

**Oxaliplatin in combination with liver-specific expression of interleukin-12
reduces the immunosuppressive microenvironment of tumors and eradicates
metastatic colorectal cancer in mice**

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Abbreviations: 5-FU: 5-Fluorouracil; Gem: gemcitabine; HC-Ad: high-capacity adenovirus; IL-12: interleukin-12; Iri: irinotecan; Mif: mifepristone; MDSC: myeloid-derived suppressor cells; OXP: oxaliplatin;

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ABSTRACT

Background and aims: New options are needed for the management and prevention of colorectal cancer liver metastases. Interleukin-12 (IL-12) is an immunostimulatory cytokine with proven antitumor effect in animal models. Despite evidences indicating its biological effect in humans, neither the recombinant protein nor gene therapy vectors expressing IL-12 have shown a relevant benefit in cancer patients. The difficulties in obtaining a suitable expression pattern and the immunosuppressive milieu in the tumors contribute to this poor performance. We propose a new approach to overcome these limitations.

Methods: A High-Capacity (“*gutless*”) Adenoviral vector carrying a liver-specific, Mifepristone (Mif)-inducible system for the expression of IL-12 (HC-Ad/RUmIL-12) was used in combination with chemotherapy. Tumors were established in the liver of C57BL/6 mice by inoculation of MC38 colon cancer cells.

Results: Intrahepatic injection of HC-Ad/RUmIL-12 and tailored induction regimes allowed the maintenance of safe and efficient levels of IL-12 *in vivo*. Individualized, step-wise increase in the dose of Mifepristone (125-4000 µg/kg) was needed to compensate the progressive but transient downregulation of the inducible system. Repeated cycles of Mif induction (every 24 hours for 10 days) were needed for optimal tumor eradication. However, complete protection against tumor re-challenge was observed in less than 25% of the animals. The administration of oxaliplatin (5 mg/kg intraperitoneally) three days before starting the induction regime achieved efficient elimination of liver metastases with a single cycle of IL-12 induction, and improved

protection against tumor re-challenge. This was associated with a shift in the tumor microenvironment towards a more pro-immunogenic phenotype, with an increase in the CD8⁺/T regulatory cell ratio and a reduction in myeloid-derived suppressor cells. These effects were not observed with 5-Fluorouracil, irinotecan or gemcitabine.

Conclusions: Long-term controlled expression of IL-12 using a HC-Ad vector in combination with oxaliplatin is effective and clinically applicable against hepatic colon cancer metastases.

SUMMARY

What is already known about this subject:

- IL-12 is an immunostimulatory cytokine with antitumor effects in animal models of colorectal cancer.
- Methods to control the location, intensity and duration of IL-12 production are needed to increase the safety and efficacy of genetic immunotherapy.
- Tissue-specific, drug-inducible expression systems can be used to modulate the expression of transgenes in specific organs, but the biological effects of IL-12 can interfere with their functions.
- High-Capacity (“gutless”) adenoviral vectors are efficient in long-term gene transfer of the liver.

What are the new findings:

- Using the HC-Ad/RUmIL-12 vector, individualized step-wise increases in the dose of inducer allowed the maintenance of therapeutic levels of IL-12 for more than 10 days, in cycles that can be repeated over a period of several months in mice.

- Oxaliplatin cooperates with IL-12 in the stimulation of an efficient immune response against cancer cells, with complete eradication of pre-established colorectal cancer liver metastases and prevention of experimental relapses in a syngeneic model.
- The mechanism of cooperation involves a reduction in the immunosuppressive microenvironment of tumors, with an increase in the ratio of CD8+/T regulatory cells and CD8+/myeloid-derived suppressor cells.
- Cooperation with IL-12 is not observed with other chemotherapeutic drugs such as 5-Fluorouracil, irinotecan and gemcitabine.

Potential impact on clinical practice in the future:

The protocol described here is compatible with conventional oxaliplatin-based chemotherapeutic regimes, and it can be applied in conjunction with surgical or ablative treatments to avoid recurrence of colorectal cancer liver metastases.

INTRODUCTION

Metastatic liver cancer is a life-threatening condition frequently observed in colorectal cancer patients. Hepatic lesions are found in 10 to 25% of cases at the time of diagnosis, and 30% of them have no evidence of dissemination in any other organ. In addition, recurrence after surgical removal of the colorectal tumor occurs mainly in the liver, with a 20-25% rate of metachronous liver metastases ¹. Overall, more than 50% of colorectal cancer patients will suffer liver involvement during the course of their disease ². Surgical resection is potentially curative only in the most favorable cases, with a general recurrence rate of 60-70%. Regional treatments achieve local control, but a significant

increase in long-term survival is not guaranteed. Standard chemotherapy combinations consisting on 5-flourouracil and leucovorin plus oxaliplatin or irinotecan (FOLFOX and FOLFIRI) achieve survival rates close to 10% in advanced colorectal cancer ³. The addition of biological agents such as cetuximab can increase the response rate ⁴ and allows resection of liver metastases that were not candidates for surgery before treatment ⁵. However, recurrence rate exceeds 70% and it is clear that new therapeutic options are needed to improve the clinical management of hepatic metastases from colon cancer. Diverse strategies aimed to stimulate the immune responses against cancer cells offer the opportunity to block progression of disseminated tumor deposits and prevent relapses ⁶. However, unraveling this potential is still a challenge in the clinical setting. Tumors avoid the attack of the immune system by promoting the development and accumulation of leukocytes with an immunosuppressive phenotype. The importance of regulatory T cells (Treg) and Myeloid-Derived Suppressor Cells (MDSC) in this process has been recently demonstrated (reviewed in ^{7, 8}). The effect of cancer treatments on these cell populations is still poorly understood, but it can greatly influence their efficacy. Exploring the opportunities of cooperation with standard treatments is especially important, in light of the recent realization that certain cytotoxic drugs can favor the immune response against tumors ⁹. These include some of the most active agents against colorectal cancer, such as oxaliplatin (OXP), 5-fluorouracil (5-FU) and gemcitabine ¹⁰⁻¹⁴.

The cytokine interleukin-12 (IL-12) can serve as a link between the innate and adaptive immune responses because it can activate the proliferation of T lymphocytes and Natural Killer (NK) cells, the secretion of other mediators such as interferon- γ (IFN- γ), and the cytotoxic activity of these effector cells ¹⁵. The capacity of IL-12 to increase the number and activity of tumor-specific lymphocytes has been verified in

humans¹⁶, and certain antitumor effect has been observed¹⁷. However, clinical responses tend to be poor, and intensification of the treatment is difficult due to the toxicity associated with systemic exposure to IL-12. The use of gene therapy vectors encoding the p35 and p40 IL-12 subunits to obtain expression of the cytokine in specific locations is an attractive alternative¹⁸⁻²⁰. However, the clinical experience indicates that refinement of these vectors is needed. Intratumoral administration of a replication-deficient adenovirus carrying the IL-12 gene was well tolerated in patients with hepatic metastases²¹. Biological effect was demonstrated, but antitumor responses were very modest. The poor performance of the vector in terms of intensity and duration of expression was recognized as a key limitation in this approach. Additional obstacles are the high heterogeneity in the infectivity of tumor nodules among different patients, and the rapid appearance of neutralizing antibodies against adenovirus, which makes subsequent administrations ineffective²². To circumvent these problems, High-Capacity adenoviral vectors (HC-Ad) have been designed. The lack of viral genes reduces their toxicity and avoids the elimination of transduced hepatocytes by the immune system²³⁻²⁶. The incorporation of a liver-specific, drug-inducible expression system in the GL-Ad/RUmIL12 vector (called here HC-Ad/RUmIL-12) allowed intense, controlled and long-term expression of the transgene upon intravenous administration of the vector²⁷. The possibility of controlling the intensity of IL-12 expression by adjusting the dose of inducer makes this approach safer and more clinically applicable. However, the function of drug-inducible expression systems is influenced by the immunostimulatory properties of IL-12 acting at the transcriptional level²⁸. This causes a progressive but reversible inhibition of transgene expression that might limit the therapeutic effect of this approach, especially in the case of pre-established liver metastases^{29, 30}. We hypothesized that optimization of the induction regime and combination with

chemotherapy would render a more efficient and clinically relevant protocol. We describe here an individualized induction scheme that prevents toxicity, compensates the downregulation of the system, and allows several rounds of sustained IL-12 expression. More importantly, we found that the combination with systemic OXP favored the immune response against tumors, alleviated the need for long-term IL-12 expression, and increased the protection from an experimental tumor re-challenge. This treatment achieved complete eradication of hepatic lesions and long-term disease-free survival of animals in a syngeneic model ²⁹.

