5. Materials and Methods

5.1. Mice

Transgenic DPE^{GFP} mice were previously generated by Dr. Weninger in the laboratory of Dr. von Andrian (CBR Institute for Biomedical Research, Boston, MA). For their generation, a 1.8 kb DNA fragment containing a 1.3 kb fragment of the distal CD4 enhancer and a 0.5 kb fragment of the proximal CD4 enhancer was isolated from a cDNA-construct provided by Dr. Siu [162, 163]. The DNA elements were subcloned into a plasmid, in which GFP is driven under the control of the CD4 promoter without the CD4 silencer [164, 165], replacing the minimal proximal CD4 enhancer with the new 1.8 kb fragment. DPE^{GFP} mice were generated by microinjection of the linearized plasmid DNA into fertilized FVB oocytes. Offspring was screened by PCR for DNA integration and by flow cytometry for GFP expression in T cells. Five independent founder lines were established. All data presented were generated using line 1 that had the brightest GFP expression in T cells and in pDCs. The mice were crossed into the C57Bl/6 background for 10 generations. After eight generations, they were also crossed with RAG-1 deficient animals in the C57Bl/6 background (DPE^{GFP}x$RAG\text{-}1^{-/-}$). For the generation of DPE^{RED} mice, GFP in the DPE^{GFP} construct was replaced with DsRED2 (Clontech, Moutain View, CA), and microinjections were performed in FVB oocytes. DPE^{RED} mice were backcrossed into the C57Bl/6 background for 3 generations.

For this project, two different $CD8^+$ T cell receptor transgenic mice were used, OT-1 mice (Tg(Tcra,Tcrb)1100Mjb mice) and P14 mice. OT-1 mice contain transgenic inserts for mouse Tcra-V2 and Tcrb-V5 genes designed to recognize ovalbumin residues 257-264 in the context of $H\text{-}2K^b$ (peptide SIINFEKL). OT-1 mice were obtained from The Jackson Laboratory (Bor Harbor, ME). P14 mice have transgenic $CD8^+$ T cells that recognize the $GP_{33\text{-}41}$ peptide from LCMV glycoprotein (GP) in context of $H\text{-}2D^b$. P14 mice crossed to Thy1.1 congenic mice were provided by Dr. Wherry (Wistar Institute, Philadelphia, PA).

Mice deficient in the type I interferon receptor (IFNAR$^{-/-}$) [166] and 129/Sv wild-type mice were obtained from The Jackson Laboratory (Bor Harbor, ME).

Ikaros$^{L/L}$ mice were generated in Dr. Chan's laboratory [167]. In brief, a fusion protein of β-gal and neomycin-phosphotransferase reporter gene was inserted into exon 2 of the Ikaros locus via homologous recombination in P1 ES cells. In the studies described here, sex- and age-matched wild-type and, in some experiments, heterozygous littermates were used as controls.

Wild-type C57Bl/6 and BALB/c mice of both sexes and generally 6-10 weeks old were purchased from Charles River (Wilmington, MA). Mice were used between 6 and 14 weeks of age unless stated otherwise.

All mice were housed and bred at the Wistar Institute under specific pathogen free (SPF)/viral antibody free conditions. All studies were performed according to animal protocols approved by the Institutional Animal Care and User Committee (IACUC) of the Wistar Institute.

5.2. Reagents

5.2.1. Viruses

A stock of the mouse-adapted influenza virus strain A/Puerto Rico/8/34 (H1N1) (PR8) was a kind gift from Dr. Palese (Mount Sinai Hospital, NY). PR8-GP33 virus (a kind gift from Dr. Wherry (Wistar Institute, Philadelphia, PA) was originally made by Dr. Webby by fusing the LCMV glycoprotein epitope 33-41 to the cytosolic part of neuraminidase of PR8 virus.

5.2.2. Cell lines

B16F10 melanoma cells modified to express recombinant FLT-3L (B16-Flt-3L) [168] were a donation from Dr. Ertl (Wistar Institute, Philadelphia, PA).

Madin Darby Canine Kidney (MDCK) cells were obtained from the American Tissue culture collection (ATCC, Manassas, VA).

5.2.3. Antibodies

5.2.3.1. Antibodies for flow cytometry

If not mentioned otherwise, antibodies were purchased from BD Biosciences (Franklin Lakes, NJ).

Table II. Antibodies against immune cell subsets

Antibody specificity	Clone	Fluorophore
CD8α (Ly-2)	53-6.7	PE[1], PerCP, APC
CD4	L3T4	PE, APC, not labeled
CD3	17A2	FITC, PerCP
CD45R/B220	RA3-6B2	FITC, PE, PerCP, APC, not labeled
CD19	1D3	PE, PerCP, not labeled
Gr-1 (Ly6C and Ly6G)	RB6-8C5	PE, not labeled
CD11b	M1/70	PE, PerCP
CD11c	HL3	PE, APC
F4/80	BM8	PE
Nk1.1	PK136	PerCP
CD49b (Pan-NK)	DX5	PE
mPDCA-1[2]	JF05-1C2.4.1	APC
Ly49Q[3]	2E6	PE
CD16/32	2.4G2	not labeled
I-A/I-E	2G9	not labeled
Ter119	Ly-76	not labeled

[1]FITC: Fluorescein isothiocyanate, PE: Phycoerythrin, PerCP: Peridinin chlorophyll protein, APC: Allophycocyanin
[2]mPDCA-1 was purchased from Miltenyi Biotec (Auburn, CA)
[3]Ly49Q was obtained from MBL International Corporation (Woburn, MA)

Table III. Antibodies against costimulatory molecules

Antibody specificity	Clone	Fluorophore
$IgG_{2a, \kappa}$	-	FITC, PE, PerCP, APC
CD40	3/23	PE
CD80 (B7-1)	16-10A1	PE
CD86 (B7-2)	GL1	PE
IA^b	AF6-120.1	PE
CD274 (PD-L1, B7-H1)	M1H5	PE
PD-L2 (B7-DC)	TY25	PE
B7H3	M32D7	PE
B7H4	Clone 9	PE

