**Myeloid derived suppressive cell expansion modulates melanoma growth and severity of autoimmunity by inhibiting CD40/IL-27 regulation in** **macrophages**

Julio C. Valencia1, Rebecca A. Erwin-Cohen1, Paul E. Clavijo2, Clint Allen2, Michael E. Sanford1, Chi-Ping Day6, Megan Hess1, Morgan Johnson1, Jie Yin1, John M. Fenimore1, Ian Bettencourt1, Koichi Tsuneyama3, Maria E. Romero4, Kimberly Klarmann1, Peng Jiang7, Heekyong R. Bae1, Daniel W. McVicar1, Glenn Merlino6, Elijah F. Edmondson5, Niroshana Anandasabapathy8 and Howard A. Young1

**SUPPLEMENTAL MATERIALS AND METHODS**

**Mice**

Mice aged between 18 – 22 weeks old, both male and female C57BL/6J WT, type 1-IFNAR sufficient ARE (ARE+/- and ARE-/-), type 1 IFNAR-deficient ARE (ifnar-/-ARE+/- and ifnar-/-ARE-/-), *Ifnar*-/- , *stat1*-/-, IL-27ra-/-, and *ifngr*-/- were bred and housed in the animal facility at the National Cancer Institute (NCI) at Frederick, Maryland. Type 1 IFNAR-deficient ARE (IFNg/IFNAR1Bactin Cre) mice were generated by cross breeding ARE (IFN-gamma Cre 1821) and ifnar-/- (B6.Cg.IFNAR1*tm1.2Eees*) as reported (1, 2). All animal care and procedures were approved by and performed in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council, Washington, D.C.) and the NCI Animal Care and use committee at NCI-Frederick (ACUC).

**Mice genotyping**

All mice were genotyped twice, first at 3-4 weeks of age and then at end of experiments to confirm their respective phenotypes by extracting DNA from tail clippings. Genotyping for ARE and *ifnar*-/-ARE mice was performed as described before (1, 2), with modifications. Briefly, the following primers were used for mouse genotyping: For ARE mice, common ARE forward: 5’-GTCAACAACCCACAGGTCCA-3’, WT ARE Reverse: 5’-ATTTAAAAATTCAAATAGTGCTGGC-3’, ARE-/- Reverse: 5’- CCGCGGTGGTACCATAACTT-3’. PCR conditions for WT: 94 °C for 2 min, 94 °C for 15 s, 55 °C for 15 s, 68 °C for 15 sec, repeat 35 cycles. For ARE-/- primers, 94 °C for 2 min, 94 °C for 15 s, 60 °C for 15 s, 55 °C for 15 sec, repeat 35 cycles. For *ifnar*-/- mice genotyping, Neo3a forward, 5’- GACCACCAAGCGAAACATCG -3’; Neo3a reverse, 5’-TAGAAGGCGATGCGCTGC-3’; Ifnar1 forward, 5’- GGCGAAGTGGTTAAAAGTGC -3’; Ifnar1 reverse, 5’- TCCACGAAGATGTGCTGTTC-3’. PCR conditions for *ifnar* primers, 94 °C for 3 min, 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 sec, repeat 30 cycles.

**Murine melanoma cell lines**

The syngeneic B16F10, HcMel1274, MEL114433, and B2905 murine melanoma cell lines were kind gifts from J. Weiss (NCI-Frederick, Frederick, MD), R. El Meskini (NCI-Frederick, Frederick, MD), and Dr. G. Merlino at NCI, Bethesda, MD), respectively. The genetic background of HcMel1274, MEL114433, and B2905 cells have been reported separately (3). All cell lines used in this study were PCR tested for mycoplasma and declared mycoplasma free. Molecular Testing of Biological Materials (MTBM) and infected agents at the Animal Health Diagnostic Laboratory (AHDL; NCI-Frederick) confirmed all cell lines were negative for other rodent pathogens before use for *in vivo* experiments. All mouse melanoma cells were cultured at 37 C in a humidified incubator with 5% CO2. Dulbecco's modified Eagle's medium (DMEM; Corning, Corning, NY) was used for B16F1, B16F10, and B2905 cells. Roswell Park Memorial Institute (RPMI; Corning) 1640 was used for HcMel1274 and MEL114433. All media were supplemented with 10% fetal bovine serum (FBS; Omega Scientific Inc. Tarzana, CA) (v/v), 100 U/ml penicillin, 100 ng/ml streptomycin (Gibco, Gaithersburg, MD), and 2 mM L-glutamine (Gibco, Gaithersburg, MD).

**Primary and immortalized bone marrow derived myeloid cells**

Primary bone-marrow cells were flushed from femurs of mice and cultured in “macrophage media” that consist of Dulbecco’s Modification of Eagle’s medium (DMEM; Corning), 10% FBS (Omega Scientific Inc), 2 mM L-glutamine and penicillin-streptomycin antibiotics (Gibco), 5nM NEA-MEM, 1 mM sodium pyruvate (GIBCO), 10 mM HEPES, 50 micromolar 2-mercaptoethanol (2-ME) and supplemented with either recombinant mouse 10 ng/ml of macrophage-colony stimulating factor (M-CSF; Peprotech) or granulocyte macrophage-colony stimulating factor (GM-CSF; Peprotech, Cranbury, NJ) for 5 to 7 days. Immortalized macrophage cell lines were established by infecting primary bone marrow cells with the J2 recombinant retrovirus as described previously (4). The packaging cell line CREJ2 was adapted from the parental J2 packaging line. CREJ2 contains the murine retroviral ecotropic coat protein, and the virus produced is replication defective. Single cell suspensions from bone marrow were prepared as described (5). Freshly isolated cells were centrifuged through a lymphocyte separation medium cushion (ICN Biochemicals, Aurora, OH). Cells were co-cultured with 0.45 micron-filtered CREJ2 supernatants in DMEM supplemented with 10% heat-inactivated FBS, Penicillin-streptomycin, 2 mM L-glutamine (complete medium) (BioSource International, Camarillo, CA), 5 mg/ml hexadimethrine bromide (Sigma-Aldrich Chemical Co., St. Louis, MO) and 1000 units/ml GM-CSF (Peprotech) for 24 h. Non-adherent cells were removed from cultures and adherent cells were cultured in complete medium with 1000 units/ml GM-CSF and without hexadimethrine bromide. After 5 to 7 days, cells were cultured in complete medium without GM-CSF and monitored for growth. Cells growing in the absence of GM-CSF were considered immortalized. Infectivity of resulting immortalized cells was tested using the LiNCAP cell line containing a green-fluorescent indicator to detect exogenous retrovirus sequence elements from XMRV, XMLV, and A-MLV (kind gift from Dr. V. K. Ramani at NCI-Frederick, MD). Resulting macrophage cell lines from WT or ARE-/- mice were designated either “WTM” or “KOM”, respectively. Cells weregrown in DMEM (Corning) supplemented with 10% FBS and 1X penicillin-streptomycin, and 2mM glutamine. Cells were stimulated with recombinant murine IFNg (IFNg, Peprotech, Cranbury, NJ) at concentrations ranging from 2 IU/ml to 100 IU/ml for short (10 minutes up to 18h) or long (up to 12 days) time periods; 25 – 50 ng/ml of murine recombinant IL-27 for 18h (R&D systems, Minneapolis, MN); or their combination for 18 h.

