2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Antibodies

Antibody	Clone/code	Procured from	Dilution/conc.
Primary			
Rat anti-hu CXCR5	8B2	our lab	1:8 (FC)
Rat anti-hu CCR7	3D12	our lab	1:8 (FC)
Rat anti-hu CCR7 R-PE	3D12	BD Biosciences	1:25 (FC)
Ham anti-hu ICOS biotinylated	C398.4A	eBioscience	1:200 (FC)
Ham IgG isotype control	n/a	eBioscience	1:200 (FC)
Mouse anti-hu CD57 purified	NK-1	BD Biosciences	1:50 (FC)
Mouse anti-hu CD8a APC	RPA-T8	eBioscience	1:25 (FC)
Mouse anti-hu CD4 R-PE	RPA-T4	BD Biosciences	1:25 (FC)
Mouse anti-hu CD4 APC	RPA-T4	BD Biosciences	1:25 (FC)
Mouse anti-hu CD4 PerCP	SK3	BD Biosciences	1:25 (FC)
Mouse anti-hu CD20 FITC	2H7	BD Biosciences	1.25 (FC)
Mouse anti-hu CD20 PerCP	L27	BD Biosciences	1:25 (FC)
Mouse anti-hu CD45RO R-PE	UCLH1	Immunotech	1:25 (FC)
Mouse anti-hu CD45RA FITC	ALB11	Immunotech	1:25 (FC)
Goat anti-hu CXCL13 Biotinylated	n/a	R&D Systems	1:500 (WB)
Goat anti-hu GAPDH	V-18	Santa Cruz Biotech	1:500 (WB)
Ms anti-hu GlycophorinA R-PE	GA-R2	BD Biosciences	1:100 (CS)
Mouse anti-hu γ/δ TCR R-PE	B1	BD Biosciences	1:40 (CS)
Mouse anti-hu CD3 purified	HIT3a	BD Biosciences	1.0μg/ml(FA)
Mouse anti-hu CD28 purified	CD28.2	BD Biosciences	1.0μg/ml(FA)
Secondary			
Mouse anti-rat FITC	212-096-168	Jackson ImmunoRes	1:200 (FC)
Rat anti-mouse FITC	415-096-100	Jackson ImmunoRes	1:200 (FC)
Goat anti-mouse R-PE	115-116-075	Jackson ImmunoRes	1:200 (FC)
Mouse anti-rat Cy5	212-176-168	Jackson ImmunoRes	1:200 (FC)
Goat anti-mouse Cy5	115-175-164	Jackson ImmunoRes	1:200 (FC)

FITC-Fluorescein Isothiocyanate, R-PE-R- Phycoerythrin, Cy-Cyanine, APC-Allophycocyanin, PerCP-Peridinin Chlorophyll, Hu-Human, ham-hamster, ms-mouse, n/a-not available, FC-Flow Cytometry, CS-Cell Sorting, WB-Western Blotting, FA-Functional Assays, Conc-Concentration

2.1.2. Recombinant proteins

Protein	Procured from
Recombinant human BLC/BCA-1/CXCL13	R&D Systems
Recombinant human SDF-1α/CXCL12	PeproTech Inc
Recombinant human B7-H2/Fc Chimera	R&D Systems

2.1.3. Chemicals

Chemical	Company
Phosphate buffered saline (PBS)	Biochrom AG
FICOLL	Biochrom AG
Trypan blue	Sigma
Gentamycin	PAA laboratories
Amphotericin B	PAA laboratories
Pencillin and streptomycin	Gibco
HEPES	Roth
Sodium pyruvate	Gibco
Sodium citrate	Roth
Citric acid	Roth
Sodium hydroxide	Roth
Ethanol	Roth
Chloroform	Roth
Phenol	Roth
Acetic acid	Roth

2.1.4. Primary human material

Human tonsils

Human palatine tonsils were obtained from patients undergoing routine tonsillectomy at the rheumaklinik, berlin-buch and Unfallkrankenhaus berlin. Freshly acquired tonsils were transported on ice and processed on the same day to isolate CD4 T cells by MACS sorting. On the following day subsets were FACS sorted and immediately used in downstream experiments.

Human peripheral blood

Freshly drawn peripheral blood from normal human donors was used. All the samples were collected between 15.00 hrs and 17:00 hrs to avoid temporal variation. From every individual approximately 70 ml of blood was drawn twice, at an interval of 1-2 weeks. First collection was used for T_{CM1} sorting and the second was used for sorting all the subsets.

