3 Materials and methods

3.1 Materials

3.1.1 Mice

Strain	Abbreviation	MHC haplotype	Supplier
B6.PL-Thy1a/Cy	B6 (Thy1.1)	H-2^{b}	The Jackson Laboratory, Bar Harbor, ME
B6.SJL-Ptprc^{a} Pep3^{b}/Boy	B6 (CD45.1)	H-2^{b}	The Jackson Laboratory, Bar Harbor, ME
B6D2F1	B6D2F1	H-$2^{b/d}$	The Jackson Laboratory, Bar Harbor, ME
BALB/c	BALB/c	H-2^{d}	The Jackson Laboratory, Bar Harbor, ME
C3FeB6F1	C3FeB6F1	H-$2^{b/k}$	The Jackson Laboratory, Bar Harbor, ME
C57BL/6	B6 and WT	H-2^{b}	The Jackson Laboratory, Bar Harbor, ME
C57BL/6.CCR2-knock-out	CCR2$^{-/-}$	H-2^{b}	W.A. Kuziel, University of Texas, Austin, TA (117)

3.1.2 Tumor cell lines

Cell line	Tissue	Strain origin	MHC	Culture medium	Cell growth	Supplier
32Dp210	Chronic myeloid leukemia	C3H/He	H-2^{k}	Complete DMEM	Non-adherent	J. Griffin, Dana Farber Cancer Institute, Boston, MA (131)
A20	B cell lymphoma	BALB/c	H-2^{d}	Complete RPMI	Non-adherent	A. Houghton, MSKCC, New York, NY
P815	Mastocytoma	DBA/2	H-2^{d}	Complete RPMI	Non-adherent	ATCC, Manassas, VA

3.1.3 Cell lines for generation of retrovirus

Cell line	Tissue	Purpose	Culture medium	Cell growth	Supplier
NIH/3T3	Fibroblast	Determination of viral titer	Complete DMEM	Adherent	ATCC, Manassas, VA
Phoenix Eco	Human embryonic kidney	Ecotropic packaging cell line	Complete DMEM	Adherent	G.P. Nolan, Stanford University, Stanford, CA

3.1.4 Chemicals and reagents

Reagent	Supplier
[^{3}H]Thymidine	NEN, Boston, MA
2-Mercaptoethanol	Invitrogen, Carlsbad, CA
^{51}Chromium	NEN, Boston, MA
Ammonium chloride	Fisher Scientific, Pittsburgh, PA
Anti-Thy-1.2	Cedarlane Laboratories Ltd., Hornby, Canada
Carboxyfluorescein diacetate succinimidyl ester (CFSE)	Molecular Probes, Eugene, OR
Cytofix/cytoperm Kit	Pharmingen, San Diego, CA
Diamidino phenylindole dihydrochloride (DAPI)	Molecular Probes, Eugene, OR
Dimethyl sulfoxide (DMSO)	Sigma, St Louis, MO
D-luciferin	Xenogen, Alameda, CA
Dulbecco's modified Eagle's medium (DMEM)	Media Lab, MSKCC, New York, NY
Ethylenediaminetetraacetic acid (EDTA)	Media Lab, MSKCC, New York, NY
Fetal calf serum (FCS)	Gemini Bioproducts, Woodland, CA
Formalin phosphate	Fisher Scientific, Pittsburgh, PA
Hank's buffered saline solution (HBSS)	Media Lab, MSKCC, New York, NY
HEPES buffer	Cellgro, Herndon, VA
Isoflurane	Baxter, Deerfield, IL
Lipofectamine 2000	Invitrogen Carlsbad, CA
Low-TOX-M rabbit complement	Cedarlane Laboratories Ltd., Hornby,

	Canada
Microscint-20	Packard, Meridan, CT
Penicillin-streptomycin-glutamine	Invitrogen, Carlsbad, CA
Percoll	MP Biomedicals, Aurora, OH
Phosphate buffered saline (PBS)	Media Lab, MSKCC, New York, NY
Polybrene	Sigma, St Louis, MO
Roswell Park Memorial Institute medium (RPMI)	Media Lab, MSKCC, New York, NY
Sodium azide	Sigma, St Louis, MO
TRIS	Gibco BRL, Grand Island, NY
Triton X-100	Sigma, St Louis, MO
Trypan blue	MP Biomedicals, Aurora, OH
Trypsin	Sigma, St Louis, MO

3.1.5 Media and buffers

Media	Recipe
BMT media	DMEM, 100 U/mL penicillin, 100 µg/mL streptomycin
Complete DMEM	DMEM, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine
Complete RPMI	RPMI, 10% FCS, 100 U/ml penicillin,100 µg/ml streptomycin, 2 mM L-glutamine
FACS buffer	PBS without Ca^{2+}/Mg^{2+}, 2% FCS, 0.05% sodium azide
Freezing media	DMEM, 20% FCS, 10% DMSO, 100 U/ml penicillin,100 µg/ml streptomycin, 2 mM L-glutamine
IEL harvest media	HBSS, 10 mM HEPES, 1 mM EDTA, 10% FCS
IEL wash media	HBSS, 10 mM HEPES, 2% FCS
MACS buffer	PBS without Ca^{2+}/Mg^{2+}, 0.05% BSA, 2 mM EDTA
MLR media	Complete RPMI, 50 µM 2-mercaptoethanol
RBC-lysing solution	0.14 M ammonium chloride, 17 mM TRIS
Sort buffer	PBS without Ca^{2+}/Mg^{2+}, 2% FCS, 2 mM EDTA
Trypsin/EDTA	HBSS, 0.1% trypsin, 0.02% EDTA