Table IV. Antibodies against homing molecules

Antibody specificity	Clone	Fluorophore
CD62L	Mel-14	PE
CD44	IM7	PE
CD49d	R1-2	PE
$\alpha_4\beta_7$	DATK32	PE
huFc region of IgG_1	-	
P-selectin Ig[1]	-	
E-selectin Ig[1]	-	

[1]P- and E-selectin (R&D Systems, Minneapolis, MN) are fused to the Fc region of human IgG_1 (huFc)

Table V. Antibodies against intracellular cytokines

Antibody specificity	Fluorophore
IFN-γ	PE
TNF-α	
IL-12	

Table VI. Antibodies against intracellular signaling molecules

Antibody specificity	Phosphorylation site
p-p38	p-T180/p-Y182
p-STAT-1	p-Y701

Table VII. Antibodies used for phenotypic analysis of T cells

Antibody specificity	Clone	Fluorophore
CD8α (Ly-2)	53-6.7	PerCP
Vα2	B20.1	PE, Biotin
Thy1.1	HIS51	APC, Biotin
CD25	PC61	PE
CD69	H1.2F3	PE
CD44	IM7	PE

APC-labeled tetramer $D^bNP_{366-374}$, which is recognized by endogenous $CD8^+$ T cells specific for the epitope of the influenza virus nucleoprotein, was a kind gift from Dr. Wherry (Wistar Institute, Philadelphia, PA).

Table VIII. Second-step reagents

Antibody specificity	Fluorophore
Streptavidin	PE, APC
goat $F(ab')_2$ anti-human (Fc sp.)	Biotin, PE

5.2.3.2. Blocking antibodies in homing studies

Monoclonal antibodies (mAbs) 9A9 and 5H1, which neutralize mouse E- and P-selectin, respectively, were kindly provided by Dr. Barry Wolitzky (ChemBridge Research Laboratories, San Diego, CA). The L-selectin neutralizing mAb Mel-14 was purchased from BD Biosciences (Franklin Lakes, NJ).

5.2.3.3. Antibodies for IFN-α ELISA

Recombinant mouse IFN-α was purchased from HyCult Biotechnology (Uden, The Netherlands).
Rabbit anti-mouse anti-IFN-α for ELISA was obtained from PBL Biomedical Laboratories (R&D Systems, Minneapolis, MN).
Peroxidase-conjugated goat anti-rabbit IgG was purchased from Calbiochem (EMD Biosciences, San Diego, CA).

5.2.3.4. Antibodies for immunoblotting

Primary antibodies for phospho-p38, phospho-ERK1/2 and anti-β-actin were obtained from Cell Signaling Technology (Danvers, MA). Anti-Iκ-Bα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary horseradish peroxidase (HRP)-linked antibodies were purchased from Pierce Biotechnology (Rockford, IL).

5.2.4. Chemokines

Chemokines CXCL12 (SDF-1α) and CCL21(SLC) were purchased from R&D Systems (Minneapolis, MN).

5.2.5. Chemicals

Table IX. Chemicals and reagents

Product name	Manufacturer
[^{3}H]thymidine	Perkin Elmer
100 bp DNA ladder	New England Biolabs
5- (and 6-) carboxyfluorescein succinimidyl ester (CFSE)	Molecular Probes
Agarose	Fisher Scientific
Ammonium Chloride	Fisher Scientific

Bovine serum albumin (Fraction V)	Fisher Scientific
Citric Acid	Fisher Scientific
Complete protease inhibitor	Roche Applied Sciences
CpG 1826 5'- tcc atg acg ttc ctg acg tt -3' Complete phosphorothioate-modified	MWG Biotech
Disodiumphosphate	Fisher Scientific
dNTPs	Roche Diagnostics
DOTAP	Roche Diagnostics
Ethidium bromide	Sigma
Gentamycin	Mediatech
Glycerol	Fisher Scientific
Incomplete Freund's adjuvant (IFA)	Sigma
Ionomycin	Sigma
Lipopolysaccharide (LPS) from E. Coli (Serotype 0127:B8)	Sigma
Magnesium Chloride	New England Biolabs
Monosodiumphosphate	Fisher Scientific
NP-40	Fluka
Paraffin	Sigma
Pertussis toxin	Calbiochem
Phorbol-13myristate (PMA)	Sigma
Phosphatase inhibitor cocktail 2	Sigma
Potassium Chloride	Fisher Scientific
Potassiumhydrogencarbonate	Sigma
Proteinase K	Roche Diagnostics
Sodium Bicarbonate	Fisher Scientific
Sodium Chloride	Fisher Scientific
Sodium Citrate	Sigma
Sodium dodecyl sulfate (SDS)	Sigma
Sucrose	Sigma
Taq-polymerase	Roche Diagnostics
Thioglycollate	BD Bioscience

Tetramethylbenzidine (TMB) substrate	Calbiochem
Tris	Fisher scientific
Triton X-100	Sigma
Tween 20	Bio-Rad

5.2.6. Cell culture reagents

Table X. Cell culture reagents

Product name	Manufacturer
0.04% Trypan Blue	Bio Whittaker
0.25% Trypsin-EDTA	Invitrogen
1 M Sodium pyruvate	Invitrogen
1 M HEPES	Invitrogen
2-Mercaptoethanol	Fisher Scientific
4% Paraformaldehyde (PFA)	Fisher scientific
5 M EDTA	Sigma
Collagenase D	Roche Diagnostics
Dimethylsulfoxide (DMSO)	Sigma
Dulbecco's phosphate buffered saline (PBS)	Invitrogen
Fetal bovine serum (FBS)	Valley Biomedical
Goat-anti-rat IgG microbeads	Miltenyi Biotec
Hanks' balanced salt solution (HBSS)	Invitrogen
Iscove's Dulbecco medium	Mediatech
L-Glutamine	Invitrogen
Methanol	Fisher Scientific
Penicillin/Streptomycin (100x)	Invitrogen
RPMI 1640 with L-Glutamine	Mediatech