**Tumor induction and monitoring**

Eighteen to twenty two-week -old WT, type 1-IFNAR sufficient ARE (ARE+/- and ARE-/-), type 1 IFNAR-deficient ARE (ifnar-/-ARE+/- and ifnar-/-ARE-/-) mice, and *Ifnar*-/- mice were randomly selected and challenged subcutaneous (SQ) or intravenous (IV) with 5 X 105 B16F10 and HcMel1274 cells suspended in HBSS. However, mice were only challenged SQ with B2905 and M114433 cells. For SQ (right flank) measurements, tumor volumes were measured with calipers twice weekly and tumor volume was calculated using the following formula: Volume= length x width^2/2 (mm2). At study end point or when animals appeared moribund, mice were humanly euthanized either for targeted tissue collection or for full necropsy. Full necropsy studies included harvest of tumors, spleen, liver, kidneys, blood, and serum samples were performed at the Laboratory of pathology (PHL, NCI-Frederick, MD). All tissues were examined blind either by board certified veterinary pathologists (EFE) or board-certified clinical pathologists (KT, MER). All procedures involving animals were approved by the NCI Animal Care and use committee at NCI-Frederick (ACUC).

**Antibody Cell depletion, cytokine blockade or stimulation, and single or combined therapy treatment**

For immune depletion of PMN, we used the Ultra-LEAF anti-mouse blocking mAb against Ly6G (Clone 1A8, Biolegend, San Diego, CA). The anti-Ly6G mAb was administered intra-peritoneal (i.p) twice weekly at the doses of 200 ug per mouse starting 10 days prior to SQ injection of 5 X 105 B16F10 cells. For blockade of IFNg, animals were treated with an anti-IFNg antibody (XMG-6) or an isotype control IgG (GL-113, kind gifts of Dr. G. Trinchieri, NCI, Bethesda, MD) i.p. starting 2 days after inoculation of B16F10 via tail vein at a dose of 0.25 mg twice a week for 14 days. Every animal received a total dose of 0.5 mg/ip/per week. For blockade of IL-27, animals were treated either with a mAb against IL-27p28 (Clone MM27-7B1, Biolegend, San Diego, CA) or rat IgG2A isotype control (Bioxcell, Lebanon, NH) i.p twice weekly at the dose of 20 ug per mouse starting 7 days after SQ. B16F10 challenge. For exogenous administration of rmIL-27, animals received rmIL-27 (100 ng per animal; carrier-free catalog # 577404; Biolegend) or isotype IgG2a (Bioxcell) i.p. every two days for 2 weeks. For single or combined immunotherapy, mouse agonist mAb against CD40 (Clone FGK115B3; MTMB-M-1083-4) and the anti-mouse PD1 (clone RPMI-14; MTM-M-R-587-1) were purified antibodies received as kind gifts from Dr. J. Weiss (NCI-Frederick, MD). Rat IgG2A antibodies from either Bioxcell (Lebanon, NH) or Sigma Aldrich (St. Louis, MI) were used as isotype controls. These antibodies were administered twice weekly at a dose of 0.25 mg i.p. starting 7 days after SQ injection of 5 X 105 B16F10 cells in HBSS at the right flank of female WT or ARE+/- mice. Tumor growth was measured twice a week with calipers as indicated above.

**Immunoblotting**

Pellets from murine cultured cell lines and mouse tissues were lysed in M-PER mammalian Protein Extraction Reagent (Thermo Scientific #78501), protease inhibitor (Thermo Scientific #87786), and phosphatase inhibitor (Thermo Scientific #78420), followed by incubation on ice for 30 minutes. Insoluble components were removed by centrifugation. Protein concentration was determined by a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific #23225). Proteins (15-50 ug per lane) were separated under reducing conditions by electrophoresis using either 10% or 4-12% NuPAGE Bis-Tris mini-gels (Invitrogen) and then transferred electrophoretically to PVDF membranes (EMD Millipore Corp #IPVH00010). The membranes were incubated at room temperature for 1 hour in blocking buffer (Thermo Scientific #37532) followed by the addition of the following antibodies: anti-Stat-1 (Cell Signaling Technology #9172), anti-Phospho-Stat-1 (Cell Signaling Technology #9167 or BD Transduction Labs #612132), anti-Stat-3 (Cell Signaling #9139), anti-Phospho-Stat-3 (Cell Signaling Technology #9145), anti-PD-1 (Abcam ab58811), anti-PD-L1 (R&D Systems MAB1019), anti-CD-40 (Santa Cruz sc-975), anti-arginase 1(Santa Cruz sc-20150), anti-iNOS (Novus Biologicals NB300-605), anti-Chitinase 3 like protein 3 (Abcam ab93034), anti-GAPDH (Novus Biologicals NB300-221) anti beta-tubulin (Sigma-Aldrich T4026), and anti-actin (Santa Cruz sc-1616). After incubation at 4°C overnight, membranes were washed three times for 5 minutes with TBS-T (Teknova #T9511) and incubated at room temperature for 1 hour with peroxidase-labeled secondary antibodies (GE Healthcare Life Sciences NA934V or NXA931 and Santa Cruz sc-2032 or sc-2033). Blots were washed three times for 5 minutes with TBS-T and protein bands were visualized using enhanced chemiluminescence (Thermo Scientific #34080). For re-probing membranes, antibodies bound to the membrane were removed using One-Minute Western Blot Stripping Buffer (GM Biosciences #GM6001).

**Multiplex measurement of inflammatory factors**

Serum samples from 20 ± 4-week-old female mice were collected from experimental animals in serum-separator tubes, processed within one hour of collection, and subsequently frozen at -80 oC until use. Serum factors were measured using two multiplex methods, luminescence (Luminex) and electro chemiluminescence assays - Meso-Scale Discovery (MSD). For Luminex assays, serum factors were measured using the Mouse Cytokine Array/Chemokine Array 32-Plex (Eve Technologies, Calgary, Canada). The array consisted of 32 targets: eotaxin, G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC (keratinocyte chemoattractant), LIF (leukemia inhibitory factor), LIX (LPS-induced CXC chemokine), MCP-1 (monocyte chemoattractant protein-1), M-CSF (macrophage colony-stimulating factor), MIG (monokine induced by IFNg), MIP-1α (macrophage inflammatory protein 1 alpha), MIP-1β, MIP-2, RANTES, TNFα, and VEGF. The levels of each cytokine and chemokine were determined using known standards for mice. Pooled data were reported in pg/ml from two independent experiments (n=10, mice / group).