3.1.6 Plasticware and other materials

Product	Supplier
Cryogenic vials	Corning Inc., Corning, NY
Flasks, pipettes, tubes, dishes, cell strainers	Becton Dickinson, Mountain View, CA
Frosted microscope slides	Fisher Scientific, Pittsburgh, PA
Heparinized micro-hematocrit capillary tube	Fisher Scientific, Pittsburgh, PA
LD MACS separation columns	Miltenyi, Auburn, CA
LS MACS separation columns	Miltenyi, Auburn, CA
Media filter 0.22 μm polyethersulfone	Corning Inc., Corning, NY
Microtainer hematology (EDTA) tubes	Becton Dickinson, Mountain View, CA
Microtainer serum tubes	Becton Dickinson, Mountain View, CA
Nylon wool	Perkin Elmer Life Sciences, Boston, MA
Virus filter 0.45 μm polyethersulfone	Nalge Nunc International, Rochester, NY

3.1.7 FACS antibodies

All supplied by Pharmingen, San Diego, CA.

Antigen	Clone	Isotype	Fluorochromes	Concentration
CD3e	145-2C11	hamster IgG_1, κ	FITC, APC-Cy7	10 μg/ml
CD4	RM4-5	rat IgG_{2a},κ	APC	10 μg/ml
CD4	GK1.5	rat IgG_{2b},κ	APC-Cy7	10 μg/ml
CD44	IM7	rat IgG_{2b},κ	FITC, PE, APC	5 μg/ml
CD45.1	A20	mouse IgG_{2a},κ	FITC, PE	10 μg/ml
CD62L	MEL-14	rat IgG_{2a},κ	FITC, PE, APC	5 μg/ml
CD8a	53-6.7	rat IgG_{2a},κ	APC, APC-Cy7	10 μg/ml
$H\text{-}2K^k$	36-7-5	mouse IgG_{2a},κ	FITC, PE	10 μg/ml
IFN-γ	XMG1.2	rat IgG1	PE	10 μg/ml
Ly-9.1	30C7	rat IgG_{2a},κ	FITC, PE	10 μg/ml
CD16/32 FcR block	2.4G2	rat IgG_{2b},κ	Purified	20 μg/ml
rat IgG_{2a},κ	LOU	rat IgG_{2a},κ	PE	Isotype
rat IgG_{2a},κ	R35-95	rat IgG_{2a},κ	APC	Isotype

rat IgG_{2b},κ	A95-1	rat IgG_{2b},κ	FITC, PE, purified	Isotype
Streptavidin			PE	1 µg/ml
Thy1.1	OX-7	mouse IgG_1,κ	FITC, PE	10 µg/ml
TNF-α	MP6-XT22	rat IgG_1	PE	10 µg/ml

3.1.8 MACS antibodies

Antigen	Clone	Isotype	Dilution	Supplier
CD5	53-7.3	rat IgG_{2a},κ	1/10	Miltenyi, Auburn, CA
CD8	53-6.7	rat IgG_{2a},κ	1/10	Miltenyi, Auburn, CA

3.1.9 ELISA kits

Kit	Supplier
Quantikine Immunoassay IFN-γ	R&D Systems, Minneapolis, MN

3.1.10 Instruments

Instrument	Manufacturer
Anaesthesia chamber	Vetequip, Pleasanton, CA
CO_2 incubator	NuAire, Plymouth, MN
FACS Vantage DiVa cell sorter	Becton Dickinson, San Jose, CA
Filtermate 196 harvester	Packard, Meridan, CT
Gammacell 40 small animal irradiator	Atomic Energy of Canada Ltd., Chalk River, Canada
Hemavet 950FS cell counter	Drew Scientific, Oxford, CT
LSR I cytometer	Becton Dickinson, San Jose, CA
Mark I cell irradiator	JL Shepard, Glendale, CA
MidiMACS Seperator	Miltenyi, Auburn, CA
MRX Revelation microplate photometer	DYNEX Technologies, Chantilly, VA
Neubauer improved counting chamber	Hausser Scientific, Horsham, PA
Sorvall Legend RT tabletop centrifuge	Kendro, Asheville, NC
Topcount NXT microplate scintillation counter	Packard, Meridan, CT
Xenogen IVIS bioluminescence imaging system	Xenogen, Almeda, CA

3.1.11 Software

Software	Supplier
CellQuest 3.1	Becton Dickinson, San Jose, CA
FlowJo 6.1	Treestar, Ashland, OR
GraphPad Prism 4.0	GraphPad Software Inc., San Diego, CA
Living Image 2.2	Xenogen, Alameda, CA

3.2 Methods

3.2.1 General procedures

3.2.1.1 Sterile technique

Cell culture and all manipulations prior to *in vitro* or *in vivo* experiments were performed in a laminar flow biosafety cabinet using sterile utensils and sterile technique.