2.1.5. Magnetic cell sorting

Materials required for Magnetic cell sorting (MACS) were purchased from Miltenyi biotech, Bergisch Gladbach, Germany. The following products were used during this study:

Magnetic beads

CD4 T cell isolation kit: The kit has two components, one is hapten-antibody cocktail consisting of monoclonal hapten-conjugated antibodies directed against

non CD4 T cells and the second one is microbeads which are colloidal super-paramagnetic microbeads conjugated to a monoclonal anti-hapten antibody.

TCRγ/δ microbead kit: Also consist of two components, one with hapten antibody against TCRγ/δ and the second one with colloidal super-paramagnetic microbeads conjugated to monoclonal anti-hapten antibody.

Goat anti-rat IgG microbeads: Colloidal super-paramagnetic microbeads conjugated to goat anti-rat IgG (H+L) $F(ab')_2$ fragments.

CD45RA microbeads: Colloidal super-paramagnetic microbeads conjugated with monoclonal mouse anti-human CD45RA antibody (Clone: L48).

Anti-PE microbeads: Colloidal super-paramagnetic microbeads conjugated with monoclonal mouse IgG1 anti-PE antibodies.

Cell separation columns

Columns for positive selection or depletion with two different cell-binding capacities were used:

LS separation columns: Positive selection of up to 10^8 positive cells labeled with microbeads from up to 2×10^9 total cells.

MS separation columns: Positive selection of up to 10^7 positive cells labeled with microbeads from up to 2×10^8 cells.

Magnetic separator

The magnetic separator used here is called VarioMACS in which both LS and MS columns fit with a removable adaptor for each type. The separation unit has a high-energy permanent magnet mounted onto a glass stand.

Principle of MACS

Cells to be enriched are prepared by labeling with a specific antibody coupled to super-paramagnetic microbeads. In case of using a cocktail of primary antibodies the secondary antibody is coupled with microbeads. First the separation column is washed and placed in the magnetic field with appropriate adaptor. A high-gradient magnetic field within the column matrix is created. Then cells are allowed to pass through the column that separates two fractions. Labeled cells (magnetic fraction) are retained and unlabeled cells (non-magnetic fraction) pass through the column into a collection tube. The matrix bound cells (magnetic fraction) are recovered from the column outside the magnetic field by simple washing. Typical enrichment

rate varies between 50 – 10,000 fold, depending on the strength and specificity of magnetic labeling and separation column used.

2.1.6. Flow cytometry and FACS sorting system

FACSVantage^{TM}SE from BD Biosciences (Heidelberg, Germany) cell sorting system was used for all the experiments. With the available configuration in our laboratory, using up to three independent laser beam spots (laser 1 = 488 nm, laser 2 = 633 nm, laser 3 = UV) a maximum of six fluorescence signals per cell can be collected. All the cell sortings were carried out with either laser 1 or laser 2 depending on the dye used for labeling cells. Typically the following were the routing settings:

Pressure: 31 PSI

Nozzle size: 70 nm

Events / second: 10,000

2.1.7. Transwell plates

For chemotaxis assay, transwell plates were used which were purchased from Corning Costar Corporation, Cambridge, MA, United states. The insert diameter was 6.5 mm with 24 wells in each plate. The set-up has a lower compartment (cluster well) and an upper compartment (transwell). The transwells used here has a polycarbonate microporous membrane with a pore size of 5.0 μm.

2.1.8. Co-culture medium

Media used for B and T cell co-culture consisted of the following constituents:

RPMI 1640 – 500 ml

Gentamycin – 50 μg / ml

Amphotericin B – 0.5 μg / ml

HEPES – 20 mM

Penicillin and streptomycin – 5 ml

FCS – 10%

Sodium pyruvate – 5 ml

2.1.9. RNA Isolation

Rnase-free DNase Set (Qiagen, Hilden, Germany).

2.1.10. Gene chips arrays

Human genome U133 Set A and B from Affymetrix, Inc. (Santa Clara, CA, United States) were used to hybridize labeled cRNA. This version of GeneChip provides a comprehensive coverage of 33,000 well-characterized human genes. Some genes have multiple probe sets; as a result there are more than 45,000 probe sets.

