SUPPLEMENTARY MATERIALS AND METHODS

Mice

For tumor induction experiments, c-myc OVA mice were crossed with tTALAP transgenic mice to generate c-myc-OVA-tTALAP double transgenic mice (c-myc OVA tg+) [1] maintaining the presence of doxycycline in drinking water during pregnancy. Doxycycline was removed at birth, to induce the expression of the c-myc oncogene and ovalbumin in the liver. C-myc OVA tg+ mice were genotyped using genomic DNA from tail biopsies and the following primers were used: human-c-myc forward 5'-AGCTTGTACCTGCAGG-ATCTGAGC-3' and reverse 5'-ATCCAGACTCTGACCTTTTGCCAG-3' primers; OVA forward 5'-GATGTTTATTCGTTCAGCCTTGCC- 3' and reverse 5'-CAATCTGGTACATCATC-TGCACAGG-3' primers; and tTALAP forward 5'-CGCTGTGGGGCATTTTACTTT-AG-3' and reverse 5'-CATGTCCAGATCGAAATCGTC-3' primers.

Rag1$^{-/-}$ transgenic mice and T cell receptor transgenic mice specific for H-2K^{b} OVA$^{257-264}$ (OT-1) and H-2IA^{b} OVA$^{323-339}$ (OT-2) were purchased from Jackson Laboratories (Bar Harbor, Maine USA). C57Bl/6 mice were purchased from Harlan Laboratories (Udine, Italy). All animal procedures were conducted under institutional guidelines that comply with national laws and policies (study number 054/10).

Reagents and cell lines

EL-4 thymoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, USA). OVA transfected EL-4 cell line, EG-7, was a kind gift from Dr. Claude Leclerc (Institut Pasteur, Paris, France). MC38 was provided by

Dr. Karl E. Hellström (Seattle, USA). These cell lines were authenticated by Idexx Radil (Columbia, MO, USA. Case 6592-2012). Hepa 1.6 hepatoma cell line was a kind gift of Dr. Rubén Hernández (CIMA, Pamplona, Spain) and MC38OVA cell line was generated by lentiviral transfection of the cDNAs encoding *ova* and *egfp* (Sancho D. *et al*. manuscript in preparation) and provided by Dr. Sancho (Madrid, Spain). JMJ cells are described below.

All cell lines were maintained in complete mouse medium with RPMI 1640 with Glutamax (Gibco, Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated FBS (Sigma-Aldrich, Dorset, UK), 100 IU/mL penicillin and 100 mg/mL streptomycin (Biowhittaker, Walkersville, MD, USA) and $5x10^5$ mol/L 2-mercaptoethanol (Gibco).

For *in vivo* treatment experiments, the anti-CD137 mAb (clone 1D8) was provided from Bristol-Myers Squibb (Princeton, NJ, USA); anti-OX40 mAb (clone OX86) was provided by Dr. Mario Colombo (IRCCS, Milan, Italy); and anti-B7-H1 mAb (clone 10B5) was kindly gifted by Dr. Lieping Chen (Yale University School of Medicine, New Haven, CT, USA). The hybridoma cell lines anti-CD4 (GK 1.5) and anti-CD8β (H35-17-2) were obtained from ATCC. Monoclonal antibodies were produced and quality controlled as described [2].

$OVA^{257-264}$ peptide was aquired from NeoMPS (Strasbourg, France). Recombinant IFNγ was purchased from Miltenyi Biotec (Pozuelo de Alarcón, Spain) and recombinant human IL-2 was acquired from Novartis (Barcelona, Spain). Percoll was from Amersham Biosciences (GE Healthcare, Uppsala, Sweden). Doxycycline hyclate was purchased from Acofarma (Terrassa, Spain) and administered to mice in drinking water at 0.1% with 2.5% of sucrose (Panreac, Barcelona, Spain).

For c-myc OVA tg+ mice genotyping, Wizard genomic DNA purification kit (Promega, Alcobendas, Madrid, Spain) and Platinum Taq DNA Polymerase (Invitrogen, Alcobendas, Madrid, Spain) were used. dNTPs were from GE Healthcare, Uppsala, Sweden. To extract the RNA from cell lines and livers, RNase mini Kit from Quiagen (Madrid, Spain) was used according to manufacturer´s instructions. For RT-PCRs, DNase I, M-MLV RT, RNase OUT, DTT 0.1M and random primers were used, all from Invitrogen (Alcobendas, Madrid, Spain).

