Non-Classic Properties of Human Cytolytic Lymphocytes: Basic and Clinical Aspects

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General Summary

The immune system comprises a complex network of specialized cells and organs that function together to recognize and remove potentially harmful "non-self", i.e. pathogens, malignant cells and –by extension– also transplanted *allogeneic* cells.

Innate –evolutionary 'old' – immune mechanisms function in a non-clonal germline-encoded manner, whereas the hallmark of the adaptive immune system is clonal expansion of cells after rearrangement of germline-encoded immune receptors.

Natural killer cells (NK cells) represent an important component of the innate immune system, whereas CD4+ ('helper') and CD8+ ('cytotoxic') T cells are key players of adaptive immunity.

Rare NK cell-deficient humans suffer from overwhelming herpes viral infections, indicative of their important role in infection biology. Mechanistic insight into their anti-viral effector function, however, remains largely based on murine data. Intriguingly, the best molecular understanding of NK cell function in humans is derived from studies of the 'man-made' setting of allo-stem cell transplantation. By contrast, in *solid organ transplantation* NK cells have, until the recent emergence of exciting murine data, been largely ignored.

To begin to understand the role NK cells may play in human solid organ transplantation, we initiated a prospective cohort-study of kidney allo-transplant recipients.

Blocking the IL-2 receptor α-chain (CD25) with mAb is an immunomodulatory treatment modality used in various clinical situations, including allo-transplantation. Of note, in patients with multiple sclerosis and uveitis administration of anti-CD25 mAb has been linked to robust expansion of $CD56^{bright}$ NK cells, which has been suggested to mediate its effect. On this background we first we enumerated NK cell-subsets in kidney transplant-recipients receiving anti-CD25 mAb (n=16) before transplantation, and at week 12, 26 and 52 post-transplantation, and in healthy controls (n=5). In healthy

controls NK cell-subsets remained stable over time. In transplant-recipients, by contrast, overall median frequencies of both $CD56^{bright}$ and $CD56^{dim}$ NK cells transiently decreased after transplantation. Expansion of $CD56^{bright}$ NK cells was observed in only 6/16 patients. These data dissociate expansion of $CD56^{bright}$ NK cells and in vivo blockade of CD25 in kidney allograft recipients, thus dismissing expansion of $CD56^{bright}$ NK cells as unifying clinical hallmark of its effect across all clinical situations (G. Zenhaeusern et al. Submitted).

Aiming next at studying *functional* allo-specific properties of NK cells, we developed a simple, paraformaldehyde fixation-based protocol that provided an accurate and robust means for assessing alloreactivity, avoiding an irradiation-induced network of various cytokines complicating interpretation of results (G. Zenhaeusern *et al*. JIM '07). Using this protocol, we found that a subset of NK cells was *activated* rather than silenced when interacting with cells expressing normal levels of autologous MHC I. Instead of inducing an inflammatory phenotype, however, activation led to the secretion of the regulatory cytokines TGF-β and IL-10. Together these data ascribe a novel pattern of reactivity to NK cells with potential implications both in autologous and allogeneic systems (G. Zenhaeusern *et al.* in revision).

The molecular understanding of the various cellular interactions involving NK cells is only emerging, whereas recognition by T cell receptor (TCR) of cognate antigen presented by HLA molecules has been analyzed in depth. On the other hand, relatively little attention has been given to *basic T cell physiological properties*, such as their random crawling activity (required to screen for antigen) or their mitochondrial energy efficiency. In order to begin to tackle these important issues, I established an experimental *in vitro* system allowing us to quantify random crawling activity of various T cell subsets, and monitor crawling under conditions mimicking the intra- and extravascular environment. Using these assays, we identified robust differences

between phenotypically distinct subsets of CD8+ T cells with regards to their random movement activity and the frequency-distribution of crawling cells. Specifically, using migration-assays and time-lapse microscopy we found (i) that CD8+ T cells lacking the lymph node homing receptors CCR7 and CD62L migrated more efficiently in trans-well assays and (ii) that these same cells were characterized by a high frequency of cells exhibiting random *crawling activity* under culture conditions mimicking the interstitial/extravascular milieu –but not when examined on endothelial cells.

With this finding at hand, we hypothesized that increased random movement activity ought to be linked to higher energy consumption. To test this hypothesis we measured mRNA expression of genes key to mitochondrial energy metabolism (PGC-1β, ERRα, Cytochrome C, ATP Synthase, and the uncoupling proteins UCP-2 and UCP-3), quantified cellular ATP contents and performed micro-calorimetric analyses. Much to our surprise, ATP contents were consistently higher in CCR7+ CD8+ T cells, the subset of T cells that showed less crawling activity, and genes involved in mitochondrial biosynthesis and ATP production (PGC-1β, ERRα, Cytochrome C, ATP Synthase) were significantly upregulated. Intriguingly, we also observed that these CCR7+ CD8+ T cells expressed uncoupling proteins UCP-2 and UCP-3 at significantly higher levels than CCR7- CD8+ T cells, which at least partly explained the higher heat flow measured in the lymph node homing T cell subset. (G. Zenhaeusern et al. Blood 2008, e-pub ahead print). Together these assays identified a phenotypically distinct 'high crawling-frequency' CD8+ T cell population, and differentially regulated heat production among non-lymphoid vs. lymphoid homing CD8+ T cells.

Introduction

The human immune system has evolved to protect us from harmful 'non-self'. The first line of immunological defence is provided by the *innate* immune system, consisting of physical and chemical barriers, the complement system, phagocytic cells, and a lymphocyte subset termed natural killer cells (NK cells). The *adaptive* immune system is composed of T cells, including CD4+ helper T cells and CD8+ cytotoxic T cells, as well as B cells that can be activated to develop into antibody secreting plasma cells.

During an infection, NK cells become activated via germline-encoded receptors to directly kill infected cells and/or secrete inflammatory cytokines. T and B cells, by contrast, recognize cognate antigen presented by HLA molecules via receptors generated by somatic recombination.

NK cells

Phenotypically NK cells can be defined as CD3 (TCR) negative, CD56 (neural cell adhesion molecule) positive lymphocytes. Based on their expression-level of CD56 and CD16 (FcγRIII), NK cells can be divided into two subsets. Approximately 90% of all circulating NK cells express low levels of CD56 and CD16 (so-called $CD56^{dim}$ NK cells).[1,2] $CD56^{dim}$ NK cells store granules containing perforin and granzyme, and are able to immediately kill virally infected cells and tumour cells (natural cytotoxicity). CD16 binds the Fc part of antibodies bound to target cells, mediating antibody-dependent cellular cytotoxicity (ADCC).[3,4] The remainder 10% of circulating NK cells expresses high levels of CD56 and are mostly CD16 negative (so-called $CD56^{bright}$ or regulatory NK cells). $CD56^{bright}$ NK cells contain low levels of perforin and granzymes, but when activated can be induced to secrete large amounts of inflammatory and, to some extent, anti-inflammatory cytokines.[2,5-8]

NK cell function is regulated by clonally distributed killer immunoglobulin-like receptors (KIR), natural killer cell receptors (NCRs) and NKG2D. KIR mediate either inhibitory or

activating signals, depending on their cytoplasmic tails. Inhibitory KIR associate with an immunoreceptor tyrosine-based inhibition motif (ITIM), and recognize conserved motifs on HLA A, B, C or G molecules.[9,10] Downregulation of HLA I molecules after viral infections or on tumour cells leads to a lack of inhibitory signals, and hence killing of target s ('missing self' recognition).[11-15] By contrast, NCR, NKG2D and KIR that associate with an immunoreceptor tyrosine-based activation motif (ITAM) mediate activating signals.[15-19]

The 'missing self' principle as outlined above for infected and malignantly transformed cells is also thought to underlie NK cell *alloreactive* killing: the HLA C locus is characterized by an asparagine/lysine polymorphism at position 80. Each HLA C allotype is recognized by a distinct set of inhibitory KIR molecules.[10,20,21] Absence of any of these two HLA C epitopes vis-à-vis a given NK cell leads to a lack of inhibition of the corresponding KIR expressed on this NK cell, and hence to its *allospecific activation*. In hematological stem cell transplantation, such 'missing self' reactions –in the sense of NK cell-mediated graft versus leukemia effects from HLA C mismatched donors– have been demonstrated to exert powerful graft-versus-leukemia effects.[22,23] In haplo-identical hematopoietic transplantation, for instance, alloreactive NK cells were shown to reduce the risk for relapse.[23]

The role NK cells play in solid organ transplantation remains largely unknown. At the University Hospital Basel, standard immunosuppression for kidney transplant recipients consists of a calcineurin-inhibitor-based triple therapy plus induction-therapy with basiliximab (anti-CD25 mAb). Anti-CD25 mAb targets the IL-2R α-chain expressed on activated T cells. In patients suffering from auto-immune diseases –such as multiple sclerosis or uveitis– anti-CD25 mAb have also been used. In these patients expansion of $CD56^{bright}$ NK cells –often termed regulatory NK cells and expressing CD25– has been documented.[24,25] Against this background we aimed at enumerating and phenotyping circulating NK cells in renal transplant recipients before and after

transplantation and relating these data to infectious episodes, allograft rejection and transplant function.

As stated, ligands for MHC I molecules actively 'silence' NK cell activity. Inversely, NK cell activity is triggered vis-à-vis cells lacking expression of autologous MHC I, a situation encountered in allogeneic transplantation ('missing-self' recognition). In mice, data of NK cells triggering an immune response in solid organ transplantation are emerging. It has, for example, been shown that NK cells can mediate transplant tolerance via killing of graft-derived antigen presenting cells (APC), thus reducing the potential for alloreactive T cells to be induced.[26] Besides their capacity to kill target cells, activated NK cells are able to produce various immunoregulatory cytokines. In addition to the secretion of IFN-γ, monokine-stimulated NK cells are also capable of secreting cytokines such as TNF-α and TNF-β, IL-10, IL-13 and GM-CSF.[2] Moreover, lymph-node homing NK cells have recently been shown to be able to secrete large amounts of IFN-γ early during the course of an immune response, hence contributing to T_H1 polarization of T cells.[27]

Here we aimed at reassessing the *ex vivo* response of NK cells vis-à-vis autologous and allogeneic target cells *without prior stimulation*. While proliferation is still used as an indicator of alloreactivity –and is routinely performed– measuring the release of cytokines is more sensitive to detect allospecific lymphocyte activation. Mixed lymphocyte reactions remain central to the characterization of cellular allo-interactions. We therefore sought to develop a protocol allowing us to measure –in a highly sensitive and specific manner– cytokine secretion induced in *unprimed* NK cells upon direct cell-cell contact.

CD8+ T cells

CD8+ T cells are crucial effectors of the adaptive immune system. Upon activation, CD8+ T cells are able to secrete inflammatory/regulatory cytokines and, similarly to NK cells, kill target cells by the release of prestored perforin and granzymes.

A central feature of the adaptive immune response is the generation of antigen-specific effector and memory T cells. Naïve T cells are induced to proliferate (clonal expansion) and differentiate upon encounter with cognate antigen presented by MHC molecules and co-stimulatory signals provided e.g. by CD80 and CD86 on dendritic cells in secondary lymphoid structures. Activation and subsequent differentiation lead to the formation of several CD8+ T-cell memory subsets with different functional and migratory properties, classified as central memory and effector memory cell subsets.[28,29] Central memory cells recirculate between the blood and the lymphoid compartment and are thought to provide a pool of antigen-experienced cells with a high proliferative capacity but a lower activation threshold than naïve cells, thus allowing for a more rapid generation of effector cells during a recall response.[30] In contrast, effector memory CD8+ T cells are thought to allow for a recall response in the tissue, as these cells survey nonlymphoid organs in search of cognate antigen.[31,32]

Homing characteristics are thought to be controlled by the expression pattern of adhesion molecules and chemokine receptors acquired by specialized T cell subsets during the process of differentiation and polarization. According to their phenotype – which is linked to specific immunological functions– CD8+ T cells can be divided into T cell subsets with distinct migratory properties: naïve and central memory T cells co-express the lymph node homing receptor CC chemokine receptor 7 (CCR7) and the adhesion molecule L-selectin (CD62L), whereas effector memory and terminally differentiated T cells lack expression of both these markers, and acquire receptors allowing homing to peripheral tissue and to sites of infection/inflammation, respectively.[28,29,33,34]

T cells move randomly, a feature that has been termed 'random walk'.[35-37] Such random lymphocyte motility allows T cells to encounter other cells of the immune system. Lymphocyte motility ('crawling') relies on the binding of motor proteins to polarized cytoskeletal filaments, an ATP-consuming process.[38] Typically T cells crawl in one direction by repeated extension and subsequent contraction of their cell-stoma, followed by pausing, in cycles of approximately 2 minutes.[39,40] The energy for crawling is generated by oxidative phosphorylation.

ATP synthase is the enzyme that catalyzes the conversion of ADP (adenosine diphosphate) and anorganic phosphate (Pi) to ATP making use of the proton gradient at the inner membrane of mitochondria.[41] Uncoupling proteins (UCP-1 to 3) are mitochondrial anion transporters that reduce the proton gradient by diverting protons thus producing heat. UCP-1 and UCP-3 are expressed mostly in skeletal muscles and brown fatty tissues,[42-44] whereas UCP-2 is more widely expressed.[45]

In our experiments, we first assessed the *ex vivo* migration of human CCR7- and CCR7+ CD8+ T cells on fibronectin and on HUVEC (human umbilical endothelial cells), mimicking intravascular and extravascular conditions in vitro respectively. In order to shed light on the energy efficiency of the distinct CD8+ T cell subsets, we related random lymphocyte motility to mitochondrial biogenesis/ATP synthesis.

References

1. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001;22:633-640.
2. Cooper MA, Fehniger TA, Turner SC, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood. 2001;97:3146-3151.
3. Leibson PJ. Signal transduction during natural killer cell activation: inside the mind of a killer. Immunity. 1997;6:655-661.
4. Anderson P, Caligiuri M, Ritz J, Schlossman SF. CD3-negative natural killer cells express zeta TCR as part of a novel molecular complex. Nature. 1989;341:159-162.
5. Fehniger TA, Cooper MA, Nuovo GJ, et al. CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. Blood. 2003;101:3052-3057.
6. Fehniger TA, Shah MH, Turner MJ, et al. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. J Immunol. 1999;162:4511-4520.
7. Trinchieri G. Biology of natural killer cells. Adv Immunol. 1989;47:187-376.
8. Trinchieri G. Natural killer cells wear different hats: effector cells of innate resistance and regulatory cells of adaptive immunity and of hematopoiesis. Semin Immunol. 1995;7:83-88.
9. Parham P, McQueen KL. Alloreactive killer cells: hindrance and help for haematopoietic transplants. Nat Rev Immunol. 2003;3:108-122.
10. Long EO, Burshtyn DN, Clark WP, et al. Killer cell inhibitory receptors: diversity, specificity, and function. Immunol Rev. 1997;155:135-144.

11. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. Immunol Today. 1990;11:237-244.
12. French AR, Yokoyama WM. Natural killer cells and viral infections. Curr Opin Immunol. 2003;15:45-51.
13. Orange JS, Fassett MS, Koopman LA, Boyson JE, Strominger JL. Viral evasion of natural killer cells. Nat Immunol. 2002;3:1006-1012.
14. Moretta L, Ciccone E, Moretta A, Hoglund P, Ohlen C, Karre K. Allorecognition by NK cells: nonself or no self? Immunol Today. 1992;13:300-306.
15. Ravetch JV, Lanier LL. Immune inhibitory receptors. Science. 2000;290:84-89.
16. Barrow AD, Trowsdale J. You say ITAM and I say ITIM, let's call the whole thing off: the ambiguity of immunoreceptor signalling. Eur J Immunol. 2006;36:1646-1653.
17. Moretta A, Bottino C, Vitale M, et al. Receptors for HLA class-I molecules in human natural killer cells. Annu Rev Immunol. 1996;14:619-648.
18. Lanier LL. NK cell receptors. Annu Rev Immunol. 1998;16:359-393.
19. Raulet DH, Held W. Natural killer cell receptors: the offs and ons of NK cell recognition. Cell. 1995;82:697-700.
20. Colonna M, Brooks EG, Falco M, Ferrara GB, Strominger JL. Generation of allospecific natural killer cells by stimulation across a polymorphism of HLA-C. Science. 1993;260:1121-1124.
21. Moretta A, Biassoni R, Bottino C, et al. Major histocompatibility complex class I-specific receptors on human natural killer and T lymphocytes. Immunol Rev. 1997;155:105-117.
22. Ruggeri L, Capanni M, Mancusi A, et al. Alloreactive natural killer cells in mismatched hematopoietic stem cell transplantation. Blood Cells Mol Dis. 2004;33:216-221.