MATERIALS AND METHODS

Cell lines.

The MC38Luc1 cell line has been previously described ^{29, 31}. They were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 µg/ml streptomycin and 100 µg/ml penicillin (all from of Gibco, Invitrogen), 2 mM glutamine (Cambrex) and 400 µg/ml G418 (geneticin, Gibco, Invitrogen).

Mice and tumor models.

Five to 8 weeks old C57BL/6 and athymic (*nu/nu*) female mice were purchased from Harlan and Charles river, respectively (Barcelona, Spain). Hepatic tumors were

established by direct implantation of 5×10^5 MC38Luc1 cells, as previously described²⁹. For subcutaneous tumor formation (rechallenge experiments), a total of 10^6 cells were injected in the right hind flank. Tumor size was monitored at indicated time points by measuring two perpendicular tumor diameters using a precision caliper. Tumor volume was calculated using the following formula: $V = \text{length} \times \text{width}^2 \times 0.5$. Survival was checked daily and mice were euthanized if general status was deteriorated or subcutaneous tumors exceeded 20 mm in diameter. All *in vivo* experiments were performed in accordance with the local animal commission.

Bioluminescence imaging.

Anesthetized animals received the substrate D-luciferin (Promega) by intraperitoneal injection at 150 mg/kg. Ten minutes later, light acquisition was performed in an IVIS CCD camera system (Xenogen) and analyzed with the Living Image 2.20 software package (Xenogen). Typically, a circular region of interest measuring 3 cm in diameter was defined in the abdomen of mice, and quantification of light emission was performed in photons/second.

Vector and treatment procedures.

The High-Capacity (*gutless*) adenoviral vector HC-Ad/RUmIL-12 (also named GL-Ad/RUmIL12) has been previously described²⁷. In this vector, all viral genes have been deleted and substituted by human non-coding DNA (“stuffer”), plus a transgene region containing a Mif-inducible system for the expression of p35 and p40 subunits of murine IL-12. The transactivator of this system is expressed under the control of a liver-specific promoter (transthyretin). Both IL-12 subunits are co-expressed due to an internal ribosomal entry site (IRES). Amplification of the vector was carried out as previously

described³². The virus was administered by direct intrahepatic injection surrounding the tumors following laparotomy³⁰. Typically, 2.5×10^8 infectious units (i.u.) of virus were mixed in a total volume of 50 μ l saline solution before injection. Activation of the inducible system was carried out by intraperitoneal injection of Mif (RU486, Sigma) dissolved in sesame oil (Sigma). Inductions started 2 weeks after implantation of cells. Administration of chemotherapy drugs was carried out by intraperitoneal injection. OXP (Eloxatin®) was from Sanofi-aventis; 5-FU was from Ferrer Farma; irinotecan was from Hospira; gemcitabine (Gemzar®) was from Lilly. Animals treated with 5-FU received 50 mg/kg folinic acid (from GES) 1 hour before 5-FU, and another dose together with 5-FU.

Determination of IL-12, IFN- γ and HMGB1.

Serum concentration of murine IL-12 and IFN- γ were determined by OptE1A mouse IL-12 (p70) and mouse IFN- γ ELISA kits (BD Bioscience PharMingen, San Diego, CA). HMGB1 in serum or supernatant of cells was quantified by ELISA kit from IBL International GmbH (Hamburg, Germany).

Flow cytometry

Spleens, healthy livers and livers with tumors were harvested and treated with 400 U/mL collagenase D and 50 μ g/mL DNase I (Roche Diagnostics). After mechanical tissue dissociation, cells were passed through a 70- μ m nylon mesh filter (BD Falcon, BD Bioscience, San Jose, CA, USA) and washed. To enrich liver cell suspension in leukocytes, hepatocytes were removed with Percoll gradients. The single cell suspension obtained was pre-treated with anti-CD16/32 (clone 2.4G2; BD Biosciences-PharMingen) to reduce non-specific binding to Fc receptors. Afterwards, cells were

stained with the following fluorochrome-conjugated antibodies: CD8 (53-6.7), CD4 (clone RM4-5), CD25 (PC61), CD11b (M1/70), Ly6C (AL-21), F4/80 (BM8) and Ly6G (1A8) from BD Pharmingen. Intracellular staining for FoxP3 was performed using mouse Regulatory T cell staining kit BD Biosciences following manufacturer's instructions. Cells were acquired on either a FACSCalibur or a FACSCanto flow cytometer (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (Tree Star). CD8/Treg or CD8/MDSC ratios were calculated as CD8 T cell count divided by either Treg cell or MDSC count.

Statistical analysis.

Two-tailed unpaired t-test was used to compare two groups of values when $n > 10$. For smaller groups, Mann-Whitney non-parametric test was used. Comparisons of more than two groups were performed using ANOVA test with Bonferroni correction. Survival curves were compared by logrank test (GraphPad Prism software).

RESULTS

Individualized and progressive adjustment of Mif compensates transduction variability and downregulation of the inducible system.

Therapeutic use of IL-12 requires efficient methods to control the plasmatic levels of this potent immunostimulatory cytokine in order to avoid toxicity. We had previously determined in our MC38 syngeneic tumor model that seric IL-12 below 20 ng/ml has no antitumor effect, and levels above 700 ng/ml are associated with toxicity²⁷. The two main obstacles for keeping this therapeutic range are the variability in transduction among individuals and the progressive silencing of the drug-inducible system mediated

by the immune system²⁸. We have focused on the favorable dose-response effect of the Mifepristone (Mif)-inducible system (figure 1A) to design a new induction protocol. Once the liver has been transduced with the vector (typically 2.5×10^8 iu), a sub-optimal amount of Mif (125 $\mu\text{g}/\text{kg}$) is administered the first two days in order to prevent toxicity. The concentration of IL-12 is measured in serum 10 hours after the first induction, and based on this information, an step-wise increase in Mif is scheduled according to table 1. In figure 1B we represent the kinetics of IL-12 production obtained with the adjusted protocol. In contrast with a fixed amount of inducer (constant protocol), doubling the dose of Mif every 3 days contributes to the stabilization of IL-12 levels for at least 10 days. Since downregulation of the system is due to a reversible inhibition of the transthyretin promoter²⁸, the induction protocols can be repeated after a resting period of at least 2 weeks in order to obtain several cycles of IL-12 expression (figure 2B). We have verified that the system is functional for more than 5 months, with a slow decrease in the intensity of expression in each cycle due to the non-integrative nature of adenoviral vectors.

Combination with OXP increases the antitumor effect of IL-12 on hepatic colon cancer metastases.

Once the induction protocol was established, we investigated if conventional OXP-based chemotherapy could enhance the antitumor effect of sustained IL-12 expression. Tumors were obtained by direct inoculation of MC38Luc1 cells in the liver of syngeneic mice²⁹. Bioluminescence detection ensured engraftment of cancer cells and homogeneous tumor load in all experimental groups (not shown). The HC-Ad/RUmIL-12 vector was injected intrahepatically in the proximity of tumors nodules, as recently described³⁰. One week later, daily inductions started following the adjusted protocol

described above. One subset of these animals received a single dose of OXP (5 mg/kg intraperitoneally) three days before the first Mif administration. Other mice were inoculated intrahepatically with saline solution instead of vector, with no additional treatment (control group), or received a single dose of OXP. A schematic representation of the experiments is provided in figure 2A. Two weeks after completing one cycle of inductions (day 35 after cell implantation), most animals from the control or OXP-only groups were dead as a consequence of tumor progression (see figure 2D). At this point, bioluminescence quantification predicted the presence of hepatic tumors in approximately half of the animals treated with intrahepatic IL-12 alone, whereas the addition of OXP achieved a stronger and more homogeneous inhibition of luciferase signal (figure 2B). To confirm this observation, all surviving animals underwent laparotomy 5 days later. In figure 2C we represent the volume of tumors determined by direct measurement at this stage, or after necropsy in animals with shorter survival. The result clearly demonstrates that the combination of IL-12 and OXP achieves the best therapeutic effect, both in terms of antitumor effect and long-term survival (figure 2D). Treatment with OXP alone caused a small, not statistically significant reduction in the final tumor volume, but no survival advantage was observed. The lack of efficacy of OXP in aggressive models based on MC38 cells has been described by other groups³³.