For MSD profiling, we used custom murine multiplex kits to measure analytes in either a 10-plex format (IFN-g, IL-1β, IL-6, IL-10, IL-12p70, IL-27p28/IL-30, IP-10, MIP-1β, MIP-2, and TNF-α; Catalog no. K15069L-2) or 29-Plex format divided in 3 plates: cytokine plate (IL-9, MCP-1,IL-33, IL-27p28/IL-30, IL-15, IL-17A/F, MIP-1α, IP-10 and MIP-2), pro-inflammatory plate (IFN-g, IL-1β,IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF-α) and a Th17 plate (MIP-3α, IL-22, IL-23, IL-17C, IL-31, IL-21, IL-17F, IL-16, IL-17A, IL-17E/IL-25). Samples were tested at least in duplicate and read on MSD QuickPlex SQ 120 imager. Raw signals generated by the instrument were analyzed using Discovery Workbench 4.0 Software, followed by statistical analysis in GraphPad Prism (v.8.01) for Windows (GraphPad, San Diego, CA). Assays performed in duplicate, or triplicate were averaged for analysis. Outlier data points were identified using the ROUT method with False Discovery Rate less than 1% (Motulsky & Brown, 2006). All cytokine values were reported in units of pg/mL, while phosho-STAT values were reported in signal intensity units that represent tyrosine phosphorylation status. Data were show as mean ± SEM.

**Analysis of IFN sensitivity of Melanoma cells by flow cytometry and Matrigel 3-D growth assay**

Cultured melanoma cells were harvested at 80% confluence and were automatically counted, and viability obtained with a Cellometer Auto T4 cell counter (Nexcelom, Lawrence, MA). For measurement of phosphorylated STAT-1, melanoma cells were placed in polystyrene round tubes at a density of 1 x 106 per ml in the presence or absence of 100 UI/ml of recombinant murine IFNg for 10 minutes, then washed with cold PBS. After fixing in 2% paraformaldehyde, cells were vortexed in ice-cold 90% methanol and then incubated for 30 to 60 minutes in dark at 4°C and washed twice with 500 l of FACS buffer (1X PBS w/0.1% sodium azide, 0.1% BSA) before incubation with AF647-Anti-Stat1 (pY701) Clone 4a or AF647-stat1 clone 1 (BD Biosciences) for 30-60 minutes at 4°C. flow cytometry experiments were performed using a BD LSRII Fortessa (BD Biosciences) analyzer with two lasers (excitation 488nm and 647nm) and 7 fluorescent detectors. For 3-D spheroid cell invasion, we used the Cultrex 3D Culture Spheroid Cell Invasion Assay (Trevigen, Gaithersburg, MD) following manufacturer’s instructions. Briefly, melanoma cells were resuspended in 1X Spheroid formation extra cellular matrix (ECM) at a density of 3X103 per well of the 3D Culture Qualified 96 Well Spheroid Formation Plate (Trevigen, Gaithersburg, MD). After centrifugation in a swinging bucket rotor, plates were incubated at 37 °C in a tissue culture incubator for 72 hours to promote spheroid formation. Invasion Matrix was thawed on ice overnight at 4°C for 24 h prior to the experiment. Working on ice, the invasion matrix was added to wells of the 3D culture qualified 96 Well Spheroid Formation Plate. Plates were centrifuged at 300xg for 5 minutes in a swinging bucket rotor to eliminate bubbles and position spheroids within the Invasion Matrix. Plates were incubated at 37 ̊C for 1 hour to promote gel formation. Then, cell culture medium alone or with 100 IU/ml of rIFNg was added to wells and plates were incubated at 37 °C in a tissue culture incubator for 3 to 6 days. Photographs of the spheroids in each well were taken every 24 h at 4X magnification using the manual mode of the Cytation 5 cell imaging reader (Biotek, Winooski, VT) and Gen5 software (Biotech). Tumor size was estimated from full images using image J software (http://rsb.info.nih.gov/ij/; NIH, Bethesda, MD).

**Flow Cytometry processing and staining**

Spleen single-cell preparations were obtained as reported previously (2). Briefly, spleens were mechanically disrupted through a filtra-bag mesh (Fisher Scientific) in 10 ml (DMEM). For tumor single-cell suspension, the mouse tumor dissociation kit (Myltenyi Biotec, San Diego, CA) was used according to manufacturer’s instructions. Briefly, tumors were cut into 2- 4 mm pieces and digested with DMEM medium supplemented with a mixture of enzymes (D, R, and A) at 37C for 40 m. Cells counts were determined from the disrupted tissues using a Sysmex KX-21 (Roche) automated cell counter. Cell suspensions were passed through a 70 -uM cell strainers to obtain single-cell suspensions. Blood and single-cells suspensions were resuspended in 5 ml or ACK red-lysis buffer (GIBCO) and lysed at RT for 5 minutes. Cell suspensions were then washed in PBS, centrifuged, and resuspended in 1 ml of FACS buffer (1X PBS with 0.5%FBS and 0.05% sodium azide). Single-cell suspensions were incubated either with 0.5% BSA (nuclear staining) or with 0.5 ug mouse CD16/32 mAb Fc block 2.4G2 (surface staining, BD biosciences or Harlan Bioproducts) for 10 min at RT. For surface staining, cells were incubated with antibodies in the dark for 30 min at 4°C and then fixed with 2% paraformaldehyde for 10 minutes at RT. The following mouse surface markers were used: FITC-CD11b clone M1/70 (BD Biosciences), AF488-CD45.2 (Biolegend); APC-Cy7 CD45.2 clone 104 (Biolegend); PE-Cy7-CD11b clone M1/70 (Biolegend); eFlour450- Ly6G (Gr-1, eBiosciences); PerCP-cyanine 5.5-CD19 clone 1D3 (eBiosciences); EF605NC-CD19 clone eBio1D3 (eBioscience); AlexaFluor 700-CD11c clone N418 (eBiosciences); APC-MHC II clone M5/114.15.2 (IA/II-E, eBiosciences); BV650- IA/IE clone M5/114.15.2 (MHC class II, Biolegend); FITC-F4/80 clone BM8 (eBioscience); APC-EF 780-CD4 clone RM4-5 (eBioscience); PE-CD4 clone RM4-4 (Biolegend); APC-CD8 clone 53-6.7 (BD Bioscience); BUV395-CD274 Clone MIH5 (BD Biosciences); PE- CD8a clone 53-1.7 (Biolegend); PE-CD40 clone 3/23 (Biolegend); PE-Dazzle 594-CD40 (Biolegend); PE-IL-27R clone 2918 (BD Bioscience); PE-IL-27p28 clone MM27-7B1 (Biolegend); PE-IL-27ra clone 2918 (BD Pharmigen); EF450-CD3e clone 17A2 (eBioscience); CD3 clone 17A2 (BioLegend), CD4 clone RM4-5 (Biolegend), CD44 clone IM7 (Biolegend), CD62L clone MEL-14 (BioLegend), NK1.1 clone PK136 (Biolegend), and TCR clone H57-597 (Biolegend). Cell viability was assessed by incubation with the fixable Viability Dye eFluor 506 (Invitrogen) or Zombie Aqua (Biolegend, San Diego, CA) for 30 minutes in the dark at room temperature (RT). For the intracellular detection of the Foxp3/Transcription Factor in splenocytes after surface staining, we used a standard staining buffer set (eBioscience) for 30 minutes on ice and the antibodies against anti-Foxp3 (FJK-16s; eBioscience) for 20 minutes on ice. All staining preparations for flow cytometry experiments were performed using a BD LSRII Fortessa (BD Biosciences) analyzer with two lasers (excitation 488nm and 647nm) and 7 fluorescent detectors. Live cell enrichment of fluorescently labelled melanoma cells and macrophages was performed using the BD FACSAria II SORP cell sorter with four lasers (excitation lines at 488nm, 647nm, 405nm and 561nm), 14 fluorescent detectors, high speed four-way bulk sorting and single cell deposition sorting. FACS sorters were located at the Flow Cytometry Core Facility at NCI-Frederick, MD or at the flow facility at NIDCD, Bethesda, MD. The geometric mean fluorescence intensity (MFI) was used to compare the expression levels of cell surface receptors and proteins among mice genotypes with or without indicated interventions. Florescence minus one (FMO) controls were used for gating analysis as reported previously (6). Compensation was performed using single color controls prepared with UltraComp eBeads (eBioscience) for cell surface staining or Aqua live/dead discrimination. The gaiting strategy for myeloid cell identification and expression analysis is shown in Supplementary Fig. S2D. All data collection and sorting were performed using BD FACS Diva Software (BD biosciences) and data analyses were performed using FlowJo 10 software (Tree Star, Ashland, CO).

