions of tumor homogenates from C57BL/6 wt mice over time from day 0 to day 6 (Figure 5a,b). Viral plaque assay of freshly isolated subcutaneous tumor homogenates of C57BL/6 wt mice that were ex vivo cultured for 1 day prior to LIVP 1.1.1 infection (MOI 0.1) revealed, that virus titers remained below the virus inoculum 24 hpi (Figure 5c). Tumor homogenates isolated from C57BL/6 wt mice or Balb/c athymic mice ex vivo cultured for 6 days supported LIVP 1.1.1 (MOI 0.1) replication 24–72 hpi (Figure 5d) in accordance with a loss of MHCII expression (Figure 5a,b). We further showed, that tumor cells from both mouse strains cultured ex vivo for 14 days, and being in a MHCII status at this time point, upregulated MHCII on their cell surface with an increase from 24 to 72 hours after stimulation with IFN-γ (Figure 5e). These results showed that imprinting of GL261 tumor cells by endogenous IFN-γ levels in C57BL/6 mice is not a permanent effect and tumor FIGURE 5 INF-γ induced antiviral state in GL261 glioma cells is a reversible effect. Single cell suspension of s.c. GL261 tumors of C57BL/6 wt and Balb/c athymic mice were prepared on d0. Flow cytometry analysis was performed on d0 (a) and after 6 days of ex vivo cultivation (b). Shown are the mean values and standard deviation (n=3 mice) of the percentages of CD11b+/MHCII+ and CD11b−/MHCII+ immune and tumor cells. Differences between the mouse strains were tested using two-sided t-test with unequal variances. (c) Tumor homogenates of subcutaneous GL261 allografts isolated from tumors of C57BL/6 wt mice (n=3) were cultured in 24-well plates for 24 hours and infected with LIVP 1.1.1 at a MOI of 0.1. Virus titers were analyzed by standard plaque assay. (d) Tumor homogenates of s.c. GL261 allografts isolated from tumors of C57BL/6 wt or Balb/c athymic mice cultured ex vivo for 6 days (n=3) were infected with LIVP 1.1.1 (MOI 0.1) and analyzed by standard viral plaque assay in triplicate. Shown are mean values and standard deviation. (e) Tumor homogenates from C57BL/6 wt and Balb/c athymic mice were cultured ex vivo for 14 days and stimulated for 24 hours with 10 ng/ml IFN-γ. Cells were analyzed by flow cytometry analysis for MHCII expression 24 and 72 hours post stimulation. Shown are mean and standard deviation of three mice per group in percentages (%). Differences between the mouse strains were tested using two-sided t-test with unequal variances. (f) Schematic overview of the experimental setup and results.
cells can be reset into the status before implantation after cultivation in cell cultures for an appropriate time period (Figure 5f).

**DISCUSSION**

In the present study, we used a comparative experimental design, considering the genetic background and the immunologic status of the host, to investigate potential microenvironmental factors which are responsible for failure or success of treatment in different GL261 glioma models (Figure 6). In our experimental setup, the genetic background—which is one of the most important determinants in the regulation of immune responses in humans and mice—could be identified to have major impact on VACV LIVP 1.1.1 replication in the subcutaneous GL261 glioma models and thus on the efficacy of oncolytic virotherapy. In case of orthotopic GL261 gliomas located within the brain there are additional cellular factors influencing the success of oncolytic virotherapy in both, C57BL/6 wt and Balb/c athymic mice (submitted manuscript, Kober and Rohn et al.). Only Balb/c athymic mice with subcutaneous GL261 tumors supported viral replication (responder) in contrast to mice with C57BL/6 background (non-responder).

In line with our findings are the in detail studied disease mechanisms of ectromelia virus infection, which is the causative agent

**Figure 6**  Schematic representation of the comparative experimental setup and key findings within the GL261 glioma model influencing oncolytic virotherapy with VACV LIVP 1.1.1.
nuclear translocation in connection with different intrinsic properties of the cancer cells. Based on the assumption that different cancer cells have a different sensitivity to respond to stimulation with cytokines,\textsuperscript{46} we assume the same is true in case of the sensitivity of the GL261 tumor cells and their response to diverse concentrations of endogenous IFN-γ levels in the tested mouse models. As shown in our study and as described by Murtas \textit{et al.},\textsuperscript{44} this concept can be used to test tumor cell lines in cell culture to make predictions about their responsiveness \textit{in vivo}. The immunologically active tumor phenotype is in contrast to the immunologically silent tumor phenotype described to be associated with immune effector functions resulting in immune mediated tissue destruction and tumor rejection and thus displaying a better prognosis and treatment responses resulting in immune mediated tissue destruction and tumor rejection.