3.2.1.2 Cell counts

Cells were counted using an improved Neubauer hemacytometer. Trypan blue was used for dead cell exclusion. About 100 cells per sample were counted.

3.2.1.3 Cell washes

To eliminate unwanted substances from single cell suspensions (e.g. excess antibodies, cell debris or proteins), cells were transferred to centrifuge tubes, tubes were filled with media/buffer and spun at 4°C for 5 minutes at 1200 rpm in a Sorvall Legend RT tabletop centrifuge. The supernatant was removed and the pellet was resuspended in new media/buffer.

3.2.1.4 Red blood cell lysis

When indicated, red blood cells (RBCs) were removed from single cell suspensions by hypotonic lysis with an ammonium chloride lysing solution. This method relies on the increased sensitivity of RBCs towards hypotonic stress in comparison to other cell types. Single cell suspensions were pelleted and resuspended in 1 ml of ammonium chloride RBC-lysing solution. Cells were vortexed briefly and incubated at room temperature for one minute. Fresh media/buffer was added and cells were washed once.

3.2.2 Cell culture

All cell lines were grown in culture medium (Chapter 3.1.5) at 37°C in a humidified atmosphere containing 5% CO_2. Before reaching confluence, cells were split at a 1/5 to 1/20 ratio (depending on cell line specific doubling time) and transferred into a new flask. Prior to transfer of adherent cell lines, flasks were washed with PBS and subsequently treated with prewarmed trypsin/EDTA until cells detached. Cells were then washed with complete medium.
For long term storage of cell lines, cells were collected from flasks, spun down and resuspended in freezing medium ($5x10^6$ cells/ml). The cell suspension was then transferred to cryovials (1 ml/vial). Cryovials were stored overnight in a cryobox at -80°C and transferred to liquid nitrogen the following day. Cells were stored in the vapor phase above the liquid at less than -140°C.

3.2.3 Mouse handling, anesthesia and euthanasia

3.2.3.1 Mouse handling

Mice were housed in sterilized microisolator cages and received normal chow and autoclaved hyperchlorinated drinking water (pH 3.0) ad libitum. Mice strains were bred according to the "Guidelines for Breeding Genetically Engineered Mice" issued by the Research Animal Resource Center (RARC) at the MSKCC. All experimental protocols were approved by the MSKCC Institutional Animal Care and Use Committee (Protocol #99-07-025). All mice used in experiments were between 8 and 12 weeks of age.

3.2.3.2 Anesthesia of mice

Mice were anesthetized for collection of peripheral blood and *in vivo* bioluminescence imaging. Anesthesia was performed according to the "Guidelines for the Utilization of Anesthetics and Analgesics in Small Laboratory Animals" issued by RARC. Isoflurane (2.5 % working concentration) was administered at an oxygen flow rate of 1.5 l/min directly into the closed induction chamber of a Vetequip mobile non-rebreathing anesthesia machine. A maximum of five mice were placed into the induction chamber until a loss of the righting reflex could be observed.

3.2.3.3 Euthanasia of mice

For organ harvests, mice were euthanized according to the "Recommended Methods of Euthanasia for Laboratory Animals" issued by RARC. 100% CO_2 (5 psi, 5-10 min) was

delivered through a euthanasia lid into the animal cages. Death was verified by absence of breathing and heart beat.

3.2.4 Preparation of single cell suspensions from mouse organs

3.2.4.1 Spleen, peripheral lymph nodes, mesenteric lymph nodes, liver, thymus

Mice were euthanized as described above. Spleen, peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN), liver and thymus were surgically removed and placed in 10 cm dishes containing complete RPMI. Organs were processed by homogenizing between frosted microslides (autoclaved slides were used if sterile work was necessary) and subsequently passed through 70 µm cell strainers. Cells were washed once and resuspended in media/buffer. Liver samples were resuspended in 5 ml of 40% Percoll and carefully layered on top of 5 ml of 70% Percoll solution. After centrifugation (30 min at 3000 rpm), mononuclear cells were recovered from the interface and washed once with PBS. Cells were then resuspended in media/buffer.

3.2.4.2 Gut

The small bowel was dissected from the gastric-duodenal junction to the ileo-cecal junction. Macroscopically visible Peyer's Patches (PP), fat and connective tissue were removed, fecal matter was expelled and the gut was cut longitudinally and washed extensively in RPMI. For separation of intraepithelial lymphocytes (IEL), the gut was cut into small pieces and incubated in 20 ml of IEL separation media on a shaker at 37°C. After 20 min, the cell suspension was vortexed for 15 sec, gut pieces were allowed to settle and IEL containing supernatant was transferred into a new tube. This procedure was repeated twice with the remaining gut pieces. IEL were washed once in media/buffer. Subsequently mononuclear cells were isolated using Percoll density gradient centrifugation as described for the liver.

3.2.4.3 Peripheral blood

Mice were anaesthetized and peripheral blood was obtained through puncture of the retro-orbital sinus with heparinized micro-hematocrit capillary tubes. For complete blood counts (CBC) blood was collected in Microtainer EDTA tubes, for the separation of serum blood was collected in Microtainer serum tubes.

