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DISSERTATION

Characterization of cytokine responses and memory cell subsets using a multicolor flowcytometry-based approach

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Abstract

The recent advances in flowcytometry have come hand-in-hand with an improved knowledge of the immune system, and with practical and clinical benefits. Given the need to answer immunological and clinically relevant questions, a flowcytometry-based approach that focuses on the assessment of cytokine secretion and memory markers in different settings was established.

The Epstein-Barr Virus (EBV)-specific T cell memory analysis revealed the existence of six subsets at distinct differentiation stages. The memory T cell profile of a protective immune-response against a chronic infection was delineated, providing a basis for analyzing T cell responses in EBV-associated diseases. The enhanced frequencies of multifunctional and cytotoxic EBV-specific T cells found in bone marrow (BM) are of great interest for adoptive immunotherapy.

A similar analysis was performed to characterize the Cytomegalovirus (CMV)-specific T cell-memory profile, and remarkable differences were observed relatively to the EBV-memory repertoire. The results uncovered that BM CMV-specific CD4+ T cells have higher proliferative and multi-cytokine producing capacities than peripheral blood (PB) cells reinforcing BM as an interesting source to generate T cells for adoptive therapy.

To elucidate the role of Toll like receptors (TLRs) in controlling the Herpes Simplex virus-1 (HSV-1), TLR function in natural killer (NK) cells of seropositive subjects with and without herpes labialis (HL) was analyzed. We found that TLR3-hyporesponsiveness of NK cells is associated with susceptibility to recurrent HL, suggesting stimulation of TLR3 as a potential strategy to prevent symptomatic HSV-1 reactivation.

T cell receptor (TCR) repertoire investigation is essential for the detection of clonal T cell expansions in physiologic and pathologic conditions. To address the impact of memory pool changes on TCR-Vβ repertoire, we characterized and quantified TCR repertoires during latent CMV infection within various T cell differentiation subsets, providing a reference for elucidating subset-specific TCR repertoires in pathologic conditions.

Adiponectin (APN) possesses immunoinhibitory effects, but little is known about its influence on T cells. We have shown that T cells express Adiponectin-Receptors and upregulate them upon TCR-

stimulation and that APN diminishes antigen-specific T cell expansion and cytokine production. Our findings suggest that APN, a cytokine abundant in human plasma, may exert an important role in systemic T cell regulation, not restricted to the interplay between immune-cells.

As a whole, the work presented herein reveals multicolor flowcytometry as a powerful tool for understanding the role of immune function in different clinical settings.

Abstrakt

Die neuesten Fortschritte in der Durchflusszytometrie gehen Hand in Hand nicht nur mit einem besseren theoretischen Verstaendnis des Immunsystems, sondern auch mit praktischem und klinischem Nutzen. Aufgrund der Notwendigkeit, immunologisch und klinisch relevante unbeantwortete Fragen zu klaeren, haben wir in verschiedenen Situationen einen durchflusszytometrischen Ansatz etabliert, der auf der Messung von Zytokinsekretion basiert.

Bei der Analyse von EBV-spezifischen Gedaechtnis-T-Zellen, haben wir die Existenz von sechs verschiedenen Untergruppen mit spezifischen Differenzierungsstadien nachgewiesen. In dieser Studie beschreiben wir das Gedaechtnis-T-Zell-Profil einer protektiven Immunantwort gegen eine chronische Infektion als Basis fuer die Analyse von T-Zell-Antworten in EBV-assoziierten Erkrankungen. Desweiteren sind die Erkenntnisse ueber höhere Frequenzen von multifunktionalen und zytotoxischen EBV-spezifischen T-Zellen im Knochenmark von großem Interesse fuer adoptive Immuntherapie.

Eine aehnliche Analyse wurde durchgefuehrt, um CMV-spezifische Gedaechtnis-T-Zellen zu charakterisieren. Dabei wurden deutliche Unterschiede zum EBV-Gedaechtnis-Repertoir festgestellt. Unsere Analysen zeigen ausserdem, dass CMV-spezifische CD4+ T-Zellen aus dem Knochenmark eine hoeheres Wachstumskapazitaet haben und mehr verschiedene Zytokine produzieren als entsprechende Zellen im peripheren Blut. Dies bestaetigt die Wichtigkeit des Knochenmarks als interessante Quelle um T-Zellen fuer die adoptive Therapie zu gewinnen.