OXP enhances the establishment of a protective immune response against a tumor rechallenge.

Protection from tumor relapse or from the appearance of new liver metastases is crucial to improve the prognosis of colorectal cancer patients. Therefore, we studied the immunological protection provided by our treatments against a tumor rechallenge. Animals that remained free from their hepatic tumors for more than one month after

being treated with HC-Ad/RUmIL-12 (with or without OXP) received a new inoculation of MC38Luc1 cells in a distant location (subcutaneous). Monitorization of tumor growth revealed that, on average, mice from both treatment groups presented a delay in tumor progression compared with naïve animals (figure 3). Interestingly, the inhibition was more intense if the treatment regime had included OXP, and the proportion of animals that were completely protected from the appearance of new tumors increased from 23 to 55%.

OXP counteracts the immunosuppressive microenvironment of tumors.

We investigated the mechanisms involved in the cooperation between IL-12 and OXP. First, we verified that IL-12 does not increase the cytotoxic activity of OXP in MC38Luc1 cells in vitro (supplementary figure 1). Although the influence of direct cytotoxicity cannot be completely ruled out in vivo, experiments performed in athymic (nu/nu) mice showed a dramatic loss of therapeutic effect (supplementary figure 2), demonstrating a critical role T cell-mediated immune responses. Exposure of calreticulin (CRT) on the cell surface and release of high-mobility group box 1 protein (HMGB1) have been proposed as key elements in the immunostimulatory properties of oxaliplatin¹⁴. Whereas we did not detect a significant increase in the exposure of CRT upon treatment of MC38Luc1 cells with OXP (data not shown), the release of HMGB1 was evident (supplementary figure 1A). However, the biological significance of this finding in our tumor model is uncertain, because tumor-bearing animals treated with HC-Ad/RUmIL-12 plus OXP did not show an increase in the serum concentration of HMGB1 compared with untreated mice (figure 4A). This is probably due to the fact that MC38Luc1 tumors spontaneously release increasing amounts of HMGB1 as they progress in the liver of the animals, in correlation with the situation observed in

advanced colorectal cancer patients³⁴. This is in contrast with other tumor models such as glioblastoma multiforme in which HMGB1 levels are low in untreated animals, but show an acute increase in response to suicide gene therapy³⁵. Next, we verified that OXP does not cause a significant increase in the production of IL-12 mediated by HC-Ad/RUmIL-12 (data not shown). However, the amount of IFN- γ produced in response of IL-12 was higher in the group treated with OXP (figure 4B), suggesting a stronger activation of the immune system. This effect was observed 6 days after the initiation of IL-12 expression, and coincides with the peak of IFN- γ stimulation. Using MHC-tetramer staining specific for an MC38 epitope we detected a moderate increase in the frequency of CD8⁺ T lymphocytes in peripheral blood in response to IL-12, but the addition of OXP did not increase significantly this population (data not shown).

Therefore, we studied the tumor microenvironment to evaluate if the combined treatment had an effect on the local immunosuppressive cell populations. A favourable balance between T-effector and T-suppressor cells has been associated with improved immune responses against tumors^{36,37}. Tumor, liver and spleen from control and treated animals were collected 3 days after the initiation of IL-12 expression, and leukocyte populations were analyzed by flow cytometry. We did not observe changes in the NK cells in any of these tissues (data not shown). However, the ratio between CD8⁺ T lymphocytes and Tregs (CD25⁺FoxP3⁺ population) was significantly increased in the tumors of mice treated with IL-12 in combination with OXP, in comparison with IL-12 alone. Interestingly, this effect was not observed neither in the surrounding liver, nor in the spleen of these animals (figure 5A, B). Similar results were obtained in samples obtained the 6th day of IL-12 induction (not shown). In addition, we found that the combined treatment caused a decrease in the monocytic Myeloid Derived Suppressor Cells (MDSC), defined as the CD11b⁺Ly6C⁺Ly6G⁻ population³⁸. The ratio

CD8⁺/MDSC was slightly elevated in the tumors of mice treated with IL-12, but it was significantly enhanced if OXP was included in the therapeutic regime (figure 5A, C). In this case, the ratio was also increased in the spleen, but not in the liver. Since monocytic MDSC have the potential to differentiate into tumor-associated macrophages (TAM) ^{7, 38}, we determined if the treatment induced changes in the expression of the macrophage differentiation marker F4/80. We found that the vast majority of myeloid-derived cells in our tumor model remained in an undifferentiated state, and no changes were observed with any of the treatment conditions (supplementary figure 3). Together, these data indicate that of OXP collaborates with IL-12 to reduce the immunosuppressive microenvironment of tumors. This effect is not shared by other agents frequently used against colorectal cancer. For instance, treatment with irinotecan alone or in combination with HC-Ad/RUmIL-12 did not increase the CD8/Treg and CD8/MDSC ratios (figure 6A and B). Interestingly, we found that 5-FU and gemcitabine caused an increase in the CD8/MDSC ratio, in accordance with recent reports ^{39, 40}. However, this effect was reversed when the drugs were combined with IL-12. Although irinotecan and gemcitabine cooperated with IL-12 in the eradication hepatic lesions and increased the survival of animals (figure 6C), they did not improve the immunological protection against tumor rechallenge (figure 6D), suggesting that alternative mechanisms are taking place.

Repeated cycles of IL-12 plus OXP are efficient in the long-term management of colorectal liver metastases.

Potential clinical application of immunochemotherapy will surely require repeated cycles of treatment not only to control the initial lesions, but also to prevent or eventually cope with relapses and the appearance of new metastases. In order to mimic

this situation, we initiated a series of experiments in which mice bearing hepatic tumors were treated with two cycles of IL-12 induction after vector administration. Then, they were subjected to a subcutaneous tumor rechallenge and finally a third cycle of induction was completed. In total, mice were evaluated for 5 months from the establishment of the initial hepatic tumors (see schematic representation in figure 7A). Three days before each one of these cycles, one group of animals received a single dose of OXP (5 mg/kg). Interestingly, we observed that two cycles of IL-12 induction separated by two weeks achieved a very efficient control of the hepatic tumors and extended survival of mice (figure 7B, left part of the graphic). In this setting, the cooperation of OXP was not apparent because most animals were cured. One month after the completion of the second cycle of induction, the subcutaneous tumor challenge was performed in mice that had rejected the hepatic lesions. Compared with the results obtained with a single cycle of induction, no increase in the immunological protection was observed with any of the treatments. Therefore, we had the opportunity to evaluate if a third cycle of induction was able to control the progression of the subcutaneous tumors, as if they were distant metastases. As shown in figure 7C, IL-12 alone was unable to inhibit the growth of the tumors that had escaped the initial immunological protection. In contrast, the combination of IL-12 and OXP obtained a response in 60% of them (figure 7C). Of note, these tumors had progressed for more than 3 weeks and some of them exceeded 1000 mm³ in volume before the third induction cycle started. As a result, the overall survival of animals, including deaths from the tumor rechallenge, was improved by the incorporation of OXP in the treatment (figure 7B, right part of the graphic).

DISCUSSION

The development of a long-term expression vector based on adenovirus enables an efficient delivery of therapeutic genes in the liver²⁵. In the current approach, the target cells for transduction are the “normal” hepatocytes, not the cancer cells. This reduces the variability of infectivity and makes this approach more predictable in terms of gene transfer. The possibility of regulating the intensity of IL-12 expression by adjusting the dose of an inducer is important for the clinical application of immunogene therapy. It allows the use of relatively high doses of vector, to ensure that the liver of all patients is efficiently transduced. Then, careful monitorization of the individual response to the inducer will guide the intensification of the regime, a concept that is common to many oncologic treatments. Regarding Mif, there is abundant information about the safety of this drug at doses higher than those used here^{41, 42}, suggesting that there is still margin to increase the potency of the system. Predicting the duration of the treatment that would be needed in patients based on our pre-clinical results is too speculative. However, we provide evidence indicating that sustained expression of IL-12 is beneficial. One cycle of Mif induction is sub-optimal in our tumor model. Repetition of short cycles does not improve the efficacy (data not shown), meaning that it is important not only the number of cycles but also the duration of each one of them, i.e. the total period covered by therapeutic levels of IL-12. This requirement will probably depend on the severity of the disease. Although suppressive mechanisms used by tumors to evade the immune attack may be activated by IL-12^{43, 44}, the net balance on tumor control is still positive. Our data suggest that OXP may enhance the immune response against tumors by tipping the balance between effector and regulatory/suppressor cells in favor of effector cells, as it has been previously described for other chemotherapeutic drugs³⁷. Stimulation of T reg development by MDSCs has been described in some