Chip design

GeneChip array is a collection of probe sets specific for each reference sequence on a glass surface encased in a plastic cartridge. Probe set is again made up of 11 probe pairs, which is a duo of Perfect Match (PM) probe and Mismatch (MM) probe. The probe is a 25-mer oligonucleotide designed to be complimentary to reference sequence. Mismatch probe consists of a single homomeric base change at the 13th position to serve as specificity control when compared to their corresponding perfect match probe. Probes are synthesized in a single square-shaped feature called as probe cell. Probes are selected from the 600 bases adjacent to the poly-A tail. For certain housekeeping genes like GAPDH and β-actin, additional probe sets in the 5' region of the transcript have also been selected. The signal intensity ratio of 3' probe set to 5' probe set is used as array quality metrics, which gives an indication of quality of starting material and efficiency of cRNA synthesis. The 3'/5' ratio for housekeeping genes should not exceed 3. However, this cutoff is not strictly applicable to all tissues, due to the presence of different isoforms of housekeeping genes and their expression pattern.

2.1.11. Real-time PCR

Instrumentation

For Real time reverse transcription polymerase chain reaction (RT-PCR), LightCycler 1.5 instrument from Roche was used. The LightCycler instrument is a combination of PCR thermal cycler and an integrated fluorescence detection device for fluorescence monitoring either continuously or once per cycle. The system has capability to detect two different types of chemistries, dye specific for double stranded DNA (e.g. SYBR green I) or sequence specific hybridization probes. Reaction was carried out in a 20 μl glass capillary. The carousel can hold a maximum of 32 capillaries for each run. The main advantages of the system over block-cycler are accurate quantification of PCR product by measuring fluorescence in log phase of the reaction, save time (approximately 30 minutes) and reduces the risk of contamination.

Primers

Gene / oligo	Oligo Sequence	Tm(°C)	$MgCl_2$(mM)	MT(°C)	Size
HEY1F	TGCCTCCTATAGCAGAAAGGTG	58	4.5	87	238
HEY1R	TTGTGAATTTGAGATCCGTGTG				
TSHRF	ACTCCTGTGCCAATCCATTC	63	4.0	88	261
TSHRR	CTGAGATTTGGCCTTGCTTC				
CXCL13F	AGAGAAAGATTCCCTGATGCTG	60	4.0	84	245
CXCL13R	CCTCCCTGATGAATAAGAATGC				
CXCR5F	TTGTCCCACTCAAGCCAAG	62	2:5	87	266
CXCR5R	TCTCTGTGCTGCCTGTACTG				
EDG1F	ATCATGTCCTGCTGCAAGTG	60	3.0	90	226
EDG1R	GCCAGCGACCAAGTAAAGAG				
NTRK3F	GTGTAGTTTCTGGCGGATTTTC	62	4.0	91	201
NTRK3R	ATGTCCGTGATGTTGATACTGG				
LIFF	GTCATCTACAAGAGCCCTGACC	60	3.0	90	247
LIFR	GTTCCTATGCCCAAGTTCTCTG				
CEBPαF	AGACCTAGAGATCTGGCTGTGG	60	4.5	89	295
CEBPαR	AGTATCCGAGCAAAACCAAAAC				
PTPN13F	ACAACAATGGTCAGCAACAGAC	60	4.5	88	293
PTPN13R	AATCCCAGAACCACATCTATGC				
VDRF	AGCATCCAAAAGGTCATTGG	60	4.5	90	280
VDRR	GCTCCTCCTCATGCAAGTTC				
CTLA4F	GGTGTTGACATGTGCTTTGG	60	4.5	87	221
CTLA4R	ACCTGCTGCCTTCTTCTGTC				
CD28F	CCTTGAAGAATGCCCTTCAG	60	4.5	86	263
CD28R	CGTCAGGGATAGGCAGAGTC				
PECAM1F	TGCCGGTTCTTAAATCCATC	60	2.5	86	222
PECAM1R	CTGTGTATGAGGGTGCATGG				
IGF1RF	GAAGTGGAACCCTCCCTCTC	60	4.5	91	241
IGF1RR	CTTCTCGGCTTCAGTTTTGG				
IL4RF	ACTCCTACAGGGAGCCCTTC	60	3.0	91	268
IL4RR	CAGGGCAAGAGCTTGGTAAG				
B2MF	CTATCCAGCGTACTCCAAAG	55	4.5	86	263
B2MR	CAAAGTCACATGGTTCACAC				

Tm - annealing temperature, $MgCl_2$ – magnesium chloride, MT – melting temperature.

Primer sequence was designed using Primer3 software suite (whitehead institute and Howard Hughes medical institute)

http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

Oligos were synthesized at BioTez GmbH, Berlin-Buch.