23. Ruggeri L, Mancusi A, Capanni M, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. Blood. 2007;110:433-440.

24. Bielekova B, Catalfamo M, Reichert-Scrivner S, et al. Regulatory CD56(bright) natural killer cells mediate immunomodulatory effects of IL-2Ralpha-targeted therapy (daclizumab) in multiple sclerosis. Proc Natl Acad Sci U S A. 2006;103:5941-5946.

25. Li Z, Lim WK, Mahesh SP, Liu B, Nussenblatt RB. Cutting edge: in vivo blockade of human IL-2 receptor induces expansion of CD56(bright) regulatory NK cells in patients with active uveitis. J Immunol. 2005;174:5187-5191.

26. Yu G, Xu X, Vu MD, Kilpatrick ED, Li XC. NK cells promote transplant tolerance by killing donor antigen-presenting cells. J Exp Med. 2006;203:1851-1858.

27. Martin-Fontecha A, Thomsen LL, Brett S, et al. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat Immunol. 2004;5:1260-1265.

28. Marzo AL, Yagita H, Lefrancois L. Cutting edge: migration to nonlymphoid tissues results in functional conversion of central to effector memory CD8 T cells. J Immunol. 2007;179:36-40.

29. Hamann D, Baars PA, Rep MH, et al. Phenotypic and functional separation of memory and effector human CD8+ T cells. J Exp Med. 1997;186:1407-1418.

30. Cyster JG. Chemokines and cell migration in secondary lymphoid organs. Science. 1999;286:2098-2102.

31. von Andrian UH, Mackay CR. T-cell function and migration. Two sides of the same coin. N Engl J Med. 2000;343:1020-1034.

32. Lefrancois L, Marzo AL. The descent of memory T-cell subsets. Nat Rev Immunol. 2006;6:618-623.

33. Guarda G, Hons M, Soriano SF, et al. L-selectin-negative CCR7- effector and memory CD8+ T cells enter reactive lymph nodes and kill dendritic cells. Nat Immunol. 2007;8:743-752.

34. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999;401:708-712.

35. Cahalan MD, Parker I, Wei SH, Miller MJ. Real-time imaging of lymphocytes in vivo. Curr Opin Immunol. 2003;15:372-377.

36. Mempel TR, Junt T, von Andrian UH. Rulers over randomness: stroma cells guide lymphocyte migration in lymph nodes. Immunity. 2006;25:867-869.

37. Miller MJ, Wei SH, Parker I, Cahalan MD. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. Science. 2002;296:1869-1873.

38. Schliwa M, Woehlke G. Molecular motors. Nature. 2003;422:759-765.

39. Miller MJ, Wei SH, Cahalan MD, Parker I. Autonomous T cell trafficking examined in vivo with intravital two-photon microscopy. Proc Natl Acad Sci U S A. 2003;100:2604-2609.

40. Wei SH, Parker I, Miller MJ, Cahalan MD. A stochastic view of lymphocyte motility and trafficking within the lymph node. Immunol Rev. 2003;195:136-159.

41. Fillingame RH. Coupling H+ transport and ATP synthesis in F1F0-ATP synthases: glimpses of interacting parts in a dynamic molecular machine. J Exp Biol. 1997;200:217-224.

42. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell. 1998;92:829-839.

43. Jacobsson A, Stadler U, Glotzer MA, Kozak LP. Mitochondrial uncoupling protein from mouse brown fat. Molecular cloning, genetic mapping, and mRNA expression. J Biol Chem. 1985;260:16250-16254.

44. Boss O, Samec S, Paoloni-Giacobino A, et al. Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. FEBS Lett. 1997;408:39-42.

45. Fleury C, Neverova M, Collins S, et al. Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. Nat Genet. 1997;15:269-272.

CD56^{bright} Natural Killer Cells Expand and Contract Irrespective from CD25 Antibody-Treatment in Kidney Allograft-Recipients

Introduction

Natural killer (NK) cells are a key element of the early innate immune response.[1,2] Little is known about possible functions of human NK cells in solid-organ transplantation. In the murine system, however, compelling evidence has accumulated suggesting NK cells play important roles in this setting.[3-5]

Phenotypically NK cells can be defined as CD3 (TCR) negative, CD56 (neural cell adhesion molecule) positive lymphocytes. Approximately 90% of all circulating NK cells express intermediate levels of CD56 (i.e. they are CD56^{dim}), whereas the remainder ~10% express high levels of CD56 (CD56^{bright}).[6,7] CD56^{dim} NK cells are efficient killer cells, CD56^{bright} NK cells contain low levels of perforin and granzymes yet can be induced to rapidly secrete large amounts of cytokines ('regulatory' NK cells) [8,9]. While the precise relation between these phenotypically distinct subsets remains unclear it has been suggested that CD56^{dim} NK cells directly derive from the CD56^{bright} subset.[10,11]

Anti-CD25 mAb are blocking the binding site for IL-2 on the α-chain of the receptor,[12,13] a principle widely used to suppress both auto- and allo-immune responses.[14-16] A detailed understanding of how blocking the binding of IL-2 to the high-affinity IL-2 receptor relates to this mAb's *in vivo* effect is lacking.

In patients with multiple sclerosis and active uveitis, blocking CD25 has recently been shown to increase the number of circulating CD56^{bright} NK cells.[17] Expansion of these CD56^{bright} 'regulatory' NK cells has been suggested to mediate the mAb's immunomodulatory effect. No data are available on how anti-CD25 mAb-treatment impacts on the frequency of NK cell-subsets in kidney allograft recipients *in vivo*.

Here we took advantage of a well-characterized prospective cohort of kidney transplant-recipients treated with anti-CD25 mAb to assess the effect of blocking CD25 with regards to the number and phenotype of circulating NK cells.

Patients and Methods

Isolation of peripheral blood mononuclear cells

Anticoagulated blood was drawn from 5 healthy blood donors and 16 renal transplant recipients before transplantation, and at week 12, 26 and 52 post-transplantation. Written informed consent was obtained from all study participants and the study was IRB approved. Peripheral blood mononuclear cells (PBMC) were isolated using standard density gradient protocols (Lymphoprep, Fresenius Kabi, Oslo, Norway).

FACScan analyses

The following antibodies were used: CD16 (3G8), CD56 (MY31), CD3 (SK7), IL-10 (JES3-19F1), IFN-γ (25723.11), and appropriate isotype control antibodies, all from BD Biosciences (San Jose, CA, USA). For staining, cells were resuspended in PBS/1% bovine serum albumin (BSA, Sigma-Aldrich, Steinheim, Germany) and incubated with relevant antibodies for 20-40 min. at 4°C. Data were acquired with a FACS Calibur flow cytometer (Becton Dickinson) gating on CD3 negative cells in the FSC/SSC lymphocyte gate, and analyzed using FlowJo software 8.6.3 (Tree Star, Inc., Ashland, USA).

Intracellular cytokine staining

After activation with PMA (25 ng/mL) and ionomycin (1 μg/mL) (both from Sigma) for 4 h, cells were labeled with anti-CD3, anti-CD16 and anti-CD56 mAbs. Cells were then fixed and permeabilized using the Cytofix/Cytoperm kit from BD BioScience according to the manufacturer's protocol, incubated for 40 min. with anti IFN-γ mAbs, or appropriate isotype control antibodies (both from BD BioScience) and washed extensively prior to data acquisition.

Statistical analysis

Results were tested for normality using the D'Agostino & Pearson omnibus normality test. Student's t-test, Mann-Whitney test and Wilcoxon's signed rank test and

Fisher`s exact test were performed to assess normally and non-normally distributed data as appropriate, using Prism4 software (GraphPad Software, Inc. San Diego, CA, USA). P values <0.05 were considered statistically significant. Results are given as mean ± standard deviation (SD) or median and range, as appropriate.

Results and Discussion

Immunomodulatory ('regulatory') effects of CD56^{bright} NK cells have been proposed to mediate the effect of anti-CD25 mAb in patients with multiple sclerosis and uveitis.[18,19] Rapid production of cytokines and access to lymphnodes (via expression of lymph-nodes-homing receptors) is thought to relate to these immunomodulatory effects of the CD56^{bright} NK cell-subset,[20-23] all characteristics that we could readily confirm in a series of set-up experiments (data not shown).

In the circulation of 16 *anti-CD25 mAb-treated* kidney-transplant recipients we then monitored the frequency of CD56^{bright} (and CD56^{dim}) NK cells before transplantation, and at week 12, 26 and 52 post-transplantation. In 5 healthy blood-donors tested at these same time-intervals circulating NK cell-subsets (CD56^{bright} vs. CD56^{dim}) remained stable ('stable' defined as <1.5-fold expansion/contraction relative to baseline) (data not shown).

Characteristics of the transplanted study population, including induction and maintenance immunosuppressive regimens, are summarized in Table 1. Overall –and in sharp contrast to what has been observed in patients treated with CD25-blocking mAb in the context of multiple sclerosis and uveitis[24,25]– median numbers of CD56^{bright} (and CD56^{dim}) NK cells *decreased* after transplantation, returning to pre-transplant levels at week 52 post-transplantation (Fig. 1A).

In only a subset of patients (6/16), relevant *expansion* of circulating CD56^{bright} NK cell-numbers was observed, either early (week 12), or late (week 26 or 52) after administering CD25-blocking mAb (Fig. 1A, red lines, expansion defined as ≥1.5-fold relative to baseline).

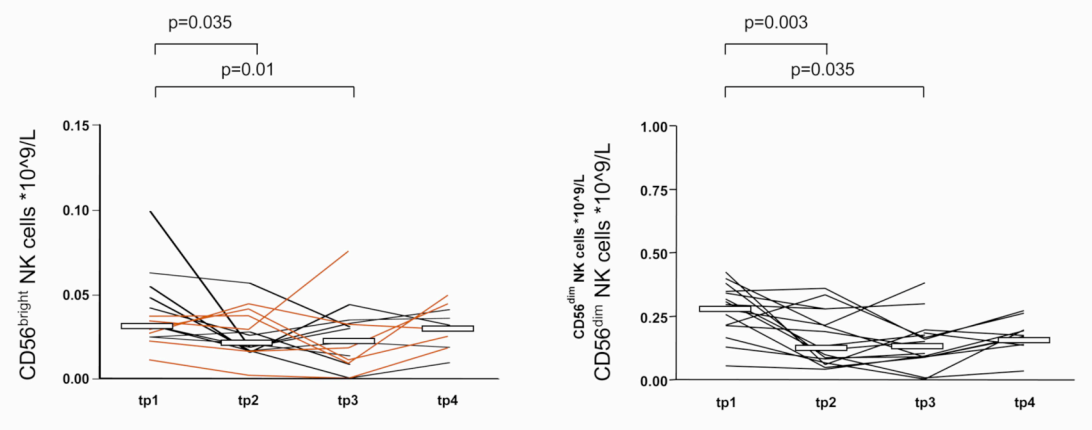
Expansion and contraction of circulating CD56^{bright} and CD56^{dim} NK cell-subsets was not necessarily linked (as exemplified by Patients 1-3, Figure 1B), and no association between total NK cell-numbers and overall lymphocyte-counts was detected (Tbl. 2).

Intriguingly, in the subset of patients that expanded circulating $CD56^{bright}$ NK cells, a trend towards an *inverse* relation with total lymphocyte counts was observed (Tbl. 2).

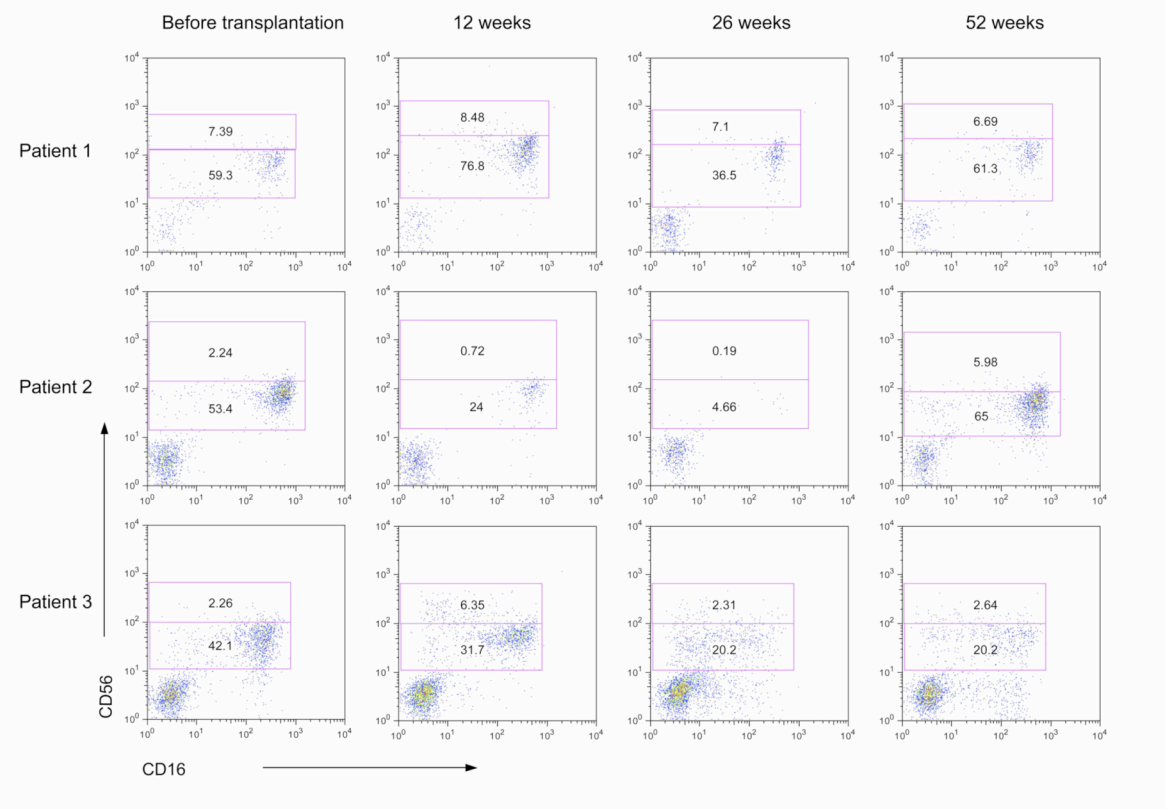
Comparing '$CD56^{bright}$ expanders' and '$CD56^{bright}$ non-expanders', the glomerular filtration rate 12 mos post-transplantation tended to be lower in patients expanding this subset, but this difference did not reach statistical significance (Tbl. 1). No similar association with regards to transplant-function was observed between patients expanding vs. not expanding the $CD56^{dim}$ NK cell-subset (data not shown).

Together these data indicate that among differing patient populations *in vivo* blockade of CD25 can be associated with variable (even opposite) effects with regards to the circulating number of NK cell-subsets. Whether this striking difference relates to specific immunological settings (e.g. *auto*-immunity vs. *allo*-immunity), or to differences in co-factors such as medication or organ-dysfunction (here renal insufficiency) remains to be determined.

Figure 1. NK phenotype in kidney transplant recipients treated with anti-CD25 mAb



(A) NK cell-subsets were enumerated before transplantation, and at week 12, 26 and 52 post-transplantation. Overall median numbers of both CD56^{bright} and CD56^{dim} NK cell contracted after transplantation. In the subset of patients that expanded CD56^{bright} NK cell-numbers (red lines), no relation to induction or maintenance immunosuppressive regimens or viral and bacterial infectious episodes was observed (Tbl. 1).



(B) Representative examples of the NK cell-phenotype in patients with stable vs. expanding / contracting CD56^{bright} and/or CD56^{dim} NK cells. Note that expansion and contraction of CD56^{bright} and CD56^{dim} NK cells was not necessarily linked.