tumor models ⁴⁵, indicating that mutual influence of these suppressive populations is possible. It has been recently described that the inhibition of MDSC accumulation by sunitinib increases the antitumor effect of IL-12 ⁴⁶, supporting the relevance of this mechanism. The need of a complementary mechanism that initiates the damage to cancer cells may be particularly important in the protocol proposed here, since expression of IL-12 takes place in the liver parenchyma surrounding the tumor. Although we did not detect a consistent increase in the exposure of CRT in MC38 cells exposed to OXP, we cannot rule out the possibility that small amounts may contribute to immunogenicity of the cells, as recently described for other murine colon cancer cells ¹⁴. Our results point to a previously unrecognized mechanism taking place when OXP is used in combination with IL-12, but further studies are needed to gain a more detailed understanding of the immunostimulatory properties of OXP. The vector and the experimental conditions described here can be easily adapted to evaluate other immunochemotherapy combinations in search of new combined protocols. Careful testing of specific combinations is required, because the effect of drugs on the immune system can be different when used as monotherapy or in the context of IL-12 stimulation, as suggested by the results obtained with 5-FU and gemcitabine. This phenomenon is currently under investigation. In summary, the finding that controlled expression of IL-12 in the liver can be a good complement for OXP-based chemotherapy regimes opens new possibilities for the management of advanced colorectal patients.

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FIGURE LEGENDS.

Table 1. Adaptation of the induction regime to the initial concentration of IL-12 in serum. Mif is administered daily by intraperitoneal injection. The first two days, a sub-optimal dose of 125 µg/kg is given to all mice. The concentration of IL-12 is determined in the serum 10 h after the first Mif injection (first column on the left). Based on this information, the dose of Mif is established for days 3 to 5, and subsequently duplicated every 3 days.

Figure 1. Progressive increase in the dose of Mif allows several cycles of sustained IL-12 expression in mice treated with the HC-Ad/RUmIL-12 vector. The vector was administered at 2.5×10^8 i.u./mouse in C57BL/6 mice by intrahepatic injection. A. Two weeks later, a single dose of Mif (125; 250; 1.000; 2.000 or 4.000 µg/kg) was administered intraperitoneally to different groups of animals (n=5). The concentration of IL-12 was measured in serum 10 h later. B. Mice received daily injections of 250 µg/kg Mif for 10 consecutive days (constant protocol, white circles, n=10). A different set of animals received an adjusted protocol (black circles, n=8) consisting on 125 µg/kg Mif days 1-2; 250 µg/kg days 3-5; 500 µg/kg days 5-7 and 1000 µg/kg days 9-11.

The concentration of IL-12 in serum was determined 10 h. after induction at the indicated days. More than 2 months after the last Mif administration, a second cycle of inductions was repeated in the same group of animals using an adjusted protocol that started at 250 µg/kg Mif. The grey area represents the range of IL-12 concentrations considered therapeutic in the MC38 tumor model. Error bars represent standard deviation.

Figure 2. OXP increases the antitumor effect of IL-12 in a syngeneic model of colorectal cancer liver metastases. A. Schematic representation of the experiment. MC38Luc1 cells (5×10^5 cells/mouse) were inoculated in the liver of C57BL/6 mice at day 0. Control animals (Cont) were left untreated (intrahepatic injection of saline solution), or treated with OXP alone (5 mg/kg intraperitoneal, group OXP). Other groups received the HC-Ad/RUmIL-12 vector by intrahepatic injection surrounding the tumors at day 5. Daily inductions with Mif started at day 12 using an adjusted protocol (group IL12). One subset of these animals received OXP (5mg/kg intraperitoneal) 3 days before the first Mif administration (group IL12+OXP). B. Bioluminescence quantification performed at day 35. At this point, most animals in the control and OXP groups were dead. C. Volume of hepatic tumors determined by direct measurement through laparotomy at day 40, or after necropsy at earlier time points in animals dead or sacrificed for ethical reasons. D Survival of animals. Statistical significance: **, $p < 0.001$; *, $p < 0.05$.

Figure 3. Immunological protection against cancer cells in animals treated with IL-12 plus OXP. MC38Luc1 cells (10^6 cells/mouse) were inoculated subcutaneously in C57BL/6 mice that were cured from their hepatic tumors after

being treated with the HC-Ad/RUmIL-12 vector in the presence (group IL12+OXP) or absence (group IL12) of OXP. The same amount of cells was inoculated in naïve mice as a control. The average tumor volume for each group is represented. The percentage of mice that remained tumor-free for the entire observation period is indicated in parenthesis. Day 0 indicates the moment of inoculation of subcutaneous cells, which corresponds to at least one month after completion of the previous treatments. *, $p < 0.05$.

Figure 4. OXP enhances the immunostimulatory action of IL-12. Mice bearing intrahepatic MC38 tumors were treated with the HC-Ad/RUmIL-12 vector, and IL-12 expression was induced with Mif following an adjusted protocol (IL12 group). A subset of animals received 5 mg/kg OXP intraperitoneally 3 days before the first induction (IL12+OXP group). Other groups were left untreated (cont), or treated with OXP only. Blood was collected 24 h after the administration of OXP (considered as day 1), and 10 h. after the 1st, 3rd and 6th Mif inductions (days 3, 5 and 8, respectively). The concentration of HMGB1 (A) and IFN- γ (B) was determined by ELISA (n=10). *, $p < 0.05$.

Figure 5. OXP counteracts the immunosuppressive microenvironment of tumors. C57BL/6 mice bearing intrahepatic MC38 tumors were treated with the HC-Ad/RUmIL-12 vector in the absence or presence of OXP, as indicated (n=10). IL-12 expression was obtained using an adjusted induction protocol with Mif, and animals were sacrificed after the 3rd day of induction. Control groups include untreated animals (Cont) and mice treated with OXP alone (n=10). Leukocytes were isolated from liver, spleen and tumor samples. CD8⁺ T cells, Tregs (CD25⁺FoxP3⁺) and monocytic MDSC (CD11b⁺Ly6C⁺Ly6G⁻) were identified by flow cytometry. (A) Countour plots showing

the percentage of CD8 T cells (CD3⁺CD8⁺), Tregs (CD4⁺CD25⁺FoxP3⁺) and MDSC (CD11b⁺Ly6C⁺Ly6G⁻) in representative mice treated with the HC-Ad/RUmIL-12 vector in the absence or presence of OXP (IL12 and IL12+OXP groups, respectively). Countour plots are gated on alive cells (CD8 T cells), CD4⁺ cells (Tregs) or CD11b⁺ cells (MDSC). (B and C) The fold increase in the ratio of CD8/Treg (B) and CD8/MDSC (C) are represented, considering the untreated group as a reference.

Figure 6. Combination of IL-12 and other chemotherapeutic drugs. Mice bearing intrahepatic MC38 tumors were treated with the HC-Ad/RUmIL-12 vector, and IL-12 expression was induced with Mif following an adjusted protocol (IL12 group). Subsets of animals received additional treatment with one of the following chemotherapeutic drugs: 25 mg/kg 5-FU plus 100 mg/kg leucovorin on 2 consecutive days, starting 3 days before the first Mif. induction (IL-12+5-FU); a single dose of 150 mg/kg irinotecan, two days before induction (IL12+Iri);. two doses of 60 mg/kg gemcitabine, 3 days and 24 h before induction (IL12+Gem). Other groups were left untreated (cont), or treated with the chemotherapeutic drugs only. Five animals from each group were sacrificed after the 3rd day of induction and tumors were processed for analysis of CD8⁺ T cells, Tregs (CD25⁺FoxP3⁺) and monocytic MDSC (CD11b⁺Ly6C⁺Ly6G⁻) by flow cytometry. The fold increase in the ratio of CD8/Treg (A) and CD8/MDSC (B) are represented, considering the untreated group as a reference. The remaining animals completed the full induction protocol and survival was monitored (C). Death of animals was due to tumor progression, except for 3 mice in the group of IL-12+Gem that died before the completion of the treatment due to toxicity and were excluded from the survival curve. Animals treated with the chemotherapy drugs without IL-12 had no survival benefit compared with the untreated group, and are not represented in the graphic. Cured

animals from the IL12, IL12+Iri and IL12+Gem groups received a subcutaneous re-challenge with MC38Luc1 cells (10^6 cells/mouse). The same amount of cells was inoculated in naïve mice as a control. The average tumor volume for each group is represented (D). The percentage of mice that remained tumor-free for the entire observation period is indicated in parenthesis. *, $p<0.05$.