2.1.12. Miscellaneous

Article / reagent	Company
Nylon membrane	Millipore
Collagen	BD biosciences
RPMI 1640	PAA laboratories
Fetal calf serum (FCS)	Biochrom AG
Annexin V-Cy5 apoptosis detection kit	abcam
7-AAD (7-amino-actinomycin D)	BD biosciences
CFSE	Molecular probes

2.1.13. Programs and software

Name (company)	Application
Cell quest (BD Biosciences)	Acquisition and analysis of flow cytometry data
Winlist (Verity Software House, inc.)	Analysis of flow cytometry data
WinMDI 2.8 (Freeware)	Analysis of flow cytometry data
Weasel v2 (WEHI)	Flow cytometry data analysis and exporting in vector formats
FCSPress 1.4 (FCSpress)	Analysis of flow cytometry data
Microarray Suite 5.0 (Affymetrix)	Scanning and fluidics control, array image acquisition and analysis
GeneSpring 7.0 (Silicon genetics)	Microarray data analysis
FileMaker pro (File maker, Inc.)	Instant access of genelists
Light Cycler (Roche)	Analysis of real time RT-PCR data
Prism 3.0 (Graphpad software, Inc.)	Biostatistics and graphing
Freehand 10 (Macromedia, Inc.)	Creating illustrations and layouts in Vector format

2.2. Methods

2.2.1. Cell separation strategies

Human material either peripheral blood or palatine tonsil was used to isolate cells for all the experimental procedures. Desired primary cells were obtained by physical sorting, which is time consuming and the cells have to stay out of the tissue causing some degree of stress. To reduce this effect on the viability of the cells, processing time was kept to minimum. CD4 T cells and B cells were isolated by MACS procedure on first day and the cells were kept at 4°C overnight. Following day, CD4 T cells were stained and the subsets were sorted on FACS. Setting up of downstream experiment was followed on the same day ensuring a uniform processing of the cells through out the study.

Ficoll gradient for isolating mononuclear cells

Peripheral blood was collected in Acid citrate dextrose (1 ml for 10 ml of blood) and diluted in equal volumes of PBS. Carefully, 20 ml of diluted blood was layered over 15 ml Ficoll in a 50 ml conical tube and centrifuged at 2000 rpm for 30 minutes at 20 °C without break. Then the peripheral blood mononuclear cell (PBMC) layer consisting of lymphocytes, monocytes and thrombocytes was carefully aspirated to a new 50 ml conical tube filled with 50 ml of PBS and centrifuged at 1200 rpm for 10 minutes. The cell pellet was resuspended in 300-500 μl of PBS and cells were diluted 1:10 in trypan blue and counted in Neubauer chamber. Cell suspension was further diluted to 10^7 cells per 40 μl of MACS buffer for magnetic sorting.

In case of tonsils, the entire tissue was wrapped in a nylon membrane and mechanically disrupted using backside of a 5 ml syringe plunger in a cell culture plate. The cell suspension was collected in a conical tube and diluted up to 50 ml. To isolate tonsillar mononuclear cells (TMNC), the cell suspension was layered over ficoll and the rest of the processing was similar to the isolation of PBMCs.

Peripheral blood CD4 T cells

All the four CD4 T cell subsets (namely T_{CM1}, Naïve, T_{CM}, T_{EM}) were obtained from every donor using a combination of multistep MACS and FACS sorting (Fig. 2). Briefly, untouched CD4 T cells were enriched from PBMCs using a modified protocol of CD4 T cell isolation kit. To achieve a higher post-sort purity (98-99%), CD4 T cell isolation kit I was combined with: (a) TCRγ/δ microbead kit and (b) PE-conjugated anti-human monoclonal glycophorin A with anti-PE microbeads using manufacturers protocol. Later, miltenyi introduced improved version of CD4 T cell

isolation kit II which was used without coupling with TCRγ/δ or glycophorin A. In the next step, CD4 T cells were labeled with rat anti-human CXCR5 (clone 8B2) and goat anti rat IgG microbeads to sort for CXCR5$^+$CD4$^+$ T cells (T_{CM1}, Central Memory 1) and CXCR5$^-$CD4$^+$ T cells. Further, CXCR5$^-$CD4$^+$ T cells were sorted based on the expression of CD45RA by employing CD45RA microbeads to yield CD45RA (Naïve CD4 T cells) and CD45RO (classical memory CD4 T cells). Finally, CD45RO were FACS sorted after labeling with rat anti-human CCR7 and PE-conjugated donkey anti-rat IgG secondary antibody, into CCR7$^+$ CD4$^+$ T cells (T_{CM}, Central Memory) and CCR7$^-$CD4$^+$ T cells (T_{EM}, Effector Memory). MACS enriched T_{CM1} and Naïve also underwent further sorting on FACS to enhance the purity above 95%, ascertaining similar treatment of all the cell subsets through out the sorting procedure. Post-sort aliquots were stained with appropriate antibodies to determine purity. Isotype-matched monoclonal antibodies were used as controls during all the staining procedures.