Table 1. Characteristics of renal transplant recipients

Patient characteristics	Expanders	Non expanders	p-value[a]
Patient number	6	10	
Age - median (range)	56 (40-67)	52 (32-65)	0.48
Male gender - no. (%)	5 (86)	6 (64)	0.71
Immunosuppressive regimen at tx			
Calcineurin-inhibitor based triple therapy	6 (100)	10 (100)	1
Induction therapy			
Basiliximab (IL-2Ra blocker) - no. (%)	6 (100)	10 (100)	1
Survival and graft function			
Patient survival, 12 mos post-tx - no. (%)	6 (100)	10 (100)	1
Graft survival, 12 mos post-tx - no. (%)	6 (100)	10 (100)	1
GFR, 12 mos post-tx - mL/min./1.73 m^2 (mean±SD)	44.6 ± 15.2	54.2 ± 14.7	0.18
Biopsy-proven rejection episodes[b]			
0-3 mos			
No (or borderline) rejection - no. (%)	6 (100)	10 (100)	1
4-12 mos			
No (or borderline) rejection - no. (%)	6 (100)	10 (100)	1
Viral and bacterial infectious-episodes			
EBV reactivation - no. (%)	0	0	
CMV reactivation - no. (%)	2 (33)	3 (30)	1
Bacterial infections - no. (%)	3 (50)	4 (40)	1

[a] Mann-Whitney U test for continuous variables and Fisher`s exact test for categorial variables

[b]Classified according to Banff-criteria [26]

Table 2. Total lymphocyte counts of renal transplant recipients

Expanders (n=6)	median (range) *10^9 lymphocytes / mL	p value[1]
Before Transplantation	1.2 (0.71 - 1.62)	
Week 12	0.98 (0.78 - 1.06)	0.09
Week 26	0.89 (0.78 - 1.14)	0.06
Week 52	1.27 (0.77 - 1.88)	0.84

Non expanders (n=10)	median (range) *10^9 lymphocytes / mL	p value[1]
Before Transplantation	1.195 (1.07 - 3.02)	
Week 12	1.075 (0.36 - 2. 07)	0.23
Week 26	1.24 (0.33 - 2.14)	1.00
Week 52	1.38 (0.71 - 1.82)	0.76

[1] p value: total lymphocytes before transplantation compared to total lymphocytes at week 12, 26 and 52

References

1. Trinchieri G. Biology of natural killer cells. Adv Immunol. 1989;47:187-376.
2. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001;22:633-640.
3. Maier S, Tertilt C, Chambron N, et al. Inhibition of natural killer cells results in acceptance of cardiac allografts in CD28-/- mice. Nat Med. 2001;7:557-562.
4. Martin-Fontecha A, Thomsen LL, Brett S, et al. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat Immunol. 2004;5:1260-1265.
5. Yu G, Xu X, Vu MD, Kilpatrick ED, Li XC. NK cells promote transplant tolerance by killing donor antigen-presenting cells. J Exp Med. 2006;203:1851-1858.
6. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001;22:633-640.
7. Cooper MA, Fehniger TA, Turner SC, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood. 2001;97:3146-3151.
8. Fehniger TA, Cooper MA, Nuovo GJ, et al. CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. Blood. 2003;101:3052-3057.
9. Fehniger TA, Shah MH, Turner MJ, et al. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. J Immunol. 1999;162:4511-4520.
10. Nagler A, Lanier LL, Cwirla S, Phillips JH. Comparative studies of human FcRIII-positive and negative natural killer cells. J Immunol. 1989;143:3183-3191.

11. Freud AG, Caligiuri MA. Human natural killer cell development. Immunol Rev. 2006;214:56-72.

12. Waldmann TA, O'Shea J. The use of antibodies against the IL-2 receptor in transplantation. Curr Opin Immunol. 1998;10:507-512.

13. Roessler E, Grant A, Ju G, Tsudo M, Sugamura K, Waldmann TA. Cooperative interactions between the interleukin 2 receptor alpha and beta chains alter the interleukin 2-binding affinity of the receptor subunits. Proc Natl Acad Sci U S A. 1994;91:3344-3347.

14. Webster AC, Playford EG, Higgins G, Chapman JR, Craig JC. Interleukin 2 receptor antagonists for renal transplant recipients: a meta-analysis of randomized trials. Transplantation. 2004;77:166-176.

15. Ben-Ari ET. Dual purpose: some cancer therapies used to treat autoimmune diseases. J Natl Cancer Inst. 2004;96:577-579.

16. Rose JW, Watt HE, White AT, Carlson NG. Treatment of multiple sclerosis with an anti-interleukin-2 receptor monoclonal antibody. Ann Neurol. 2004;56:864-867.

17. Cooper MA, Fehniger TA, Turner SC, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood. 2001;97:3146-3151.

18. Bielekova B, Catalfamo M, Reichert-Scrivner S, et al. Regulatory CD56(bright) natural killer cells mediate immunomodulatory effects of IL-2Ralpha-targeted therapy (daclizumab) in multiple sclerosis. Proc Natl Acad Sci U S A. 2006;103:5941-5946.

19. Li Z, Lim WK, Mahesh SP, Liu B, Nussenblatt RB. Cutting edge: in vivo blockade of human IL-2 receptor induces expansion of CD56(bright) regulatory NK cells in patients with active uveitis. J Immunol. 2005;174:5187-5191.

20. Cooper MA, Fehniger TA, Turner SC, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood. 2001;97:3146-3151.

21. Anfossi N, Andre P, Guia S, et al. Human NK cell education by inhibitory receptors for MHC class I. Immunity. 2006;25:331-342.

22. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001;22:633-640.

23. Vitale M, Della Chiesa M, Carlomagno S, et al. The small subset of CD56brightCD16- natural killer cells is selectively responsible for both cell proliferation and interferon-gamma production upon interaction with dendritic cells. Eur J Immunol. 2004;34:1715-1722.

24. Bielekova B, Catalfamo M, Reichert-Scrivner S, et al. Regulatory CD56(bright) natural killer cells mediate immunomodulatory effects of IL-2Ralpha-targeted therapy (daclizumab) in multiple sclerosis. Proc Natl Acad Sci U S A. 2006;103:5941-5946.

25. Li Z, Lim WK, Mahesh SP, Liu B, Nussenblatt RB. Cutting edge: in vivo blockade of human IL-2 receptor induces expansion of CD56(bright) regulatory NK cells in patients with active uveitis. J Immunol. 2005;174:5187-5191.

26. Solez K, Colvin RB, Racusen LC, et al. Banff '05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). Am J Transplant. 2007;7:518-526.

Detection of Alloreactive NK cells in Mixed Lymphocyte Reactions using Paraformaldehyde-Silenced Target Cells

Text

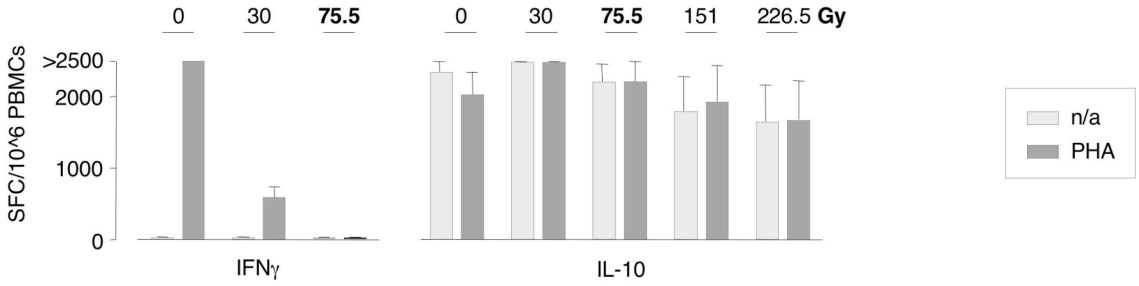
Since its introduction in 1963/64,[1-3] studies based on the mixed lymphocyte reaction (MLR) have contributed significantly to our understanding of allo-specific immunity.[4] In response to allo-antigen exposure, reactive cells may proliferate, mature and/or secrete various cytokines. To evaluate alloreactive proliferation selectively, target cell-division can be blocked efficiently (e.g. with mitomycin C or via irradiation).[5] While proliferation is still used as read-out for alloreactivity, measuring the release of cytokines may be more sensitive to detect allo-specific lymphocyte activation, and is performed routinely. When investigating one-way, allo-specific induction of cytokines, irradiation is widely used to inhibit protein-secretion in the target cell-population.

Using ELISpot analyses, we reassessed the effect of irradiation with the classic 'silencing'-dose (30 Gy) on the capacity of peripheral blood mononuclear cells (PBMCs) to secrete IFN-γ and IL-10 after 12 h of activation (Material and Methods for details). PBMCs irradiated with 30 Gy, and subsequently activated with PHA at a concentration of 1.8 μg/ml, still secreted readily detectable amounts of IFN-γ (non-irradiated PBMCs; > 2500 SFC/10^6 cells, PBMCs irradiated with 30 Gy; 650 [±202] SFC/10^6 cells). By contrast, irradiation of PBMCs with 75.5 Gy completely abolished secretion of IFN-γ (Fig. 1A, left panel). Unlike secretion of IFN-γ, the release of IL-10 was – as observed in this short-time incubation assay – constitutive in nature, rather than PHA-induced. Importantly, this constitutive release of IL-10 was largely unaffected by irradiation with 75.5 Gy (the dose that consistently abolished secretion of IFN-γ), and was readily detectable upon 12 h of incubation even after irradiation with 226.5 Gy (Fig. 1A, right panel). By use of a sensitive chemiluminescence-based cytokine detection system (see Material and Methods for details) we expanded our assessment of irradiated PBMCs

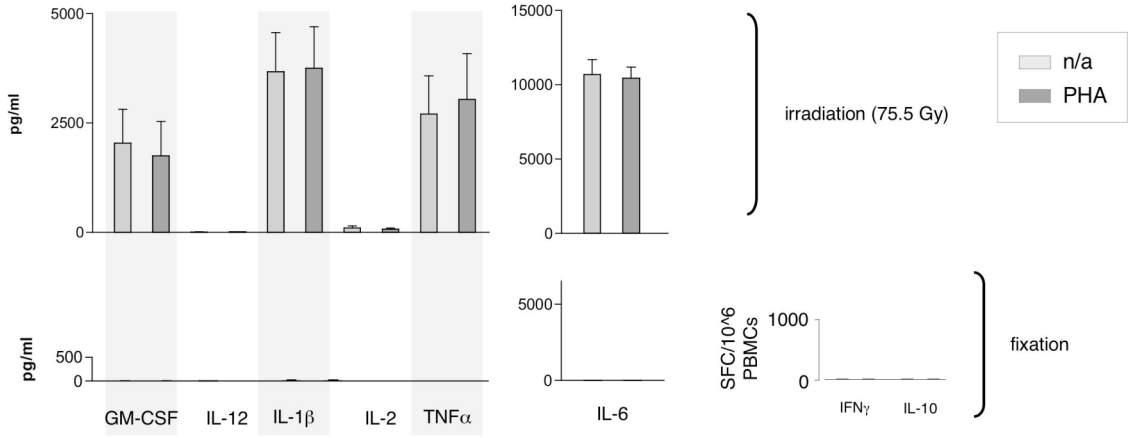
(75.5 Gy) with regards to their capacity to secrete GM-CSF, IL-12, IL-1β, IL-2, TNF-α, and IL-6. These experiments identified that a significant amount of GM-CSF, IL-1β, IL-2, TNF-α, and IL-6 are produced by PBMCs after exposure to a dose of irradiation that is 'silencing' the release of IFN-γ (Fig. 1B, upper panel). In an ideal one-way MLR concerned with the release of cytokines, the 'target' or 'stimulatory' cell-population would, with the exception of being unable to secrete cytokines, remain intact with regards to its biological/immunological behavior. As evidenced here, as well as by large amount of literature (for review see Ref. 6-8) irradiation is far from inducing such a state of selective secretory anergy.

We then tested the impact of paraformaldehyde 0.1% (10 min. exposure) on the secretion of cytokine by PBMCs (see Material and Methods for details). This mild and short-term fixation-protocol completely abolished secretion of all cytokines tested (Fig. 1B, lower panel).

Figure 1. Effect of irradiation and formaldehyde-exposure on the release of cytokines by PBMCs



(A) γ-irradiation reduced the frequency of IFN-γ-producing cells detectable after 12 h of activation with PHA (1.8 μg/ml) in a dose-dependent manner (left panel) (30 Gy: n=5, 75.5 Gy: n=15). Secretion of IL-10 was constitutive in nature and not further induced upon over-night activation with PHA (1.8 μg/ml). Note that increasing doses of γ-irradiation slightly reduced, yet were unable to abolish, constitutive secretion of IL-10 (right panel) (n=3-7).

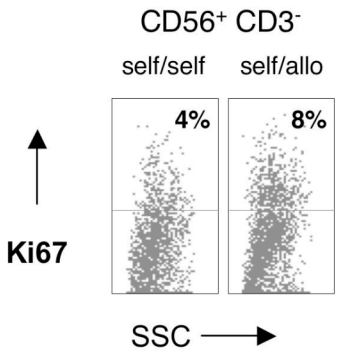


(B) Using a sensitive chemiluminescence-based detection system, secretion of GM-CSF, IL-12, IL-1β, IL-2, TNF-α, and IL-6 by PBMCs was detected even after exposure to a dose of irradiation that is 'silencing' the release of IFN-γ (upper panel) (n=3). Mild and short-term exposure of PBMCs to paraformaldehyde (0.1% for 10 min.) abolished both PHA-inducible as well as constitutive secretion of all cytokines assessed (lower panel) (n=6). n/a=non-activated, PHA=activated with phytohemagglutinin.

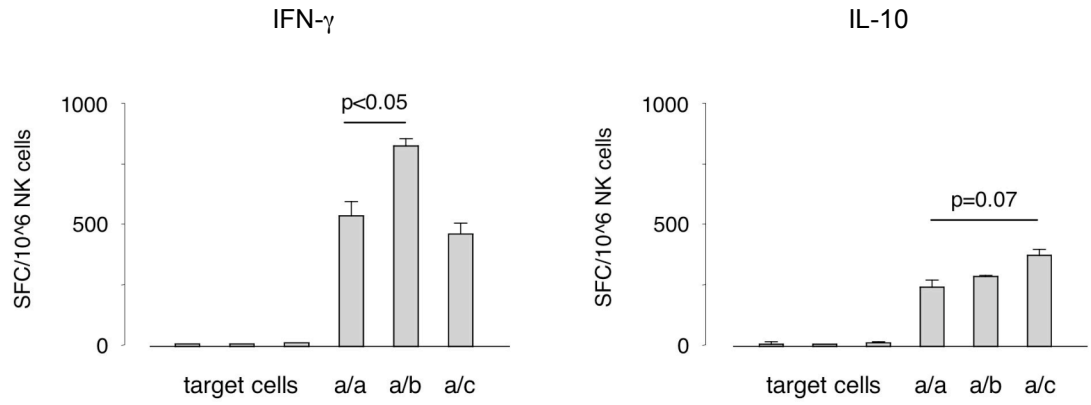
Natural killer (NK) cells, a subset of lymphocytes, are a key-element of the early innate immune-response, and capable to rapidly produce significant amounts of various cytokines.[9] NK cells express a complex set of receptors for HLA class I molecules (HLA-A, -B, -C, -G, -E) that belong either to the family of killer-cell immunoglobulin-like receptors (KIRs), or the lectin-like receptor family.[10] NK cell alloreactions seem to be mediated via mismatched HLA class I ligands and their receptors. Of these, the dominant pattern of alloreactivity is thought to be due to two epitopes on HLA-C, determined by a simple amino-acid dimorphism. Absence of any of these two HLA-C epitopes vis-à-vis a given NK cell is leading to a lack of inhibition of the corresponding KIRs expressed on this NK cell, and hence to its allo-specific activation.

Aiming at exploiting this NK cell alloreactivity to test the usefulness of paraformaldehyde-fixed (and hence truly silenced) target cells in MLRs, NK cells were isolated from PBMCs (see Material and Methods for details). Purity of NK cell-preparations were assessed by flow-cytometry, and contained 96% (±2%) CD56+ CD3- cells with only 2% (±2%) contaminating CD3+ cells (data not shown). First we confirmed that target cells treated according to the here-developed protocol retain their capacity to induce NK cell allo-specific proliferation (Fig. 2A, representative of n=3). Attempting next to assess NK cell alloreactivity in strict one-way MLRs, we then tested whether paraformaldehyde-treated – and hence truly silenced – target cells still were capable of triggering allo-specific secretion of cytokines. Importantly (and to our knowledge for the first time), allo-specific IFN-γ-production was readily detected (Fig. 2B, left panel, representative of n=5). In addition, our modified experimental conditions also allowed assessing the frequency of IL-10-secreting NK cells, which would have been hindered in MLRs using irradiated target cells (see Fig. 1A/B for levels of cytokines secreted by 75.5 Gy-irradiated PBMCs).