Figure 7. Long-term management of colorectal cancer. Cooperation of OXP and IL-12 for the control of experimental relapses in distant locations. A. Schematic representation of experiments. C57BL/6 mice bearing hepatic tumors were treated with the HC-Ad/RUmIL-12 vector and received two cycles of Mif induction preceded or not with OXP (5 mg/kg, intraperitoneal). Animals cured from their hepatic tumors were subjected to a subcutaneous challenge with the same tumor cells (MC38Luc1), and received a third cycle of induction during the indicated days. B Overall survival of mice. The grey areas indicate the duration of each induction cycle. Most animals in the OXP and OXP+IL12 groups were cured from their hepatic tumors (not shown). The day of subcutaneous tumor rechallenge is marked with a vertical dotted line. “Control rechallenge” refers to naïve animals that were inoculated with tumor cells and received no treatment. Mice were sacrificed when tumor burden reached the ethical end point. Survival after rechallenge was significantly higher in all treated groups versus control ($p<0.01$). Survival of animals in the IL12+OXP group was significantly higher than in the IL-12 group ($p<0.04$). The progression of individual subcutaneous tumors in the rechallenge experiment is represented in panels C (group IL12) and D (group IL12+OXP). The duration of the induction protocol (Mif) and the day of administration of OXP are indicated. All naïve animals inoculated with tumor cells developed tumors that progressed in the absence of treatment (not shown).

Supplementary figure 1. Effects of IL-12 and chemotherapeutic agents on MC38Luc1 cells in vitro. A. MC38Luc1 cells were treated for 24 h with 200 μ M OXP, and 24 h later the amount of HMBG1 released in the supernatant was analyzed by an ELISA kit, as indicated by the manufacturer. (B-E) Cells were treated for 24 h with increasing doses of OXP (B) Iri (C), Gem (D), or 5-FU (E), as indicated. Then, the chemotherapeutic agents were removed and cells were incubated for additional 48 h with 500 ng/ml IL-12. Viability was analyzed by crystal violet staining at the end of the experiment. The graphic represents the percentage of cells that survive to the treatment.

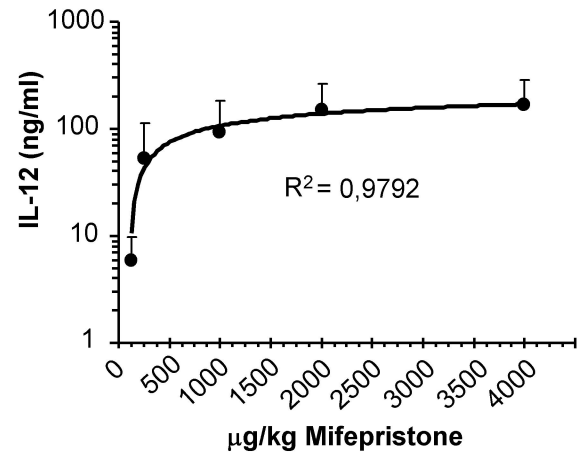
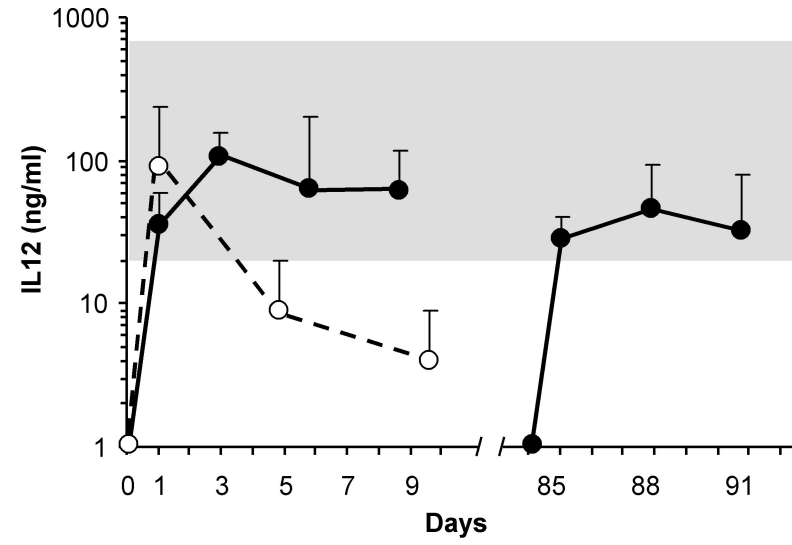
Supplementary figure 2. Treatment of colorectal cancer liver metastases with IL-12 plus OXP in athymic mice. MC38Luc1 cells (5×10^5 cells/mouse) were inoculated in the liver of athymic (nu^{-}/nu^{-}) mice at day 0. Control animals (Cont) were left untreated (intrahepatic injection of saline solution), or treated with OXP alone (5 mg/kg intraperitoneal, group OXP). Other groups received the HC-Ad/RUmIL-12 vector by intrahepatic injection surrounding the tumors at day 5. Daily inductions with Mif started at day 12 using an adjusted protocol (IL12 group). One subset of these animals received OXP (5mg/kg intraperitoneal) 3 days before the first Mif administration (group IL12+OXP). The survival of animals is represented.

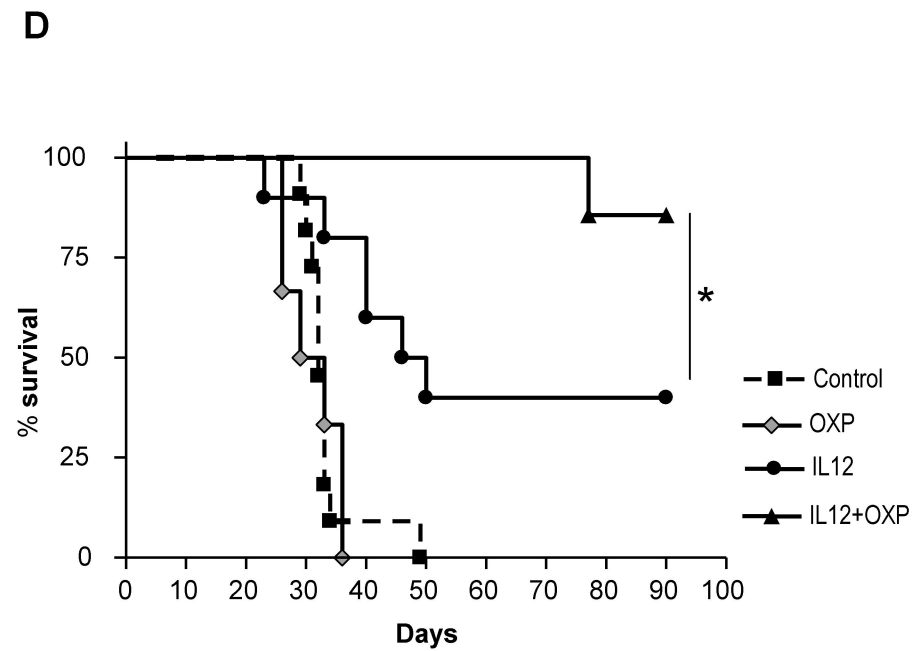
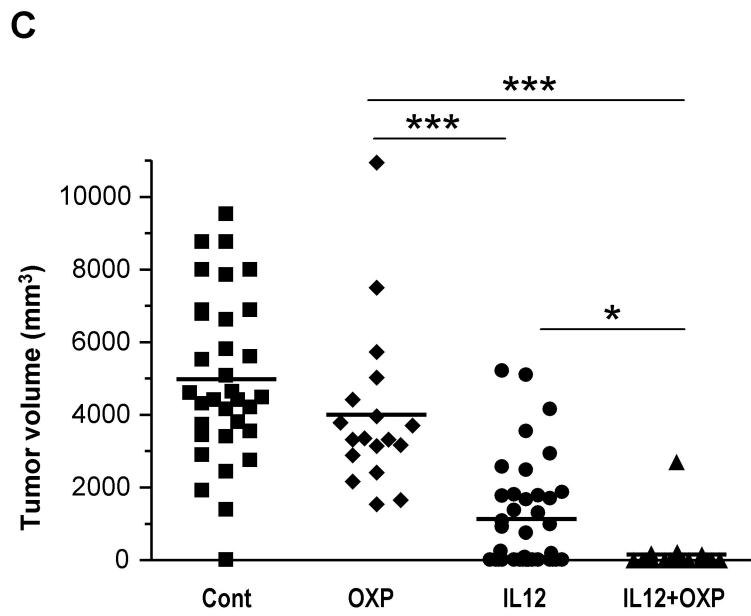
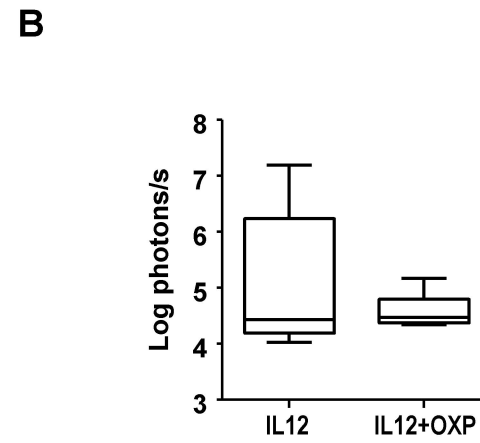
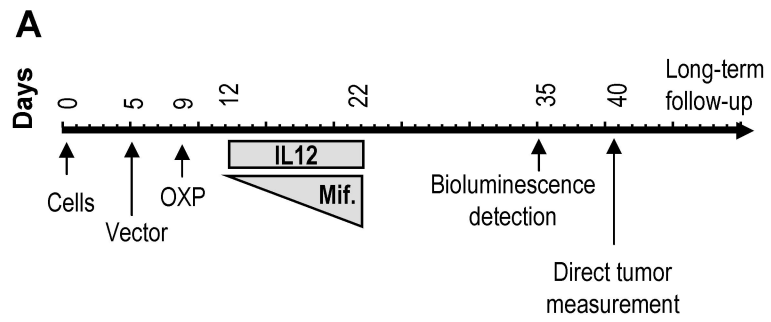
Supplementary figure 3. Analysis of TAM. C57BL/6 mice bearing intrahepatic MC38 tumors were treated with the HC-Ad/RUmIL-12 vector in the absence or presence of OXP, or received an intrahepatic injection of saline as a control. IL-12 expression was obtained using an adjusted induction protocol with Mif, and animals were sacrificed