5.2.7. Buffers and media

Staining buffer: PBS, 1% FBS

Citrate solution: PBS, 5% Sodiumcitrate, 1.6% Citric Acid

Ammonium chloride lysis buffer (ACK): 0.15M NH_4Cl, 0.1 mM EDTA, 10 mM $KHCO_3$

Tail lysis buffer: 50 mM Tris, pH 8.0, 50 mM KCl, 2.5 mM EDTA, 0.45% Tween 20, 0.45% NP-40

Cell lysate buffer (HNTG): 0.1% Triton X-100, 20 mM HEPES, 10% Glycerol, 150 mM NaCl

Wash buffer: 25 mM Tris, pH 7.5, 500 mM NaCl, 0.1% Tween 20

Stripping buffer: 2 M Tris, 10% SDS, 14.4 M 2-Mercaptoethanol

Tris-Acetate-EDTA (TAE) buffer: 40 mM Tris-Acetate, pH 8.3, 1 mM EDTA

10x PCR-Amplification buffer: 0.1 M Tris, pH 8.4, 0.5 M KCL, 25 mM $MgCl_2$

HAS-buffer: 3.8 mM NaH_2PO_4, 6.7 mM Na_2HPO_4 x $7H_20$, 140 mM NaCl

Complete medium: RPMI supplemented with 10% heat-inactivated FBS, Penicillin/Streptomycin, 10 mM HEPES, 2 mM L-Glutamine, 10 mM Sodium pyruvate, 50 μM 2-Mercaptoethanol

Labeling medium: RPMI, 20 mM HEPES, 1% FBS

5.2.8. Peptides

GP_{33-41}: NH_2-KAVYNFATM-COOH

$OVA_{257-264}$: NH_2-SIINFEKL-COOH

Peptides were a kind gift from Dr. Wherry, Wistar Institute, Philadelphia, PA.

5.3. Methods

5.3.1. Genotyping and Phenotyping of mice

5.3.1.1. Tailbleeds

DPE^{GFP}, DPE^{DsRED} and TCR-transgenic mice were screened at weaning age by analysis of peripheral blood. In brief, tail veins of mice were nicked with a sterile scalpel blade and 5-7 μl blood were collected into tubes containing 30 μl citrate solution. Subsequently, erythrocytes were lysed with ammonium chloride lysis buffer. OT-1 and P14 mice were identified by staining with antibodies against the Vα2-chain of the TCR and CD8α. Samples were analyzed by flow cytometry on a FACSscan or FACSCalibur (BD Biosciences) (see 5.3.11.). Fluorescent-reporter transgenic mice were identified by expression of GFP or DsRED on T cells in the blood. OT-1 and P14 mice were positive for the transgene, when all $CD8^+$ T cells co-expressed the Vα2-chain.

5.3.1.2. PCR (Polymerase chain reaction)

5.3.1.2.1. PCR of $Ikaros^{L/L}$ mice

For genotyping, a small tail clip (0.2 cm) was incubated overnight in 100 μl tail-lysis buffer containing 1 μl Proteinase K at 55°C. On the next day, tails were heated to 100°C for 10 min., and 1 μl was used for the PCR reaction.

Genotyping of mice was performed by PCR with the following oligonucleotide primers:

A) 5’ GAA GCC CAG GCA GTG AGG TTT TCC 3’

B) 5’ GGC AAA GCG CCA TTC GCC ATT CAG 3’

C) 5’ CAT GCC TCG ATC ACT CTT GGA GTT C 3’

The reaction was mixed as follows:

1 μl tail DNA

5 μl 10x PCR reaction buffer containing 15 $MgCl_2$

0.5 μl dNTPs (10 mM)

0.2 µl of 1 mg/ml oligonucleotide A, B, C

0.4 µl 5 U/µl Taq polymerase

at 50 µl H_20

PCR was performed in a Mastercycler gradient (Eppendorf, Westbury, NY):

94°C 5:00

94°C 0:30

65°C 0:30

72°C 0:30 (35 cycles)

72°C 10:00

4°C hold

The PCR products were separated by gel electrophoresis on a 2% agarose gel containing 0.3 mg/ml ethidium bromide in 1x Tris-Acetate-EDTA (TAE)–buffer. PCR-product of Ikaros$^{L/L}$ mice: 250 bp (base pairs); PCR-product of wild-type mice: 200 bp; PCR-products of Ikaros$^{+/L}$ mice: 250 bp and 200 bp.

5.3.1.2.2. PCR of DPE^{GFP}xRAG-1$^{-/-}$ mice

Tails were prepared as described above.

Genotyping of DPE^{GFP}xRAG-1$^{-/-}$ mice was performed by PCR with the following primers:

A) 5' TGG ATG TGG AAT GTG TGC GAG 3'

B) 5' GAG GTT CCG CTA CGA CTC TG 3'

C) 5' CCG GAC AAG TTT TTC ATC GT 3'

The reaction was performed according to the protocol provided by the Jackson Laboratories for RAG-1^{tm1Mom} mice:

2 µl tail DNA

5 µl 10x amplification buffer

2.5 µl dNTPs (2 mM)

0.2 µl of 1 mg/ml oligonucleotide A, B, C

0.4 µl 5 U/µl Taq polymerase

at 50 µl H_20

PCR was performed in a Mastercycler gradient (Eppendorf Westbury, NY):

94°C 3:00

94°C 0:30

58°C 0:45

72°C 0:45 (37 cycles)

72°C 2:00

4°C hold

The PCR products were separated by gel electrophoresis on a 1.5% agarose gel in 1x TAE-buffer. Mutant: band 530 bp, Wild-type: band 474 bp, Heterozygous: bands at 530 bp and 474 bp.

5.3.2. Cell culture

MDCK cells were grown in Iscove's Dulbecco medium supplemented with 5% fetal bovine serum and 50 μg/ml Gentamycin in 175 cm^2 flasks (BD Falcon™, BD Biosciences, Franklin Lakes, NJ). For the passage of cells, cultures were washed once with PBS and then treated with 0.25% Trypsin-EDTA. After detachment, cells were transferred into a new flask containing fresh media.

B16-Flt-3L cells were grown in culture in RPMI medium containing 10% FBS and Penicillin/Streptomycin. The passage was performed as described for the MDCK cells.