3.2.5 Fluorescence activated cell sorting

3.2.5.1 Overview

FACS relies on the detection of fluorochrome-labeled antibodies, which are bound to cell surface or intracellular antigens. FACS allows a semiquantitative measurement of antigen expression levels and detection is achieved on a single cell level. Fluorochromes are small organic molecules with ring structure, which can be promoted to an excited electronic state after absorption of photons from a laser source. When fluorochromes return to their ground state, they emit energy in the form of fluorescent light of a specific wavelength. As different fluorochromes have different excitation and emission maxima, several fluorochromes can be detected at the same time. For analysis, fluorochrome-stained cells are subjected to hydrodynamic focusing and single cells are directed through the laser beam for excitation. The emitted light is split into frequencies of a specific bandwidth by a set of optical filters and is subsequently detected by photomultiplier tubes. Problems with spectral overlap of fluorochromes can be overcome by electronic compensation. Excitation light scattered by the cell in a forward angle (forward scatter (FSC)) or at a right angle (side scatter (SSC)) is used to determine size and granularity and these parameters are used to identify mononuclear cells. The detected signals are amplified logarithmically or linearly and can be displayed in a variety of different diagrams (e.g. histogram, contour plot or dot plot).
The LSR I cytometer used in this study is equipped with two lasers (argon ion laser at 488 nm, helium-neon laser at 633 nm) for the detection of up to 5 different fluorochromes plus an additional helium-cadmium UV laser (325 nm) for detection of DAPI. DAPI is a fluorescent dye, which specifically stains double strand DNA and can therefore be used for dead cell discrimination.

3.2.5.2 Staining protocol

All staining steps were performed at 4°C to prevent receptor internalization and normal membrane turnover. Cells were kept in FACS buffer throughout the procedure. Final staining concentrations for individual antibodies are listed in chapter 3.1.7. Appropriate isotype controls were used in all experiments.

One-step staining
After preparation of single cell suspensions, cells were washed with FACS buffer and subsequently 10^6 cells per stain were incubated for 20 minutes with rat anti-mouse CD16/CD32

FcR block. Mastermixes of fluorochrome-labeled antibodies were prepared and 25 µl of mixture was added to 25 µl of FcR-blocked cell suspension. After 30 min incubation, cells were washed twice and then resuspended in FACS buffer for subsequent analysis. In some experiments, DAPI (5 µg/ml) was added for dead cell discrimination.

Two-step staining

In the case of a 2-step-staining procedure, FcR-blocked cells were incubated with the primary biotin-labeled antibody for 30 min. Cells were washed twice and subsequently stained for 30 min with a mix of fluorochrome-labeled antibodies and fluorochrome-labeled Streptavidin. Cells were prepared for analysis as described for one-step stainings.

3.2.5.3 Acquisition and analysis

Cells were acquired on a LSR I cytometer with CellQuest software. Data was analyzed using FlowJo. Mononuclear cells were identified according to FSC/SSC properties and subpopulations were identified using gates set according to the isotype controls. If DAPI was used, dead cells were excluded from the analysis.

3.2.6 Magnetic cell sorting

3.2.6.1 Overview

In some experiments T cells or T cell subsets were highly purified from single cell suspensions by magnetic cell sorting (MACS). MACS relies on the detection of cell surface antigens with monoclonal antibodies labeled with superparamagnetic particles (MACS MicroBeads). Labeled cells are retained in the high-gradient magnetic field of a MACS column (the column matrix is composed of ferromagnetic spheres) placed in the permanent magnet of a MACS Separator. Unlabeled cells are washed out of the columns by rinsing with buffer. The labeled fraction can be eluded after removal of the column from the magnetic field. MACS separation is a gentle procedure, which maintains cell viability and function of target cell populations.

3.2.6.2 Separation protocol

A single cell suspension of splenic cells was prepared as described above. After hypotonic lysis of RBCs, cells were washed once in MACS buffer and incubated with rat anti-mouse CD16/CD32 FcR block (0.5 mg/ml in a total volume of 9 µl/10^6 cells for 15 min at 4°C). Subsequently 1 µl/10^6 cells of antibodies (CD8 or CD5) coupled to MACS MicroBeads was

added and incubated on a shaker at 4°C for another 15 min. Cells were then washed once, and up to 300x10^6 cells were loaded on top of a MACS LS positive selection column, which was placed in the magnetic field of a MACS separator. The unlabeled negative fraction was washed out with 3x3 ml of MACS buffer and collected in a separate tube. Subsequently, the MACS column was removed from the magnetic field and positive cells were eluded with 5 ml of MACS buffer. Cells were washed once and an aliquot was stained with FACS antibodies for analysis of purity. Generally, the purity for positive selection ranged from at least 88% to 95%.

3.2.7 Generation of tumor cell lines for *in vivo* bioluminescence imaging

3.2.7.1 Overview

For accurate assessment of the GVT effect mediated by WT and CCR2$^{-/-}$ CD8$^+$ T cells, tumor burden of transplanted mice was quantified by *in vivo* bioluminescence imaging (BLI) (132, 133). BLI relies on the light-emitting properties of reporter enzymes such as firefly luciferase, which catalyses the transformation of its substrate D-luciferin into oxyluciferin leading to the emission of photons (134). The emission peak of firefly luciferase (560 nm) is contained within the spectrum of visible light and can be detected and quantified with low light imaging systems such as ultra-sensitive cooled charge coupled device (CCD) cameras (135).
For the present study, the P815 mastocytoma and A20 B cell lymphoma cell lines were transduced with a Moloney murine leukemia virus (MoMLV) based triple-modality reporter construct.