**Purification of Ly6Chi monocytes and *Ex vivo* stimulation**

Single-cell suspensions of naïve blood or BM were processed for flow cytometry analysis as indicated above and incubated with mixtures of monoclonal antibodies (mAbs) containing anti-FcIII/II, mouse serum, rat IgG (Jackson Immunoresearch), and 7-AAD viability staining solution (eBioscience) for 15 minutes on ice. Purified Ly6Chi monocytes were cultured in complete RPMI at a concentration of 150,000 cells/mL (for monocytes derived from BM) or 30,000 cells/mL (for monocytes derived from blood) in a 96-well round-bottom tissue culture plate. In some experiments, cells were incubated with 10 IU/ml mouse recombinant IFN- (Peprotech) for 6 hours and washed prior to stimulation with LPS. After staining and stimulation, cell suspensions were washed, filtered, resuspended in complete medium without phenol red and sorted on a BD FACS Aria II.

***MDSC inhibition of T cell proliferation***

Spleens from WT, ARE, ifnar-/-ARE+/-, ifnar-/-ARE-/-, and ifnar-/- female mice were processed into single-cell suspension and assessed for accumulation of live CD45.2+CD11b+Ly6GlowLy6Chi cells as described for flow cytometry. Expression of TGF-b and PD-L1 on these cells was quantified by MFI. For functional T cell proliferation assays, spleen or BM Ly6Ghi cells were sorted by positive magnetic selection (AutoMACS Pro, Miltenyi) and assessed for ability to suppress the proliferation of CFSE-labelled naïve T cells stimulated with CD3 and CD28 mAbs. Percent inhibition of T cell proliferation was quantified for CD8+ and CD4+ T cells co-cultured with Ly6Ghi cells sorted from the spleen or whole BM using a weighted formula that quantified the percentage of T cells within each daughter population.

**RNA preparation, Nano String data and quantitative RT-PCR**

Protocols for messenger RNA (mRNA) preparation, library construction, and nano string sample processing were reported previously (2, 7). Briefly, tissues were excised from ARE mice immediately upon euthanasia and frozen in liquid nitrogen. Tissues were grinding with a pestle and mortar chilled with liquid nitrogen and then transferred into a 15 ml Falcon tube. RNA from tissues was extracted with Trizol per manufacturer’s instructions and dissolved in DEPC treated water. For cultured cells, total RNA was isolated with the RNeasy plus kit following manufacturer’s instructions (Qiagen, Valencia, CA) and cDNA was amplified using the SuperScript III First-Strand Synthesis System for RT (Invitrogen). RNA qualities and DNA measures were analyzed using Nanodrop ND-1000 spectrophotometer (Thermo Fisher). All validated gene target TaqMan probes were processed using TaqMan universal Master Mix II no UNG (Invitrogen) according to manufacturer’s instructions. The following TaqMan (Applied biosystems) gene probes were used: IFNg (Mm01168134\_m1), IL-27(Mm00461162\_m1), IL-27ra (Mm00497259\_m1), CD40 (Mm00441891\_m1), NOS2 (Mm00440502\_m1), and ARG1 (Mm00475988\_m1). HPRT1 (Mm03024075\_m1) or Beta-actin (Mm02619580\_m1) was used to normalize gene expression and measure fold change differences among treatments and groups. Quantitative PCR was performed on a Light Cycler 480 (Roche) or GeneAMP 7500 (Applied Biosystems) systems.

**Histology and immunohistochemical analysis**

Mice underwent full necropsy and tissues, blood, and serum were collected for clinical and anatomic pathology assessment. For fixation, tissues were placed either in 10% neutralized buffer formalin (NBF) for 72 hours at room temperature or 2% paraformaldehyde for 12–16 h at 4 °C. Then, samples were embedded in paraffin and 5 – 8 um-thick serial sections were cut. For frozen tissue blocks embedded in Tissue Tek O.C.T (Sakura Finetek) under dry ice, blocks were mounted on a MicroM HM550 cryostat (MICROM International GmbH) and 5–8-μm-thick sections were cut. Sections were transferred to positively charged slides or Superfrost/Plus adhesive slides (Fisher brand). Representative sections were stained with hematoxylin and eosin stain and aniline blue stain following protocols at the Molecular Histopathology Laboratory, NCI-Frederick, MD, USA.