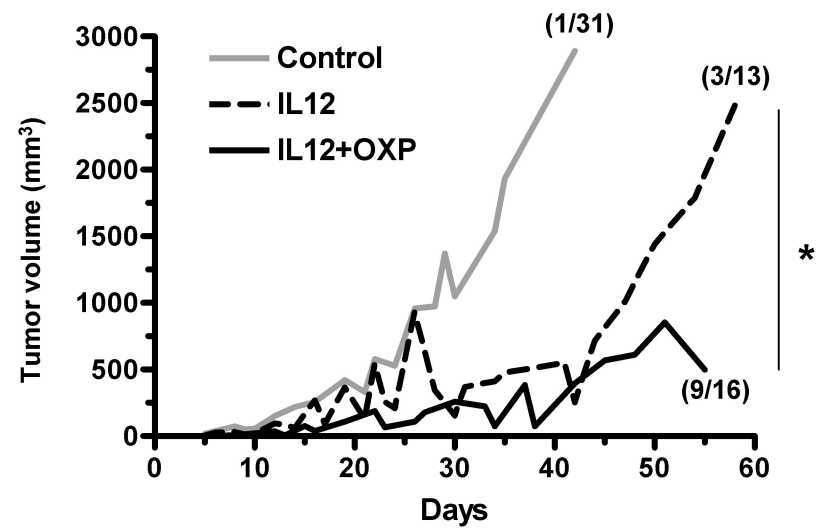
after the 3rd day of induction. Leukocytes were isolated from tumor samples, and TAM were quantified by flow cytometry using anti-F4/80 and CD11b antibodies.

Table 1.

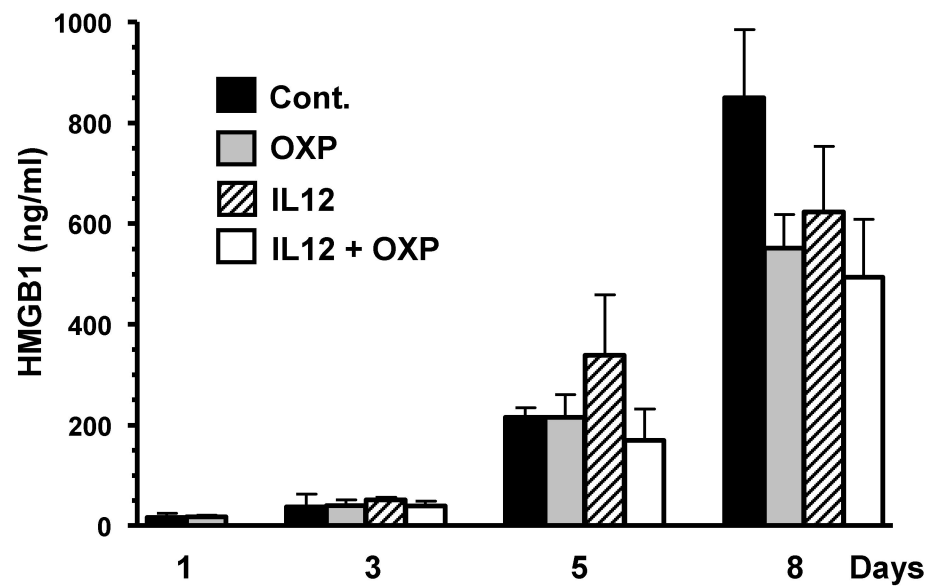
Initial IL-12 (ng/ml)	<i>Days of induction</i>			
	3-5	6-8	9-11	
<5	1000	2000	4000	Mif. (µg/kg)
5-20	500	1000	2000	
20-75	250	500	1000	
>75	125	250	500	