Adoptive T cell therapy and antibody treatment

Spleens from OT-1 and OT-2 transgenic mice were mechanically disrupted and cell suspensions were cultured with 5μg/ml of the cognate OVA peptides for 48h ($OVA^{257-264}$ and $OVA^{323-339}$, respectively). $2x10^6$ OT-1 cells and $2x10^6$ OT-2 cells were i.v. transferred to c-myc OVA tg+ mice on day 21 or 28 after birth, depending on the experiment. For survival experiments, a dose of $2x10^4$ IU of i.p. IL-2 was administered to mice transferred with activated OT-1 and OT-2 on day 21. For treatment with mAbs, Combo3 (100 μg of each anti-CD137 [3], anti-OX40 [2] and anti-B7-H1) or control rat IgG was administered i.p on the indicated days. For depletion experiments, 3 doses of 200μg of anti-CD4 or anti-CD8β mAbs were i.p. injected starting one day before Combo3 treatment onset and every three days. Efficacy of depletion was checked in sentry mice.

Tumor burden was monitored by Vevo 770 ultrasound system (Visualsonics, Toronto, Canada) equipped with a real time micro-visualization scan head probe (RMV-707B) and measurements were performed offline using dedicated Vevo770 quantification software (Vevo 770 v. 3.0.0).

Phenotypic analyses of tumor infiltrating lymphocytes

For analysis of tumor infiltrating lymphocytes, mice were treated with two doses of control rat IgG, the single immunostimulatory mAbs, possible doublets of the three mAbs or the Combo3 antibody combination and sacrificed on the indicated days. Livers were excised, weighted and enzymatically disrupted with DNase I and collagenase D (both from Roche, Madrid, Spain) for 15 minutes at 37ºC. To obtain unicellular cell suspensions, livers were mechanically disrupted and passed through a 70-µm cell strainer (BD Falcon, BD Bioscience, San Agustín de Guadalix, Spain) pressing with a plunger. To remove non-mononuclear cells, unicellular cell suspensions were pelleted, resuspended in a 35% Percoll gradient and centrifuged for 10 min, 500g at room temperature. Erythrocytes were lysed with ACK buffer (Gibco, NY, USA). Single cell suspensions were treated with FcR-Block in a PBS-based buffer containing 10% of FCS to avoid unspecific staining.

Flow cytometry and antibodies

Cells were stained with the following antibodies: CD3-APC, CD8-PECy7, CD8-PB, CD8-PE, CD4-PB, streptavidin-APC, H-2K^{b}-FITC, H-2D^{b}-FITC, H-2IA^{b}-FITC and anti-CD16/32 FcR-Block from BD Pharmingen (San Agustín de Guadalix, Spain); CD45.2-PerCPCy5.5, CD137-biotin, PD-1-FITC, OX40-PE, B7-H1-APC, granzyme B-AF647, FoxP3-AF647, CD107a-FITC, TRAIL-PE, FasL-PE, Rat IgG2a-FITC, Rat IgG2a-PE and mouse IgG1-AF647 from Biolegend (London, UK); and EpCAM-PE, Perforin-FITC, hamster-biotin, Rat IgG1-PE and Rat IgG2a-FITC from eBioscience (Barcelona, Spain). Cytofix/Cytoperm (BD Biosciences, San Agustín de Guadalix, Spain) was used

for granzyme B and perforin intracellular staining and anti-mouse/rat FoxP3 Staining Set for Foxp3 intracellular staining (Biolegend, London, UK).

FACS-Canto II and FACSCalibur (BD-Biosciences, San Agustín de Guadalix, Spain) were used for cell acquisition and data analysis was carried out using FlowJo software (Tree Star Inc).

Immunohistochemistry

3-week old healthy or c-myc OVA tg+ mice were sacrificed and livers excised and processed as described below. Mice treated with single or Combo3 mAbs were treated on day 28 and sacrificed on day 34. Liver fragments were formalin-fixed for 24h and embedded in paraffin. 2 µm paraffin sections were deparaffinized and rehydrated by the following series at room temperature: 10 min. xylol; 2 min. absolute alcohol (2 times); 2 min. 96% alcohol (2 times); 2 min. 90% alcohol; 2 min. 80% alcohol; 2 min. 70% alcohol; 2 min. aqua dest. (2 times). Slides were pretreated 20min at 95°C using heat-mediated epitope retrieval with sodium citrate buffer pH6 for 20 minutes; incubation with the primary antibody was at room temperature for 15 minutes using monoclonal rabbit anti mouse CD3 (SP7, DCS, Hamburg) at 1:100 dilution. An enzyme-polymer system (Medac, Wedel Germany) was used for sensitive detection with polymer-HRP (Medac, Germany) as a chromogen. Sections were counterstained by incubation in Mayer's haemalaun for 5 minutes. Negative-controls were included in each staining series omitting the primary antibody. CD3 was exclusively expressed in a membranous staining pattern. Tunel stains were performed using the ApopTag in situ apoptosis kit (Chemicon, Nürnberg, Germany) according to manufacturer's conditions. For quantitative analysis of T-cells in situ: the number of CD3 positive cells per mm^2 tumor area was