For FFPE sections, IHC staining was performed on Leica Biosystems’ BondMax autostainer with heat induced epitope retrieval with EDTA 10’ and CD31 / PECAM-1 (Abcam ab28364, rabbit polyclonal) at 1:100 60’ and the Bond Polymer Refine Detection Kit (Leica Biosystems #DS9800) with omission of the Post-primary reagent. For MHCII (D.R, 1:10; BD Biosciences, San Jose, CA), Ly6G/Gr1 (D.R: 1:100; Origene, Rockville, MD), F4/80 (Clone BM8, D.R. 1:200; eBioscience/Thermo-Fisher, Waltham, MA), CD206 (D.R. 1:200; Abcam, Cambridge, MA), NOS (clone ON4C, D.R. 1:200; Abcam) immunohistochemistry, epitope retrieval was performed with citrate buffer for 10-20’ at 100°C or proteinase K (Dako/ Agilent, Santa Clara, CA) for 5 min at RT. All sections were incubated overnight at 4C. Isotype control reagents were used in place of the primary antibodies for negative controls. For acetone-fixed frozen sections, sections were rinsed with PBS and incubated for 60’ with biotinylated CD4 (BD Bioscience), diluted 1:50; CD8a (BD Bioscience), diluted 1:50; F4/80 (eBioscience #14-4801), diluted 1:400. A biotinylated rabbit anti-rat IgG, mouse adsorbed (Vector Labs) was then applied for 30’. Following an endogenous peroxidase block with 0.6 % H2O2/methanol, sections were rinsed, and ABC Elite Reagent (Vector Labs) was applied for 30’. Visualization was accomplished with DAB. Melanin pigment was bleached with 0.5% KOH/3% H2O2 and a 1% acetic acid rinse in all tumor slides before antibody staining. Slides were counterstained with hematoxylin and cover slipped. Bright-field images were acquired either with SPOT 5.2 software using a Nikon Eclipse E600 microscope (Nikon) and SPOT RT3 camera (Digital Instruments) or with CellSens imaging software (Olympus of America, Center Valley, PA) using a DX41 microscope (Olympus) and DP71 camera (Olympus).

**Digital slide scans and quantitative measurement of histochemical staining**

Slides containing FFPE tissue sections were digitized with either an Aperio ScanScope XT (Leica) or Axio Scan.Z1 scanner (Zeiss) at 200X. For the Aperio whole-slide scan, images were evaluated for staining intensity by a board-certified veterinary pathologist (EFE). Images of the entire organ or tumor section were manually segmented to evaluate superficial tumor micro vessel density (1-millimeter depth) in comparison to sections of the tumor immediately subjacent to selected superficial regions. Regions of intratumoral necrosis and of stroma surrounding the tumor were excluded from micro-vessel density analysis. For quantification of IHC positive cells, automated algorithms were run to assess the total number of positive cells as well as a percentage of positive cells to total cells within a tumor; surrounding epidermal and subcutaneous tissues were not included for cell counts. In addition to positive immune cells, some tumors displayed multifocal membranous positivity within tumor cell populations for MHCII. These foci were often associated with small vessels or in regions of high vessel density and was semi-quantitatively assessed as follows: 0 = none, 1 = minimal cells, 2 = multiple large foci, 3 = focally extensive regions of marked positivity. For the Axio Scan.Z1, whole lung sections or tumor sections stained for antibody F4/80 or CD31 were scanned and subsequently analyzed using the Indica HALO software. Indica Labs’ Tissue Classifier module utilizes a machine-learning algorithm to identify tissue types based on color, texture, and contextual features and the module runs within a HALO platform. Regions within sections were defined as tumor or non-tumor region by board-certified clinical pathologists. Within tumor regions, stained areas were characterized for the presence of clusters, nests, or cell aggregates that are morphologically compatible with melanoma. Defined areas were measured and compared against the measured total tissue area. DAB (brown) stained cells or structures were quantified using the area quantification module to determine the overall positive staining. Measured area was reported in um2, percentage of area, and in some cases degree of staining intensity (weak, moderate or strong).

***Analysis of transcriptomic data of human melanoma***

RPKM data were retrieved from published RNA sequencing result of pre-treatment melanoma from 2 Gene Expression Omnibus (GEO) public databases: GSE78220 (8) and GSE85898 (9). Full access was granted to GM to a third database of 42 patients in clinical study of ipilimumab (10). Previously, we identified a 45-gene melanocytic plasticity signature (MPS) which quantitates differentiation status of melanoma and predicts of patient outcome in response to immune checkpoint blockade (3). It has been shown that immune response regulated differentiation status of melanoma (11). Therefore, we used the expression pattern of MPS genes to evaluate the intensity of cytokine signaling in human melanoma. The z-score of MPS genes across all genes were calculated in each patient’s melanoma samples as input to **CytoSig**, and the output is the z-score of each cytokine, representing the intensity of signaling on the melanoma. **CytoSig** is a web application that predicts the cytokine signaling activities based on a sample's gene expression profile (https://cytosig.ccr.cancer.gov) under development in NCI. This platform contains more than 20 thousand profiles of treatment response data manually curated from the NCBI GEO, SRA, and EBI ArrayExpress databases. For each transcriptomic input, the expression levels of given genes were deconvoluted to the aggregated regulatory effects (induction or repression) of factors, based on the treatment response profiles included in the public data collection, and the output is the predicted activities of cytokine, chemokine, and growth factors whose. The **CytoSig** Platform will be published soon. Technical information is available upon request made to the author (J.P.). The deconvolution for identifying cell types associated with each melanoma sample was performed following the instructions of CIBERTSORT (12) at <https://cibersort.stanford.edu/>.

**Statistical Analysis**

Statistical analyses and graphical presentations were performed using GraphPad Prism 8 software for Windows (Graph Pad Software). Differences between means were obtained either with an unpaired, 2-tailed Student’s T-test for individual comparisons and 1-way or 2-way ANOVA for 2 or more groups comparisons with Holm-Sidak correction for multiple comparisons. Survival percentages were generated using the Kaplan-Meier method. The log-rank (Mantel-Cox) test was used for statistical analysis. All statistical tests were conducted at the 0.05 (alpha) level. All data are presented as mean ± SEM. The following p values were considered significant (\*) for differences: p < 0.05 (\*), p< 0.01 (\*\*), p < 0.001 (\*\*\*) and p< 0.0001 (\*\*\*\*).

**SUPPLEMENTARY FIGURES**

**Diagram

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**Supplementary Figure S1 (related to Fig.1):** **ARE autoimmune landscape delays growth of IFNg-sensitive** m**elanoma cells.** (**A**) Histograms show p-stat1 induction by B16, HcMel, and M114433 melanoma cells after exposure to 100 IU/ml of mouse rIFNg for 15 min, as assessed by flow cytometry. Orange overlays represent the untreated control. (**B, C**) Spheroids of B16F1, B16F10, and HcMel1274 melanoma cells suspended in Matrigel alone or containing 100 UI/ml (black line) or 200 UI/ml (blue line) of mouse recombinant IFNg for up to 7 days. (**B**) Representative images of spheroids after 96 h. Note HcMel spheroids retain metastatic projections after exposure to IFNg (black arrows). Original magnification 5X, Scale bar 100 microns. (**C**) Spheroid growth progression plots. (**D-F**) Tumor growth curves for B16 and HcMel1274 (**D**), and B2905 (**E**) cells in WT and ARE+/- mice (n=5). (**F**) Tumor growth curves for M114433 cells in ARE and type-1 ifnar deficient ARE mice (n=6). (**G**) Pie charts showing percentage of indicated immune cells infiltrating B16 tumors in WT, ifnar-/-ARE+/-, ifnar-/-ARE-/-, and ifnar-/- mice. Data are shown as mean ± SEM. Two-way ANOVA, Asterix denotes \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001.