We could identify IFN-γ to be responsible not only for the immune-cell-like upregulation of MHCII on the tumor cell surface but also for the induction of an antiviral state in these cells resulting in reduced viral replication and virus-mediated cell death. In the present study, the IFN-γ-induced antitumor effects described for the immunological active phenotype do not seem to be strong enough to lead to therapeutic success.

Taken together, this study nicely illustrates that for successful development of cancer therapeutics the genetic and immunologic background of the patient should be taken into account. Further, the concept of personalized medicine and pretesting of patients before starting a particular therapy could be highly advised. One possible biomarker for oncolytic virotherapy of cancer could be the endogenous IFN-γ-level or MHCII expression on cancer cells respectively and the sensitivity of the particular cancer type to this cytokine.

\textbf{MATERIALS AND METHODS}

\textbf{Viruses strains}

LIVP 1.1.1 was isolated from a wild-type stock of the VACV vaccine strain LIVP originated from the Lister strain (Institute of Viral Preparations, Moscow, Russia). LIVP 1.1.1 represents a “native” virus where no genetic manipulations were conducted. Sequence analysis revealed a naturally occurring disruption of the thymidine kinase (Tk) gene locus.\textsuperscript{44} Consequences of a Tk gene deletion in VACVs are a preferential replication in dividing cells such as tumor cells resulting in enhanced tumor specificity and reduced virulence.\textsuperscript{105} Toxicity and efficiency of LIVP 1.1.1 was tested in various cell lines in cell culture and \textit{in vivo}.\textsuperscript{13,101}

\textbf{Cell lines}

African green monkey kidney fibroblast (CV-1) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Murine GL261 glioma cells were kindly provided by A. Pagenstecher (Department of Neuropathology, University Hospital of Marburg, Germany) and the cell line was authenticated by the Leibniz Institute DSMZ (Braunschweig, Germany). CV-1 and GL261, were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Sigma-Aldrich, Steinheim, Germany) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories, Coelbe, Germany) and penicillin G/streptomycin solution (100 U/ml, Sigma-Aldrich). Cells were maintained and incubated at 37 °C with 95% humidity and 5% CO\textsubscript{2}, and growth medium was changed every third day until confluence.

\textbf{Cell culture experiments}

GL261 cells or single cell suspensions were seeded in wells of a 24-well plate in 1 ml culture medium with 2% FBS supplemented with 10 ng/ml recombinant murine (rm)-IL-4 (ImmunoTools GmbH, Friesoythe, Germany) or rm-IFN-γ (ImmunoTools GmbH, Friesoythe, Germany) or without stimulation (w/o) as control. Twenty-four and 72 hours later, cells were analyzed for the expression of MHCII by flow cytometry or were infected with LIVP 1.1.1 at a MOI of 0.1 for viral replication and MTT-assay, respectively. After infection, cells were either cultured with culture medium (10% FBS) or in culture medium supplemented with the particular cytokine (10 ng/ml). In addition, 30, 60, and 100 ng/ml rm-IFN-γ were used for stimulation of GL261 cells to test MHCII expression by flow cytometry analysis 24 and 48 hours postinfection (hpi).

\textbf{Flow cytometry analysis of cultured cells}

Cells were detached with trypsin/EDTA (EDTA, PAA Laboratories, Coelbe, Germany) and centrifuged at 2,000 rpm for 3 minutes. The pellets were resuspended and stained in 200 μl phosphate buffered saline (PBS, PAA, Pasching, Austria) + 2% FBS and 0.2 μl labeled monoclonal antibody anti-mouse MHCII-PE (clone M5/114.15.2, ebioscience, Frankfurt, Germany) for 45–60 minutes at 4 °C. Afterwards, cells were washed once and resuspended in PBS + 2% FBS. To distinguish dead and living cells, 1 μl propidium iodide (PI, Sigma-Aldrich, Steinheim, Germany) was added to the samples 5 minutes prior to measurements. For analysis, an Accuri C6 Cytoometer (BD, Franklin Lakes, NJ) or flow cytometry analysis software FlowJo Version 10.227.4 (Accuri Cytometers, Ann Arbor, MI) were used. 10,000 events per sample were counted. Experiments were performed in triplicate and repeated twice.