assessed using a Carl Zeiss objective and an automatic slide scanner (3D Histech midi) and the accompanying software histoquant 14.1 (3D Histech, Budapest, Hungary). Data were expressed as the mean ± standard deviation.

JMJ cell lines, generation and characterization

A bulky tumor-bearing mouse was sacrificed and tumor liver was finely minced with a scalpel blade. Small tumor pieces were then disrupted with GentleMACS Dissociator (Miltenyi Biotec, Pozuelo de Alarcón, Spain) in a medium containing Dispase II (Roche, Madrid, Spain), Collagenase I (Sigma-Aldrich, Madrid, Spain) and DNase I (Calbiochem, Darmstadt, Germanny) according to manufacturer's instructions. Resulting tissular material in suspension was cultured in flasks precoated with collagen type I (BD Biosciences, San Agustín de Guadalix, Spain) at 50 μg/ml in 0.02N acetic acid. Primary cell cultures were maintained in complete mouse medium. When 80% of confluence was reached, cells were sequentially passed to flasks without collagen I (BD Biosciences, San Agustín de Guadalix, Spain) precoating, Two hepatocarcinoma-derived variants of JMJ (JMJ7 and JMJ9) were routinely used because of adaptation and growth in culture.

JMJ7 and JMJ9 cells RNA, as well as tumoral and healthy liver-extracted RNA, were used to perform RT-PCR analyses of β-actin (forward 5'-CGCGTCCACCCGCGAG-3' and reverse 5'-CCTGGTGCCTAGGGCG-3'), albumin (forward 5'- GAAGTGGGTAACCTTTCTCC-3' and reverse 5'-ACAGCAGTCAGCCAGTTCACC-3'), HNF4 (forward 5'-CTGCACCCTCACCTGATGC-3' and reverse 5'- GGCTGGGGGATGGCAGAG -3'), OVA and human-c-myc (both described previously). JMJ7 and JMJ9 cells

cultured with or without recombinant IFNγ at 1000 U/ml for 48h were subjected to flow cytometry to assess surface expression of EpCAM, B7-H1, H-2K^b/D^b and H-2IA^b.

Cytotoxic activity of activated OT-1 T cells against JMJ9 cells (precultured with or without recombinant IFNγ at 1000 U/ml for 48h) was measured by the standard sodium [^{51}Cr] release assay. Splenocytes retrieved from OT-1 transgenic mice were activated with the cognate SIINFEKL peptide at 5μg/ml for 48h. JMJ9 or control EL-4 and EG-7 target cells were labeled with 50 μCi [^{51}Cr] (PerkinElmer, Waltman, MA, USA) for 1 h at 37°C, then washed three times and resuspended in complete mouse medium. 3x10^3 target cells were cocultured with activated OT-1 T cells at indicated ratios for 5h.

Release of [^{51}Cr] into the supernatant was quantified with a microplate scintillation counter (Packard TopCount; PerkinElmer, Waltman, MA, USA). The percentage of cytotoxicity was calculated as the percentage of [^{51}Cr] released using the following equation: (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100.

JMJ7 and JMJ9 culture images were taken with an inverted microscope ECLIPSE Ti-U (Nikon, Barcelona, Spain) equipped with a CCD digital camera Orca ER (Hamamatsu, Massy, France) and the NIS-Elements 3.0 AR software (Nikon).