All cell cultures were kept in a humidified incubator at 37°C with 5% CO_2 (Thermo Fisher Scientific, Waltham, MA).

5.3.3. Viral assays

5.3.3.1. Propagation and purification of viruses

Influenza viruses were propagated in the allantoic fluid of 10-day-old embryonated SPF-grade hen chicken eggs (B&E Eggs, York Springs, PA). Eggs were candled for identification of the air sac and vessels and marked for a clear area. A small hole was drilled into the shell

at the mark without scarifying the membrane. Infectious PR8 stock was diluted in sterile PBS (1:1000) containing 50 μg/ml gentamycin, and 50 μl were injected per egg. Heated paraffin was applied afterwards to seal the injection hole. Eggs were placed into a humidified incubator at 37°C for 2 days and then transferred into a cold room overnight before harvesting the allantoic fluid. Allantoic fluid was obtained after removal of the eggshell and membrane and pooled from several eggs. To pellet debris, fluid was centrifuged at 3000 rpm for 10 min. at 4°C. Aliquots were stored in a -80°C freezer (Thermo Fisher Scientific, Waltham, MA) until further use.

For purification of virus, allantoic fluid was pelleted in an ultracentrifuge (Beckmann Coulter, Fullerton, CA) at 18,000 rpm for 2 hours. Supernatant was discarded, and pellet kept overnight at 4°C. 30% sucrose in HAS-buffer was frozen and thawed twice resulting in a 15-45% gradient. Resuspended virus-pellet was placed on top of the sucrose gradient and centrifuged at 18,000 rpm for 45 minutes. Virus was harvested and pelleted at 25,000 rpm for 45 minutes. Finally, concentrated purified virus was resuspended in HAS and stored in aliquots at -80°C.

For UV-inactivation, virus was placed under a ultraviolet (UV)-lamp (UVG-54, UVP) and treated for 1 min. with short wave UV (254 nm). Virus was subsequently kept in a glass vial at 4°C.

5.3.3.2. Titration of viral preparations and lung homogenates on MDCK cells

For determination of viral titers (viral infectivity) in virus preparations and tissues, $5x10^4$ MDCK cells in 100 μl Iscove's medium supplemented with gentamycin and 0.1% BSA were plated in 96 well flat bottom plates (Costar Corning, Inc., Acton, MA), and incubated at 37°C. Four hours later, dilutions of viral stocks or tissue homogenates were prepared.

For lung titers, organs were obtained at several days post infection and frozen at -20°C until further processing. In brief, frozen lungs were homogenized in PBS in a sterile mortar. The debris was pelleted at 1300 rpm for 5 min., and the supernatant was collected. Lung extracts were tested for infectious virus by titration in replicate MDCK cell microcultures (detection threshold of $10^{1.4}$ to $10^{1.8}$ 50% tissue culture infectious dose ($TCID_{50}$)/ml). Fifty μl

of sample-dilutions were transferred to plated MDCK-cells and incubated overnight at 37°C. On the next day, 50 µl of 2.5% Trypsin-EDTA, diluted 1:3000 in Iscove's media was added to each well and incubated for an additional 2 days. Supernatants were tested for hemagglutinating activity of chicken red blood cells (see below).

5.3.3.3. Hemagglutination assay

This assay uses the ability of influenza virus to cause red blood cell-agglutination as a read-out. In brief, 25 µl of supernatants from MDCK microcultures were transferred into round bottom plates (Costar Corning, Inc., Acton, MA) and mixed with 25 µl 1% chicken red blood cells (CRBC) (B&E Eggs, York Springs, PA) in HAS. After incubation for 30 min. at room temperature (RT), presence of virus in wells was determined by agglutination of CRBC. Titers were calculated as 50% $TCID_{50}$/ml and hemagglutinating units (HAU).

5.3.3.4. Hemagglutination inhibition assay

This assay is used to detect and quantify the presence and amount of anti-HA antibodies in plasma or serum of infected mice, which will prevent the hemagglutination of CRBC by the virus. In brief, plasma or serum of mice was collected at several time points following infection. For preparation of serum, peripheral blood was collected by cardiac puncture (see 5.3.11.1) into a 1.5 ml reaction tube and allowed to clot overnight at 4°C. After centrifugation at 13,000 rpm for 5 min., supernatants were transferred to new tubes and stored frozen at -20°C. Prior to the assay, samples were heat inactivated for 30 min. at 56°C. In round bottom 96 well plates, serial dilutions of samples in HAS buffer (25 µl) were incubated in equal shares with of a standard (4 agglutination units) of virus. After 1 h, 50 µl of 1% CRBC were added and incubated for 30 min. before determining the agglutination. The hemagglutination inhibition titer for each sample was calculated from the endpoint (greatest dilution) at which the hemagglutination was inhibited.

5.3.4. *In vivo* Flt-3L-treatment

B16-Flt-3L cells were grown as described above. For injection, cells were trypsinized, washed once, counted by trypan blue exclusion and resuspended in PBS. $3x10^6$ tumor cells in 100 μl PBS were injected into the skinfold of the neck of $DPE^{GFP}xRAG\text{-}1^{-/-}$ mice. Mice were sacrificed at days 12-14 after tumor-injection.

5.3.5. Isolation and activation of plasmacytoid dendritic cells and myeloid dendritic cells

5.3.5.1. Isolation of DC subsets by flow cytometry activated cell sorting (FACS)

pDCs and mDCs were purified from spleens of B16-Flt-3L-bearing (FLT-3L-treated) $DPE^{GFP}xRAG\text{-}1^{-/-}$ mice. Spleens were harvested from mice that had been euthanatized by CO_2-inhalation. Single cell suspensions were prepared by passing organs through a 70 μm nylon mesh (BD Biosciences, Franklin Lakes, NJ). After centrifugation at 1300 rpm for 5 min., red blood cells were lysed in ACK-lysis buffer for 1 minute. Lysis buffer was neutralized by washing once in staining buffer. For Enzyme-linked ImmunoSorbent Assays (ELISA) to measure IFN-α and microarray experiments, pDCs were sorted solely based on GFP expression using a MoFlo™ (DakoCytomation, Carpinteria, CA). For coculture experiments with T cells, splenocytes from Flt-3L-treated $DPE^{GFP}xRAG\text{-}1^{-/-}$ mice were incubated with the antibodies CD11c-APC and CD11b-PE and subsequently sorted into $CD11c^+$ $CD11b^+$ (mDCs) and $CD11c^{int}$ GFP^{hi} (pDCs) cells. For testing the type I interferon dependence during pDC activation, splenocytes from Flt-3L-treated 129/Sv WT and $IFNAR^{-/-}$ mice were stained with anti-CD11c and mPDCA-1 and the $CD11c^{int}$ $PDCA\text{-}1^+$ population was sorted. The purity of isolated cell subsets was generally 95-98%.