Figure 2. Reactivity of NK cells vis-à-vis formaldehyde-exposed target cells



(A) Allo-specific proliferation of NK cells was readily detected after 5 days incubation with target cells silenced according to the here described fixation-protocol.



a: A*24, A*30, B*18, B*27, Cw*02, Cw*07 (**C1/C2**)
b: A*24, A*31, B*18, B*35, Cw*04, Cw*05 (**C2/C2**)
c: A*11, A*68, B*15, B*51, Cw*07, Cw*15 (**C1/C2**)

(B) Allo-specific secretion of IFN-γ was detected exposing individual **a** (HLA C1/C2 haplotype) to target cells from individual **b** (HLA C2/C2 haplotype). No reactivity was seen exposing individual **a** (HLA C1/C2 haplotype) to target cells from individual **c** (HLA C1/C2 haplotype). A tendency towards an increase in the frequency of IL-10 producing NK cells was observed exposing individual **a** (HLA C1/C2 haplotype) to target cells from individual **c** (HLA C1/C2 haplotype).

Avoiding irradiation of target PBMCs in MLRs offers the obvious advantage of getting rid of a complex, target cell-derived cocktail of cytokines potentially impacting (both directly and possibly also indirectly) on the assay. Continuously reevaluating the limits and potential pitfalls of (even long-standing) protocols is important. In the case of the one-way MLR, a simple modification in preparing target cells offers a means to avoiding the complexities introduced via their irradiation, and enhances the scope of the assay.

Material and Methods

Isolation of PBMCs / NK cells

Anticoagulated blood was drawn from healthy donors after written informed consent and IRB-approval. PBMCs were isolated from buffy coats using standard density gradient protocols (Lymphoprep™, Fresenius Kabi, Oslo, Norway). NK cells were negatively selected using a human NK cell isolation kit (Miltenyi Biotecs GmbH, Bergisch-Gladbach, Germany), according to the manufacturers protocol. After negative selection, purity of cell-preparations were assessed via flow cytometry.

FACScan analyses

The following antibodies were used: CD16 (3G8), CD56 (MY31), CD3 (SK7), CD4 (RPA-T4), and appropriate isotype control antibodies, all from BD Biosciences (San Jose, CA, USA), Ki67 (clone Ki67) (DakoCytomation, Dako Schweiz AG, Switzerland). Cells were resuspended in phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA), and incubated with relevant antibodies for 30-45 min. at 4°C. Data were acquired with a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed using CellQuest software (Becton Dickinson).

ELISpot assay

MultiScreen HTS™ IP 96 well plates (MSIPS4510, Millipore AG, Volketswil, Switzerland) were coated with 1:2000 diluted mAb anti-human IFN-γ (1-D1K) and anti-human IL-10 (9D7) (both from Mabtech AB, Stockholm, Sweden). Cells were added to a final volume of 130 μl/well. After incubation, plates were washed with PBS and, prior to developing, incubated with PBS containing 1% FCS for 10 min. Plates were incubated for 2 h with anti-human IFN-γ mAb (1:200) coupled with alkaline phosphatase (7-B6-1-ALP), and biotinylated anti-human IL-10 mAb (1:2000) (12G8-Biotin) (both from Mabtech) for 2 h at RT, washed and incubated with Streptavidin-HRP (1:2000) (Mabtech). Spots were developed using HistoMark® RED phosphatase system (KPL, Gaithersburg, Maryland, USA) and HistoMark® True BLUE peroxidase system, and

counted by direct visualization with the AID CytoSpot Reader System (CSR01, AID GmbH, Strassberg, Germany) using the ELISpot 3.5 software (AID GmbH). The mean of the negative controls plus 3 standard deviations (SD) was considered as cut-off for a positive result.

Mixed lymphocyte reaction

Irradiation of target cells: PBMCs were resuspended in PBS for irradiation (30 Gy to 226.5 Gy, as indicated [Cs source]). After irradiation, cells were washed twice in PBS, and resuspended at $3.3x10^7$ cells/ml in R10 (RPMI 1640 containing 10% heat inactivated FCS, 50 U/ml penicillin and 50 μg/ml Streptomycin [all from GIBCO™, LuBioScience GmbH, Luzern, Switzerland]).

Paraformaldehyde-treatment of target cells: PBMCs were resuspendend in freshly prepared PBS 0.1% paraformaldehyde (Merck KGaA, Darmstadt, Germany), and incubated for 10 min. at RT. Cells were then washed twice in PBS and quenched with 0.1M L-lysine buffer (Sigma-Aldrich, St Louis, MO) pH 8 for 30 min. at 4°C. Prior to use cells were washed 3 times in PBS and were resuspended at $1x10^7$ cells/ml in R10.

Cytokine-secretion assays were performed in MultiScreen HTS™ IP 96 well plates (Millipore AG). Isolated NK cells ($1x10^5$ for MLRs using paraformaldehyde-treated target cells, $2.5x10^5$ using irradiated target cells), and target PBMCs were added to the wells at a ratio of 1:5 (optimal ratio [data not shown]), and incubated in R10 overnight at 37°C, 5% CO_2. Non-stimulated NK cells were used as negative control, NK cells stimulated with purified PHA (1.8 μg/ml) (Lenexa) as a positive control.

To assess proliferation, NK cells were incubated with autologous and allogeneic paraformaldehyde-fixed target cells in a ratio 1:5 (ratio as optimized for the ELISpot assays) for 5 days at 37°C 5% CO_2 (n=3). Cells were then stained with surface antibodies (anti-CD3, anti-CD16, and anti-CD56) before being fixed and permeabilized using an intracellular staining kit according to the manufacture's protocol

(Cytofix/Cytoperm Kit, BD Biosciences). After permeabilization, cells were stained with anti-Ki67 mAb for 30 min. at 4°C and analyzed by flow cytometry.

Quantification of cytokines

Relevant supernatants from target cell-cultures were transferred to MSD® 96-well MULTI-SPOT® plates (Meso Scale Discovery, Gaithersburg, MD, USA), and cytokines quantified according to the manufacturers protocol. Plates were analyzed with the SECTOR Imager 6000 (MSD).

HLA typing

HLA-A, -B, -C typing of the responder and stimulator cells was performed by reverse PCR-SSOP (sequence-specific oligonucleotide probe) hybridization (LabScreen, OneLambda), using genomic DNA extracted from PMBCs. Assignment to C1 and C2 alleles was based on the presence of Asn80 and Lys80, respectively, on the α-helix of HLA-C antigens.

Statistical analysis

Mann-Whitney U tests and Student t-tests were performed for assessing normally and non-normally distributed data, respectively, using Prism3 software (GraphPad Software, Inc. San Diego, CA, USA). *P* values < 0.05 were considered statistically significant. Results are given as mean ±SD.

References

1. Bach F, Hirschhorn K. Lymphocyte Interaction: A Potential Histocompatibility Test in Vitro. Science. 1964;143:813-814.

2. Bain B, Vas MR, Lowenstein L. The Development of Large Immature Mononuclear Cells in Mixed Leukocyte Cultures. Blood. 1964;23:108-116.

3. Hirschhorn K, Bach F, Kolodny RL, Firschein IL, Hashem N. Immune Response and Mitosis of Human Peripheral Blood Lymphocytes in Vitro. Science. 1963;142:1185-1187.

4. McDevitt HO. Discovering the role of the major histocompatibility complex in the immune response. Annu Rev Immunol. 2000;18:1-17.

5. Lightbody J, Kong YC. Comparison of 137Cs irradiation and mitomycin C treatment of stimulator cells in the mixed lymphocyte culture reaction. Cell Immunol. 1974;13:326-330.

6. Barcellos-Hoff MH, Park C, Wright EG. Radiation and the microenvironment - tumorigenesis and therapy. Nat Rev Cancer. 2005;5:867-875.

7. Skorski T. Oncogenic tyrosine kinases and the DNA-damage response. Nat Rev Cancer. 2002;2:351-360.

8. Safwat A. The immunobiology of low-dose total-body irradiation: more questions than answers. Radiat Res. 2000;153:599-604.

9. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001;22:633-640.

10. Parham P, McQueen KL. Alloreactive killer cells: hindrance and help for haematopoietic transplants. Nat Rev Immunol. 2003;3:108-122.

HLA C Allotypes Differentially Support Regulatory Allo-Specific NK Cell-Function

Introduction

Natural killer cells (NK cells) are a key element of the early innate immune response. Based on their expression of CD56 (neural cell adhesion molecule) and CD16 (FcγRIII), NK cells can be divided into two subsets. Approximately 90% of all circulating NK cells express CD56 and CD16, the remainder ~10% are $CD56^{bright}$ $CD16^{dim/negative}$.[1] Whereas $CD56^{dim}$ NK cells are efficient killer cells, $CD56^{bright}$ NK cells contain low levels of perforin and granzymes, but, when activated by cytokines *in vitro*, can be induced to secrete large amounts of inflammatory and –to some extent– anti-inflammatory cytokines.[2,3]

Downregulation of HLA I molecules after viral infections or on tumor cells leads to a lack of inhibitory signals and hence killing of the target cell ('missing self' recognition).[4-7] The same 'missing self' principle is thought to underlie NK cell *allo*-reactive killing. The dominant pattern of NK cell alloreactivity is due to recognition by the NK cell of two HLA C allotypes, determined by an asparagine/lysine polymorphism at position 80.[8-10] Lack of recognition of these two HLA C epitopes vis-à-vis a given NK cell leads to a loss of inhibition of the corresponding NK cell, and hence to its allospecific activation. In hematological stem cell transplantation, such 'missing self' reactions –in the sense of NK cell-mediated graft versus leukemia effects from HLA C mismatched donors– have been demonstrated to exert powerful graft-versus-leukemia effects.[11,12] The role human allo-specific NK cells play in solid-organ transplantation remains largely unknown.[13,14]

Material and Methods

Isolation of peripheral blood mononuclear cells and natural killer cells

Anticoagulated blood was drawn from healthy blood donors after written informed consent was obtained from all study participants and IRB approval of the study. NK cells were negatively selected from PBMC using NK cell isolation kits (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) according to the manufacturer's protocol. The purity of the NK cells was assessed by flow cytometry and was ≥98% (data not shown).

HLA genotyping

HLA genotyping was performed by SSO (sequence-specific oligonucleotide probe) hybridization (Labtype SSO, OneLambda, Montpellier, France), using genomic DNA extracted from PBMC. Assignment to C1 and C2 allotypes was based on the presence of Asn 80 and Lys 80, respectively, on the α-helix of HLA C molecules.[15]

NK cell cloning and chromium release assays

HLA C1/C2 NK cell-clones were generated by limiting dilution, using irradiated PBMC as feeder cells. NK cells were plated at a concentration of 1-20 cells/100 μl, feeder cells were irradiated with 25 Gy and added at a concentration of $1x10^6$ cells/mL. NK cells were activated with 1 μg/mL phytohaemaglutinin (PHA) (Remel Europe Ltd, Dartford, UK) on day 1, and 500 IU of IL-2 (Proleukin® Novartis Pharma, Basel, Switzerland) from day 2 on. A total of 100 NK cell-clones were tested for alloreactivity by (51)Cr release cytotoxicity assays at a ratio of 1:5. Target cell lysis of >20% was considered significant.

Mixed lymphocyte reaction using paraformaldehyde-silenced target cells

Target cells were prepared as previously described.[16] Briefly, PBMC were resuspended in freshly prepared phosphate-buffered saline (PBS) 0.1% paraformaldehyde (Merck KGaA, Calbiochem, Darmstadt, Germany), and incubated for 10 min. at room

temperature. Cells were then quenched with 0.1M L-lysine buffer (Sigma-Aldrich, St Louis, MO, USA). MHC I expression was not influenced by this procedure (data not shown). Prior to use, cells were resuspended at $1x10^7$ cells/mL in R10 (RPMI 1640 containing 10% heat-inactivated FCS, 50 U/mL penicillin and 50 μg/mL streptomycin [all from GIBCO, LuBioScience GmbH, Luzern, Switzerland]). When mixed lymphocyte reactions (MLRs) were followed by qPCR experiments, target cells were depleted from CD56-expressing cells by use of CD56 MACS beads (>98% depletion-efficiency, data not shown) (Miltenyi Biotec).

MLRs were performed in MultiScreen HTS™ IP 96 well plates (MSIPS4510, Millipore AG, Volketswil, Switzerland) or in Microtest tissue culture 96 well plates (Becton Dickinson, Mountain View, CA, USA). Effector cells ($1x10^5$ isolated NK cells) were incubated with PBMC as target cells at a ratio of 1:5 (=optimal ratio [data not shown]). All MLRs were performed overnight in R10 at 37°C, 5% CO_2. As negative control, fixed target cells were used. NK cells stimulated with phorbol myristate acetate (PMA) (20 ng/mL) and ionomycin (2 μM) (both from Merck KGaA, Calbiochem) served as positive control.

Cell-densities of all experimental settings ('autologous–low density' [$1x10^6$ NK cells/mL]; 'autologous–high density', 'allo-C1/C1', 'allo-C1/C2' and 'allo-C2/C2' [=all 'high density', $1x10^6$ NK cells plus $5x10^6$ autologous or allogeneic cells/mL; 100 μl plated in 6.35 mm diameter flat bottom plates]) were documented using an Olympus IX50 Inverted Phase Contrast microscope (Olympus Schweiz AG, Volketswil, Switzerland).

ELISpot assay

MultiScreen HTS™ IP 96 well plates (Millipore AG) were coated with 1:2000 diluted mAb anti-human IFN-γ (1-D1K) and anti-human IL-10 (9D7) (both from Mabtech AB, Stockholm, Sweden). Cells were added to a final volume of 130 μl/well. After incubation, plates were incubated for 2 h with anti-human IFN-γ mAb (1:200) coupled

with alkaline phosphatase (7-B6-1-ALP), and biotinylated anti-human IL-10 mAb (1:2000) (12G8-Biotin) (both from Mabtech) for 1.5 h at room temperature, washed and incubated with Streptavidin-HRP (1:2000) (Mabtech). Spots were developed using HistoMark® RED phosphatase system and HistoMark® True BLUE peroxidase system (both from KPL, Gaithersburg, Maryland, USA), and counted by direct visualization with the AID CytoSpot Reader System (CSR01, Autoimmun Diagnostika (AID) GmbH, Strassberg, Germany) using the ELISpot 3.5 software (AID GmbH).

Quantitative PCR (qPCR)

Total RNA was extracted from NK cells that were either isolated directly from PBMC, or negatively selected after MLRs (NK cell purity was always >98%, data not shown), using the RNeasy Mini kit (QIAGEN GmbH, Hilden, Germany). Total RNA was used for reverse transcription (Promega, Madison, WI, USA). For qPCR, 2-4 μl of cDNA were used on an ABI 7900 instrument, using TaqMan chemistry (Applied Biosystems, Foster City, CA, USA). Values are given as copies over copies of β2-microglobulin. Primers and probes used in this study are listed in Table 1.

Table 1. Primers and probes

Assay	Primers	Probe
IL-10	F: GCCTTGTCTGAGATGATCCAGTT	ATGCCCCAAGCTGAGAACCAAGACC
	R: TCACATGCGCCTTGATGTCT	
TGF-β	F: CGAGAAGCGGTACCTGAAC	CAGCACGTGGAGCTGTACCAGAAATACAGC
	R: TGAGGTATCGCCAGGAATTGT	

Statistical analysis

Results were tested for normality using the D'Agostino & Pearson omnibus normality test. Mann-Whitney U test and Wilcoxon's signed rank test were performed as appropriate, using Prism4 software (GraphPad Software, Inc. San Diego, CA, USA). P values <0.05 were considered statistically significant. Results are given as mean ± standard deviation (SD) or median and range, as appropriate.