Um die Bedeutung von TLR bei der Kontrolle von HSV-1 besser zu verstehen, analysierten wir den TLR-Antworten von NK-Zellen in seropositiven Patienten. Wir haben gesehen, dass eine verminderte TLR3-Antwort der NK-Zellen wiederkehrendem HL assoziiert ist. Unsere Erkenntnisse deuten darauf hin, dass die Stimulierung von TLR3 eine potentielle Strategie zur Verhinderung von symptomatischen HSV-1-Reaktivierungen sein koennte.

Mittels TZR Repertoire Analyse werden klonale T cell Expansionen nachgewiesen, das wird normalerweise an Gesamt-T-Zellen durchgeführt.. Um beurteilen zu können, inwieweit sich das TZR Repertoire in T Zell-Gedächtniszellen ändert, quantifizierten wir das TZR Repertoire in CMV–spezifischen T-Zell-Subpopulationen. Unsere Analyse kann als Referenz für zukünftige Studien dienen.

Adiponectin (APN) besitzt immuninhibitorische Effekte in verschiedenen Krankheitsmodellen, dennoch ist wenig ueber dessen Einfluss auf T-Zellen bekannt. Wir konnten nachweisen, dass T-Zellen Adiponectinrezeptoren exprimieren und nach TCR-Stimulierung hochregulieren. Ausserdem zeigten wir, dass APN die antigen-spezifische T-Zell-Expansion und Zytokinproduktion vermindert. Da APN im menschlichen Plasma in grossen Mengen vorhanden ist, deuten unsere Ergebnisse darauf hin, dass dieses Zytokin eine wichtige Rolle bei der systemischen T-Zell-Regulierung spielt, die nicht auf den direkten Kontakt zwischen Immunzellen beschraenkt ist.

Unsere Arbeit bestaetigt die Wichtigkeit, Zytokinantworten in verschieden Zellpopulation mittels Vielfarben-Durchflusszytometrie zu charakterisieren. Diese ist ein wichtiges Werkzeug fuer das bessere Verstaendnis der Rolle des Immunsystems in verschiedene klinischen Situationen.

1 Introduction

The aim of this research work was to unveil the potential of multicolor flowcytometric analysis for a better understanding of the human immune system. In the five different studies described herein, this approach allowed us to answer clinically relevant questions about immune cell function in healthy people and its association with infectious diseases.

1.1 EBV and CMV-specific T cell memory

EBV (Epstein-Barr Virus) and Cytomegalovirus (CMV) are human herpes-virus infections (HHV-4 and 5, respectively), leading to life-long viral persistence. Although most carriers remain disease-free as a result of effective control by T cells, EBV infection can result in chronic disease and malignant transformation. In addition, CMV reactivation in immunocompromised patients, especially in the setting of transplantation, can lead to life-threatening infections. However, the profile of a protective immune response to the different chronic viral diseases is yet to be well established.

Adoptive transfer of virus-specific cytotoxic T lymphocytes has been increasingly used as a promising therapeutic tool for the control of viral reactivation [1] but it is still not clear which are the ideal cells to infuse and neither the most appropriate response monitoring in order to correlate them with clinical outcome. The combined analysis of differentiation markers, cytokine-profiles and homing brought new insights into the complexity of CD4 and CD8 T cell memory affinity, allowing the identification of new T cell subsets. Central Memory T cells (TCM) are especially attractive due to their high proliferative potential and long-lasting persistence and, more recently, the so called multifunctional memory T cells gained interest for their enhanced functionality [2].

Therefore, the first two studies correspond to the development of a multiparameter flowcytometric approach to comprehensively delineate the EBV and CMV-specific memory, an outcome of great interest for adoptive immunotherapy. Using 15-mer overlapping protein spanning peptide pools as targets against EBV and CMV, we characterized viral-specific CD4+ and CD8+ T cells based on cytokine-producing capacity, differentiation markers expression, degranulation ability and proliferation potential in both bone marrow (BM) and peripheral blood (PB) paired samples.

1.2 TLR responses of NK cells to HSV-1

Herpes Simplex virus-1 (HSV-1) infection is prevalent in 80–90% of the population worldwide. However, only one-third of the infected people suffer from recurrent orolabial reactivations or herpes labialis (HL), while the majority stays asymptomatic. A minority of patients suffer from herpes encephalitis. In these patients, genetic defects in Toll like receptors (TLRs) pathways have been found.