5.3.5.2. *In vitro* activation of pDCs and mDCs

Sorted DCs were resuspended at $1x10^6$/ml in complete media. PDCs were cultured in 15 ml Falcon tubes in media alone or with 5 μg/ml CpG 1826 or 300 HAU/ml UV-inactivated sucrose-gradient purified influenza virus A/PR/8/34 (PR8). Tubes were placed in a humidified incubator at 37°C with 5% CO_2. $CD11c^{hi}$ cells were cultured in media with or without 5 μg/ml LPS. Cell pellets and supernatants were collected at different time points as indicated below.

5.3.5.3. *In vivo* activation of pDCs

20 μg CpG 1826 oligonucleotides were mixed with 15 μl DOTAP in 200 μl PBS and incubated for 30 min. on ice before intravenous injection into DPE^{GFP} mice. Twenty hours later, the phenotype of GFP^{hi} $B220^+$ pDCs was assessed by flow cytometry.

5.3.6. Influenza virus infection

Mice were anesthetized intraperitoneally with Ketamine/Xylazine (70 mg/7 mg per kg). Subsequently, mice were inoculated intranasally with 500 $TCID_{50}$ live virus (allantoic fluid containing PR8 or PR8-GP33 virus diluted in 50 μl sterile PBS). Mice were monitored daily for signs of disease (ruffled fur, labored breathing, wasting). Morbidity was quantified daily by measure of body weight.

5.3.7. Cytospins and Microscopy

Cytospins of cells were performed using a Cytospin3 centrifuge (Thermo-Shandon, Pittsburgh, PA). 10^5 cells in 100 μl medium were placed into the centrifugation units assembled with a slide and centrifuged for 5 min. at 2000 rpm. Slides were subsequently fixed and stained using the Kwik-Diff stain kit (Thermo-Shandon, Pittsburgh, PA) (5 min. fixation, 5 min. eosin-stain, 1 min. methylene blue-stain). Slides were washed with water, air-

dried and embedded with a cover slip. Images were taken on a Nikon E600 upright microscope (40x magnification). Freshly sorted pDCs on a slide were imaged with a Nikon TE300 inverted microscope.

5.3.8. Supernatant analysis of activated dendritic cell cultures

5.3.8.1. ELISA

Supernatants from overnight DC cultures (see 5.3.5.2.) were collected and stored at -80°C. The ELISA for IFN-α was performed as described [42]. In brief, round bottom plates (Costar Corning, Inc., Acton, MA) were coated overnight at 4°C with 50 µl of 2 µg/ml monoclonal anti-mouse IFN-α in 1 M $NaHCO_3$, pH 8.2. Plates were then extensively washed with PBS, 0.05% Tween. After a blocking step with 200 µl PBS, 3% BSA per well for 2 h at RT, plates were washed again and then incubated with 50 µl of samples or a recombinant mouse IFN-α standard for 2 h at RT. After repeated washing, 100 µl of a 1:1000 dilution of polyclonal rabbit anti-mouse IFN-α in PBS, 3% BSA was added for 1 h and incubated at room temperature. Subsequently, plates were washed and developed with 100 µl of a 1:4000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h. After repeated washing, 100 µl TMB peroxidase substrate was added to wells and incubated for 20 minutes. Reaction was stopped with 50 µl of 250 mM HCl and absorbance was measured at 450 nm with a Microplate ELISA reader (BioTEK Instruments, Inc., Winooski, Vermont).

5.3.8.2. Multianalyte profiling

Supernatants were collected from cultured pDCs (see 5.3.5.2.) after 24 h and stored at -80°C. For multianalyte profiling, samples were submitted to Rules-Based Medicine (Austin, Texas). This technology uses differentially color-labeled microspheres that are covered with capture antibodies that bind to specific target proteins. In a multiplex sandwich immunoassay, identification and quantification is subsequently performed with a laser system.

5.3.9. Preparation of cell lysates and immunoblotting

Whole cell lysates were prepared from $1x10^6$ pDCs cultured in medium or activated as described above. Cells were washed with ice-cold PBS, and resuspended in HNTG buffer supplemented with complete protease inhibitor and phosphatase inhibitor cocktail 2 according to manufacturer's instructions. Whole cell lysates were stored at -20°C until used for immunoblotting. Protein concentrations were determined using the Micro BCA protein assay kit (Pierce, Rockford, IL). As previously described [169], lysates were then resolved on a 10% polyacrylamide gel containing sodium dodecyl sulfate and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) using a Trans-Blot Semi-Dry cell (Bio-Rad, Hercules, CA). Nitrocellulose filters were then incubated with wash buffer containing 5% milk protein for 1 h to block nonspecific protein binding. Primary antibodies were diluted in wash buffer, 0.5% milk protein (1:1000 p-ERK, 1:500 Iκ-Bα, 1:1000 β-actin) and applied to the blots overnight at 4°C. Following washing, primary antibodies were detected by incubation with horseradish peroxidase-linked anti-rabbit IgG for 45 min. at room temperature. Immunoreactive bands were visualized using Enhanced Chemiluminescence (ECL) (Amersham Pharmacia Biotech, Piscataway, NJ) solution. To determine total loading, immunoblots were washed and incubated in stripping buffer for 30 min. at 65°C, and then reprobed for total ERK, total p38, or β-actin proteins.