Results

Allo-specific killing and secretion of IFN-γ and IL-10 by NK cells

Ex vivo expanded and activated NK cell-clones readily kill allo-target cells (Ref. 17 and Fig. 1). To begin to understand how allo-specific NK cell-mediated killing relates to their secretion of cytokines, we applied a recently developed method to completely inhibit the cytokine-secretion capacity of target cells used in MLRs.[18]

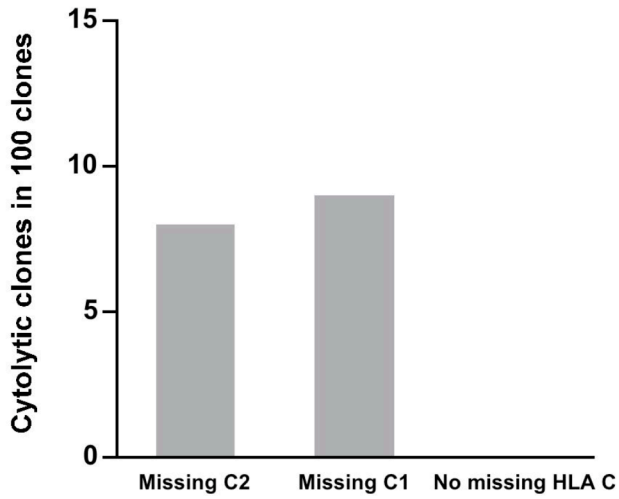


Figure 1. Allo-specific killing by NK cells

NK cells expressing HLA C1 and C2 were cloned and tested for alloreactivity in chromium release assays. Cytotoxicity was defined as ≥20% killing of target cells. Cytotoxic clones were exclusively detected when target cells lacked expression of HLA C1 or HLA C2.

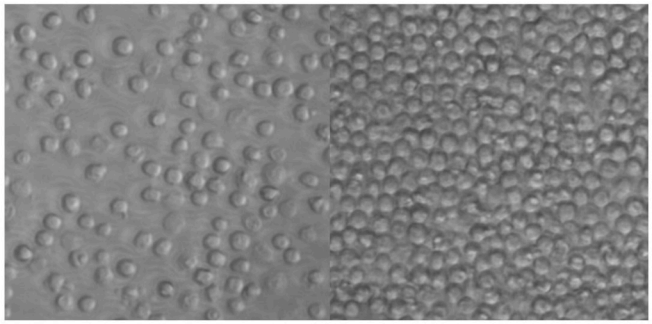
Surprisingly, using this system to assess NK cells directly *ex vivo*, a population of NK cells secreting the regulatory cytokine IL-10 was identified. This secretion of IL-10 was not constitutive, since NK cells plated at a concentration not allowing for cell-cell interaction ('low-density' plating, Fig. 2A, left panel) did not secrete IL-10, or did so only at very low levels. However, when plating NK cells at a density promoting extensive cell-cell interactions ('high-density' plating, Fig. 2A, right panel), a population of NK cells

secreting IL-10 was consistently induced (Fig. 2B, left panel). The median frequency of this population increased when increasing the target to effector (i.e. NK) cell-ratio –with a plateau being reached at a target to effector cell-ratio between 5:1 and 10:1– and was induced irrespective of whether PBMC or isolated NK cells were used as target cells (data not shown). Variability of the population-size of IL-10 secreting NK cells was not related to their own HLA C genotype, nor was the HLA C genotype of NK cells related to the frequency of IL-10 secretion in allogeneic reactions (data not shown). Interestingly however, frequencies of IL-10 secreting NK cells were found to differ depending on the HLA C genotype of the *allo-target cell population*. Exposure to target cells with a C1/C2 genotype even *increased* the median frequency of IL-10 secreting NK cells as compared to the 'autologous-high density' condition (p=0.03), and was higher than after exposure to allo-target cells with a C1/C1 or a C2/C2 genotype (Fig. 2B, right panel). The relation between the frequency of IL-10 secreting NK cells and the HLA C genotype of the allo-target cell population became even more evident when directly comparing reactions of NK cells against both allo-target cells with a C1/C2 genotype *and* allo-target cells with a C1/C1 genotype, or –alternatively– allo-target cells with a C1/C2 genotype *and* allo-target cells with a C2/C2 genotype. In 13/14 (93%) experiments the frequency of NK cells secreting IL-10 was higher when reacting against allo-target cells with a C1/C2 genotype than when reacting against allo-target cells with a C1/C1 genotype (p=0.0005), and in 4/5 (80%) reactions the frequency of NK cells secreting IL-10 was higher when reacting with allo-target cells with a C1/C2 genotype as compared to reactions against allo-target cells with a C2/C2 genotype.

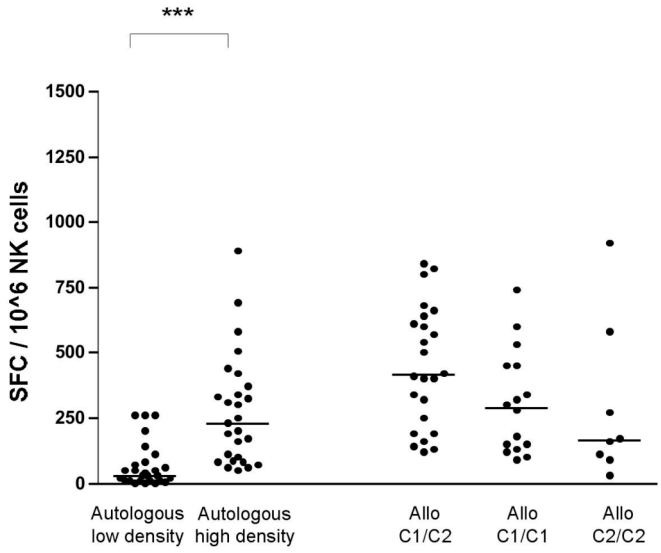
Dual-color ELISpot analyses allowed discriminating the source of IL-10 vs. IFN-γ at the single-cell level and indicated that NK cells never simultaneously secreted both IL-10 *and* IFN-γ (Fig. 2C). Flow-cytometric experiments gating on CD3- CD56+ lymphocytes and staining for intracellular IL-10 confirmed these ELISpot based observations (data not shown).

Together these experiments indicated that a distinct population of unprimed NK cells could be induced to secrete IL-10 directly *ex vivo*. The relative frequencies of IL-10 secreting NK cells related to the HLA C allotype of allo-target cells.

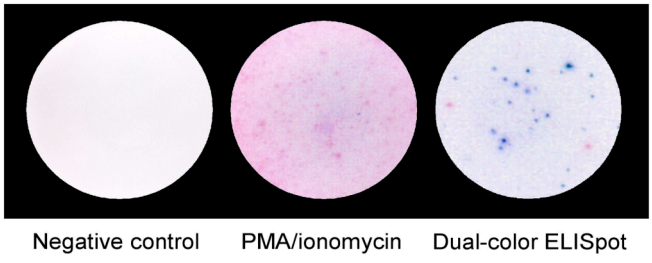
Figure 2. Killing-activity and secretion of IFN-γ and IL-10 by NK cells interacting with autologous and allogeneic target cells



(A) Throughout the manuscript secretion of cytokines by NK cells was tested either under experimental conditions allowing only for limited cell-cell contacts (termed 'autologous–low density') (left panel=representative), or conditions promoting extensive cell-cell contacts (termed 'autologous–high density', 'allo C1/C2', 'allo C1/C1' or 'allo C2/C2') (right panel=representative).



(B) The impact of NK cell–target cell interactions on NK cell-derived secretion of IL-10 was assessed using ELISpot assays. Under 'autologous–low density' conditions, NK cells secreted little, if any, IL-10. By contrast, 'autologous–high density' conditions consistently induced a population of NK cells to secrete IL-10. Under allo-conditions, NK cell–target cell interactions also induced an IL-10 secreting NK cell-population. Grouping allo-target cells according to their HLA C genotype, cells expressing both HLA C1 *and* C2 epitopes best supported –or even enhanced– secretion of IL-10 by NK cells. Horizontal lines indicate median values. ***P<0.0001



(C) In dual-color ELISpot assays, secretion of IL-10 (blue spots) and IFN-γ (red spots) was assessed at the single-cell level. Only single-positive NK cells were detected.

Secretion of TGF-β by NK cells after mixed lymphocyte reactions

TGF-β is a pleiotropic cytokine with immunoregulatory properties.[19] Using a sandwich ELISA-system we measured the concentration of TGF-β in the supernatant of NK cells plated at low density ('autologous–low density'), exposed to silenced autologous cells ('autologous–high density'), as well as silenced 'allo-C1/C2', 'allo-C1/C1', and 'allo-C2/C2' target cells. In the supernatant of NK cells plated at a concentration preventing extensive cell-cell contact ('autologous–low density'), concentrations of TGF-β were at –or below– background levels of the assay. By contrast, autologous cellular interactions ('autologous–high density') induced NK cells to release considerable amounts of TGF-β (Fig. 3, left panel). In line with the data obtained for secretion of IL-10, interaction with allo-target cells differentially supported secretion of TGF-β by NK cells. Whereas similar levels were detected after exposure of NK cells to autologous cells and 'allo-C1/C2' target cells, less TGF-β was released after exposure of NK cells to 'allo-C1/C1' target cells and 'allo-C2/C2' target cells (Fig. 3, right panel).

Thus, autologous cell-cell interactions actively induced NK cells to secrete TGF-β. Under allo-conditions this secretion was only supported by target cells expressing both HLA C1 *and* C2 epitopes.

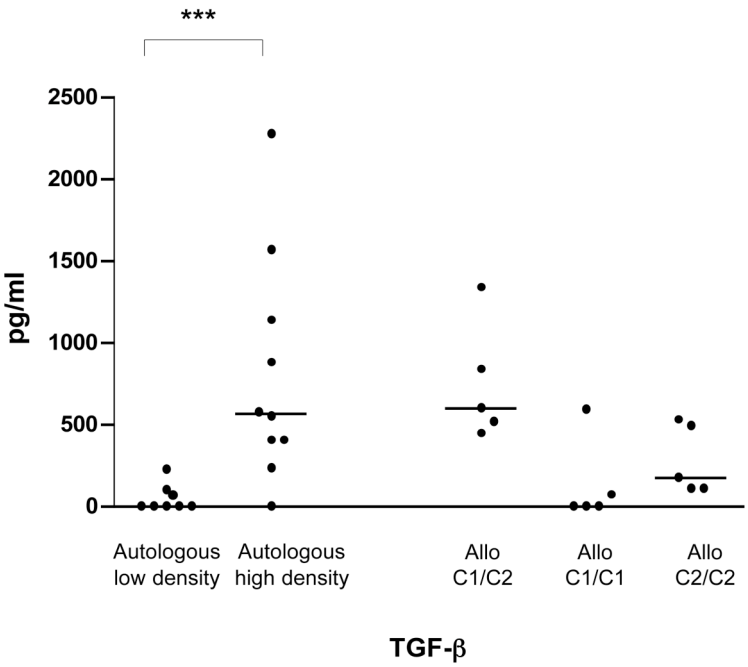


Figure 3. Secretion of TGF-β by NK cells interacting with autologous and allogeneic target cells

The concentration of TGF-β secreted by NK cells was measured by ELISA after overnight MLRs. Significantly more TGF-β was detected in the supernatant from NK cells plated at 'autologous-high density' conditions as compared to NK cells plated at 'autologous–low density' conditions. 'Allo C1/C2' target cells best supported this cell-contact mediated secretion of TGF-β. Horizontal lines indicate median values. ***P<0.0001

mRNA levels of IL-10 and TGF-β in NK cells after mixed lymphocyte reactions

Using standard qPCR technology we also quantified NK cell-derived mRNA for IL-10 and TGF-β after MLRs. Analogous to the above experiments that assessed IL-10 and TGF-β at the protein level, mRNA was quantified in NK cells that were plated at concentrations not supporting extensive cell-cell interactions ('autologous–low density', n=3), and compared to levels in NK cells plated so as to allow for extensive cell-cell contact (either 'autologous–high density' or 'allo C1/C2', 'allo C1/C1', and 'allo C2/C2'; n=3 for each MLR-constellation).

In NK cells plated at low density, no message for IL-10 and only low levels of message for TGF-β was detected. Plating NK cells under 'autologous–high density' conditions induced detectable amounts of IL-10 mRNA, whereas levels for TGF-β mRNA remained detectable at low levels. Importantly, after exposure of NK cells to 'allo C1/C2' target cells –i.e. the MLR-constellation best supporting or even enhancing production of IL-10 and TGF-β at the protein level– message for both IL-10 and TGF-β was best induced (Fig. 4).

These experiments supported a model in which –also at the mRNA level– autologous cell-cell contacts, and interactions with allo-target cells co-expressing C1 *and* C2 epitopes, induced/supported production by NK cells of the regulatory cytokine IL-10, and revealed that mRNA for TGF-β was best induced after exposure of NK cells to allo-target cells co-expressing C1 *and* C2 epitopes.

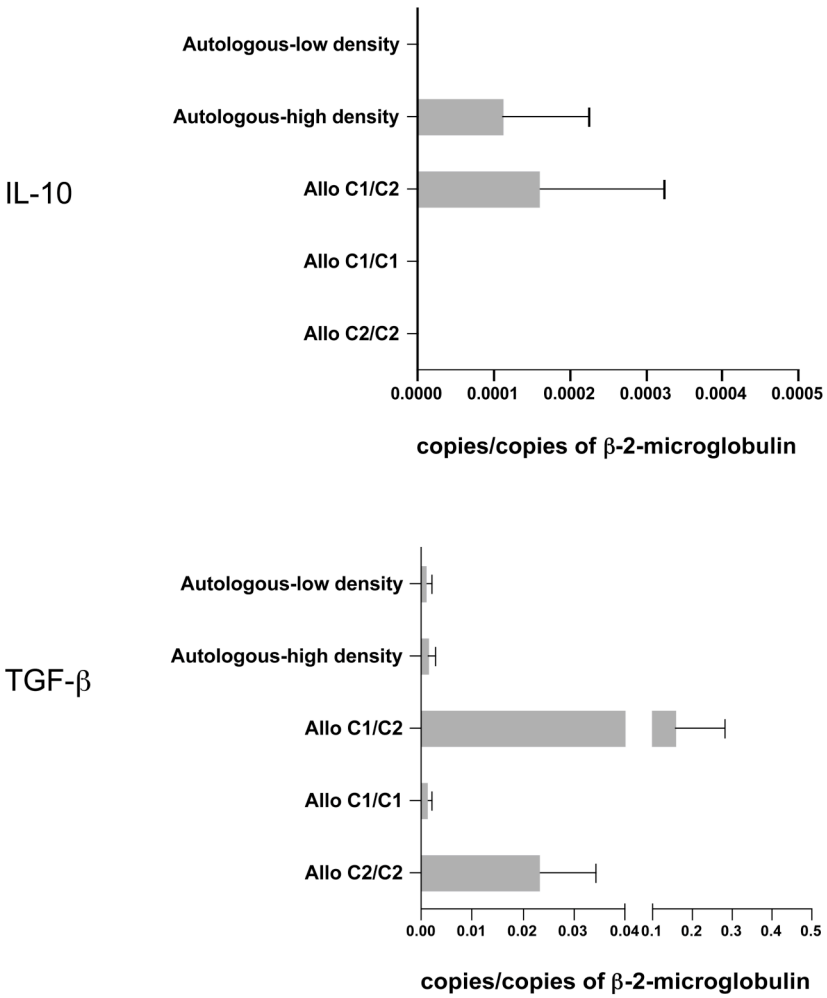


Figure 4. mRNA-levels of TGF-β and IL-10 in NK cells interacting with autologous and allogeneic target cells

IL-10 and TGF-β mRNA levels from NK cells were quantified after overnight MLRs. In non-stimulated NK cells plated at 'autologous–low density' conditions, no message for IL-10 and low amounts of message for TGF-β were detected. Plating NK cells at 'autologous–high density' conditions induced mRNA for IL-10, mRNA levels for TGF-β remained unchanged. 'Allo C1/C2' target cells further induced IL-10 mRNA and also most strongly induced TGF-β mRNA. Results are given as copies over copies of β2-microglobulin. Data are presented as mean ± SD.