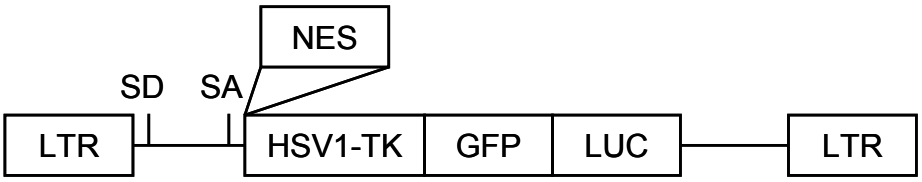

Figure 4: MoMLV-based TGL triple-modality reporter construct.
LTR: Long terminal repeat, SD: Splice-donor site, SA: Splice-acceptor site, NES: Nuclear export signal, HSV-TK: Herpes simplex virus 1 thymidine kinase, EGFP: Enhanced green fluorescent protein, LUC: Luciferase

This construct contains the coding sequences for herpes simplex virus 1 thymidine kinase (HSV1-TK, for PET imaging), Aequorea victoria enhanced green fluorescent protein (EGFP, for optical fluorescence imaging) and firefly luciferase (LUC, for optical bioluminescence imaging) and forms a single fusion protein with three functional subunits (HSV1-TK-EGFP-LUC (TGL))

(kindly provided by V. Ponomarev, MSKCC, New York, NY (136)) (Figure 4). Fluorescence imaging was used for the selection of single tumor clones with high EGFP expression by FACS and bioluminescence imaging was used for *in vivo* quantification of tumor burden. PET imaging was not used in this study.

3.2.7.2 Production of retrovirus

Phoenix Eco producer cells were grown to a 90% confluent monolayer in T75 tissue culture flasks. Cells were split 1/5 and transferred to a new flask containing 15 ml of complete DMEM without penicillin/streptomycin. The next day, the TGL plasmid was transfected into Phoenix Eco cells using Lipofectamine 2000 following the manufacturer's guidelines. Briefly, 80 µl Lipofectamine 2000 and 33 µg of plasmid were each diluted in 1.5 ml of DMEM and incubated at room temperature for 5 min. Solutions were mixed and incubated at room temperature for another 20 min until DNA-Lipofectamine complexes formed. DNA-Lipofectamine complexes were then added to a 90% confluent monolayer of Phoenix Eco cells. The next day, the transfection medium was exchanged for complete DMEM and the flask was placed at 32°C. 24 hours later, the viral supernatant was collected from flasks and filtered though 0.45 µm polyethersulfone filters. Viral supernatant was frozen in 4.5 ml aliquots at -80°C until use.

3.2.7.3 Titration of retrovirus

$1x10^5$ NIH/3T3 cells/well were plated in 6-well plates and placed in an incubator at 37°C. The next day, cells from one well were trypsinized and counted. Medium was removed from the other wells and serial dilutions of viral supernatant (1/10, 1/50, 1/100, 1/200) were added in 1 ml of media containing 8 µg/ml polybrene. The next day, viral supernatant was aspirated from the plates and 2 ml of fresh medium was added. Cells were grown for another 2 days and subsequently analyzed by FACS for EGFP expression. The viral titer was determined according to the following formula: titer = $1x10^5$ x fraction of $EGFP^+$ cells x dilution. Titers were consistently between $5x10^5$ and $1x10^6$ infectious viral particles per ml.

3.2.7.4 Transduction of tumor cell lines

For transduction of tumor lines, $2x10^5$ P815 or A20 cells/well were seeded in 6-well plates and 2 ml of viral supernatant containing polybrene (8 µg/ml) was added. After 24 hours, retroviral supernatant was exchanged for fresh culture medium.

3.2.7.5 Selection of single clones

After transduction, cells were expanded and individual clones with high EGFP expression were sorted into 96-well plates using a FACS Vantage DiVa cell sorter. Cell sorting equipment was operated by MSKCC flow cytometry core facility staff. Cells were fed using filtered (0.22 μm) conditioned media from untransduced cells. 24 clones of each cell line were expanded and subsequently analyzed for EGFP expression (by FACS) and bioluminescence intensity. The clones with the brightest BLI signal and a clear single peak in the FACS analysis (termed P815 TGL and A20 TGL) were used for subsequent experiments.

3.2.8 Bone marrow transplantation, graft-versus-host disease and tumor induction

Bone marrow transplantation and induction of GVHD was performed in well-defined murine bone marrow transplantation models (B6→C3FeB6F1, B6→B6D2F1, B6→BALB/c). To exclude potentially confounding variables associated with CCR2-deficiency on host cells or BM-derived cells, allografts were designed as a combination of T cell-depleted (TCD) WT BM mixed with either WT or $CCR2^{-/-}$ $CD8^{+}$ donor T cells. Mice which received TCD WT BM without $CD8^{+}$ donor T cells served as control groups, which only developed radiation induced damage but no GVHD. The $CCR2^{-/-}$ mice used in this study were viable, fertile, exhibited normal growth and development and were not severely immunocompromised (117).