A**B**

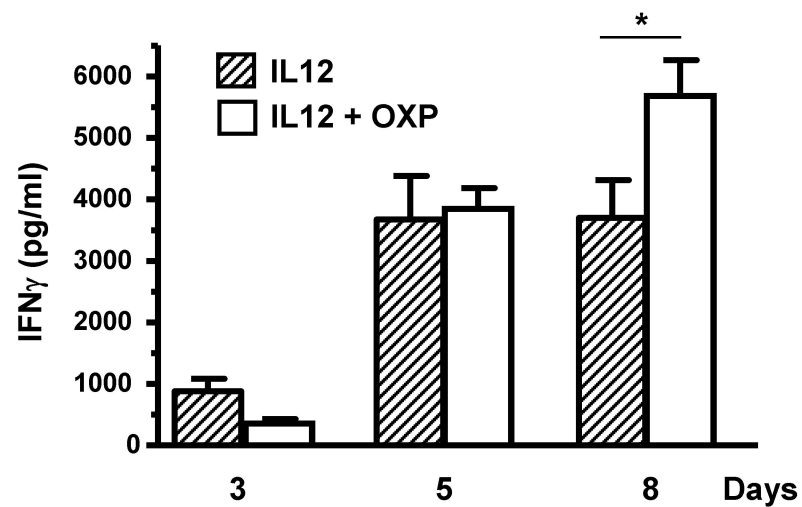


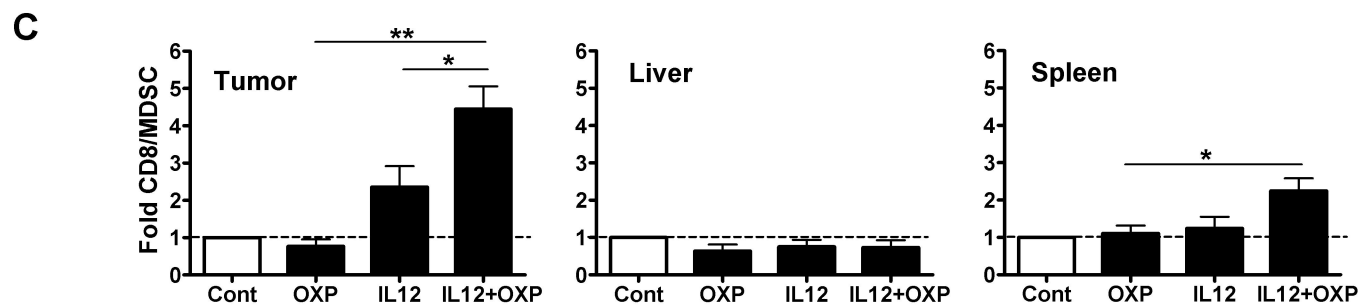
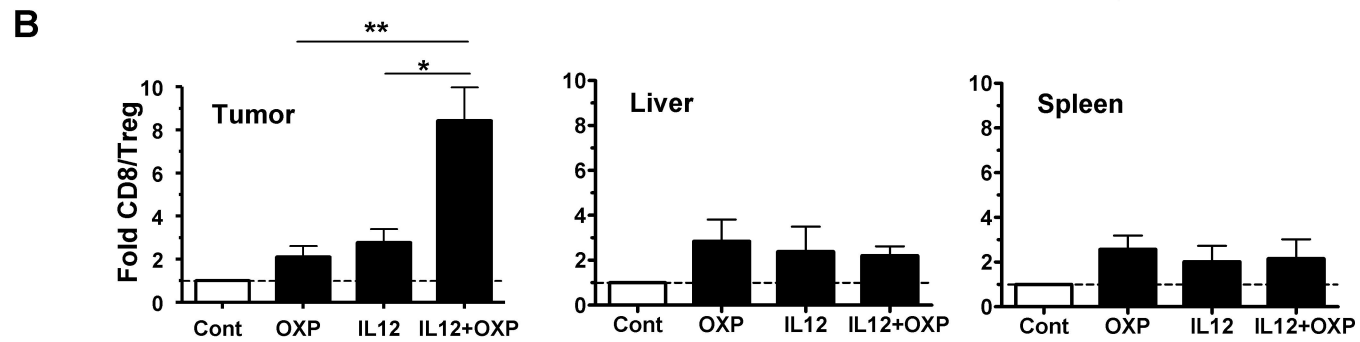
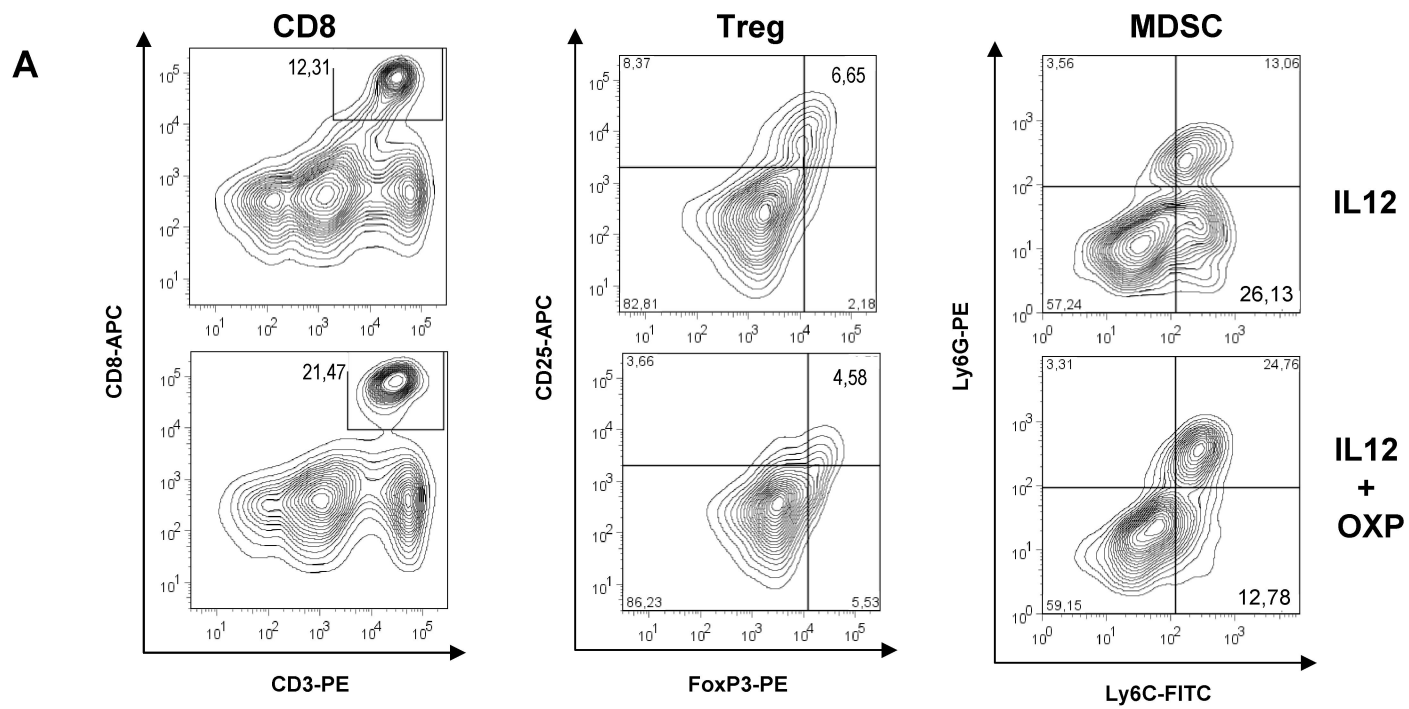