**Diagram, engineering drawing

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**Supplementary Figure S2 (related to Fig.2): Suppressive TME conditions corelates with the severity of autoimmune disease in ARE mice.** (**A-B**) Comparison of grade of glomerular damage (**A**) and Ly6G infiltration in kidneys and glomeruli determined by IHC (**B**) between tumor-free (n=3) and IV challenged B16-bearing ARE mice (n=6 organs per genotype). (**C**) Representative H&E and IHC images of glomeruli (dotted circles) and immune infiltrates (CD3+ and Ly6G+) cells in ARE-/- mice from A. (**D**) Pooled serum levels of indicated cytokines and growth factors from tumor-free, B16-, and HcMel-bearing ARE mice (n= 6 per group). Serum samples from tumor-bearing mice correspond to end point of experiment (**E**) Representative images of IHC staining for indicated immune cell markers in B16 SQ tumors grown in WT, ARE+/- or ARE-/- mice. (n = 5 - 6 mice per genotype) (\*) denotes vascular structures; “N” denotes necrotic regions. Scale bars 100 microns. Every dot represents the measure within whole tumor sections per slide. **(F)** Gating strategy for identification of Ly6GhiLy6Clo PMN and Ly6GloLy6Chi monocyte population in spleens from ARE mice using flow cytometry. Cell debris was excluded with the viability dye Zombie aqua by FSC-A, followed by cell selection with SSC-A vs FSC-A and the single cell population by FSC-H vs FSC-A. After selection of CD45+ immune cells, CD11b+ cells were selected by sub-gating on CD11b+ cells. Finally, CD11b+ myeloid cells were sub-gated by Ly6G (Gr-1) and Ly6C expression. The resulting populations were then analyzed for specific markers such as: IL-27ra, CD40, PD-L1, and MHC II expression. (**G**-**H**) Flow cytometry analysis done using gating strategy from **F** to determine the percentage of CD45.2+Ly6CloLy6Ghi, CD45.2+Ly6Chi Ly6Glo, and CD45.2+ CD11b+ F4/80+ within B16 and MEL114433 tumor cells in WT and ARE+/- mice. (**I**) Levels of indicated cytokines in lysates from tumors grown in ARE and WT mice (n = 5 mice per group), 2 dots per sample. Original magnification for histology slides: 200X, **C**; 50X, **E**. Data are shown as mean ± SEM. Two-way ANOVA, Asterix denotes \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001.

**Diagram

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**Supplementary Figure S3 (related to Fig. 3): IFNg regulates production of IL-27 in ARE mice.** (**A-B**) Expression and protein levels of *IFNg* and *Il27* mRNA from age-matched tumor-free ARE-/- and WT mice using Nanostring analysis. (**A**) Comparison of mRNA expression for IFNg and IL-27 in organs (n=5 per genotype). (**B)** Endogenous serum levels of IL-27 and IFNg, obtained by MSD. Each dot represents 2 technical replicates from one mouse (n = 6 mice per genotype). (**C-D**) Comparison of cytokine levels in serum taken from WT and ARE+/- mice 1 week before (Pre) challenge (n=4 per treatment/genotype) or 2 weeks (Post) after injection of anti-IFNg mAb and IV challenge with B16 cells. Summary representation of the serum levels of IFNg (**C**) and IL-27 (**D**). (**E**) Blood monocyte cell count (K/ul) after 2 weeks (Post) end point from (C). Dotted line indicates normal values in mouse. (**F**) Variation of serum levels of IL-27 levels (pg/mL) after 18-hour culture media of BMDM with or without stimulation with LPS, as measured by MSD assay (n=4). (**G**)Expression of IL-27ra (MFI) among spleen T cells in tumor-bearing ARE and WT mice, as measured by flow cytometry. Data are shown as mean ± SEM. When compared groups significant differences are shown with \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001 as indicated by the horizontal lines.

**Diagram

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**Supplementary Figure S4 (related to Fig. 4): Existent subpopulation of Ly6G+ PMN are MDSC in ARE mice.** (**A**) Representative H&E and IHC images of immune infiltrates (Ly6G+, F4/80, iNOS+, and PD-L1+) cells within lymph nodes from tumor-bearing ARE+/- mice (n= 6 organs per genotype). Arrows indicate overlap localization between markers. (**B)** Expression levels of CXCR2 (mean florescence intensity; MFI) in CD11b+Ly6GhiLy6Clo PMNs (n=4), as measured by flow cytometry. **(C-D)** Summarized quantitation of the percentage of proliferation inhibition induced by Ly6Ghi cells isolated from ARE mice (**C**) or type 1 ifnar-deficient mice (**D**) over CD8+ and CD4+ T cells. Note that Ly6Ghi cells isolated from BM had no inhibitory effect on the T cell proliferation (C, right) (n = 3 mice per group). (**E-F**) Statistical comparison of the percentage populations and expression (MFI) of indicated markers in CD45.2+Ly6GloLy6Chi monocytes from tumor-free ARE mice. (**G**) Percentage expression of Ly6Chi monocytes expressing IL-27ra in bone marrow (BM) and spleen from WT and ARE-/- mice. Data are shown as mean ± SEM. When compared groups significant differences are shown with \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001 as indicated by the horizontal lines.

**Diagram, engineering drawing

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**Supplementary Figure S5 (related to Fig. 5): IL-27 modulates the effect of IFNg over ARE macrophages.** (**A-B**) Comparison of the percentages of macrophages expressing CD206 (**A**) and tumor infiltrating CD3+ T cells (**B**) from CD45.2+ viable cells in anti-Ly6G-treated or isotype-treated ARE+/- and WT mice.(**C**) Expression of CD40 mRNA before and after co-stimulation with IFNg and IL-27 for 18h in immortalized BMDM from ARE-/- mice (KOM) and C57BL/6 WT mice (WTM). **(D)** Expression of the IFNg regulated genes CXCL9, CIITA, and TNFa in WTM and KOM exposed to IFNg, IL-27, or their combination at 18h of treatment. (**E-F**) Summary quantitation of each marker per tumor (**E**) and representative H&E and IHC images showing the intratumoral distribution of indicated immune cell markers within or around necrotic areas (“N”) from isotype and anti-IL-27p28-treated tumors (**F**). (**G**) Comparison of the variability of cytokine levels per ARE+/- mouse prior (black dots) and post (red dots) anti-IL-27p28 treatment and tumor challenge (PRE challenge; n = 5 animals per group). (**H-I**) Percentage of F4/80+ macrophages (MACS), CD11C+dendritic cells, Ly6Ghi neutrophils, and Ly6Chi monocyte populations within the tumors from isotype or rmIL-27-treated tumors (**H**) and spleens (**I**), as determined by FACS. Experiments were conducted in duplicate. Unless indicated, pooled data from two independent experiments are shown as mean ± SEM. Data are shown as mean ± SEM. When compared, groups significant differences are shown with \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001 as indicated, while groups not significant differences are shown as n.s.