3.2.8.1 Preparation of donor bone marrow

WT mice were euthanized and hind legs were surgically removed. Femurs and tibias were isolated by removing fur, and muscles and bones were cut at proximal and distal ends. BM was recovered by flushing bones with complete RPMI using a 10 ml syringe with a 25G5/8 needle. Next, donor BM was T cell-depleted by incubation with anti-Thy-1.2 for 30 minutes at 4°C, followed by incubation with Low-TOX-M rabbit complement for 40 minutes at 37°C. Finally, BM cells were washed in BMT media.

3.2.8.2 Preparation of $CD8^{+}$ donor T cells

WT and $CCR2^{-/-}$ $CD8^{+}$ donor T cells were purified by MACS as described above and washed with BMT media. Cells were then mixed with $5x10^{6}$ TCD WT BM cells at varying doses (see respective figure legends).

3.2.8.3 Transplantation and tumor induction

Recipient mice were conditioned with total body irradiation on day 0 (BALB/c: 900 cGy, B6: 1100 cGy, C3FeB6F1 and B6D2F1: 1,300 cGy). Radiation was administered from a ^{137}Cs source as a split dose with a 3-hour interval between doses to reduce gastro-intestinal toxicity. 2 hours after the second irradiation, mice were injected via tail vein with the BM/CD8$^+$ T cell mixture in a volume 250 µl BMT media. In GVT experiments, animals received a separate intravenous injection of tumor cells (P815, P815 TGL or A20 TGL) on day 0 (Figure 5).

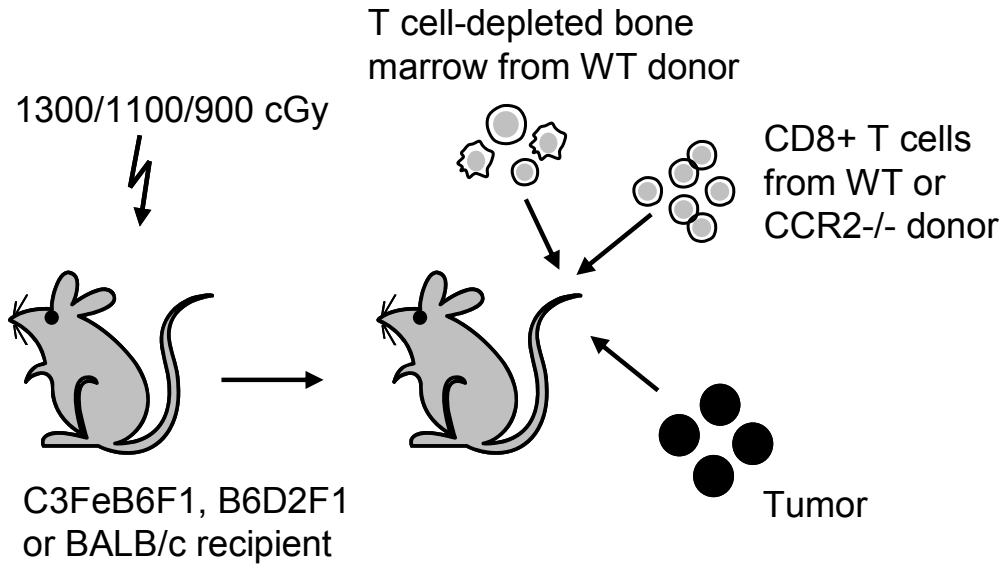

Figure 5: Murine bone marrow transplantation model.

Lethally irradiated recipients were transplanted with TCD WT BM alone (control group which does not develop GVHD) or with TCD WT BM and CD8$^+$ T cells from WT or CCR2$^{-/-}$ mice (experimental groups). In GVT experiments mice received a separate injection of tumor cells on day 0.

3.2.9 Assessment of graft-versus-host disease and graft-versus-tumor activity

3.2.9.1 Scoring and necropsies

Survival of transplanted mice was monitored daily and mice were individually scored weekly for 5 clinical parameters (weight loss, hunched posture, activity level, fur ruffling, and skin lesions) on a scale from 0 to 2. A clinical GVHD score was generated by the summation of the 5 criteria scores as first described by Cooke et al. (137) and mice scoring 5 or greater were sacrificed (Table 5).

In GVT experiments, cause of death (tumor versus GVHD) was determined by clinical assessment, necropsy and histopathology. Presence of hindleg paralysis at the time of death (caused by compression of the spinal cord and its roots by tumor), splenomegaly (spleen weight >300 mg) or liver metastases on macroscopic examination were used as specific criteria for death from tumor. Liver and spleen of animals that did not exhibit any gross evidence of tumor were histopathologically examined for evidence of lymphoma/mastocytoma by a veterinary pathologist (K. La Perle, Cornell University Medical College, New York, NY) and cause of death was subsequently determined. Death from GVHD was defined as the absence of tumor death and the presence of GVHD symptoms.