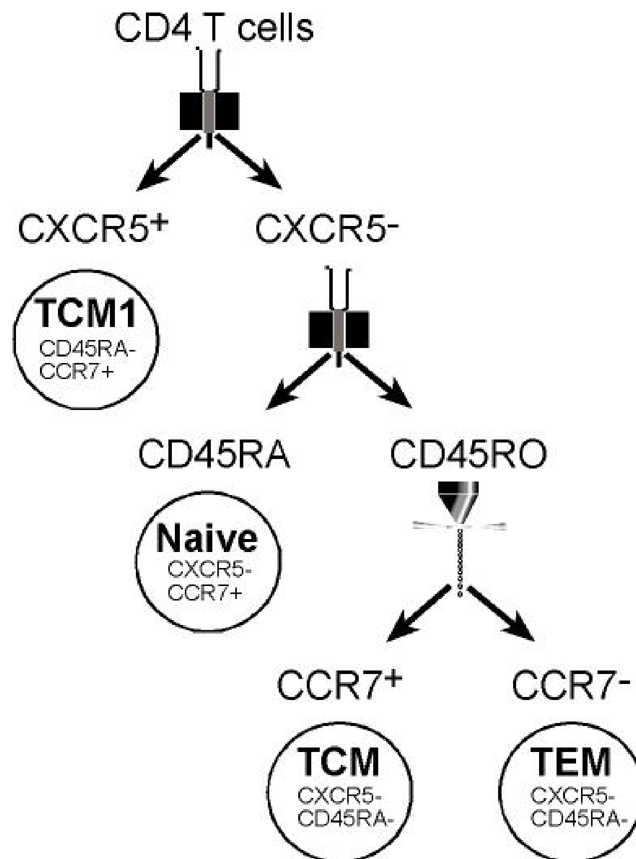

Fig. 2: Sorting strategy for isolation of peripheral blood subsets. Based on the expression of chemokine receptors in combination with CD4 and CD45RA, peripheral blood CD4 T cells were MACS and FACS sorted into four subsets – CD45RA$^+$CCR7$^+$ Naïve, CD45RO$^+$CCR7$^+$CXCR5$^+$$T_{CM1}$, CD45RO$^+$CCR7$^+$ T_{CM} and CD45RO$^+$CCR7$^-$ T_{EM} cells.

Tonsillar CD4 T cells

TMNC were divided into two unequal aliquots, small one (15-20%) is used for B cell isolation and the rest was used for CD4 T cell isolation. B and T cells were isolated using the respective kits from miltenyi. CD4 T cells were stained with rat anti-human-CXCR5 and FITC labeled anti-rat IgG secondary antibody; biotin labeled hamster anti-human ICOS and PE-conjugated streptavidin. Based on the surface expression of CXCR5 and ICOS, three subsets (Fig. 2A and B) were sorted on FACS Vantage™ SE System. Sorted subsets were used throughout the downstream experiments.

2.2.2. Functional assays

Co-cultivation of B and T cell subsets

To study the interactions of autologous B and T cell subsets, *in vitro* Co-cultures were used. Cells were used for staining with markers to assay apoptosis or proliferation and the supernatant was used for ELISA to determining the amount of immunoglobulin or CXCL13. Equal numbers (1×10^5) of B and each of the T cell subsets were used for all the co-cultures. MACS sorted B cells and FACS sorted CD4 T cells or the subsets were placed in 150 μl of co-culture medium. The cells were incubated at 37°C with 5% CO_2 overnight; following day they were treated with anti-human-CD3/CD28 antibodies at a concentration of 1.0 μg/ml. Further incubation varied based on the type of assay performed. For immunoglobulin and CXCL13 determination supernatant was collected at day 4, 7 and 10. For apoptosis and proliferation cells were harvested at day 3, 4 and 5, washed once in PBS with 0.5% BSA and stained with appropriate protocols.