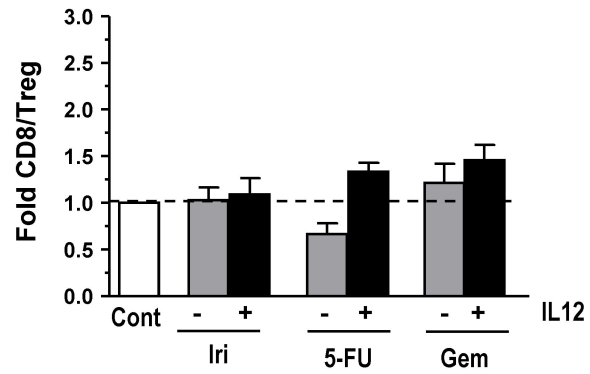
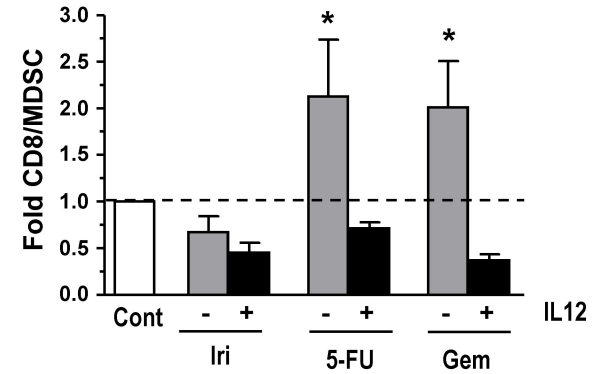
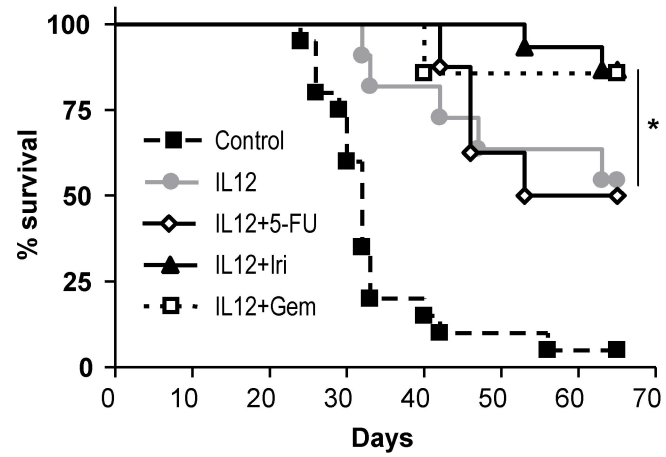
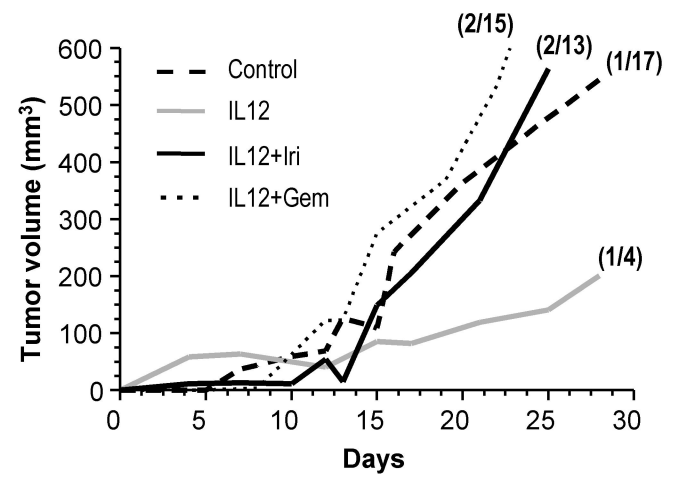
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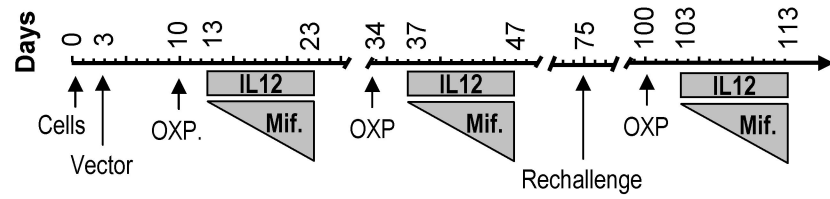
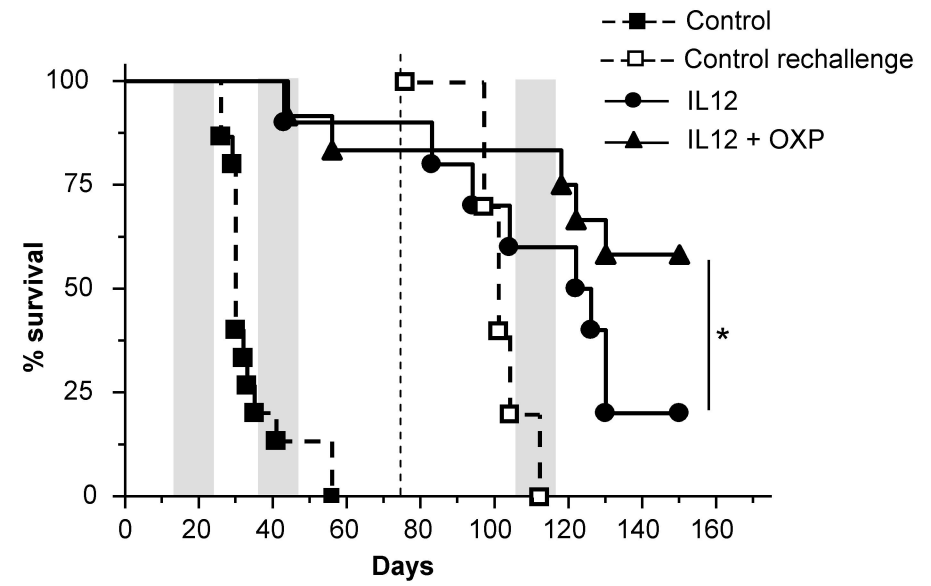
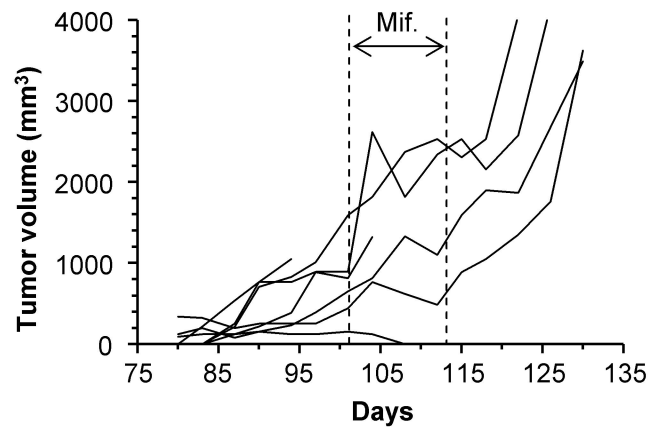
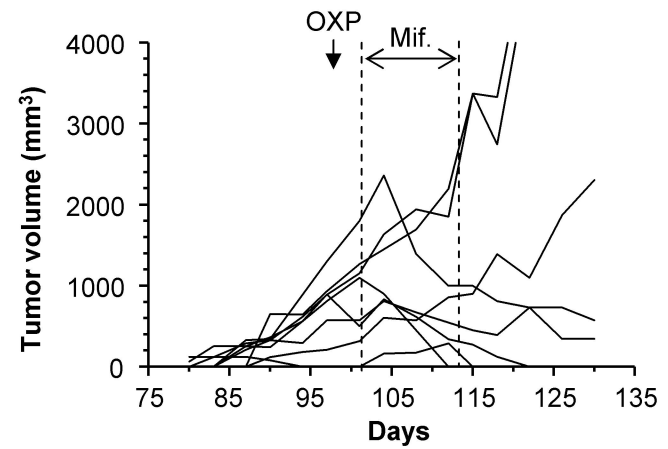


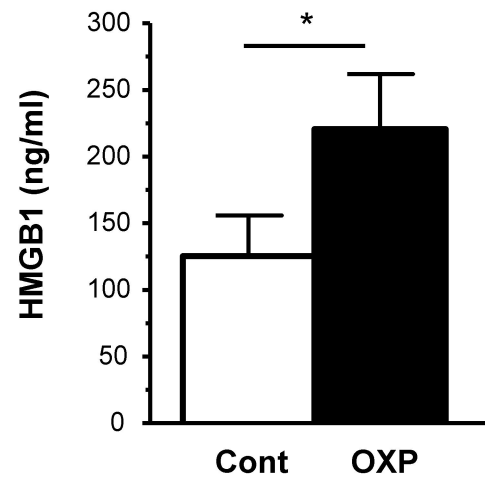
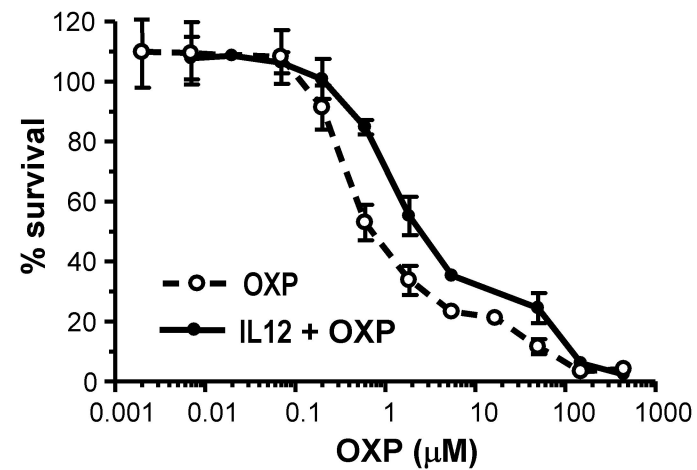
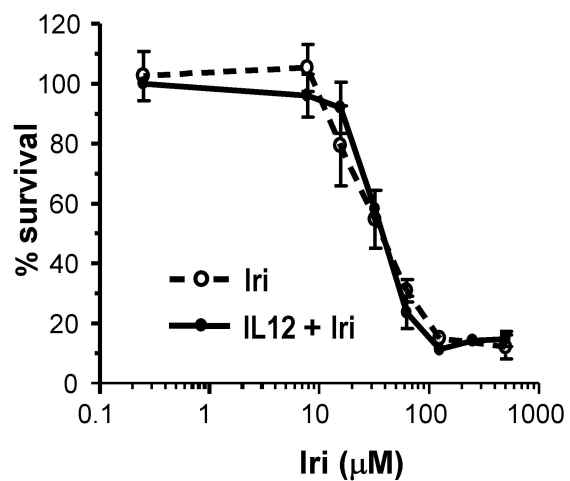
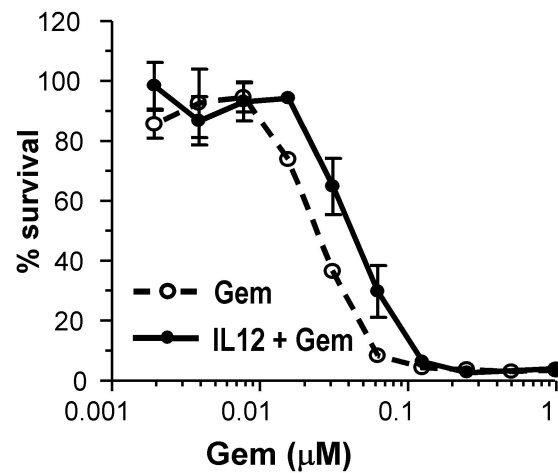
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A**B****C****D**

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