Discussion

The key observation of the current study was that, in absence of activating cytokines, 'constitutive' cell–cell interactions induce –in a subset of NK cells– an *active regulatory response*. Exposing NK cells to allo-target cells we found that *specific allogeneic cell-cell interactions* support –or even enhance– 'constitutive' cell-contact dependent cytokine secretion (Fig. 2B). This regulatory NK cell activity was operational directly *ex vivo* (i.e. in unprimed cells), and confined to a population distinct from IFN-γ secreting NK cells (Fig. 2C).[20]

Pro-inflammatory and cytotoxic NK cell-function has been implicated in promoting allograft injury.[21] Somewhat unexpectedly therefore, more recent murine data indicated that NK cells may also play important roles inducing certain forms of allograft tolerance.[22] Whether NK cells impact allo-immunity in the setting of human solid organ transplantation remains largely unknown, and a detailed understanding of the reactivity of *unprimed* human NK cells vis-a-vis allo-target cells is lacking. Our preliminary observation of a dominant impact of the recipient's HLA C allotype on the pattern of cytokines induced in unprimed allo-NK cells might thus stimulate –and direct– research aiming at elucidating the molecular basis of this phenomenon in humans. A provocative hypothesis could state that certain allo-constellations efficiently induce regulatory NK cells, hence providing T cells a non-inflammatory –perhaps tolerogenic– environment. From a more clinical point-of-view our data lend support to current efforts aiming at reassessing the role of HLA C in kidney allograft survival/function.

In summary, our data identify a distinct subset of NK cells that –upon cell-cell interaction– are activated, rather than silenced, and secrete regulatory cytokines. This novel type of NK cell reactivity may have important implications with respect to various aspects of NK cell-biology –and particularly NK cell allo-reactivity– *in vivo*.

References

1. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001;22:633-640.

2. Fehniger TA, Cooper MA, Nuovo GJ, et al. CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. Blood. 2003;101:3052-3057.

3. Fehniger TA, Shah MH, Turner MJ, et al. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. J Immunol. 1999;162:4511-4520.

4. French AR, Yokoyama WM. Natural killer cells and viral infections. Curr Opin Immunol. 2003;15:45-51.

5. Moretta L, Ciccone E, Moretta A, Hoglund P, Ohlen C, Karre K. Allorecognition by NK cells: nonself or no self? Immunol Today. 1992;13:300-306.

6. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. Immunol Today. 1990;11:237-244.

7. Ravetch JV, Lanier LL. Immune inhibitory receptors. Science. 2000;290:84-89.

8. Colonna M, Brooks EG, Falco M, Ferrara GB, Strominger JL. Generation of allospecific natural killer cells by stimulation across a polymorphism of HLA-C. Science. 1993;260:1121-1124.

9. Moretta A, Biassoni R, Bottino C, et al. Major histocompatibility complex class I-specific receptors on human natural killer and T lymphocytes. Immunol Rev. 1997;155:105-117.

10. Long EO, Burshtyn DN, Clark WP, et al. Killer cell inhibitory receptors: diversity, specificity, and function. Immunol Rev. 1997;155:135-144.

11. Ruggeri L, Capanni M, Mancusi A, et al. Alloreactive natural killer cells in mismatched hematopoietic stem cell transplantation. Blood Cells Mol Dis. 2004;33:216-221.

12. Ruggeri L, Mancusi A, Capanni M, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. Blood. 2007;110:433-440.

13. Kitchens WH, Uehara S, Chase CM, Colvin RB, Russell PS, Madsen JC. The changing role of natural killer cells in solid organ rejection and tolerance. Transplantation. 2006;81:811-817.

14. Vilches C, Parham P. Do NK-cell receptors and alloreactivity affect solid organ transplantation? Transpl Immunol. 2006;17:27-30.

15. Colonna M, Brooks EG, Falco M, Ferrara GB, Strominger JL. Generation of allospecific natural killer cells by stimulation across a polymorphism of HLA-C. Science. 1993;260:1121-1124.

16. Zenhaeusern G, Gasser O, Saleh L, Villard J, Tiercy JM, Hess C. Investigation of alloreactive NK cells in mixed lymphocyte reactions using paraformaldehyde-silenced target cells. J Immunol Methods. 2007;321:196-199.

17. Ruggeri L, Capanni M, Casucci M, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. Blood. 1999;94:333-339.

18. Zenhaeusern G, Gasser O, Saleh L, Villard J, Tiercy JM, Hess C. Investigation of alloreactive NK cells in mixed lymphocyte reactions using paraformaldehyde-silenced target cells. J Immunol Methods. 2007;321:196-199.

19. Wahl SM. Transforming growth factor-beta: innately bipolar. Curr Opin Immunol. 2007;19:55-62.

20. Campbell JJ, Qin S, Unutmaz D, et al. Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. J Immunol. 2001;166:6477-6482.

21. Maier S, Tertilt C, Chambron N, et al. Inhibition of natural killer cells results in acceptance of cardiac allografts in CD28-/- mice. Nat Med. 2001;7:557-562.

22. Beilke JN, Kuhl NR, Van Kaer L, Gill RG. NK cells promote islet allograft tolerance via a perforin-dependent mechanism. Nat Med. 2005;11:1059-1065.

A 'High-Mobility Low-Cost' Phenotype Defines Human Effector-Memory CD8+ T cells

Introduction

Phenotypic and immune-functional analyses have defined differences in the nature of CD8+ T cell populations, differing homing being one of the key distinctions among subsets. Naïve and central-memory CD8+ T cells express the lymphnode homing receptors CCR7 and CD62L, and screen lymphoid tissue for cognate antigen. Effector-memory CD8+ T cells, by contrast, lack expression of CCR7 and CD62L and primarily home into non-lymphoid tissues.[1-5] Screening for cognate antigen via random lymphocyte motility ('random-walk' activity) –an obviously energy consuming biological feature– has been shown to be characteristic of both, lymphoid as well as non-lymphoid homing CD8+ T cells.[6-11] Mimicking intravascular and extravascular conditions *in vitro*, we sought here to (i) characterize random lymphocyte motility among lymphoid and non-lymphoid homing CD8+ T cells, and (ii) relate random lymphocyte motility and mitochondrial biogenesis/ATP synthesis.

Material and Methods

Isolation of peripheral blood mononuclear cells and T-cells

Anticoagulated blood was drawn from healthy donors after written informed consent. Peripheral blood mononuclear cells (PBMC) were isolated using standard density gradient protocols (Lymphoprep™, Fresenius Kabi, Oslo, Norway). CD4+ and CD8+ T cells were positively selected using MACS beads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). Purity of CD4+ and CD8+ Tcells, as assessed by flow cytometry, was always >95% (data not shown).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured in 24 or 48 well cell culture plates BD BioScience (San Jose, CA, USA) or on 5 μm pore size polycarbonate transwell inserts (Costar®, Corning Incorporated, Corning, NY, USA) in endothelial cell medium (EGM® - 2MV, Lonza, Basel, Switzerland) supplemented with 5% fetal calf serum (GIBCO™, Luzern, Switzerland). The medium was changed daily and cells used when a confluence of ~90% was reached.

FACScan analysis

The following antibodies were used: CCR7 (FAB197F / FAB197P) and appropriate isotype control antibodies from R&D systems (R&D systems Europe, United Kingdom), CD3 (SK7), CD4 (RPA-T4), CD8 (RPA-T8 / SK1), CD27 (M-T271), CD28 (CD28.2), CD44 (G44-26), CD45RA (HI100), CD62L (Dreg 56), CD69 (L78) and appropriate isotype control antibodies from BD Biosciences. For staining, cells were resuspended in phosphate buffered saline / 1% bovine serum albumin (Sigma-Aldrich, Steinheim, Germany) and incubated with relevant antibodies for 20 min. at 4°C. After extensive washing, data were acquired with a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed using CellQuest (Becton Dickinson) or FlowJo 8.6.3. software (Tree Star, Inc., Ashland, OR, USA).

FACS sorting

CD8+ T cell subsets (as indicated) were sorted using the BD FACSVantage SE System (Becton Dickinson). Purity of CD8+ T cell subsets was assessed by post-sort flow cytometry and always was >98% (data not shown).

Migration assay

Bulk lymphocytes or sorted CD8+ T cell subsets were resuspended at 6x10^5 cells/mL in RPMI 1640 containing 10% fetal calf serum, 50 U/mL penicillin and 50 μg/mL Streptomycin (all from GIBCO™, Luzern, Switzerland) (R10) and loaded in duplicates into uncoated, fibronectin coated (10 μg/mL) (Human Plasma Fibronectin, Temecula, CA, USA) or HUVEC coated 5 μm pore size polycarbonate transwell inserts (Costar®, Corning, NY, USA). 1 mL R10 or EGM® - 2MV for HUVECs, respectively, was added to the lower well. After 16 h of incubation at 37°C, cells from both compartments were stained with the appropriate antibodies.

ELISpot

After transwell migration assays, CD8+ T cells from the upper and lower compartments were tested separately for EBV-specific IFN-γ production using ELISpot assays. Briefly, MultiScreen HTS™ IP 96 well plates (Millipore AG, Volketswil, Switzerland) were coated over night with 1:2000 diluted mAb anti-human IFN-γ (1-D1K) (Mabtech AB, Stockholm, Sweden). Isolated migrating and non-migrating CD8+ T cells were enumerated, and autologous CD8+ T cell depleted PBMC were added back to provide antigen presenting cells (APC) at a ratio of 1:5. APC were loaded with EBV-infected –and as a control EBV naïve– B cell extract at a concentration of 20 μg/mL (Virusys corporation, Randallstown, MD, USA). After over night incubation, plates were washed with PBS and, prior to developing, incubated with PBS containing 1% FCS for 10 min. Plates were incubated for 2 h with anti-human IFN-γ mAb (1:200) coupled with alkaline phosphatase (7-B6-1-ALP) (Mabtech) for 1.5 hours at room temperature. After washing, spots were developed using HistoMark® RED phosphatase system (KPL, Gaithersburg, Maryland,

USA), and counted by direct visualization with the AID CytoSpot Reader System (CSR01, Autoimmun Diagnostika (AID) GmbH, Strassberg, Germany) using the ELISpot 3.5 software (AID GmbH).

Time-lapse microscopy

CCR7- CD8+ and CCR7+ CD8+ T cells were FACS sorted and resuspended in R10 and plated in uncoated 24 or 48 well cell-culture dishes (Costar®), coated with fibronectin (10 μg/mL), or on HUVEC coated plates. After an incubation period of 1 hour, pictures were taken every minute using an Olympus IX 81 motorized inverted microscope (Olympus Schweiz AG, Volketswil, Switzerland). Data were analyzed using the cell^P and cell^R software (professional imaging software, Olympus) and ImageJ software (Image Processing and Analysis, http://rsbweb.nih.gov/ij).

ATP quantification

FACS sorted CD8+ T cell subsets were resuspended at 1x10^6 cells/mL in purified water, transferred to cryotubes (Nunc A/S, Roskilde, Denmark) and snap frozen in liquid nitrogen. After thawing, cells were incubated for 10 min. in boiling water and centrifuged for 5 min. at 20,000xg. The supernatant was kept on ice until ATP measurements were performed according to the manufacturer's protocol (FL-AA, Sigma-Aldrich, Steinheim, Germany).

Gene expression analysis

Total RNA was isolated from FACS sorted CCR7- and CCR7+ CD8+ T cells using TRIzol® Reagent according to the manufacturer's protocol (Invitrogen™, Basel, Switzerland). After DNA digestion, 1 μg RNA was reverse transcribed. 2 μl of a 1:3.5 cDNA dilution were used to perform the qPCR reaction in a Stratagene cycler (Stratagene, La Jolla, CA) using SYBR Green master mix (Sigma). Gene expression levels are given as relative gene expression compared to the housekeeping gene TBP (TATA-Box-binding protein). Primers are listed in Table 1.

Table 1. Primers

Assay	Primers[1]
hPGC-1beta (NM_133263)	R: AGGTGGCCGAGTCAAAGTC
	L: CAACTATCTCGCTGACACGC
hERRalpha (NM_004451)	R: CGCTTGGTGATCTCACACTC
	L: GCTACCACTATGGTGTGGCA
hAtp5o (NM_001697)	R: CGGATCAGTCTTAGCCTCCA
	L: GCACAGTGACCTCTGCATCT
hCycs (NM_018947)	R: AGATTTGGCCCAGTCTTGTG
	L: TTGTGCCAGCGACTAAAAAG
hUCP2 (NM_003355)	R: CTGATTTCCTGCTACGTCCC
	L: CCGTGAGACCTTACAAAGCC
hUCP3 (NM_003356)	R: ACATCACCACGTTCCAGGAT
	L: CCTCCAGGCCAGTACTTCAG

[1] R: Right primer sequence, L: Left primer sequence

Calorimetry

Bulk lymphocytes and FACS sorted CCR7- and CCR7+ CD8+ T cells were resuspended at 1.2x10^6 or 2.4x10^6 cells/mL in R10. After a calibration period of 24 hours, heat production of bulk and sorted lymphocytes was recorded every minute during 12 hours using an isothermal calorimeter (Thermal Activity Monitor 3102 TAM III, TA Instruments, New Castle, DE; USA).

Statistical analysis

Results were tested for normality using D'Agostino&Pearson omnibus normality test. Student's t-tests, Mann-Whitney U-tests and Wilcoxon's signed rank-tests were performed using Prism4 software (GraphPad Software, Inc. San Diego, CA, USA). P-values <0.05 were considered statistically significant. Results are given as mean±standard deviation (SD), or median and range, as appropriate.

Results and Discussion

Random cellular movement of CD8+ T cells

Distinct anatomical compartments are screened for cognate antigen by specialized subsets of T cells.[1-5] 'Antigen screening efficiency' of T cells –both in lymphoid and non-lymphoid organs– is thought to be increased by random cellular movement ('random-walk').[6,7,10,12-15] Aiming at assigning random cellular movement-activity to distinct cell-populations we first compared the phenotype of CD8+ T cells *ex vivo* with that of CD8+ T cells that –in absence of a chemotactic gradient– migrated across 5 μM pore-inserts coated with either fibronectin (Fig. 1A, upper panel) or human umbilical vein endothelial cells (HUVECs) (Fig. 1A, lower panel). Irrespective of whether CD8+ T cells were interacting with fibronectin or with HUVECs, the phenotype of transmigrating CD8+ T cells became skewed towards cells lacking expression of the lymphnode homing receptors CCR7 and CD62L (Fig. 1A and Tbl. 2).

CD8+ T cells were also stained for expression of the maturation-marker CD45RA, the cell-surface glycoprotein CD44 (involved in lymphocyte homing), and the activation-marker CD69.[4,16-20] Neither of these molecules was differentially expressed on non-migrating vs. migrating CD8+ T cells (Fig. 1A). We also used CD27 and CD28, two phenotypic markers of CD8+ T cell differentiation,[1] to characterize cells accumulating in migration assays. As for expression of CCR7 and CD62L, also according to the expression of CD27 and CD28 accumulation of cells with an effector-memory phenotype (CD27-CD28- ; 'late differentiated' cells) was observed (data not shown).

We then quantified migration of *sorted* CCR7- and CCR7+ CD8+ T cells, by relating the starting number of cells to the number of cells that had migrated after 16 h of incubation. Indicating their higher intrinsic random movement-activity, the percentage of migrating cells was almost 3-fold higher among sorted CCR7- CD8+ T cells (median % of cells

migrating after 16h: CCR7- 27.7% [range 23.7-49.0%], n=5, CCR7+ 10.1% [range 1.3-14.4%], n=6, p=0.004).

Lastly, we compared the size (forward scatter histogram) of migrating vs. non-migrating cells, thus testing –and dismissing– the hypothesis that accumulation of CD8+ T cells with an effector-memory phenotype merely reflected selection for smaller cells (data not shown).

In parallel, migration experiments were performed using isolated CD4+ T cells. Increased migration of CD4+ T cells with an effector-memory phenotype, analogous to CD8+ T cells, was observed, and –also analogous to CD8+ T cells– no difference in the expression of CD45RA, CD44 or CD69 was seen (data not shown).

In order to monitor random migration *at the antigen-specific level* we then compared the frequency of EBV-specific IFN-γ producing CD8+ T cells among non-migrating and migrating populations. EBV-specific CD8+ T cells were readily detected both among non-migrating and migrating cells, and –on each occasion– accumulation of effector-memory cells among the migrating cells (as shown in Fig. 1A) was reflected by an increase in the frequency of cells releasing the effector-cytokine IFN-γ (Fig. 1B).