Score	Skin	Fur	Posture	Mobility	Weight
0.5	Flaking: ears, tail, paws	Ventral ruffling	Slight kyphosis	Decreased activity	-
1	Erythema: ears, tail	Ventral lines, slight back ruffling	Obvious kyphosis	Stationary >50% of time	10-25% weight loss
1.5	Open lesions	Ruffling >50% of body	Severe kyphosis	Movement if stimulated	-
2	Multiple open lesions	Ruffling entire body, denuded skin	Extreme kyphosis	No movement	>25% weight loss

Table 5: Clinical GVHD scoring system.

3.2.9.2 Bioluminescence imaging

In GVT experiments using P815 TGL or A20 TGL cells, bioluminescence intensity of tumor bearing mice and controls was determined at least once weekly. 15 minutes after intra-peritoneal injection of D-luciferin (3 mg/mouse in 200 µl PBS), mice were anaesthetized and placed into the light tight chamber of a Xenogen IVIS bioluminescence imaging system. This system consists of a CCD camera, an imaging chamber, and a cryogenic refrigeration unit supported by a camera controller, a computer and Living Image software. Grayscale photographic images of the mice were acquired first, then the lights in the chamber were automatically turned off and low-level bioluminescence signal was recorded for 5 sec to 5 min (depending on signal intensity). Pseudocolor images showing the whole body distribution of bioluminescence signal

were superimposed on conventional photographs and total flux (photons/sec) was determined for individual mice using manually selected regions of interest (ROI).

3.2.10 Assessment of graft-versus-host disease target organ damage

In some experiments, mice were sacrificed at different time points for histopathologic analysis of GVHD target organs (small and large bowel, liver and skin). Organs were harvested, formalin-preserved and subsequently paraffin-embedded, sectioned and hematoxylin- and eosin-stained by K. La Perle (Cornell University Medical College, New York, NY). Skin samples were then examined by G.F. Murphy (Harvard Medical School, Boston, MA) and the number of apoptotic cells per millimeter of epidermis was determined (138). Liver and gut samples were analyzed by C. Liu (University of Florida College of Medicine, Gainesville, FL) and a semiquantitative score consisting of 19 to 22 different parameters associated with GVHD was calculated (22).
To assess thymic damage due to irradiation and GVDH, the total thymic cellularity and the number of $CD4^+CD8^+$ thymocytes was determined by FACS on day 21 after BMT. To assess the effects of CCR2-deficiency of donor $CD8^+$ T cells on the recovery of white blood cells, red blood cells and platelets, peripheral blood was obtained 1, 3, 5 and 9 weeks after BMT and was analyzed on a Hemavet 950FS cell counter.

3.2.11 Competitive migration assay

For quantitative assessment of the migratory properties of $CCR2^{-/-}$ $CD8^+$ T cells, a modified BMT with a mix of WT (Thy1.1, CD45.2) and $CCR2^{-/-}$ (Thy1.2, CD45.2) donor $CD8^+$ T cells and WT BM (Thy1.2, CD45.1) was performed (139). Donor $CD8^+$ T cells were separated by MACS as described above. WT and $CCR2^{-/-}$ $CD8^+$ T cells were mixed in equal numbers and a total of $4x10^6$ mixed $CD8^+$ donor T cells was injected via tail vein into lethally irradiated C3FeB6F1 recipients. On days 6, 14, and 28 after transplant, the mice were euthanized and organs (liver, small bowel, spleen, PP, PLN, MLN) were harvested. Single cell suspensions of organs were prepared as described above. Cells were counted and stained for FACS analysis as described above. Fluorochrome-labeled antibodies against Thy1.1, CD45.1 and $H2K^k$-PE were used for discrimination of WT BM, WT and $CCR2^{-/-}$ donor and C3FeB6F1 host cells.

3.2.12 Mixed lymphocyte reaction

Ability to proliferate against foreign MHC *in vitro* was compared for WT and $CCR2^{-/-}$ $CD8^+$ T cells in a mixed lymphocyte reaction (MLR). $CD8^+$ splenic T cells from WT and $CCR2^{-/-}$ mice

were isolated by MACS as described above and $1x10^5$ T cells/well were incubated with irradiated (2000 cGy) RBC-lysed allogeneic (C3FeB6F1 or BALB/c) splenocytes ($2x10^5$/well) for 5 days in 96-well plates. 1 μCi/well of tritiated thymidine ([^{3}H]thymidine) was added for the last 18 h of stimulation. [^{3}H]thymidine incorporates in the DNA of dividing cells and allows the quantification of cell proliferation by measurement of incorporated radioactivity. Cells were harvested with a Filtermate 196 harvester and after addition of Microscint-20 scintillation fluid, counts per minute were measured with a Topcount NXT microplate scintillation counter. The basal proliferation rate of T cells without splenic stimulators was subtracted from specific proliferation against allogeneic stimulators. All experiments were done in multiple replicate wells and mean specific proliferation ± SEM was calculated.

3.2.13 CFSE assay

To determine the *in vivo* proliferation kinetics of T cells, lymphocyte division can be visualized by flow cytometry using cells labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) as described by Lyons and Parish (140). This method is based on the sequential halving of the intracellular dye CFSE with each round of cell division. Cell cycles (up to nine) can be analyzed for different T cell subsets and can be correlated with the expression of other markers of interest.