TLRs are present in natural killer (NK) cells, which play an important function in HSV control [3]. Thus, we investigated if functional differences and genetic polymorphisms in TLR responses are associated with susceptibility to recurrent HL. Using a multicolour flowcytometric assay, cytokine responses of NK cells to TLR agonists in people with HL were assessed.

1.3 TCR repertoire of distinct T cell subsets

Flowcytometry based analysis of T cell receptor (TCR) repertoires is an essential tool for the detection of clonal T cell expansions in physiologic and pathologic conditions. They have been extensively studied in the setting of infections, autoimmune disorders, neoplasia and transplantation, but these studies have only focused on cytotoxic and T helper cell populations. In the present study, we systematically investigated the TCR repertoire of distinct T cell subsets. Moreover, in order to address the impact of memory pool changes on TCR-Vβ repertoire, we precisely characterized and quantified TCR repertoires during latent CMV infection within the T cell differentiation pathway. Our study provides a reference for future work concerning the elucidation of subset-specific TCR repertoires in pathologic conditions.

1.4 APN as an immunomodulatory cytokine

Adiponectin (APN), an adipocyte-derived cytokine, is abundantly present as different oligomeric forms in human plasma [4], possessing profound immunoinhibitory effects in several disease models such as sepsis, inflammatory diseases and transplantation. It is known to exert its biological effects via binding to two receptors Adiponectin-Receptors (AdipoRs) expressed by a variety of cell types including immune-cells [5]. The effect of APN on monocytes and macrophages has been already analyzed in many studies but little was known about its role in systemic regulation of T cell responses. Therefore, we have focused on studying the immunomodulatory effect of APN on T cells.

2 Methods

2.1 Samples

CMV/EBV: peripheral blood mononuclear cells (PBMCs) were obtained from autologous blood banking of healthy carriers undergoing total hip arthroplasty. Paired BM samples were collected from resected femoral heads; HSV-1/TLR: PBMCs from HSV-1-seropositive immunocompetent volunteers with a history of recurrent HL and from another group of donors with no history of HL; TCR: PBMCs for healthy donors, including cord blood samples; APN: human PBMCs from healthy donors.

2.2 PBMCs isolation, T cell expansion, NK and T cell stimulation

PB and BM mononuclear cells were isolated by density gradient centrifugation.

CMV/EBV: Virus-specific T cells were expanded for 10 days with a combination of Interleukin (IL)-2 and IL-7 to 15-mer overlapping protein-spanning peptide pools from EBV (latent EBNA-1 and lytic BZLF-1) or CMV (lytic phase viral tegument protein pp65 and immediate early protein IE-1). For both PB and BM samples, T cell differentiation status and cytokine responses were assessed upon stimulation with the corresponding peptides at day 0 (*ex vivo*) or restimulation after expansion. HIV-peptide was used as negative control. For the analysis of the degranulation potential of expanded lymphocytes, cells were restimulated for 5h with the corresponding peptide pool in the presence of anti-human CD107a antibody and a protein transport inhibitor (Monensin) was added after 1h. DMSO was included as a negative control for spontaneous degranulation.

HSV-1/TLR: For the assessment of TLR induced gamma-interferon (IFN-γ) responses, NK cells were incubated in the presence of IL-12 together with TLR ligands for 24h with the addition of Brefeldin A for the last 6h. Responses to IL-12 alone have been subtracted. TLR ligand agonists were titrated to optimal doses [1/2 (Pam3Cys), 3 (poly(I:C)), 4 (LPS), 7/8 (R-848) and 9 (CpG)] or HSV-1 viral lysate for 24h. To evaluate NK cells cytotoxic function in response to TLR3, isolated cells were incubated with IL-2 or IL-2 + poly(I:C) for 12h, followed by co-incubation with K562 cells and CD107a antibody for another 6h. In a subset of experiments, NK cells were isolated from PBMCs with MACS NK cell isolation kit.

TCR: Freshly isolated mononuclear cells were analyzed immediately after staining.

APN: Influenza-specific T cell responses were assessed *ex vivo* in freshly isolated PBMCs after 24h of stimulation with influenza peptide in the presence or absence of APN. Influenza and SEB-specific

T cells were generated from freshly isolated PBMCs following stimulation with the corresponding peptides and cultured for 9-10 days with IL-2 and IL-7. To evaluate APN effect on T-specific cells, expansions were performed in the presence or absence of the cytokine. At day 10 and upon 6h restimulation with the corresponding peptide, we assessed the cytokine producing profile or CD137 expression of the specific T cells. HIV peptide was used as negative control.