\textbf{Replication analysis and standard viral plaque assay}

Cells were infected with LIVP 1.1.1 (MOI 0.1) diluted in infection medium with 2% FBS for 1 hour. Infection medium was collected and replaced by culture medium. Infected and uninfected cell lines were resuspended in 1 ml PBS after harvesting. Supernatants were collected separately 24, 48, and 72 hpi. Prior to analysis three freeze and thaw cycles in liquid nitrogen were accomplished to release viral particles. All samples were infected in triplicate. Serial dilutions of the samples were titrated on 100% confluent CV-1 monolayers in duplicate for standard viral plaque assay. The replication capacity in percentage (%) was calculated by the following formula ((actual viral load (pfu/ml)/virus inoculum (pfu/ml)) * 100%). The initial infection dose (inoculum) was set 100%.

\textbf{MTT cell viability assay}

Cells were infected with LIVP 1.1.1 at a MOI of 1.0 and 1.0. Cell viability was determined 24, 48, and 72 hours after viral infection. For this purpose, culture medium was replaced by 500 μl sterile filtrated 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT 2.5 mg/ml; Sigma-Aldrich) dissolved in medium without phenol red (Sigma-Aldrich). After an incubation time of 2 hours at 37 °C in the 5% CO\textsubscript{2} incubator the MTT-solution was removed. The color reaction and thus the cell viability was measured after adding 400 μl 1 N HCl (Sigma-Aldrich) diluted in isopropl alcohol (Roth, Karlsruhe, Germany). The optical density was measured at a wavelength of 570 nm in an Elisa Photometer Sunrise (TECAN Group, Männedorf, Germany). Uninfected cells were used as positive control, defined as 100% viable. The experiment was performed two or three times in triplicate.

\textbf{Animal studies}

All animal experiments were carried out in accordance with protocols approved by the Regierung von Unterfranken, Germany (permit number: Az. 55.2-2531.01-30/12 and AZ 55.2-2531.01-62/11).

\textbf{Subcutaneous implantation of GL261 cells and tumor growth analysis}

Five to 6-week-old female Balb/c athymic nude (Hsd:ATHymic Nude-Foxn1\textsuperscript{nu}, C57BL/6 wt (C57BL/6 J;JoIaHsd) mice ordered from Harlan Winkelmann GmbH (Borchern, Germany), C57BL/6 athymic nude (B6.Cg-NuTc Foxn1\textsuperscript{nu}) mice from
Taconic Europe A/S (Lille Skensved, Denmark) and C57BL/6 IFN-γ knockout (KO) (B6.12957-Ifngtm1Ts/J) mice purchased from Charles River Laboratories (Sulzdorf, Germany) were implanted subcutaneously (s.c.) with 1 × 10^6 GL261 cells in 100 µl PBS into the abdominal right hind flank. Tumor growth was monitored every third day. Tumor volume was measured from two directions with a digital caliper and calculated as follows (length × width^2) × 0.52. At a tumor volume between 200–300 mm³ mice were injected intratumorally (i.t.) with 5 × 10^5 pfu LIVP 1.1.1 or mock control in a volume of 100 µl PBS.