JMJ and HCC tumor fragments growth in vivo

5x10^5 or 1x10^6 JMJ7 or JMJ9 cells were injected s.c. with or without matrigel into WT or *Rag1*$^{-/-}$ mice. Tumor growth was monitored up to 3 months. 5x5 mm (approximately) HCC tumor fragments were initially implanted s.c. into *Rag1*$^{-/-}$

mice and passed sequentially to other WT or *Rag1*$^{-/-}$ mice. For experiments with HCC tumor pieces in *Rag1*$^{-/-}$ mice, a previously implanted *Rag1*$^{-/-}$ mouse was sacrificed and the tumor was excised and cut into small 5x5 mm tumor. These tumor fragments were implanted into other *Rag1*$^{-/-}$ mice. HCC bearing *Rag1*$^{-/-}$ mice were treated with a single dose of i.v. $5x10^6$ naïve WT splenocytes and i.p. control rat IgG or Combo3 antibodies on day 23 after implantation. Tumor growth was monitored every 3-4 days.

In vivo killing experiments

For syngeneic WT mice immunization, 5x5 mm HCC tumor fragments were surgically implanted s.c. Mice were treated as indicated in each experiment. Splenocytes from WT mice were recovered and divided into two samples. One sample was pulsed with the $OVA^{257–264}$ peptide (10 μg/mL) for 30 min at 37ºC in 5% CO_2, washed extensively, and subsequently labeled with high concentration 1.25 μM of CFSE (Sigma, Madrid, Spain). The non-pulsed control sample was labeled with 0.125 μM of CFSE. Then, both $CFSE^{high}$- and $CFSE^{low}$-labeled cells were mixed at a 1:1 ratio and injected i.v. into tumor-bearing mice. 24 h after transfer spleens were harvested and specific cytotoxicity was analyzed by flow cytometry. Specific cytotoxicity was calculated as follows: 100–[100x(%$CFSE^{high}$ tumor-bearing mice/%$CFSE^{low}$ tumor-bearing mice)/(%$CFSE^{high}$ control mice/%$CFSE^{low}$ control mice)]. As control mice, we used naïve WT mice (in figure 5C) and rat IgG treated c-myc OVA tg+ mice (in figure 5D).

In vitro degranulation and IFNγ production assay

TILs were isolated as described above. 10^4 JMJ7 cells were incubated with IFNγ (10^3 U/ml) for 48h in a P96 plate. Then, cells were washed and cocultured with TILs for 5h in the presence of anti-CD107a antibody (1:100 v/v), 2 μg/ml monensin (Sigma-Aldrich, Dorset, UK) and 10 μg/ml brefeldin A (Sigma-Aldrich, Dorset, UK). $CD107a^+$ cells were quantified by flow cytometry. For IFNγ production assay, JMJ7 cells or MC38 cell line preincubated with IFNγ for 48h were irradiated with 30.000 rads in a Nordion Gammacell 3000 irradiator (Nordion, Ottawwa, Canada). 10^4 irradiated JMJ7 or MC38 cells were incubated with TILs at a 1:100 ratio (target:effector) for 72h and IFNγ in the supernatant was measured with Mouse IFN-γ (AN-18) ELISA Set (BD Biosciences, San Agustín de Guadalix, Spain). To test $CD8^+$ TILs and splenocytes reactivity against JMJ7, JMJ9, MC38 and MC38OVA, $CD8^+$ T cells were immunomagnetically purified with CD8α MicroBeads (Miltenyi Biotec, Pozuelo de Alarcón, Spain) according to manufacturer´s instructions. Restimulation experiments were performed incubating 150.000 $CD8^+$ T cells with 10^4 irradiated target cells and IFNγ in the supernatant was measured 72h after coculture. PMA (100ng/ml) and Ionomycin (1 μg/ml) (both from Sigma-Aldrich, Dorset, UK) were used to stimulate T cells as positive control.

For restimulation with human-c-myc, OVA and tTA derived peptides, TIL cellular suspension was incubated with at 20 μg/ml of each peptide for 72h to measure IFNγ in the supernatant.

Mouse H-$2K^b$ and H-$2D^b$ binding putative peptides were predicted *in silico* by the artificial neural network (ANN) algorithm provided by the Immune Epitope Database and Analysis Resource. Peptides were synthesized manually in a

multiple-peptide synthesizer using 9-fluorenylmethoxy carbonyl (Fmoc chemistry) [4]. The ninhydrin test of Kaiser was used to monitor every step. At the end of the synthesis they were cleaved and deprotected with trifluoroacetic acid and washed with diethyl ether. Purity of the peptides was always above 90%.

Statistical analysis

Prism software (Graph Pad Software, La Jolla, CA, USA) was used to analyze tumor infiltrating lymphocytes, specific lysis and IFNγ production between groups by applying unpaired Student's t-tests or U-Mann–Whitney tests. Survival curves were analyzed by Kaplan-Meyer plots and log rank tests. P-values < 0.05 were considered significant.

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