2.3 Multiparameter flowcytometry

Phenotypic and functional analysis of human PBMCs was performed by staining with fluorescent-conjugated mAb, tetramers or dyes. Lineage markers: CD3, 4, 8, 14, 19, 56; Differentiation markers: CCR7, CD45RA, CD27, CD57; Functional markers: IFN-γ, IL -2, -10, -13, tumor necrosis factor-alpha (TNF-α); degranulation capacity (CD107a), activation marker (CD137); Study specific markers: TLR-3, 24 different TCR-Vβ antibodies (IO Test Beta Mark TCR Beta-Repertoire Kit, Beckman Coulter) and TCRVβ6.7 mAb, AdipoR -1,-2, tetramers (influenza A, HIV); Viability: amine reactive dye for discrimination between live/dead cells (LIVE/DEAD stain kit). Data was acquired on a BD LSRII or Canto flow cytometer and then analyzed with FACSDiva (Becton Dickinson GmbH) and FlowJo software (Treestar, USA).

2.4 Statistics

Statistical analysis was performed with the appropriate tests (single and two-tailed Wilcoxon's signed ranks test, one-tailed Mann–Whitney U test, Fisher's exact test and the non-parametric Spearman's test, ANOVA). Calculations were done using SPSS software and graphing with GraphPad Prism 5 software.

3 Results

Results are divided in five sections. The first two correspond to the study of EBV and CMV memory-repertoire, in the 3rd section the analysis of TLRs function in NK cells in HSV-1 infection is presented, the 4th section concerns the TCR repertoires along the T cell differentiation pathway and in the 5th and last section the immunomodulatory effect of APN on T cells was analyzed.

3.1 EBV-specific T-cell repertoire in latent infection reveals distinct memory T cell subsets

In the study of EBV-specific T cell responses, after stimulation with the peptide pools for 24h, we were able to examine the *ex vivo* EBV-T cell memory. Low frequency CD4+ and CD8+ EBV-specific T cells were detected by the production of at least one cytokine (TNF-α, IFN-γ, IL-2) in all eight studied donors. In the BM, numbers of EBV-specific CD4+ T cells were increased compared to PB. Importantly, a major fraction of *ex vivo* EBV-reactive EBNA-1 and BZLF-1 CD4+ T cells as well as EBNA-1-reactive CD8+ T cells were IL-2 and/or TNF-α-producing cells, suggesting that this least differentiated population may represent memory T cells serving as a T cell reservoir. In a subset of patients with a higher frequency of EBV-specific T cells, we further analyzed *ex vivo* cytokine profiles and CCR7 expression levels. The majority of EBNA-1 and CD4+ BZLF-1 responses (TNF-α/IL-2 single or double producers) were CCR7+ TCM phenotype. In contrast, CD8+ BZLF-1 responses were dominated by a CCR7- effector-memory T cells (TEM) IFN-γ/TNF-α double–producing population.

To further characterize the proliferative and differentiation capacity of EBV-specific T cells, we extended our study to the peptide pool expanded T cells. In PB, while for total CD8+ T cells a better expansion was observed, the total CD4+ T cells barely increased, expanding only in the BM. Upon peptide restimulation at day 10, the two most prominent populations observed within both the CD4+ and CD8+ compartments and against both antigens were the IFN-γ+/TNF-α+ and the TNF-α+ subsets. Furthermore, in response to EBNA-1 a substantial fraction of EBNA-1-specific triple cytokine CD4+ T cells was predominantly found in BM. Concerning the differentiation status after expansion, a considerable number of EBV-specific CD4+ and CD8+ T cells retained a TCM phenotype which is in accordance with our previous findings and supports the idea that TCM cells serve as reservoir for EBV-specific effector T cells. Due to the potential interest of using BM as a source to generate EBV-specific T cells for adoptive immunotherapy, we have also evaluated the cytolytic potential of the expanded cells by CD107a surface mobilization. Both BZLF-1-specific CD8+ and CD4+ BM T cells exhibited a higher cytotoxic potential compared to PB T cells as assessed by the degranulation assay.

Taken all together, six different EBV-specific T cell subsets could be distinguished with TNF-α-single or TNF-α/IL-2-double producing cells expressing the highest CCR7 levels, with expansion potential and resembling early differentiated memory T cells. The finding of enhanced frequencies of multifunctional and cytotoxic EBV-specific T cells in BM is of great interest for adoptive

immunotherapy of EBV disease. Our study delineates the memory T cell profile of a protective immune response providing a basis for analysing T cell responses in EBV-associated diseases.