Orthotopic implantation of GL261 cells

Four to 5-week-old Balb/c athymic and C57BL/6 wt mice were stereotactically implanted intracranially with 1 × 10^6 GL261 cells. Mice were anesthetized with a mixture of ketamine (Ketavet, Pharmacia GmbH, Berlin, Germany) and xylazine (Xylavet, CP-Pharma GmbH, Burgdorf Germany) by inhalation on d0 or 1 dpi. Blood was taken and tumors were excised and frozen in liquid nitrogen. Fixation, embedding and cryosections were performed as described in ref. 11. Slides were fixed in ice-cold acetone for 10 min and afterwards stained with anti-mouse MHC class II (I-A/I-E) or anti-mouse MHC class II (I-A/I-E) functional grade biotin (clone M5/114.15.2; eBioscience) and rat-anti-mouse CD68 (FA-11; Serotec) class II (I-A/I-E) or anti-mouse MHC class II (I-A/I-E) functional grade biotin (clone M5/114.15.2; eBioscience), F4/80-APC (clone BM8; eBioscience). The following isotype controls have been used: Rat IgG2a K-APC/FITC/PerCP-Cy5.5 (clone eBR2a; eBioscience), Rat-IgG2b-PE/ APC/FITC/PerCP-Cy5.5 (clone eB149/104H5; eBioscience), Hamster IgG-APC (clone HTR888; BioLegend, London, UK), Rat-IgM-APC (eBioscience), Rat-IgG1-APC (clone EBRG1; eBioscience). For the intracellular staining of CD68 and CD206 cells were fixed with 2% paraformaldehyde/PBS solution (AppliChem, Darmstadt, Germany) for 15 min at RT under continuous agitation. The reaction was stopped with PBS + 2% FBS. Afterwards CD206-FITC (clone C068C2, BioLegend) or CD68-FITC (Serotech, Puchheim, Germany) were diluted in permeabilization buffer (5% FBS, 0.1% NaN₃, 0.2% Saponin in PBS) in the appropriate concentration and stained for 1 hour at 4 °C. Prior to the analysis, cells were washed once, resuspended in an appropriate volume of PBS + 2% FBS. Stained cells were analyzed using an Accuri C6 Cytometer and flow cytometry analysis software CFlow Version 1.0.227.4 (Accuri Cytometers). 10,000 events per sample were analyzed. Cell debris was excluded by FSC/SSC gating. Double stainings were conducted to avoid false positive or unspecific results. Calculated percentages below 1% were defined as not detectable (<1).

Preparation of single cell suspensions from subcutaneous tumors

For flow cytometry analysis or cell culture studies, 3–4 C57BL/6 wt, C57BL/6 athymic, Balb/c athymic, or C57BL/6 IFN-γ KO mice were sacrificed by CO₂ inhalation on d0 or 1 dpi. Blood was taken and tumors were removed. Single cell suspensions of tumors were prepared as described by Gentschev et al. In brief, tumor tissues were minced and transferred into a 50 ml tube containing 5 ml Roswell Park Memorial Institute (RPMI, Sigma-Aldrich) + 2% FBS, 150 µl 10,000 CDU/ml Collagenase I (Sigma-Aldrich) and 5 µl 5 MU/ml DNase I (Calbiochem, Darmstadt, Germany) for 30 minutes. Cells were passed through a 70 µm nylon mesh filter (BD Biosciences, Erembodegem, Belgium). Suspension was centrifuged at 1,000 rpm for 10 minutes and washed once with 20 ml PBS + 2% FBS. For the blood samples, 0.1 ml blood/2 ml 1× lysis buffer (10× stock: 8.29 g NH₄CL + 1.09 g KHCO₃ + 41 mg EDTA-Na, dissolved in 100 ml H₂O) were incubated for 10 minutes at RT. The samples were then centrifuged at 1,200 rpm for 5 minutes and washed twice.