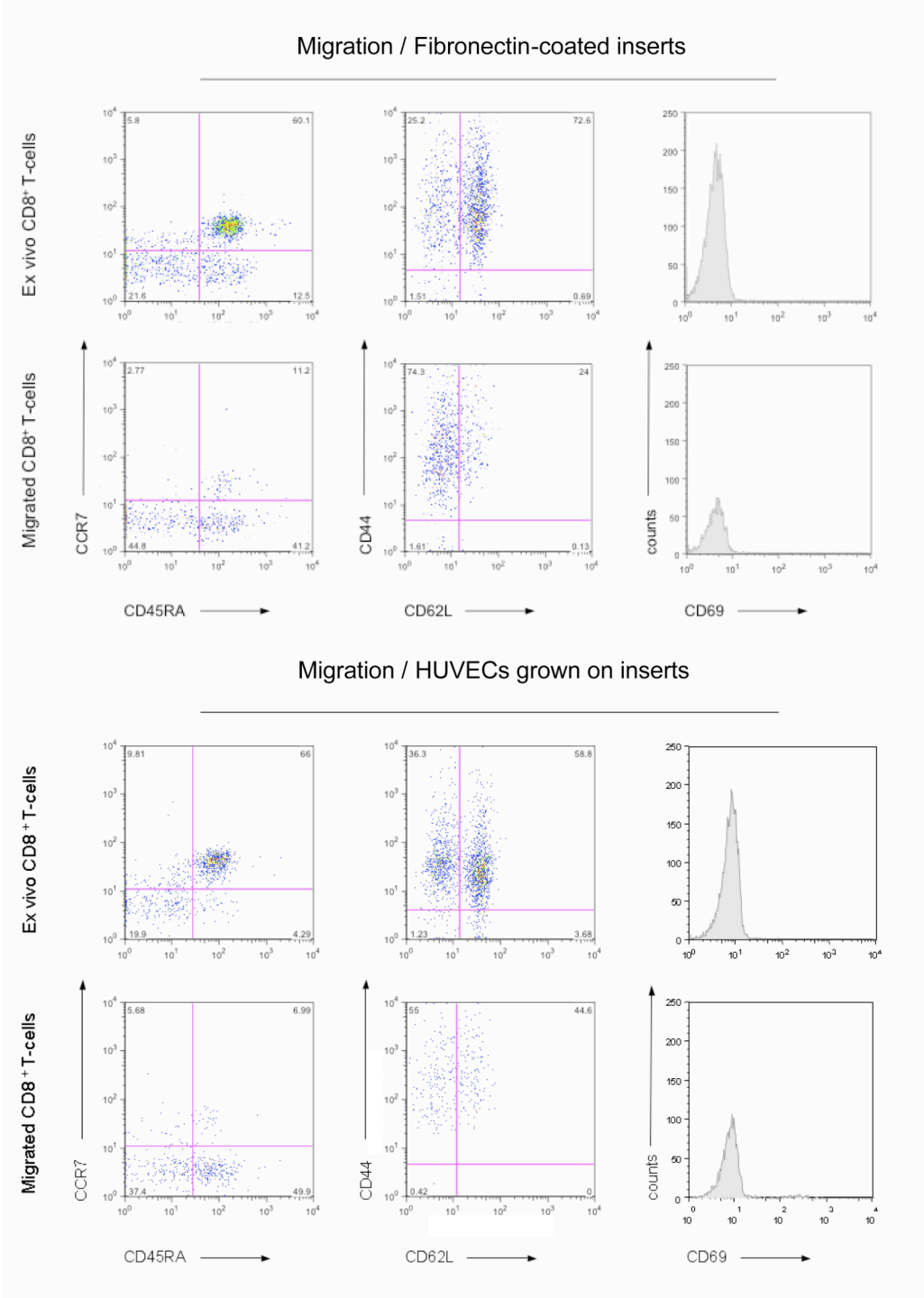
We next aimed at *visualizing* random migration of CD8+ T cells on fibronectin –mimicking the extra-vascular/interstitial milieu– and on HUVECs. Both on fibronectin and on HUVECs –at any given time-point– (i) crawling and (ii) non-crawling

CD8+ T cells could be readily discerned (crawling defined here as flattening of the cell-stoma with extension of pseudopodia; see insert in Fig. 1D). The average speed of crawling cells (CCR7- and CCR7+) was similar on fibronectin and on HUVECs, whereas non-crawling cells (CCR7- and CCR7+) moved slightly more rapid on fibronectin –possibly reflecting a moving-restrain imposed by the bold relief of HUVECs (Fig. 1C, upper panels). Intriguingly, comparing the percentage of crawling vs. non-crawling CD8+ T cells on fibronectin vs. HUVECs, a striking difference was observed between

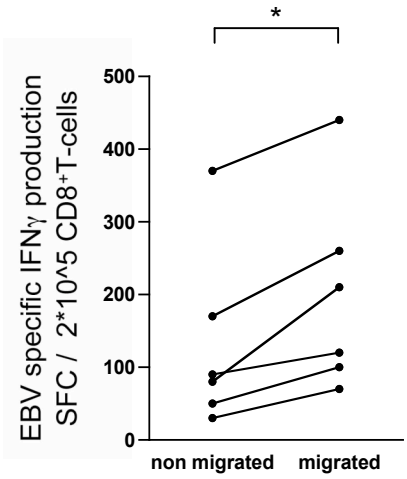
cells expressing vs. not expressing the lymphnode homing chemokine-receptor CCR7: While for each recorded time-point on fibronectin ~10% of all CCR7- CD8+ T cells exhibited a crawling phenotype, the frequency of CCR7+ CD8+ T cells crawling on fibronectin was always <2%. By contrast, on HUVECs the percentage of crawling cells was higher among CCR7+ CD8+ T cells, yet it always remained <5% for both subsets (Fig. 1C, lower panels). Representative live-microscopy images of CCR7- and CCR7+ CD8+ T cells on fibronectin and HUVECs are shown in Figure 1D, time-lapse movies are available at http://bloodjournal.hematologylibrary.org First edition, Supplemental Videos.

Together these experiments identified robust differences between phenotypically distinct subsets of CD8+ T cells with regards to their random movement activity, and the frequency-distribution of crawling cells. These differences may imply that stochastic detection of cognate antigen in the extravascular milieu *at a given rate* requires higher crawling-activity in structurally more variable non-lymphoid tissues than within anatomically highly defined secondary lymphnodes.

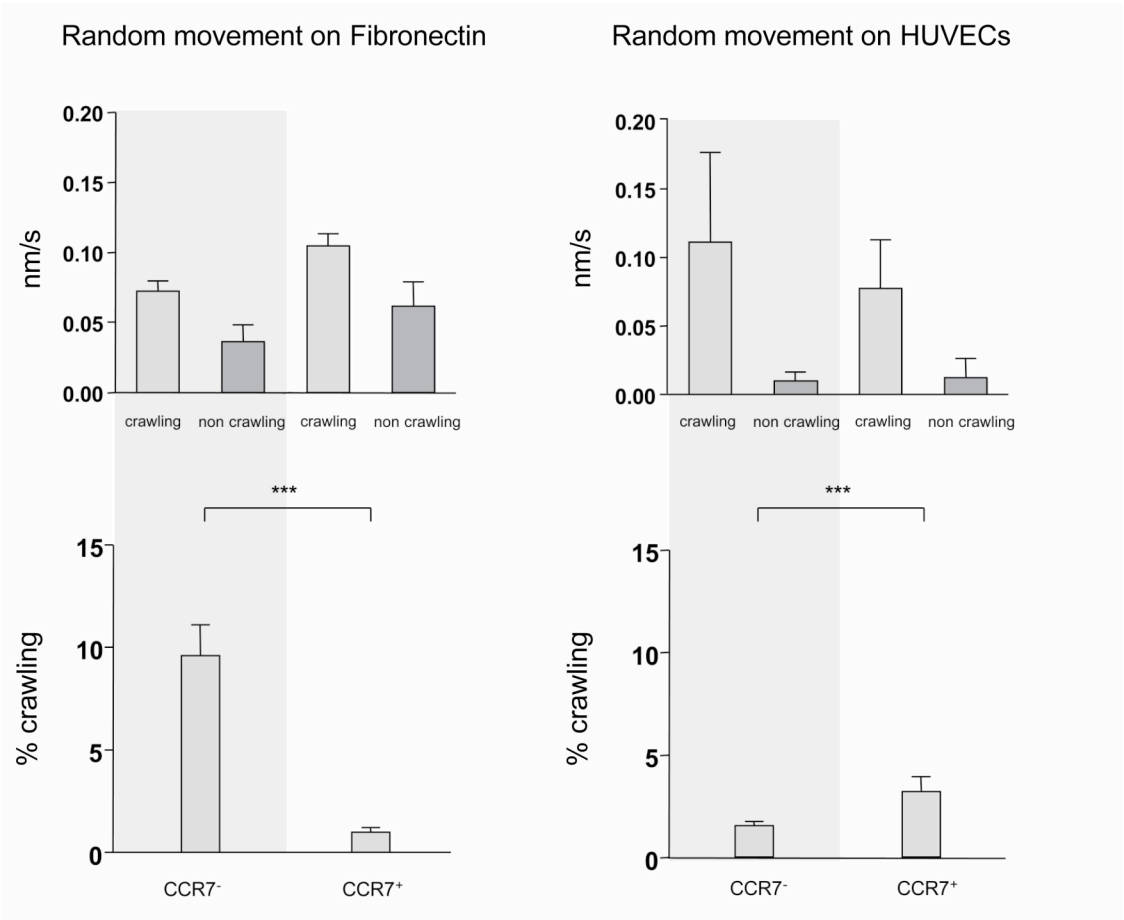
Figure 1. Phenotypic and functional characteristics of randomly moving of CD8+ T cells



(A) In transwell migration experiments, migration of CD8+ T cells through fibronectin coated 5 μM pore membranes (upper panel) and through HUVECs grown on 5 μM pore membranes (lower panel) was assessed in absence of a chemotactic gradient. Ex vivo expression of CCR7 and CD45RA, CD44 and CD62L, and the activation marker CD69 was compared to expression on migrating cells (16 hours migration). Migrating CD8+ T cells were skewed towards a CCR7- and CD62L- phenotype.

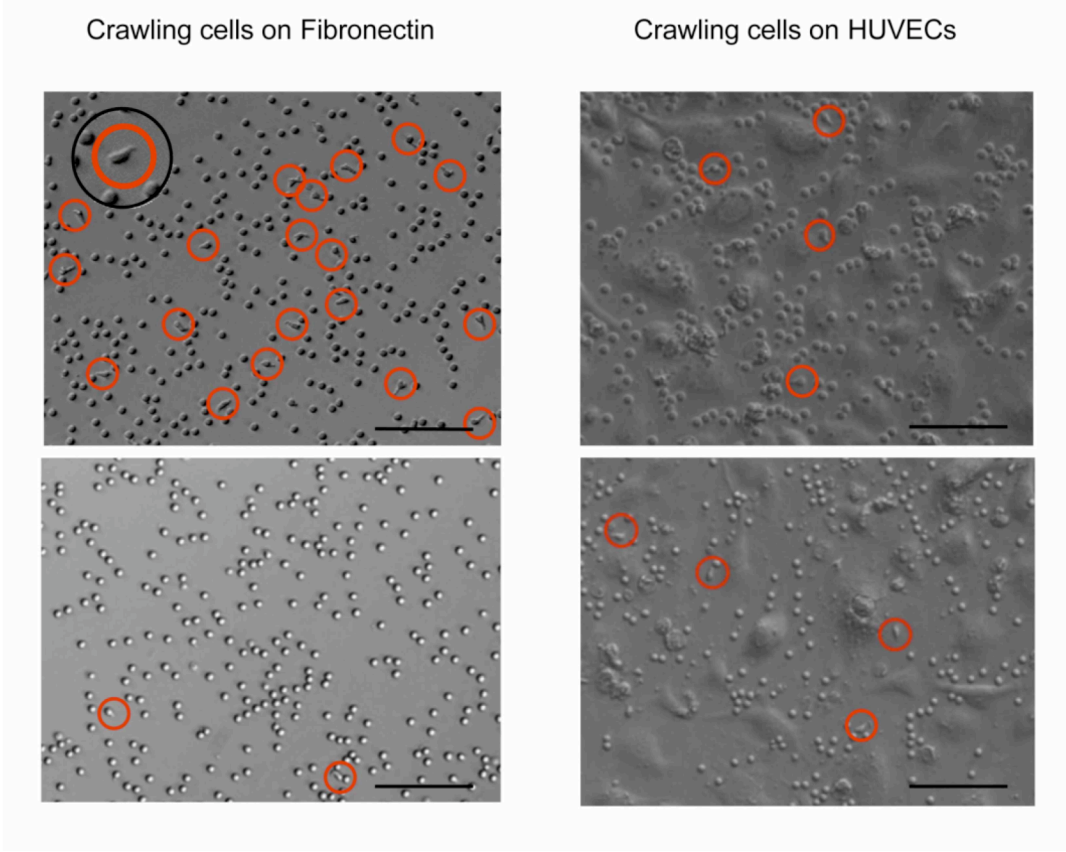


(B) As assessed in transwell migration experiments, the frequency of EBV-specific CD8+ T cells producing IFN-γ was higher amid migrating than non-migrating cells. *P<0.05



(C) Using time-lapse microscopy, the movement-pattern of sorted CCR7- and CCR7+ CD8+ T cells on fibronectin-coated cell culture plates and on HUVECs was visualized. The velocity of crawling and non-crawling cells was analyzed assessing 1 min.-spaced pictures. The average speed of crawling cells was similar among subsets on both fibronectin and HUVECs, whereas non-crawling cells moved slightly more rapid on fibronectin (upper panel). Crawling and non-

crawling cells were enumerated on 5 min.-spaced pictures throughout a 1 hour observation period, and the mean percentage of crawling cells compared among CCR7- and CCR7+ CD8+ Tcells. Crawling was a prominent feature of CCR7- CD8+ T cells on fibronectin (~10% of all cells). On HUVECs the frequency of crawling $CD8^{+}$T-cells was higher among the CCR7+ subset, but always remained <5% (lower panel). ***P<0.001



(D) On representative time-lapse video-pictures crawling CD8+ T cells on fibronectin and HUVECs are marked with a circle. The insert shows the typical features of a crawling cell at higher magnification. — = 0.1 mm

Table 2. Phenotype of transmigrating CD8+ T cells

Migration through Fibronectin	Ex vivo [median (range)]	Migrated [median (range)]	p-value
CCR7- CD8+ T cells, n=9	37.9% (29.5-63.5)	86.4% (75.7-93.0)	0.0007
CCR7+ CD8+ T cells, n=9	62.1% (36.5-73.2)	13.5% (7.0-24.3)	0.0007
CD62L- CD8+ T cells, n=13	38.1% (26.7-49.7)	67.2% (48.1-74.3)	0.0002
CD62L+ CD8+ T cells, n=13	64.1% (23.8-66.4)	23.6% (11.7-43.6)	0.0002

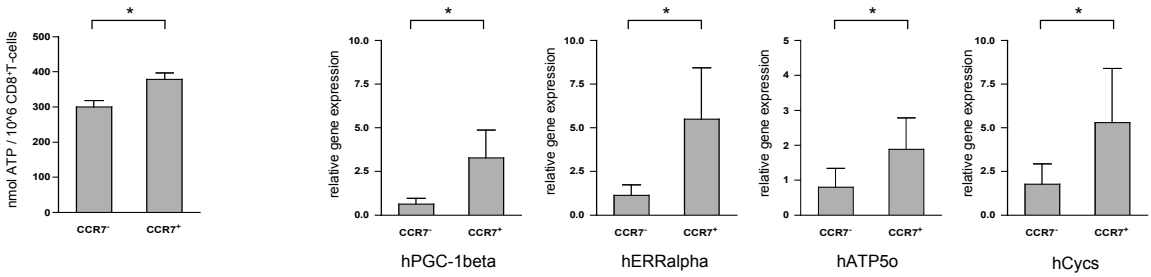
Migration through HUVECs	Ex vivo [median (range)]	Migrated [median (range)]	p-value
CCR7- CD8+ T cells, n=5	30.5 % (24.19-46.5)	51.1% (42.6-87.3)	0.04
CCR7+ CD8+ T cells, n=5	69.5% (53.5-85.9)	48.9% (12.7-57.4)	0.04
CD62L- CD8+ T cells, n=5	32.3% (20.1-34.4)	43.2% (28.0-73.8)	0.03
CD62L+ CD8+ T cells, n=5	70.2% (57.9-73.0)	46.9% (23.6-65.4)	0.03