3.2 BM as source to generate multifunctional CMV-specific CD4+ T Cells

In the study of CMV-specific T cells, *ex vivo* pp65 and IE-1-specific T cell responses, defined once more as producing IFN-γ, TNF-α or IL-2 cytokine upon peptide stimulation, were assessed and detected in all 6 donors but not all subjects had CD4+ and CD8+ responses to both antigens. Cytokine response patterns varied among the 6 subjects without any relevant difference between PB and BM, in contrast with the results from the EBV study. The differentiation phenotype of *ex vivo* CMV-specific CD4+ or CD8+ T cells in PB and BM was compared in the donors with cytokine responses greater than 0.4%. In accordance with the previous study, higher frequencies of T cells with a "naïve-like" T phenotype were found in BM in all 7 responses. However, these cells were functionally not naïve, most likely representing a less differentiated memory subset. In comparison to PB, higher frequencies of TCM cells were also found in 5 BM samples, which implies, as in the EBV study, BM as an important compartment for memory T cell homeostasis.

To evaluate the proliferative and differentiation capacities of PB and BM to expand CMV-specific T cells, a parallel analysis was performed after 10 days of expansion. Interestingly, both total CD4+ and CD8+ T cells could be more rapidly expanded from BM while in the EBV study this was only observed for the CD4+ population. After expansion, the predominant CMV-specific T cell responses consisted of IFN-γ+/TNF-α+ producing cells and the second largest fraction were IFN-γ+ or TNF-α+ single-producers, a different pattern from the one determined for the EBV responses. At day 10, both in PB and BM, a predominant TEM phenotype was found in CMV-specific IFN-γ-producing CD8+ and CD4+ T cells and in the triple cytokine producing CD4+ T cells. IL-2-producing T cells were mostly found among the fraction of triple cytokine producers and were nearly 3-fold higher in CMV-specific CD4+ T cells in BM than in PB. The analysis of the mean fluorescence intensity of the triple cytokine producing CMV-specific CD4+ T cells revealed a 2- to 4-fold increase of IFN-γ, TNF-α, and IL-2 production when compared to the single-producers, suggesting a higher functional potential. In contrast with the EBV data, the degranulation assay in response to CMV peptides has shown comparable cytotoxic potential in PB and BM CMV-specific T cells.

These data suggest that a subset of CMV-memory CD4+ T cells in BM has increased proliferative potential and the ability to retain a multi-cytokine profile with higher functionality. This finding

supports once more the BM as an interesting source for the generation of T cells for adoptive therapy.

3.3 TLR3-hyporesponsiveness of NK cells associated with HL susceptibility

In the NK analysis in order to unveil if functional differences of TLR responses are associated with susceptibility to develop HL, we evaluated TLR ligand-specific responses in subsets of PBMCs from subjects with HL histories (without active lesions) and HSV-1-seropositive asymptomatic controls. To analyze the responses of monocytes, myeloid DCs, plasmacytoid DCs and B cells, we stimulated PBMCs with TLR 1/2, 3, 4, 7/8 and 9 and measured intracellular TNF-α by cytometry. NK cells have been shown to express all known TLR mRNAs and to produce IFN-γ in response to TLR ligands after pre-activation with IL-2 or IL-12. Therefore, to determine the responses of this subset and CD8+ T cells, we stimulated PBMCs with IL-12 together with TLR ligands and assessed for IFN-γ production. Great inter-individual variations of TLR ligand-induced cytokine responses in unfractionated monocytes, DCs, NK cells and CD8+ T cells were observed, but the responses were similar between HL subjects and asymptomatic controls. Since it is reported that certain TLR responses might be redundant in blood DCs but not in NK cells, we separated NK cells from HL subjects and asymptomatic HSV-1 carriers and repeated the analysis. Remarkably, it was found that the frequencies of IFN-γ-producing NK cells in response to the TLR3 agonist were significantly reduced in the HL group, in contrast to the responses to agonists of TLR2/1 and TLR7/8 that were similar for the two groups. In addition, a reduced percentage of NK cells in lymphocytes was observed in people with HL histories.

Enhanced NK cells cytotoxic function by TLR3 stimulation has been found, but in contrast to the IFN-γ production, no significant difference in NK cell cytotoxic activity was detected between the control and HL groups. This analysis was further extended to compare the effect of the single nucleotide polymorphism of TLR3 on NK cells responsiveness. The poly(I:C)-induced IFN-γ production of purified TLR3 412F/F NK cells was significantly decreased as compared to the IFN-γ response of wild type NK cells.