Chemotaxis

Prior to the assay the transwell membrane is coated with mouse collagen type IV (20 μg/ml in 0.05M HCl) for 1 hour at room temperature to promote attachment and spreading of the cells. RPMI 1640 supplemented with 2.5% fetal calf serum was used as chemotatic medium. CD4 T cells isolated from magnetic sorting were directly used. A small aliquot of the cell suspension stained with CXCR5-ICOS and measured on flow cytometer served as a control to compare the distribution of the subsets after the assay. Before inserting the transwell, 500 μl of media with 300 nM recombinant human CXCL13 was placed in the lower compartment. Media without any chemokine served as a negative control. 5×10^5 cells suspended in 100 μl of media was placed in the upper chamber. The transwell plates were incubated for 3 hours at 37°C in a 5% CO_2 incubator. During this time, CXCR5 expressing cells are attracted to CXCL13 and start to migrate in the lower

chamber. After the incubation the media from the lower chamber was collected and centrifuged at 1200 rpm for 10 minutes. The cells were stained with CXCR5-ICOS and measured on flow cytometer. Chemotaxis index was calculated using the values derived from the distribution of the subsets before the assay and migrated cells after the assay.

Apoptosis assay

For apoptosis assay CD4 T cell subsets were sorted based on the following secondary antibody staining:

Rat anti human CXCR5 – anti rat FITC

Hamster anti human ICOS – streptavidin R-PE

Co-cultured Cells were harvested at day 3, 4 and 5 were washed in PBS with 0.5% BSA and a 3 step staining was performed

1) rat anti human CXCR5

   hamster anti human ICOS

incubated for 30 mins on ice, and washed two times with PBS (0.5% BSA)

2) anti rat FITC

   streptavidin R-PE

incubated for 30 mins on ice, and washed two times with PBS (0.5% BSA)

3) 7-AAD (Amino Actinomycin D)

   Annexin V Cy5

After adding the reagents of step 3, cells were directly measured on the cytometer.

Proliferation assay with CFDA SE

For proliferation assay CD4 T cell subsets were sorted based on the following secondary antibody staining:

Rat anti human CXCR5 – anti rat CY5

Hamster anti human ICOS – streptavidin R-PE

MACS sorted CD4 T cells were labeled with CFDA SE (Vybrant CFDA SE cell tracer kit, Molecular Probes) at a concentration of 1 μM before setting up the co-culture. Cells were suspended in 1 ml of PBS (with 5% FCS) in a 15 ml conical tube. To this 1 ml of 2μM CFDA SE in PBS (with 5% FCS) was added simultaneously mixing on a vortexer ensuring uniform labeling of the dye. The cells

were allowed to stand at room temperature for 5 minutes and then washed three times, once with PBS (5% FCS) and twice with native RPMI 1640. High concentration of protein was used in PBS (5% FCS), to reduce the amount of labeling. Higher levels of CFDA SE labeling are toxic to the cells. Washed cells were used to set up co-cultures. The cells were collected after the defined incubation and stained as follows:

1) rat anti human CXCR5

   hamster anti human ICOS

30 minutes incubation on ice, washed two times with PBS (0.5% BSA)

2) anti rat CY5

   streptavidin R-PE

   anti-human Per CP

30 minutes incubation on ice, washed two times with PBS (0.5% BSA)

2.2.3. RNA level

RNA Isolation

Total RNA was prepared using RNeasy mini kit from approximately 4 x 10^6 cells. On column DNase digestion was performed with Rnase-free Dnase set to avoid genomic DNA contamination. RNA from three individuals was pooled to achieve a total amount of about 3-4μg. Antisense RNA (cRNA) was synthesized using single cycle amplification, RNA was reverse transcribed to cDNA using a T7-promotor tagged polyT primer and biotin labeled cRNA was synthesized.

Synthesis of labeled cRNA

For first strand synthesis, total RNA was vacuum concentrated to 3.0μl and 1.0μl of polyT-T7-primer (10μM) was added. RNA-primer mix was incubated at 70°C for 5 minutes followed by 2 minutes on ice and 2 minutes at room temperature. First strand cocktail was added to the RNA which includes, 1.0μl of RNase-free water, 1.0μl of 100mM DTT, 0.5μl of RNasin® Ribonuclease Inhibitor (40u/μl), 0.4μl dNTPs (25mM), 2.0μl of 5x buffer and 1.0μl of Superscript™ II Reverse Transcriptase incubated at 42°C for 1.0 hour and 5 minutes on ice.

Second strand synthesis was carried out by adding the following reagents (ice cold) to the first strand mix: 45.5μl of water, 1.0μl of 25mM dNTPs, 15.0μl of 5x second strand buffer, 20 units of E. coli DNA polymerase I, 10 units of E.coli DNA Ligase, and 1.0 unit of RNase H, Incubated at 16°C for 2.0 hours.

cDNA clean-up was performed using neutral phenol/Chloroform and Phase Lock Gel^{TM} light tubes at 14,000 rpm for 2 minutes at room temperature. The upper phase was transferred to a fresh tube and precipitated using 37.5ul of 7.5M NH_4OAc (0.5 volumes), 187.5μl of ethanol (2.5 volumes) and 0.5μl of linear acrylamide carrier. Centrifuge at 14,000 rpm for 30 minutes at room temperature. The cDNA pellet was washed twice with 80% ethanol, air-dried and dissolved 4μl of water.