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**Supplementary Figure S6 (related to Fig. 6): Anti-CD40 immunotherapy decreases tumor growth with limited organ inflammation in ARE+/- mice.** (**A**) Representative weight variation curves from tumor-bearing animals in survival studies. Arrows indicate the start and end of indicated immunotherapies. (**B**) Representative IHC images of F4/80+ staining distribution in brown color (n= 4-5). Arrows point to F4/80 clusters around necrotic regions. Scale bar 200 um (Original magnification 10X). (**C-D**) Global inflammatory responses in tumor-treated ARE+/- and WT mice after immunotherapy with the antiPD1/CD40 combination that increased pro-inflammatory cytokines IL-1a, TNFa, and IP-10 (**C**) and lobular inflammation in liver (**D**). (**E**) Individual tumor growth curves in ARE+/- and WT animals SQ challenged that received IgG2a isotype controls or anti-PD1 alone after 36 days (n= 4 per intervention). (**F**-**G**) Summary representation of end weights for tumors (**F**) and spleens (**G**) per intervention from **E**. (**H**) Summarized quantitation of flow cytometry data showing the percentage of live infiltrating cells within tumors (**top**) and spleens (**bottom**) from anti-PD1 or isotype-treated WT and ARE+/- mice. Experiments were repeated twice with similar results. Data are shown as mean ± SEM. When compared groups significant differences are shown with \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001 as indicated by the horizontal or vertical lines.

**Diagram, schematic

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**Supplementary Figure S7 (related to Fig. 7): IL-27/CD40 signature is unaltered in melanoma cells and tumors with an IFNg signature.** (**A**) Schemes detailing study comparisons and source of transcriptomic data from human melanoma tumors before and after treatment with anti-PD1 (left) or anti-CTLA4 (right). (**B**) Global differential expression of CD40, IL27, or IFNg between responder (R) and non-responder (NR) patients either before (pre) and after (post) anti-PD1treatment in biopsies from human metastatic melanoma, as reported by (Hugo et al 2016).

Table S1 (related to Fig. 3) show the induction of tissue-specific genes in organs from ARE-/- mice relative to organs in WT mice.

Table S2 (related to Fig. 7) show the histological grading of kidney, liver and lung autoimmune disease in tumor-bearing ARE mice.