Summarizing, the collected data on cytokine production indicates that TLR3-hyporesponsiveness of NK cells, along with reduced NK cell percentage is associated with susceptibility to recurrent HL. Our findings warrant a larger study for the direct comparison of clinical phenotype with NK TLR3

function and TLR3 genotype. Stimulation of TLR3 is likely to be a potential strategy to prevent symptomatic HSV-1 reactivations.

3.4 Effector T cells show a strongly biased TCR repertoire

To analyze the TCR repertoire in T cells we devised a strategy for the simultaneous detection of 12 parameters and 10 cell surface antigens using polychromatic flowcytometry. The antibody panel included 25 different Vβ mAbs and by staining for CD45RA, CD27, CCR7, CD57 we delimited eight T cell subsets already proven to be robust. These included one definition for naïve T cells, three for various memory T cell subsets and four for effector T cells.

To investigate the Vβ repertoire, the first step consisted on determining reference values for subsequent analysis. Therefore, PBMCs acquired from 66 healthy volunteers were separately analyzed for CD4+ and CD8+ cells. A clonal expansion in the cohort was defined as the number of Vβ families exceeding normal values of the respective Vβ family (mean + 2SD). We showed that CD4 Vβ repertoire exhibits smaller standard deviations than CD8 Vβ repertoire suggesting a higher variation in clonal expansion among CD8+ T cells.

Next we wondered whether the enhanced frequencies of antigen-specific T cells among memory and effector subsets are associated with a differentially biased TCR-Vβ repertoire. We observed that effector cells have expansions with higher values on both CD4+ and CD8+ cells than naïve or memory cells. Interestingly, we found small numbers of expansions on CD8+ or none on CD4+ long-lived central memory cells.

The influence of CMV serostatus was also investigated. In the CD8+ cell compartment, we observed more TCR expansions and with higher expansion factor in CMV positive donors. Moreover, higher frequencies of effector cells were identified in all CD8 effector subsets of CMV positive donors. Due to the small cohort number these differences were not statistically significant and additional multivariate analysis was not possible.

Our data demonstrate that effector T cells show a strongly biased TCR repertoire but, surprisingly, central memory T cells display a naïve-like distribution. These data suggest that antigen-driven expansion among long-lived T central memory is predominantly more polyclonal while for short-lived terminally differentiated T effector cells the expansion is more oligoclonal. Our results also serve as reference for future studies elucidating clonal TCR dominance of T cell subsets.

3.5 Negative regulation of T cells by APN

To analyze the effect of APN on PB T cells we first demonstrated AdipoR1 and AdipoR2 expression on both CD4+ and CD8+ T cells and in the cytoplasm of most T cells. Furthermore, CD137 and influenza-tetramer staining revealed that AdipoRs are upregulated on the cell surface of activated antigen-specific T cells. To study the direct effect of APN on antigen-specific cytokine production, influenza-specific T cell responses were assessed *ex vivo* after influenza peptide stimulation in the presence or absence of APN. The frequencies of IFN-γ- and TNF-α-producing T cells were impaired by addition of APN. In line with the previous findings, we examined the influence of this adipocytokine on the generation of influenza-specific CD8+ T cell lines. APN was added, at day 0 together with influenza peptide, and influenza specificity of T cells was determined by a tetramer staining technique without the need of previous restimulation. In the presence of APN, significant lower numbers of antigen-specific CD137+CD8+ T cells were observed, however total cell counts were not diminished. To further evaluate the influence of APN on the expansion of antigen-specific T cells, cytokine production upon restimulation with influenza peptide was determined after 9–10 days of culture. Significant lower numbers of IFN-γ, TNF-α and IL-2-producing influenza specific T cells were observed on the cell lines raised in the presence of APN. Neither IL-10 nor IL-13-influenza-specific T cells were observed under both culture condition revealing that the inhibitory effect is not due to these suppressive cytokines.

Taken together, these data show that AdipoRs are also expressed by antigen-specific T cells and that APN negatively influences their generation. Furthermore, APN exerts a direct inhibitory effect on antigen-induced cytokine production. In contrast to other immunoregulatory receptor–ligand interactions known to be restricted to the interplay between immune cells, the ligand of APN receptors, adipocytokine, is abundant in human plasma and may exhibit an important role in systemic T cell regulation.