ATP content and energy-efficiency of CCR7- versus CCR7+ CD8+ T cells

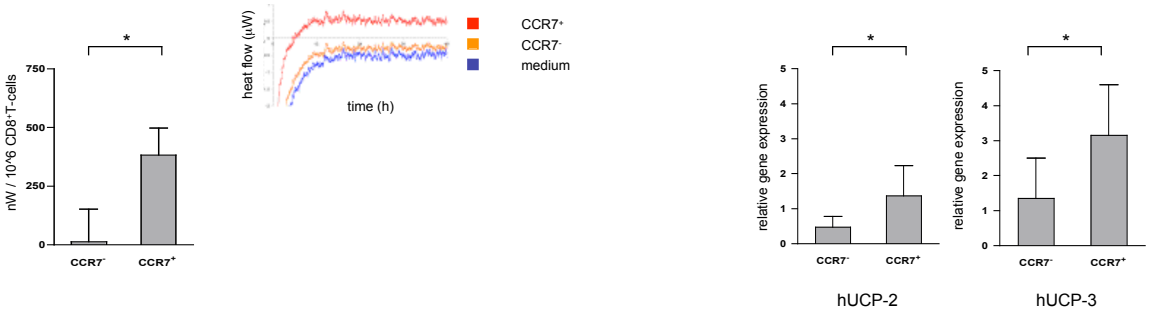
Cellular locomotion relies on transient interactions of motor proteins with microtubules or actin filaments, which then activates the binding and hydrolysis of ATP.[21] Using a robust luciferin-based assay, ATP contents were found to be consistently lower in CCR7- as compared to CCR7+ CD8+ T cells (CCR7- CD8+ T cells: median ATP content 299±62 nmol/10^6 cells, CCR7+ CD8+ T cells: median ATP content 378±62 nmol/10^6cells, n=10, p=0.025) (Fig. 2A, left panel). Intriguingly however, lower ATP contents did not simply reflect increased ATP consumption by crawling cells, since messenger RNA of PGC-1β (a strong activator of mitochondrial biogenesis/ATP synthesis), ERRα (an important partner of PGC-1), Cytochrome C (Cycs) und ATP Synthase (Atp5o) were all expressed at a higher level in the CCR7+ CD8+ T cell subset as well (Fig. 2A, right panels).[22] CCR7- CD8+ T cells thus seem indeed able to maintain a higher crawling frequency despite lower overall ATP generation.

To further investigate the energy-efficiency of CCR7- CD8+ T cells we next compared the heat-flow of sorted CCR7- and CCR7+ CD8+ T cells using microcalorimetry assays.[23] In line with a postulated higher energy-efficiency, CCR7- CD8+ T cells produced a median of only 68 nW/10^6 cells (range 0-530 nW) (n=8), whereas CCR7+ CD8+ T cells produced a median of 390 nW/10^6 cells (range 2-1050 nW) (n=8) (Fig. 2B, left panel). Surprisingly, at least part of the higher energy-efficiency of CCR7- CD8+ T cells might be brought about via *upregulation of uncoupling proteins* of the oxidative chain (UCP-2 und UCP-3) in the CCR7+ CD8+ T cell subset (Fig. 2B, right panels).

Figure 2. ATP content and energy-efficiency of CCR7- versus CCR7+ CD8+ T cells



(A) ATP contents of sorted CCR7- and CCR7+ CD8+ T cells were measured in a luminescence based assay (left panel); mRNA levels of PGC-1β, ERRα, Cytochrome C (Cycs) und ATP Synthase (Atp5o) were quantified using RT-PCR technology (right panels). CCR7- CD8+ T cells contained less ATP and expressed less mRNA for PGC-1β, ERRα, Cycs und Atp5o than their CCR7+ counterparts. *P<0.05



(B) In calorimetric analyses heat production of sorted CCR7- and CCR7+ CD8+ T cells was quantified. After a calibration period of 24 hours heat production was quantified every minute for 12 hours. The insert shows the heat flow diagram of a representative experiment (left panel). mRNA levels of the uncoupling proteins 2 and 3 of the respiratory chain (UCP-2 and UCP-3) were quantified by RT-PCR (right panels). CCR7- CD8+ T cells contained significantly less mRNA for both UCP-2 and UCP-3 than their CCR7+ counterparts. *P<0.05

As any biologically costly system, immunity has evolved to minimize energy expenditure in performing its task,[24,25] and screening for cognate antigen within lymphoid and non-lymphoid/peripheral tissues is bound to comply with this principle. Our data regarding non-lymphoid homing CD8+ T cells recognizably comply with that principle; regulated

uncoupling of the respiratory chain via upregulation of uncoupling proteins, on the other hand, seems counterintuitive and suggests as yet unrecognized properties of lymphnode homing CD8+ T cells.

References

1. Appay V, Dunbar PR, Callan M, et al. Memory CD8+ T cells vary in differentiation
phenotype in different persistent virus infections. Nat Med. 2002;8:379-385.
2. Hamann D, Baars PA, Rep MH, et al. Phenotypic and functional separation of memory and effector human CD8+ T cells. J Exp Med. 1997;186:1407-1418.
3. Lefrancois L, Marzo AL. The descent of memory T-cell subsets. Nat Rev Immunol. 2006;6:618-623.
4. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999;401:708-712.
5. Schluns KS, Lefrancois L. Cytokine control of memory T-cell development and survival. Nat Rev Immunol. 2003;3:269-279.
6. Cahalan MD, Parker I, Wei SH, Miller MJ. Real-time imaging of lymphocytes in vivo. Curr Opin Immunol. 2003;15:372-377.
7. Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. Nature. 2004;427:154-159.
8. Miller MJ, Wei SH, Parker I, Cahalan MD. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. Science. 2002;296:1869-1873.
9. Mempel TR, Junt T, von Andrian UH. Rulers over randomness: stroma cells guide
lymphocyte migration in lymph nodes. Immunity. 2006;25:867-869.
10. Bajenoff M, Egen JG, Koo LY, et al. Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. Immunity. 2006;25:989-1001.
11. Mrass P, Takano H, Ng LG, et al. Random migration precedes stable target cell interactions of tumor-infiltrating T cells. J Exp Med. 2006;203:2749-2761.

12. Sumen C, Mempel TR, Mazo IB, von Andrian UH. Intravital microscopy: visualizing immunity in context. Immunity. 2004;21:315-329.
13. Nombela-Arrieta C, Mempel TR, Soriano SF, et al. A central role for DOCK2 during interstitial lymphocyte motility and sphingosine-1-phosphate-mediated egress. J Exp Med. 2007;204:497-510.
14. Wei SH, Parker I, Miller MJ, Cahalan MD. A stochastic view of lymphocyte motility and trafficking within the lymph node. Immunol Rev. 2003;195:136-159.
15. Miller MJ, Wei SH, Parker I, Cahalan MD. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. Science. 2002;296:1869-1873.
16. Champagne P, Ogg GS, King AS, et al. Skewed maturation of memory HIV-specific CD8 T lymphocytes. Nature. 2001;410:106-111.
17. Lesley J, Hyman R, Kincade PW. CD44 and its interaction with extracellular matrix. Adv Immunol. 1993;54:271-335.
18. DeGrendele HC, Estess P, Siegelman MH. Requirement for CD44 in activated T cell extravasation into an inflammatory site. Science. 1997;278:672-675.
19. Hara T, Jung LK, Bjorndahl JM, Fu SM. Human T cell activation. III. Rapid induction of a phosphorylated 28 kD/32 kD disulfide-linked early activation antigen (EA 1) by 12-o-tetradecanoyl phorbol-13-acetate, mitogens, and antigens. J Exp Med. 1986;164:1988-2005.
20. Cebrian M, Yague E, Rincon M, Lopez-Botet M, de Landazuri MO, Sanchez-Madrid F. Triggering of T cell proliferation through AIM, an activation inducer molecule expressed on activated human lymphocytes. J Exp Med. 1988;168:1621-1637.
21. Wanka F, Van Zoelen EJ. Force generation by cellular motors. Cell Mol Biol Lett. 2003;8:1017-1033.
22. Kelly DP, Scarpulla RC. Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes Dev. 2004;18:357-368.

23. Karlsson H, DePierre JW, Nassberger L. Energy levels in resting and mitogen-stimulated human lymphocytes during treatment with FK506 or cyclosporin A in vitro. Biochim Biophys Acta. 1997;1319:301-310.

24. Moret Y, Schmid-Hempel P. Survival for immunity: the price of immune system activation for bumblebee workers. Science. 2000;290:1166-1168.

25. Read AF, Allen JE. Evolution and immunology. The economics of immunity. Science. 2000;290:1104-1105.

Future perspectives

The key findings of the 'NK cell part' of my PhD-thesis were that (i) blocking CD25 in vivo has a differential impact on NK cell-phenotype and -numbers in kidney transplant recipients vs. patients with autoimmune disease, and (ii) regulatory cytokine secretion can be induced in unprimed NK cells in a cell-cell contact dependent manner –both in autologous and allogeneic systems. It is now interesting to speculate about the potential function of NK cells in solid organ transplant settings, from homing to secretion of immunoregulatory cytokines.

Ad (i) In kidney transplantation, efforts are made to match, in donor and recipient, the loci HLA A, HLA B and HLA DR. However, it is the non-matched HLA C locus that is most involved in NK cell alloreactivity.[1] In mice, there is evidence that NK cells influence the outcome of solid organ transplantation. In CD28-/- mice, for instance, it was shown that cardiac allo-transplants were rejected via NK cell graft infiltrations and subsequent T cell stimulation.[2,3] Also, it was shown that NK cells can home into the lymph nodes to stimulate the adaptive immune response, both via direct cell-cell contact with DCs[4] and by providing an early source of IFN-γ upon activation.[5] It will now be interesting to investigate the migration pattern of allo-specific NK cells. Specifically, it would be interesting to establish the chemokine receptor expression-profile of allo-specific NK cells in patients with differing grades of allo-graft specific immunity and differing HLA C matching/missmatching.[6]

Ad (ii) The molecular mechanism(s) leading to secretion by NK cells of the regulatory cytokine IL-10 are not clearly defined. It has been shown that IL-12 and IL-15 can induce NK cells to secrete IL-10,[7] and only recently it was observed that membrane-bound IL-15 signals development of an IL-10 secreting regulatory NK cell subset.[8,9] In our experiments, we show that –in a cell-cell contact-dependent manner– IL-10 is induced under autologous and allogeneic conditions in a small subset of unprimed NK cells. The size of this population induced to secrete IL-10 was found to relate to the

HLA-C allotype of the allo-target population. Since IL-10 was detected among the $KIR^{dim/-}$ NK cell subset, it is tempting to speculate that this regulatory response is mediated in a non-HLA dependent manner. It will now be interesting to characterize the molecular mechanisms inducing NK cells to secrete IL-10, and whether this cytokine impacts development of alloreactivity.

The key findings of the 'CD8+ T cells part' of my PhD-thesis were (i) that CCR7+ and CCR7- CD8+ T cells exhibit a distinct migration pattern when examined under conditions mimicking the interstitial vs. the extravascular milieu and (ii) that these same CD8+ T cell-subsets differentially regulate their heat production.

Ad (i) Intravital microscopy has been used quite extensively to study migration patterns of CD8+ T cells in mice.[10,11] In these systems it has been shown that T cells screen for antigen while randomly migrating through lymph nodes –along anatomically defined pathways consisting of a network of fibroblastic reticular cells.[12-14] The here established fact that CCR7- CD8+ T cells contain a higher frequency of crawling cells than their CCR7+ counterparts may indicate that cells screening for antigen in comparably ill-organized interstitial tissues are forced to crawl more to detect antigen at the same rate than cells screening highly organized lymphoid tissues.

Ad (ii) From the literature it is known that immune responses most efficiently develop at a temperatures of 37°C, while developing sub-optimally when induced at lower temperatures.[15,16] An exciting hypothesis –that we are currently testing– now states that induced (UCP-2 mediated) heat production by lymphnode homing T cells may raise the local (i.e. draining) lymph node-temperature to the level required for the best possible immune response to ensue.

References

1. Parham P, McQueen KL. Alloreactive killer cells: hindrance and help for haematopoietic transplants. Nat Rev Immunol. 2003;3:108-122.

2. Maier S, Tertilt C, Chambron N, et al. Inhibition of natural killer cells results in acceptance of cardiac allografts in CD28-/- mice. Nat Med. 2001;7:557-562.

3. McNerney ME, Lee KM, Zhou P, et al. Role of natural killer cell subsets in cardiac allograft rejection. Am J Transplant. 2006;6:505-513.

4. Yu G, Xu X, Vu MD, Kilpatrick ED, Li XC. NK cells promote transplant tolerance by killing donor antigen-presenting cells. J Exp Med. 2006;203:1851-1858.

5. Martin-Fontecha A, Thomsen LL, Brett S, et al. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat Immunol. 2004;5:1260-1265.

6. Gregoire C, Chasson L, Luci C, et al. The trafficking of natural killer cells. Immunol Rev. 2007;220:169-182.

7. Cooper MA, Fehniger TA, Turner SC, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood. 2001;97:3146-3151.

8. Cooper MA, Fehniger TA, Turner SC, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood. 2001;97:3146-3151.

9. Giuliani M, Giron-Michel J, Negrini S, et al. Generation of a novel regulatory NK cell subset from peripheral blood CD34+ progenitors promoted by membrane-bound IL-15. PLoS ONE. 2008;3:e2241.

10. Wei SH, Parker I, Miller MJ, Cahalan MD. A stochastic view of lymphocyte motility and trafficking within the lymph node. Immunol Rev. 2003;195:136-159.

11. Cahalan MD, Parker I, Wei SH, Miller MJ. Real-time imaging of lymphocytes in vivo. Curr Opin Immunol. 2003;15:372-377.

12. Mrass P, Takano H, Ng LG, et al. Random migration precedes stable target cell interactions of tumor-infiltrating T cells. J Exp Med. 2006;203:2749-2761.

13. Bajenoff M, Egen JG, Koo LY, et al. Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. Immunity. 2006;25:989-1001.

14. Wei SH, Parker I, Miller MJ, Cahalan MD. A stochastic view of lymphocyte motility and trafficking within the lymph node. Immunol Rev. 2003;195:136-159.

15. Hanson DF. Fever and the immune response. The effects of physiological temperatures on primary murine splenic T-cell responses in vitro. J Immunol. 1993;151:436-448.

16. Hanson DF. Fever, temperature, and the immune response. Ann N Y Acad Sci. 1997;813:453-464.

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Gasser O, Schmid T A, **Zenhaeusern G**, Hess C. Cyclooxygenase regulates cell-surface expression of CXCR3/1-storing granules in human $CD4^+$T cells. *J. Immunol.* 2006. 177:8806-12.

Zenhaeusern G, Gasser O, Saleh L, Villard J, Tiercy J-M, Hess C. Investigation of alloreactive NK cells in mixed lymphocyte reaction using paraformaldehyde-silenced target cells. *J. Immunol. Meth.* 2007. 10:196-9.

Eken C, Gasser O, **Zenhaeusern G**, Oehri I, Hess C, Schifferli J A. PMN-ectosomes interfere with the maturation of monocyte-derived dendritic cells. *J. Immunol.* 2008. 180:817-24.

Gasser O, Berger C, **Zenhaeusern G**, Elsässer H, Oehri I, Schaub S, Hess C. Testing the 'Cytokine-ome' Theory: a Longitudinal Pilot Study in Renal Transplant-Recipients. *Transplantation* 2008. 85:491.

Zenhaeusern G, Gubser P, Eisele P, Gasser O, Steinhuber A, Trampuz A, Handschin C, Luster A D, Hess, C. A 'High Mobility Low-Cost' Phenotype Defines Human Effector-Memory $CD8^+$ T cells. *Blood*. e-pub ahead print.

Zenhaeusern G, Gasser O, Hoenger G, Durovic B, Stern M, Hess C. HLA C Allotypes Differentially Support Regulatory Allo-Specific NK Cell-Function. In Revision.

Zenhaeusern G, Hoenger G, Gasser O, Steiger J, Schaub S, Hess C. $CD56^{bright}$ Natural Killer Cells Expand and Contract Irrespective from CD25 Antibody-Treatment in Kidney Allograft-Recipients. Submitted.