RBC-lysed splenic T cells of WT and $CCR2^{-/-}$ mice were positively selected using MACS columns and anti-CD5 microbeads. After separation, cells were washed in PBS, counted and resuspended in 2.5 μM CFSE in PBS ($50x10^6$ cells/ml). Cells were incubated for 20 min at 37°C and subsequently spun down without washing. Cells were then washed twice in PBS and resuspended in injection media. $10\text{-}20x10^6$ stained cells were transferred into sublethally (750 cGy) irradiated C3FeB6F1 recipients via tail vein injection. Recipient mice were euthanized at indicated time points and organs were analyzed by FACS.

3.2.14 Intracellular cytokine staining

To measure intracellular cytokine production of *in vivo* activated $CD8^+$ donor T cells from WT and $CCR2^{-/-}$ mice, splenocytes were harvested on day 7 from recipients of allogeneic BMT ($5x10^6$ B6 TCD BM + $3x10^6$ splenic $CD8^+$ T cells into lethally irradiated C3FeB6F1 recipients). Cells were restimulated *in vitro* with irradiated (2000 cGy) syngeneic (B6 (CD45.1)) or allogeneic (C3FeB6F1) TCD stimulators. After 12 h, cells were washed and stained for FACS analysis of surface antigen expression. Next, cells were fixed and permeabilized with the

Cytofix/Cytoperm Kit from Pharmingen according to the manufacturer's protocol. Cells were then stained with intracellular cytokine (IFN-γ and TNF-α) PE-conjugated antibody and acquired on a LSR I cytometer. Activated (CD44 high) donor $CD8^+$ T cells were analyzed for expression of IFN-γ and TNF-α.

3.2.15 Enzyme-linked immunosorbent assay

A quantitative sandwich enzyme immunoassay technique was used to measure serum IFN-γ levels of WT and $CCR2^{-/-}$ $CD8^+$ T cell recipients after BMT. All assays were performed using the mouse IFN-γ Quantikine ELISA Kit from R&D Systems. At three different time points (days 7, 14, 21), peripheral blood was obtained by retro-orbital puncture from mice in GVHD experiments. Blood in Microtainer serum tubes was allowed to clot for 30 min and serum was separated by centrifugation (12,000 rpm, 1.5 min) and stored at -80°C until analysis. Undiluted serum samples and serial dilutions of IFN-γ standards (600 pg/ml, 300 pg/ml, 150 pg/ml, 75 pg/ml, 37.5 pg/ml, 18.8 pg/ml, 9.4 pg/ml) were pipetted in duplicates into single wells of mouse IFN-γ mAb precoated 96-well plates. Plates were incubated at room temperature for 2 h to allow binding of IFN-γ to the plate-bound IFN-γ mAb. Plates were then washed rigorously to remove any unbound substances. Subsequently, an enzyme-linked polyclonal antibody specific for mouse IFN-γ was added to the wells. After 2 hours of incubation at room temperature, unbound antibody-enzyme reagent was removed by a washing procedure. A substrate solution, which can be metabolized by the now IFN-γ bound enzyme, was added to each well. The enzymatic reaction yields a blue product that turns yellow when a specific stop solution is added. The intensity of the color was determined on a MRX Revelation microplate photometer. The IFN-γ concentration of the sample is proportional to the color intensity and was determined by comparison to a standard curve, which was automatically generated for the serial dilutions of IFN-γ standards.

3.2.16 Cytotoxicity assay

Effector splenocytes were harvested on day 7 from recipients of allogeneic BMT ($5x10^6$ B6 TCD BM + $3x10^6$ splenic $CD8^+$ T cells into lethally irradiated C3FeB6F1 recipients). Target cells (32Dp210 and P815) were labeled for 2 hours with 100 μCi ^{51}Cr at $2x10^6$ cells/ml at 37°C and 5% CO_2. After 3 washes, labeled targets were plated at $5x10^3$ cells/well in 96-well plates. Effector cells were added at various effector-to-target ratios (corrected for % donor $CD8^+$ T cells as determined by FACS) in a final volume of 200 μl and plates were incubated for 4 hours at

37°C and 5% CO_2. Subsequently, 30 µl supernatant was removed from each well and counted in a Topcount NXT microplate scintillation counter to determine experimental release. Spontaneous release was obtained from wells receiving target cells and medium only and total release was obtained from wells receiving 10% Triton X-100. Spontaneous release was less than 10% of total release. Percentage cytotoxicity was calculated by the following formula: % cytotoxicity = 100 x (experimental release - spontaneous release) / (total release - spontaneous release).

3.2.17 Statistics

All values shown in graphs represent the mean ± SEM. All group-wise comparisons except for the migration, survival and GVHD score analysis were performed with the non-parametric unpaired Mann-Whitney U test. Survival data was analyzed with the Mantel-Cox log-rank test. For GVHD scores, the statistical analysis performed to test whether a differential change occurred between treatment groups was the pair-wise difference in the area under curve (AUC) between groups, using all possible pair-wise contrasts. Not all the mice were followed for the full length of the study. In order to account for informative dropouts, the AUCs were calculated up to the minimum follow-up time for each pair-wise difference (141). To assess differences between groups in the competitive migration assay, a permutation test based on the Wilcoxon rank sum statistic was used. $p<0.05$ was considered statistically significant. Only statistically significant differences are annotated in the graphs.