4 Discussion

4.1 Advantages of flowcytometry for studying the immune system

Multicolor flowcytometry specificities have a broad spectrum of advantages over other techniques for the understanding of the human immune system. The fact that only a small amount of donor or patient blood is required and that cells retain their viability and functionality after cryopreservation can be crucial (e.g. in clinical trials). This procedure allows study orientation and synchronisation of data assessment from several patients and time points, minimizing inter-experimental variability. Being based on single cell data acquisition, very low frequency of cytokine-producing populations can be detected with accuracy by this technique.

In our studies, we were able to identify and characterize: (i) EBV-specific T cells subsets consisting of only 0.2% of total CD8+ T cells, (ii) IE-1-specific triple-producers CD4+ T cells with mean frequencies of 0.1% and (iii) 0.43% CD56br NK cells secreting IFN-γ+ after HSV-1-lysate stimulation. Even if present in very small percentages, the lower HSV-1-triggered IFN-γ-production by NK cells correlated well with cytokine levels in the supernatant determined by a cytometric bead array, which reflects the sensitivity of this approach. As the crosstalk between the different cell subtypes is a core feature of our immunity, analysing responses in a more physiologic setting is of paramount importance. The fact that reduced TLR-3-triggered NK responses in the HL group could not be detected in unfractioned PBMCs clearly supports that cell interaction may largely influence results. Moreover, cytometry allows comparative analysis of antigen surface expression levels between different cell sorts from the same sample. It is important to state that the analysis performed for AdipoRs in CD14+ and CD3+ cells could not have been done by RT-PCR of the corresponding mRNAs even after a T cell depletion step. In addition to the classical lineage markers, an increasing number of antibodies and fluorochromes are becoming available to help in the characterization of cell subsets. As such, in the APN study, the staining of the activation marker CD137 identified memory cells responding to SEB and influenza-peptide without the need to stain several cytokines in order to obtain a more global picture of this population. The specificity of this antibody for the influenza-memory was confirmed by co-staining with a T cell specific-tetramer that through TCR conformation allows the identification of this same population without requiring peptide-stimulation.

The staining of membrane receptors such as TLR3 also enabled us to clarify if different NK responses in a variant allele correlated with the expression levels of this antigen. Likewise, it was

possible to carefully study the expression or co-expression of AdipoR 1 and 2 on T cells, their kinetics upon stimulation and to further localize these receptors in the membrane and/or cytoplasm. Another important parameter for an accurate analysis of the immunoregulation is to determine the number of proliferating and apoptotic cells. By co-staining for BrdU or Annexin we could describe APN effect over the cell cycle phase of antigen-specific T cells, helping to further understand the biological relevance of this adipocytokine. As for the study of TCR-Vβ families, by employing a set of 25 antibodies we were able to characterize and enumerate approximately 85% of the total human T cell repertoire. This approach becomes appealing as it is time and cost efficient compared to other methods such as single cell analysis or next-generation sequencing. Collectively, the increasing multiplicity of measurable parameters makes cytometry an attractive approach to dissect our immune system.

4.2 Characterization of T cell differentiation status

T lymphocytes transit through progressive stages of differentiation characterized by a stepwise loss of functional and therapeutic potential. Thus, it is important for T cell-based immunotherapies to identify and better characterize subsets of mature T cells that may exhibit the stem cell-like characteristics as enhanced self-renewal and the multipotent capacity to form all memory and effector subsets. These properties may allow T cells to sustain a prolonged immune attack by giving rise to more differentiated, highly lytic effector cells while maintaining a continuous supply of less differentiated cells that can refresh the pool of cytotoxic cells [6]. But the task to delineate these different T cell subsets is becoming more complex as an increasing variety of differentiation markers combinations are available.

In the study of TCR repertoire, being the effector population especially interesting, we selected CD27, CD45RA, CCR7 and CD57 to allow the characterization of four effector T cells subsets. In the other two studies, by staining just for CCR7 and CD45RA we were able to characterize EBV and CMV-specific memory T cells in distinct differentiation subsets, revealing the different profiles of the immuneprotective memory. As an example, *ex vivo* CMV specific cells were mainly TEM and terminal-effector T cells (TEFF) in CD4+, and only TEFF in the CD8+ population, while for EBV almost no terminally differentiated effector cells could be identified as the majority of memory cells were CD45RA negative. These observations may reflect an increased CMV replication level, consequent antigen stimulation, leading to terminal differentiation. As previously noticed,