**Table S1: Top differentially expressed immune-regulated genes in organs from ARE-/- vs. WT mice**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Kidney** | | **Lymph Node** | | | | **Thymus** | | | | **Liver** | | | | **Spleen** | | | | **Bone Marrow** | | | |
| **Gene ID** | **fold-change** | **Gene ID** | | **fold-change** | | **Gene ID** | | **fold-change** | | **Gene ID** | | **fold-change** | | **Gene ID** | | **fold-change** | | **Gene ID** | | **fold-change** | |
| **Cxcl9** | 24.62 | **Fcgr4** | | 65.14 | | **Fcgr4** | | 36.39 | | **Stat1** | | 7.76 | | **Fcgr4** | | 7.01 | | **Cxcl9** | | 13.65 | |
| **Ccl8** | 23.45 | **Ccl4** | | 16.36 | | **Cxcl9** | | 13.61 | | **Fcgr4** | | 4.92 | | **Ccl8** | | 6.78 | | **Ccl8** | | 8.05 | |
| **Cxcl10** | 20.58 | **Cxcl9** | | 11.78 | | **Ccl8** | | 8.72 | | **Cxcl9** | | 6.92 | | **Cxcl9** | | 5.67 | | **Stat1** | | 4.65 | |
| **C4b** | 17.17 | **Ccl3** | | 7.37 | | **Ccl4** | | 4.94 | | **C1qa** | | 2.22 | | **Ccl24** | | 4.70 | | **Pawr** | | 4.49 | |
| **Stat1** | 16.11 | **Ifng** | | 6.66 | | **C4b** | | 4.77 | | **Tnfaip3** | | 2.23 | | **Ifng** | | 4.07 | | **Il27** | | 4.20 | |
| **C4a** | 15.02 | **Il27** | | 5.60 | | **C4a** | | 4.30 | | **Cxcl10** | | 5.85 | | **C3ar1** | | 3.59 | | **Irf1** | | 4.01 | |
| **Fcgr4** | 14.44 | **Fos** | | 5.57 | | **Ifng** | | 3.94 | | **Sftpd** | | 2.82 | | **Il27** | | 3.43 | | **C4b** | | 3.63 | |
| **Socs1** | 11.34 | **Cxcl10** | | 5.37 | | **Stat1** | | 3.71 | | **Socs1** | | 7.18 | | **Stat1** | | 3.25 | | **Ccl20** | | 3.13 | |
| **Irf1** | 9.50 | **C3ar1** | | 5.36 | | **Ccl3** | | 3.67 | | **Ptpn22** | | 2.44 | | **Socs1** | | 3.03 | | **Il18bp** | | 2.96 | |
| **Il18bp** | 9.22 | **C1qa** | | 5.11 | | **Fcer1g** | | 3.47 | | **Il18bp** | | 4.36 | | **Ccl4** | | 2.72 | | **Cd4** | | -2.88 | |
| **Fcer1g** | 7.83 | **Stat1** | | 4.88 | | **Cxcr3** | | 3.28 | | **Ptprc** | | 3.30 | | **Ccl3** | | 2.56 | |  | |  | |
| **Tbx21** | 6.87 | **Fcer1g** | | 4.55 | | **Cxcl10** | | 3.18 | | **Ccl8** | | 9.72 | | **Cxcl11** | | 2.46 | |  | |  | |
| **Cxcr3** | 6.77 | **Igsf6** | | 4.07 | | **Igsf6** | | 2.99 | | **Ccr2** | | 2.99 | | **Spp1** | | 2.37 | |  | |  | |
| **Il27** | 6.42 | **Ccl8** | | 3.82 | | **C1qa** | | 2.74 | | **Fcer1g** | | 3.38 | | **Cxcl10** | | 2.37 | |  | |  | |
| **Ptprc** | 6.26 | **Socs1** | | 3.82 | | **Irf1** | | 2.69 | | **Igsf6** | | 2.94 | | **C1qa** | | 2.33 | |  | |  | |
| **C3ar1** | 6.22 | **Il18bp** | | 3.74 | | **Fos** | | 2.64 | | **Il27** | | 2.74 | | **C2** | | 2.19 | |  | |  | |
| **C1qa** | 5.65 | **Cxcl11** | | 3.31 | | **Il27** | | 2.62 | | **Irf1** | | 2.70 | | **Blk** | | -2.11 | |  | |  | |
| **Igsf6** | 5.57 | **Cxcr3** | | 3.28 | | **C3ar1** | | 2.60 | | **Scube1** | | 2.34 | | **Tnfrsf13b** | | -2.16 | |  | |  | |
| **Ccl20** | 5.32 | **Nos2** | | 3.13 | | **C2** | | 2.59 | | **Ccl4** | | 3.71 | | **Tnfrsf13c** | | -2.43 | |  | |  | |
| **Ccl4** | 4.95 | **Irf1** | | 2.98 | | **Il17a** | | 2.50 | | **Cxcr3** | | 2.14 | | **Il22ra2** | | -20.63 | |  | |  | |
| **Ccl3** | 4.92 | **Pdcd1** | | 2.83 | | **Tbx21** | | 2.39 | | **Pdcd1** | | 3.40 | |  | |  | |  | |  | |
| **Ccr7** | 4.82 | **Tbx21** | | 2.71 | | **Bank1** | | 2.37 | | **Ccr7** | | 3.92 | |  | |  | |  | |  | |
| **Il12rb2** | 4.60 | **Il12b** | | 2.65 | | **Itgam** | | 2.33 | | **Il7** | | 3.06 | |  | |  | |  | |  | |
| **Cd5** | 4.37 | **Ccl7** | | 2.50 | | **Nos2** | | 2.31 | | **Ccl3** | | 2.13 | |  | |  | |  | |  | |
| **C3** | 4.28 | **Socs3** | | 2.49 | | **Tlr6** | | 2.28 | |  | |  | |  | |  | |  | |  | |
| **Cxcl11** | 4.25 | **Tlr6** | | 2.31 | | **Il18bp** | | 2.22 | |  | |  | |  | |  | |  | |  | |
| **Cd4** | 4.01 | **Itgam** | | 2.14 | | **Lyn** | | 2.20 | |  | |  | |  | |  | |  | |  | |
| **Tlr6** | 3.82 | **Spp1** | | 2.12 | | **Tnfsf13b** | | 2.20 | |  | |  | |  | |  | |  | |  | |
| **Ctla4** | 3.76 | **Tnfrsf8** | | 2.05 | | **Cxcl11** | | 2.11 | |  | |  | |  | |  | |  | |  | |
| **Ifng** | 3.71 | **Tnfsf13b** | | 2.05 | | **Ccl20** | | -2.43 | |  | |  | |  | |  | |  | |  | |
| **Cd40lg** | 3.54 | **Il13** | | -2.05 | | **Il22ra2** | | -4.81 | |  | |  | |  | |  | |  | |  | |
| **Ptpn22** | 3.53 | **Il7** | | -2.37 | |  | |  | |  | |  | |  | |  | |  | |  | |
| **Ccr2** | 3.49 | **Tlr5** | | -2.70 | |  | |  | |  | |  | |  | |  | |  | |  | |
| **Pdcd1** | 3.43 | **Ccl20** | | -3.21 | |  | |  | |  | |  | |  | |  | |  | |  | |
| **Tnfsf13b** | 3.40 | **Il22ra2** | | -42.90 | |  | |  | |  | |  | |  | |  | |  | |  | |
| **Stat4** | 3.08 |  | |  | |  | |  | |  | |  | |  | |  | |  | |  | |
| **Fasl** | 3.06 |  | |  | |  | |  | | | |  | |  | |  | |  | |  | |
| **Fos** | 3.02 |  | |  | |  | |  | |  | |  | |  | |  | |  | |  | |
| **Cxcl2** | 2.58 |  | |  | |  | |  | |  | |  | |  | |  | |  | |  | |
| **Il12b** | 2.55 |  | |  | |  | |  | |  | |  | |  | |  | |  | |  | |
| **Itgam** | 2.43 |  | |  | |  | |  | |  | |  | |  | |  | |  | |  | |
| **Ccl7** | 2.41 |  | |  | |  | |  | |  | |  | |  | |  | |  | |  | |
| **Tlr7** | 2.41 |  |  | |  | |  | |  | |  | |  | |  | |  | |  | |

**Table S2: Overview of metastasis and severity of kidney and liver pathology in tumor-bearing ARE mice after immunotherapy**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Group** | **WT** | | | | **ARE+/-** | | | |
| **Immunotherapy** | **IgG2** | **Anti-PD1** | **Anti-CD40** | **Anti-PD1/CD40** | **Isotype IgG2D** | **Anti-PD1** | **AntiCD40** | **Anti-PD1/CD40** |
| Mice per group | 11 | 10 | 10 | 10 | 11 | 12 | 12 | 11C |
| Mice with Grafts | 11 | 10 | 10 | 10 | 11 | 12 | 12 | 11C |
| * w/o metastasis | 6 | 7 | 10 | 8 | 10 | 9 | 11 | 8 |
| * With metastasis | 5 | 3 | 0 | 2 | 1 | 3 | 1 | 3 |
| Mice with Metastases in: |  |  |  |  |  |  |  |  |
| * Lung | 4 | 2 | 0 | 0 | 0 | 2 | 1 | 3 |
| * LN A | 1 | - | - | 2 | 1 | 2B | - | - |
| **Kidney (examined)** | **6** | **5** | **5** | **3** | **9** | **12** | **12** | **11** |
| Glomerulonephropathy  Membrane-proliferative | 3 | 1 | 2 | 1 | 9 | 12 | 12 | 11 |
| **Negative** | 3 | 4 | 3 | 2 | 0 | 0 | 1 | 0 |
| **+** | - | 1 | 2 | 1 | 4 | 2 | 4 | 3 |
| **++** | 3 | - | - | - | 2 | 6 | 4 | 2 |
| **+++** | - | - | - | - | 3 | 4 | 3 | 4 |
| **++++** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| **Liver (examined)** | **7** | **5** | **5** | **5** | **9** | **12** | **12** | **11** |
| Coagulative Necrosis | **0** | **1** | **0** | **0** | **1** | **4** | **5** | **2** |
| **++** | - | 1 | - | - | 1 | 3 | 3 | 1 |
| **+++** | - | - | - | - | - | 1 | 2 | 1 |

\* Severity: +, minimal; ++, mild; +++, moderate and ++++, severe.

A – Lymph nodes (LN) were only examined if noted to be enlarged at time of necropsy.

B – One animal had metastasis in both the lung and lymph node.

C – One animal had no evidence of graft at time of necropsy.

D – One animal died from a dissecting aneurysm in the aorta and was excluded from study.

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