5.3.10. Migration assays

5.3.10.1. Chemotaxis assay

This *in vitro* assay determines whether a specific cell subset is chemoattracted by a certain reagent. In principle, cells are separated by a membrane from a chemotactic agent that is tested for its ability to induce cell migration through the membrane towards the chemotactic agent.

In brief, $5x10^5$ splenocytes from $DPE^{GFP}xRAG\text{-}1^{-/-}$ mice in 100 µl RPMI 1640/10% FBS were loaded in Transwell filters with 5 µM pores (Costar Corning, Inc., Acton, MA), which

were placed in 24 well plates containing 600 µl medium without additives or with chemokines at different concentrations. After incubation for 3 h at 37°C, cells in the bottom well were collected, counted by flow cytometry, and the percentage of transmigrated cells was calculated.

5.3.10.2.Homing assays

5.3.10.2.1. Adoptive transfer of pDCs

Spleens from Flt-3L-treated $DPE^{GFP}xRAG\text{-}1^{-/-}$ mice were harvested as described above. $5x10^7$ cells containing $2\text{-}4x10^6$ GFP^+ pDCs were injected into the tail vein of C57BL/6 recipients. After 4, 24, or 96 h, organs of recipient mice were collected and analyzed for homed cells. Single cell suspensions from various organs were prepared as described below (see 5.3.11.1.), stained with αB220 and analyzed for the presence of GFP^+ $B220^+$ pDCs by flow cytometry. To correct for differences in the number of injected cells between individual experiments, all data were normalized to the number of homed pDCs/10^6 injected pDCs.

5.3.10.2.2. Homing of endogenous pDCs in response to Thioglycollate-induced inflammation

To investigate the accumulation of endogenous pDCs at sites of inflammation, BALB/c mice were injected with 1 ml Thioglycollate (3% w/v in H_20) into the peritoneal cavity. At various time points thereafter, peritoneal exudate cells (PECs) were harvested by lavage (see 5.3.11.1.). PDCs were identified by staining with the antibodies CD45R/B220 and mPDCA-1 and analyzed by flow cytometry.

For blocking experiments, mice were injected i.v. with mAb (100 µg in 200 µl PBS of Mel-14 (anti-L-selectin), 5H1 (anti-P-selectin), or 9A9 (anti-E-selectin) alone or in combination) at the time of Thioglycollate injection and again after 8-9 h (each mAb at 50 µg).

For intracellular cytokine production, PECs were harvested after 68 h and incubated for 4 h with 375 HAU/ml purified and UV-inactivated PR8 virus or 5 μg/ml CpG 1826 in the

presence of Brefeldin A. pDCs were identified by staining with αB220. Intracellular cytokine staining for IFN-γ, IL-12 and TNF-α was performed as described below (see 5.3.11.3.).

5.3.10.2.3. Competitive homing assay in a peritonitis-model

For competitive homing experiments, peritonitis was induced in C57Bl/6 WT mice by i.p. injection of 0.5 ml emulsified incomplete Freund's adjuvant (IFA) in PBS (1+1, v/v). Twenty four hours later, $5x10^7$ splenocytes from Flt-3L-treated $DPE^{GFP}xRAG\text{-}1^{-/-}$ mice were mixed with 10^7 splenocytes from DPE^{DsRED} mice and injected intravenously into recipient mice. The input was analyzed by flow cytometry for the ratio of GFP^+ pDCs and $DsRED^+$ T cells. For treatment with pertussis toxin (PTX), cells were incubated with 100 ng/ml PTX for 1 h at 37°C in complete media prior to injection. Twenty four hours later, mice were sacrificed and PECs harvested. At the same time, peripheral blood (PBL) was obtained by cardiac puncture, and peripheral lymph nodes and spleens were also harvested. To correct for differences in the number and composition of injected cells between individual experiments, the homing index (HI) was calculated according to the following equation:

$HI = pDC_{organ}/T\ cells_{organ} : pDC_{input}/T\ cells_{input}$.

5.3.11. Flow cytometry analysis

5.3.11.1. Preparation of tissue samples

Mice were either euthanatized by CO_2 inhalation or anesthetized by intraperitoneal injection of Ketamine/Xylazine (70 mg/7 mg per kg). Depth of anesthesia was monitored by checking the hind leg withdrawal reflex. Peripheral blood lymphocytes (PBL) were obtained by cardiac puncture and the addition of 50 µl citrate solution to the blood sample to avoid clotting. Organs collected for homing assays included peripheral lymph nodes (PLN: inguinal, brachial and axillary), mesenteric LNs (MLN), spleen, liver and lung. In addition, the draining LNs of the upper and lower respiratory tract, cervical LNs (cervLNs) and the mediastinal LN (medLN), were collected in some experiments. In some cases, lungs were perfused retrogradely with 5 ml of PBS/1% FBS (staining buffer) through the left ventricle of

the heart. Bone marrow (BM) was harvested from the femura and tibiae of hind legs. PECs were collected by flushing the peritoneal cavity with 3x3 ml cold staining buffer. In the model of lung infection, bronchioalveolar lavage (BAL) was performed by flushing the airway compartment via the trachea with 3x0.8 ml staining buffer.

Single cell suspensions from explanted organs were produced by passing them through a 70 µm nylon mesh (BD Biosciences, Franklin Lakes, NJ) or a metal mesh and resuspending them in staining buffer. Lungs and the left superior liver-lobe were cut into small pieces and digested in Hank's buffered saline solution (HBSS) supplemented with 400 U/ml Collagenase D for 30 min. at 37°C. Cell suspensions from organs containing red blood cells (PBL, spleen, lung, liver and bone marrow) were lysed for 1 minute. Single cell suspensions of organs were kept in staining buffer on ice unless stated otherwise.