In vitro Transcription

Amplification and biotin labeling was done using MEGAscript ^{TM} High Yield Transcription Kit. In short, half standard setup was used, 1.75μl of each Biotin-11-CTP and Biotin-16-UTP was dried under vacuum and 4.0μl of cDNA, 1.0μl of each T7 ATP (75mM) and T7 GTP (75mM), 0.75μl each of T7 CTP (75mM) and 0.75μl of T7 UTP (75mM), 1.0 μl of 10X reaction mix and 1.0 μl of Enzyme mix was added. This was incubated for 5 hours at 37°C. Finally, the cleanup of labeled cRNA was carried out using RNeasy mini columns.

The cRNA was quantified and 15μg was fragmented as described in affymerix expression analysis technical manual.

Hybridization and scanning

Hybridization and Scanning was performed at central facility of MDC, Berlin, Germany. To check the integrity of the target, 5μg was used to hybridize to Affymetrix Test Chip3. Once the quality of the labeled cRNA was confirmed, rest 10μg was used to hybridize Affymetrix GeneChip® Human genome U133 Set (HG-U133A and B). Hybridization was done at 45°C for 16 hours and then the chip was washed and stained with streptavidin-phycoerythrin using the fluidics station. Last step was to scan the genechip array using gene array scanner. DAT and CEL files were generated using Microarray Suite from the gene chips with global scaling to a factor of 200, and the CHP files were copied to Excel (Microsoft, Redmond WA) as Pivot tables. Three biological replicates were generated for each subset and data from respective chips (U133A and U133B) was merged before analysis.

Data analysis

Microarray data was analyzed using GeneSpring GX (formerly GeneSpring from Silicongenetics), Agilent Technologies, Inc. Palo Alto, CA, United States.

Definition of terms used in the Gene Expression analysis

Experiment: set of replicates combined together

Replicate: set of samples combined together

Sample: data set generated for a single chip

Probe: set of complementary oligonucleotides designed for each transcript sequence

Probe ID: an alphanumeric name designated to each probe

Signal log ratio: the change in expression level for a transcript between a baseline and an experiment array. This change is expressed as the $\log_2$ ratio. A signal log ratio of 1 is the same as a fold change of 2

p-value: The probability that a certain statistic is equal or more extreme to the observed value when the null hypothesis true. The null hypothesis is that the two samples are the same

Detection call: A qualitative measurement indicating if the transcript is detected (present), not detected (absent), or marginally detected (marginal)

One-way ANOVA:

Main aim of the data analysis is to generate a list of genes that are two fold up regulated, statistically significant and differentially regulated. Key features of the GeneSpring analysis are summarized below:

Loading experimental data and specifying data set-up

The software has pre-loaded settings for commercially available chips with standard genomes. For example, all human genome chips from Affymetrix are already preset and upon loading the data file GeneSpring will identify the genome. In the first step, raw data files (pivot tables) generated from Microarray Suite for every data set were loaded into GeneSpring under the corresponding genome.

The data was normalized using the recommended settings for Affymetrix with the following order: data transformation, per chip (normalize to 50th percentile) and per gene (normalize to median).

Each experiment consists of several replicates; these in turn are made of several data sets. Experiment Parameters were described based on the replicates. This ensures that data sets forming a replicate are grouped together.

Experiment interpretation allows making settings for displaying data modes, log or log of ratio. Log mode is used for filtering and fold regulation (up/down); where as log of ratio is used for statistical analysis and clustering. The experiment with all the replicates was saved in both the modes.

Genespring analysis

Defined data analysis algorithm was used with sequential steps listed below:

- Probes with “present flags” in all the samples from a given replicate were filtered and individual lists were merged.
- Using the above list probes having two fold up regulation using all the pair-wise combinations were filtered and the resulting lists were merged.
- This list was used for statistical analysis by one-way ANOVA.
- Probe list satisfying the ANOVA was again broken down using two fold criteria and present flags.

Thus the list generated at this stage represents the probes, which are statistically significant differentially regulated among the CD4 T cell subsets.