Flow cytometry of single cell suspensions of tumor homogenates

Cell suspensions were blocked with purified anti-mouse CD16/CD32 (1 µl/slide) stained cells (clone 93; eBioscience, Frankfurt, Germany) for 30 minutes at 4 °C. Cells were stained in appropriate antibody solutions for 40 minutes at 4 °C. The following monoclonal anti-mouse antibodies were used in this study: CD3-PE (clone145-2C11; eBioscience), CD4-APC (clone GK1.5; eBioscience) CD8-FITC (clone S3-6-7; BD Bioscience Heidelberg, Germany), CD11b-APC (clone M1/70; eBioscience), CD11b-PE (clone M1/70; BD Biosciences), CD11b-PerCP-Cy5.5 (clone M1/70; eBioscience), CD14-PerCP (clone SA2-8; eBioscience), Ly6c-PerCP-Cy5.5 (AL-21; BD Bioscience, Heidelberg, Germany), Gr1-FITC (clone RB6-8C5; eBioscience), CD49b-APC (clone DX5; eBioscience), CD19-PerCP/Cy5.5 (clone 6D5; eBioscience), CD11c-PerC (clone N41.C1; BioLegend, London, UK), Ly6G-PE (clone A8; BD Biosciences), CD45-PerCP-Cy5.5 (clone 50-F11; eBioscience), HC-M1/5/14.15.2; eBioscience, F4/80-APC (clone BM8; eBioscience). The following isotype controls have been used: Rat IgG2a K-APC/FITC/PerCP-Cy5.5 (clone eBR2a; eBioscience), Rat-IgG2b-PE/ APC/FITC/PerCP-Cy5.5 (clone eB149/104H5; eBioscience), Hamster IgG-APC (clone HTR888; BioLegend, London, UK), Rat-IgM-APC (eBioscience), Rat-IgG1-APC (clone EBRG1; eBioscience). For the intracellular staining of CD68 and CD206 cells were fixed with 2% paraformaldehyde/PBS solution (AppliChem, Darmstadt, Germany) for 15 minutes at RT under continuous agitation. The reaction was stopped with PBS + 2% FBS. Afterwards CD206-FITC (clone C068C2, BioLegend) or CD68-FITC (Serotech, Puchheim, Germany) were diluted in permeabilization buffer (5% FBS, 0.1% NaN₃, 0.2% Saponin in PBS) in the appropriate concentration and stained for 1 hour at 4 °C. Prior to the analysis, cells were washed once, resuspended in an appropriate volume of PBS + 2% FBS. Stained cells were analyzed using an Accuri C6 Cytometer and flow cytometry analysis software CFlow Version 1.0.227.4 (Accuri Cytometers). 10,000 events per sample were analyzed. Cell debris was excluded by FSC/SSC gating. Double stainings were conducted to avoid false positive or unspecific results. Calculated percentages below 1% were defined as not detectable (<1).

Immunochemistry of cryosections

For immunohistochemistry, mice were sacrificed by CO₂ inhalation. Tumors were excised and snap frozen in liquid nitrogen. Fixation, embedding and sectioning were performed as described in ref. 11. Slides were fixed in ice-cold acetone for 10 min and afterwards stained with anti-mouse MHC class II (I-A/I-E) or anti-mouse MHC class II (I-A/I-E) functional grade biotin (clone M5/114.15.2; eBioscience) and rat-anti-mouse CD66 (PA-11; Serotec) in single or costainings for 1 hour at RT. After fixation with PBS sections were labeled with secondary antibodies (Cy3/Cy2-conjugated AffiniPure Donkey Anti-Rat IgG (H+L); Jackson ImmunoResearch Laboratories. West Grove, PA) Streptavidin-Cy3 (from Streptomyces avidinii, buffered aqueous solution; Sigma-Aldrich) and Hoechst 33258 (Sigma-Aldrich) for 1 hour at RT. After three washing steps and an ethanol step, the slides were mounted in Moviol 4-88 (Roth, Karlsruhe, Germany). Images were taken with an Axiovert 200M inverse microscope (Zeiss, Jena, Germany) and edited with the software program Axiovision 4.8.2 (Zeiss, Feldbach, Switzerland). Ten images per tumor slice (five images from tumor rim and five from tumor center) were taken at a magnification of 20× with identical settings. RGB-Images were converted into 8-bit gray scale images and the fluorescence intensity of CD66 and MHCII stained images was measured by Image J and represents the average brightness of staining related pixels.
were incubated with the secondary antibodies (Streptavidin-Cy3 and donkey anti-rabbit-Cy5; Dianova, Hamburg, Germany) for 5 hours. Nuclei were Hoechst 33342-labelled. Then, sections were washed with PBS and mounted onto glass slides in Mowiol 4-88. The fluorescent-labelled preparations were examined using the MZ16 FA Stereo-Fluorescence microscope (Leica, Wetzlar, Germany) equipped with a digital CCD camera (DC500, Leica, Wetzlar, Germany). The Iba-1-Cy5 staining was converted to a green signal by Photoshop CS5 (Adobe Systems).

Statistics
To determine significance between two independent groups and normal distribution, a two-tailed t-test with unequal variances was used (Excel 2010 for Windows). P values were defined as follows: **P < 0.05, ***P < 0.001, ****P < 0.0001. For calculating boxplot diagrams, a template from Vertex42 LLC has been used. The optimal sample size (n = 17) to detect a difference of d = 1.02 for the viral replication in CS75BL/6 wt and CS75BL/6 IFN-γ KO mice was calculated to assure an adequate power to detect statistical significance. We used a power of 80% and an α-level of 0.05. Power analysis was performed using the software G*Power.

CONFLICT OF INTEREST
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