5.3.11.2.Staining procedure for flow cytometry

For immunofluorescence staining, cells were resuspended in staining buffer and 1-5x10^6 cells were transferred into a 96 V-bottom well plate (Costar Corning, Inc., Acton, MA). Fc-receptors were blocked by 20 min. incubation on ice with unlabeled αCD16/αCD32 antibody (2.5 µg/ml). After washing, cells were resuspended in 50 µl staining buffer containing the first antibody in the appropriate concentrations (see Tables II-IV, Table VII) and incubated for 20 min. on ice. When P- or E-selectin-Ig chimera were used, staining and washing steps were performed in HBSS containing Ca^{2+}- and Mg^{2+}-ions followed by PE-labeled $F(ab')_2$ anti-human Ig. When biotinylated antibodies were used, cells were washed and incubated with fluorescently labeled streptavidin. After washing, cells were analyzed by flow cytometry on a FACScan or a FACSCalibur (BD Biosciences, Franklin Lakes, NJ). Data were processed using the FlowJo software (Tree Star, Inc., Ashland, OR) or the Cell Quest software (BD Biosciences, Franklin Lakes, NJ).

5.3.11.3. Staining procedure for intracellular cytokines

Intracellular cytokine staining procedure was performed using the commercially available Cytofix/Cytoperm Kit (BD Biosciences, Franklin Lakes, NJ). Before fixation, cells from restimulation cultures were stained for CD8α (and Thy1.1) or B220 (pDCs). For fixation, cells were then resuspended in 100 µl Cytofix/Cytoperm solution containing paraformaldehyde (PFA) and incubated for 20 min. on ice. Cells were washed twice in Perm/Wash buffer. This buffer contains the detergent saponin, which permeabilizes the cell membranes to allow for intracellular staining. Fluorescently labeled antibodies against the cytokines TNF-α, IFN-γ and IL-12 (see Table V) as well as the respective isotype control antibody were diluted in 1x Perm/Wash buffer, and cells were incubated in a total volume of 50 µl for 20-30 min. on ice in the dark. Cells were washed twice and finally resuspended in staining buffer for the analysis by flow cytometry.

5.3.11.4. Staining for intracellular phosphoproteins

The protocol was previously described by Krutzik at al. [170]. In brief, PFA was added to cells in a final concentration of 1.6% and incubated for 10 min. at RT. Cells were pelleted by centrifugation and supernatant aspirated. Then, cells were resuspended in 500 μl ice cold methanol and incubated on ice at least 10 minutes. Cells were washed once with PBS/1% FBS and incubated with antibodies against phosphoproteins (see Table VI) and αCD11c for 30-45 min. on ice.

5.3.12. Gene chip microarrays

Gene expression profile of immature and activated pDCs was studied by using mouse gene chip MOE430v2 microarrays (Affymetrix, Santa Clara, CA). This chip is comprised of over 45,000 probe sets representing more than 34,000 mouse genes. A gene chip array is a collection of probe sets specific for a gene sequence. Eleven pairs of oligonucleotide probes allow for precise determination of the level of transcription for each gene. By extracting and

labeling nucleic acids from experimental samples, and then hybridizing them to the array, the amount of label can be determined for each sequence.

5.3.12.1.Sample preparation

PDCs of spleens of Flt-3L-induced $DPE^{GFP}xRAG\text{-}1^{-/-}$ mice were subjected to cell-sorting based on GFP expression (see above). $0.5x10^6$ of sorted cells were stimulated with CpG 1826 or PR8 virus as described above. Cell pellets were collected after 1 h and 4 h. Subsequently, total RNA was isolated using the RNeasy-Kit (Qiagen, Valencia CA). RNAs were stored at -80°C until further processing.

5.3.12.2.Target preparation and gene chip hybridization

Total RNA samples were submitted to the University of Pennsylvania Microarray Facility (Philadelphia, PA) for purity check, RNA-target preparation and hybridization to gene chips. In brief, samples were run on an Agilent Bioanalyzer to determine RNA integrity and concentration. 5-8 µg of total RNA was converted to first-strand cDNA using Superscript II reverse transcriptase primed by a poly(T) oligomer that incorporates the T7 promoter. cRNA synthesis was performed by *in vitro* transcription for linear amplification of each transcript and incorporation of biotinylated CTP and UTP using the Enzo High-Yield amplification kit. The cRNA products were fragmented into 200 nucleotide sequences, denatured at 99ºC for 5 min. and hybridized for 16 h at 45ºC to gene chips. The microarrays were then washed at low (6x SSPE) and high (100 mM MES buffer, 0.1 M NaCl) stringency, and stained with streptavidin-phycoerythrin (SA-PE). Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional SA-PE stain. The Gene Chip 3000 scanner was used to collect fluorescence signal intensities after excitation at 570 nm. All protocols were conducted as described in the Affymetrix Gene Chip Expression Analysis Technical Manual.

5.3.12.3.Data Analysis

The initial data analysis including microarray quality control, quantification, background correction and normalization was performed by the University of Pennsylvania Microarray Facility (Philadelphia, PA) using the Affymetrix software program MAS 5.0. In brief, default values provided by Affymetrix were applied to all analysis parameters. Border pixels were removed, and the average intensity of pixels within the 75th percentile was computed for each probe. The average of the lowest 2% of probe intensities occurring in each of 16 microarray sectors was set as background and subtracted from all features in that sector. For further analysis, Cel files of probe level data were imported into the GeneSpring software program (version 7.0, Silicon Genetics). First, GCRMA (Robust Multi-array analysis (RMA) with correction for GC content) algorithm was used to summarize and normalize data. Per gene normalization was applied by using the median of each gene. Principal Component Analysis ensured that replicates were similar to each other. Then, a 3-way (time, treatment, experiment) mixed ANOVA (Analysis of variance) with a 5% FDR (false discovery rate) was applied for statistical variance using Partek software (Partek, Inc. St. Louis, MO). Subsequently, >2-fold differences were calculated on replicates in GeneSpring to further increase the stringency of filtering the differentially expressed genes. Subsequently, specificity of a gene for one condition was defined as change ≥3-fold compared to the other condition. Genes of interest were grouped based on their known immunological functions and gene ontology.

5.3.13. T cell assays

5.3.13.1.T cell enrichment by magnetic activated cell sorting (MACS)

In principle, cell suspensions are incubated with antibodies that are subsequently bound to magnetic beads. The cells are then applied to a column that is placed within a magnetic field and rinsed with an appropriate volume of washing buffer. Cells labeled with the beads

are retained in the column and the non-bound fraction, which is depleted of magnetically labeled cells, is collected in the flow-through.