Reverse transcription polymerase chain reaction

Total RNA of the tonsillar subsets from three individual tonsils was reverse transcribed into cDNA with Superscript™ II Reverse Transcriptase (Invitrogen, life technologies) primed with random hexamer. RT-PCR was performed in LightCycler using SYBR green I format (Roche, Basel, Switzerland). Data analysis was carried out using LCDA software (Roche) using the analysis mode of second derivative maximum and arithmetic as baseline adjustment. B2M expression levels were used to normalize the cDNA samples under interrogation. Relative levels of each of the differentially expressed genes were obtained from individual runs using the normalized cDNA volumes. Specificity of the amplicon was confirmed by agarose gel electrophoresis and melting curve readouts from LCDA software.

2.2.4. Secondary validation

Western blotting

Sample preparation

Sorted cells were pelleted by centrifugation and PBS is completely removed. 300 μl of PBS with 0.5% NP40 was added and placed on ice for 15 minutes. Then the lysate was centrifuged at 4 °C for 10 minutes at 14,000 rpm. The supernatant was

collected into another tube and 4x sample storage buffer was added to a final volume of 1x and stored at –20 °C

SDS-Polyacrylamide gel electrophoresis, blotting and exposing to film

The sample was denatured at 95°C for 5 minutes and resolved on polyacrylamide gel containing 15% of acrylamide. The gel was electrophoresed at 4W for 30 minutes and 12W for 1 hour. The resolved proteins were elcetroblotted from the gel by electrical transfer onto the nitrocellulose membrane at 300 mA, 70V for 1 hour in cold transfer buffer. Before staining, the membrane was blocked by incubating in 2.5% milk powder solution in PBS for 20 minutes at room temperature. Then the membrane was placed in a plastic pouch and filled with primary antibody appropriately diluted in blocking solution and incubated at 4°C overnight on a shaker. The membrane was removed and washed with 4 changes of PBST and finally with PBS. Diluted secondary antibody (peroxidase conjugated) was added to the membrane and incubated for 1 hour at room temperature. Subsequent washing was similar to the one carried out after primary antibody. Drain the solution and place on a saran wrap. The membrane was treated with Enhanced Chemiluminiscence Membrane (ECL) solution A and B following manufacturers instructions. The treated membrane was prepared for exposure and to a film by supporting on a piece of whatmann paper and wrapped in saran wrap. Based on the intensity of the bands the films were exposed to the membrane from 30 seconds to 1 hour and placed in developing solution for 1 minute. After washing and fixing they were dried at 45°C for 30 minutes.

Solutions

Polyacrylamide gel

Solution	separating gel (15%)	stacking gel (5%)
Running buffer	1.5 ml	
Stacking gel		0.9 ml
Polyacrylamide	3.0 ml	0.6 ml
Distilled water	1.5 ml	2.0 ml
10% ammonium per sulphate	50.0 µl	50.0 µl
TEMED	5.0 µl	5.0 µl

Sample buffer, 4x (higher ß-ME) 1M Tris.Cl, pH 6.8, 1 ml

	20% (w/v) SDS, 4 ml
	Glycerol, 2 ml
	ß-mercaptoethanol (ß-ME), 2 ml
	distilled water, 2 ml
	bromophenol blue, 40 mg
	filter sterilized, stored at RT w/o ß-ME
	before using, added 250 ul of ß-ME to 1 ml of buffer
Separating gel buffer:	0.1% SDS, 1.5M Tris/HCl, pH 8.8
Stacking gel buffer:	0.1% SDS, 1M Tris/HCl, pH 6.8
5x SDS electrophoresis buffer:	15.1 g Tris base
	72.0 g glycine
	5.0 g SDS
	Distilled water to 1000 ml
	Diluted to 1x working solution
10x Western transfer buffer:	240 mM Tris-base
	2 M Glycine
1x Western transfer buffer:	10% (v/v) 10x western transfer buffer
	23% (v/v) ethanol
Blocking solution:	2.5% skimmed milk powder
	Prepared in TBST
Tris-Buffered Saline Tween (TBST)	100 mM Tris.Cl, pH 7.5
or staining buffer:	0.9% (w/v) NaCl (150 mM)
	0.05% Tween20

2.2.5. Buffers

Acid citrate dextrose (pH 5.2)

Sodium citrate – 37.3 g

Citric acid – 8 g

Distilled water – 450 ml

Adjusted the pH to 5.2 with NAOH, and then the volume to 500 ml with distilled water. Sterilized by filtration through 0.22 μm filters and stored at 4°C

MACS buffer

PBS – 4.775 g

Bovine serum albumin – 0.5%

EDTA – 2 mM

pH 7.2

Dissolved the contents in 450 ml of distilled water and made the final volume to 500 ml. Sterilized by filtration through 0.45 μm filters and stored at 4°C.