$CD8^+$ T cells were isolated from spleens and PLN of donor OT-1 or P14xThy1.1 mice and single cell suspensions were prepared as described. Cells were resuspended in staining buffer. For $CD8^+$ T cell isolation, total cell suspensions were incubated for 10 min. on ice with purified rat anti-mouse antibodies αI-A/I-E (5 μg/ml), αCD4, αGr-1, αB220, αCD19 and αTer 119 (all 2.5 μg/ml) to deplete dendritic cells, $CD4^+$ T cells, granulocytes, B cells and red blood cell precursors, respectively (see Table II). Next, cells were washed, centrifuged and resuspended in 80 µl buffer per 10^7 cells. 20 µl goat anti-rat IgG coupled to magnetic microbeads per 10^7 total cells were added. Incubation was allowed for 15 min. at 6-10°C. In the meantime, columns with the capacity to retain an appropriate number of cells attached to the magnetic beads were placed into a magnetic field and rinsed with a total of 10 column volumes with cold buffer according to manufacturer's instructions. Cells were washed and resuspended in 500 µl buffer (10^8 cells) and then loaded to the columns. After the cells had entered the column, they were washed with 5 column volumes. The flow-through containing non-retained $CD8^+$ T cells was collected. The purity of the cells obtained was determined by staining an aliquot of the flow-through with αCD8 and subsequent analysis by flow cytometry. The purity of the isolated $CD8^+$ population was usually 90-95%.

5.3.13.2.Labeling procedure with CFSE

$2x10^7$ cells/ml splenocytes from OT-1 mice or P14xThy1.1 mice were labeled with 5 µM 5- (and 6-) carboxyfluorescein succinimidyl ester (CFSE) in labeling media for 8 min. at room temperature. Labeled cells were subsequently centrifuged over a layer of prewarmed FBS at 1300 rpm for 10 min. at room temperature. Supernatant was aspirated and cells washed twice with complete media.

5.3.13.3.*In vitro* proliferation assay

$CD11c^+$ $CD11b^+$ (mDC) cells and GFP^{hi} $CD11c^{int}$ (pDCs) cells were sorted and stimulated for 4 h as described above (see 5.3.11.1.). During the last hour, SIINFEKL-peptide was added at a final concentration of 10 ng/ml. DCs were washed extensively and their viability was determined by trypan blue exclusion. DCs were resuspended in complete media and plated in serial dilutions in 96 well round bottom plates (Costar Corning, Inc., Acton, MA). $5x10^4$ of purified $CD8^+$ T cells from OT-1 mice were added to each well. Cultures were pulsed after 72h with 1 μCi [^{3}H]thymidine/well. Sixteen hours later, cells were transferred to a glass fiber filter (Packard Instrument, Meriden, CT) and [^{3}H]thymidine incorporation was measured using a Matrix 96 beta counter (Packard Instrument, Meriden, CT). In some experiments CFSE-labeled $CD8^+$ T cells were used instead of radioactive pulsing. After 72 h, cultures were harvested and stained for activation marker CD25, CD69 and subsequently analyzed for CFSE dilution by flow cytometry.

5.3.13.4.*In vivo* proliferation assay

A) For the *in vivo* proliferation assay, 1 day prior to DC-preparation, WT C57Bl/6 recipients received CFSE-labeled splenocytes containing $5x10^6$ $V\alpha_2^+$ $CD8^+$ T cells from OT-1 mice intravenously. Mice received 3-$5x10^5$ non-stimulated or stimulated DC subsets that were pulsed with peptide or remained unpulsed within 24 hours. Mice were sacrificed after 72 h and single cell suspensions from spleen and LNs analyzed by flow cytometry for $V\alpha_2^+$ $CD8^+$ T cells and their respective CFSE-profile.

B) For the experiments in $Ikaros^{L/L}$ mice, $2x10^6$ CFSE-labeled $V\alpha_2^+$ $CD8^+$ T cells from P14xThy1.1 mice were injected intravenously in $Ikaros^{L/L}$ mice or WT littermates. Some $Ikaros^{L/L}$ mice received 3-$4x10^6$ pDCs 24 h later. Within additional 24 h, mice were infected with influenza virus PR8-GP33 (see 5.3.6.). Mice were sacrificed at day 4 p.i. and spleen and draining cervLNs were harvested. $CD8^+$ $Thy1.1^+$ donor cells were analyzed for CFSE-dilution and activation markers (see Table VII).

5.3.13.5. Peptide restimulation for cytokine production

$1x10^6$ cells splenocytes or lung cells were plated in 96 round bottom wells in complete media. Cells were restimulated for 5 hours at 37°C by adding PMA (Phorbol-13myristate) (50 ng/ml), a protein kinase activator, and Ionomycin (500 ng/ml), a Ca^{2+}-ionophor, to activate intracellular signaling cascades. For restimulation with peptide, SIINFEKL (for OT-1 T cells) or GP_{33-41} (for P14 T cells) peptide was added at a concentration of 0.4 μg/ml. Brefeldin A or Golgiplug (BD Biosciences) was added to cultures in a final concentration of 1 μM. The function of these reagents is to interfere with the vesicular transport of proteins from the endoplasmic reticulum to the Golgi apparatus, therefore preventing secretion and leading to intracellular accumulation of cytokines. Following restimulation, cells were transferred to a V-bottom plate, washed once in PBS/1% FBS and stained for intracellular cytokines (see 5.3.11.3.).

5.3.14. Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical significance between two groups was calculated using the unpaired Student's t-test. For multi-group comparison, one-way ANOVA with Bonferroni's correction was applied. Significance was defined at $p < 0.05$. In some graphs, the following symbols were used to express the statistical significance p value thresholds: ns = non significant ($p > 0.05$), * = $p(0.01 \leq 0.05)$, ** = $p(0.001 \leq 0.005)$, *** = $p < 0.001$. All statistical tests were performed using the Prism software (GraphPad Software, San Diego, CA).