differentiation assessment revealed BM as source of less differentiated memory cells with a CD45RA+CCR7+ ‘‘naïve-like" phenotype for CMV and TCM for both viruses. The biological relevance of these findings was confirmed by sorting different cell populations based on several markers, while keeping cell viability and functionality. Using this approach, we isolated T cell subsets at different maturation status prior to the *in vitro* expansion revealing that, indeed, the majority of EBNA-1-specific T cells could be expanded from the TCM compartment, suggesting an increased self-renewal capacity. However, after a 10 day expansion CMV and EBV-specific T cells become predominantly TEM both in PB and BM, indicating that our *in vitro* expansion may corrupt the beneficial attributes of the less-differentiated T cell subsets after isolation. Assessing the differentiation phenotype of *ex-vivo* memory populations and end-culture cells is essential for the fine tuning of our expansion protocol and to better correlate the characteristics of the infused cells with their clinical effect in adoptive therapies.

4.3 Cytokine profile analysis

The differentiation status is insufficient to precisely define which subsets of cells are functionally similar and relevant in disease pathogenesis or vaccination. Several relationships between functional markers and disease were not apparent in earlier studies due to the fact that only one or two parameters could be assessed at a time [7, 8]. Additionally, literature is increasingly supporting the importance of polyfunctional T cells (cells simultaneously expressing a majority of the measured functional markers, including e.g. IFN-γ, IL2, TNF-α, MIP1β, and CD107a) as the frequency of these cells has been associated with (i) less pathogenic disease (HIV in long-term non-progressors, HIV-2), (ii) protective immunity (Leishmania, Vaccinia, Yellow Fever, SIV), and (iii) the identification of critically-relevant cells from the standpoint of immune monitoring and optimization of vaccine efficacy. More recently, multifunctional CMV-specific cells were shown to protect against high level replication after liver and lung transplantation. In our studies, as dissected in the results section, the analysis of 4 functional markers (IFN-γ, IL-2, TNF-α and CD107a) has shown that cytokine-producing memory T cell differ between CMV, with a IFN-γ+/TNF-α+ and IFN-γ+ predominant population, and EBV with a majority of *ex vivo* memory T cells producing TNF-α+/IL-2+ or TNF+. Consistent with our previous differentiation data, after expansion for both viral peptides most cells acquired a TEFF cytokine profile with increased numbers of IFN-γ+/TNF-α+ cells. This information is of major relevance for adoptive therapies as the transfer of TCM and

effector cells correlate better with clinical outcome [9]. Interestingly, BM T cell analysis revealed an increased content of multifunctional T cells even after expansion and, by the use of mean fluorescence intensity we could also estimate the relative amount of cytokines being synthetized by each cell, revealing multifunctional T cells as high-producers, compared to single or double producers, which probably contributes for their enhanced biological functionality. Due to the potential interest of using BM as a source to generate EBV and CMV specific T cells for the adoptive immunotherapy, the cytolytic potential of the expanded was evaluated by CD107a surface mobilization. Compared with PB, BM revealed higher numbers of EBV-specific cytotoxic T cells, while CMV expanded cells have shown similar cytotoxic potential, demonstrating that less differentiated BM memory cells can give rise to a population with degranulation capacity which is important for killing virus infected cells. Peptide-induced cytokine detection also helped in clarifying the regulatory effect of APN on the expansion of antigen-specific T cells from the lower numbers of IFN-γ, TNF-α, and IL-2-producing cells at the end of the culture. In parallel, by staining for other functional markers as the anti-inflammatory cytokines IL-10 and IL-13, we could show that the impaired expansion was not due to the priming of type 2 responses. Likewise, this approach is very solid, allowing the extension of the analysis to other cell lineages, as the assessment of IFN-γ-production by NK cells in response to TLR ligands, while for monocytes, dendritic and B cells, TNF-α is considered a more appropriate cytokine to screen. The fact that the degranulation assay demonstrated enhanced cytotoxic responses by NK cells to K562 cell line following TLR3 stimulation, in clear contrast with IFN-γ secretion data, strongly supports the need for measuring different functional markers for a broader view of the complex cell functionality.

4.4 Integrate data to analyze biological relevance

By combining multicolor assessment of both cytokines and differentiation status, it is possible to identify various subsets with potential clinical interest. For example, we observed that after *in vitro* expansion, a subset of CD4+ and CD8+ EBV-specific T cells retains a TNF-α single or TNF-α/IL-2 central memory phenotype. This is an important result as previous studies, mainly focusing on IFN-γ-production to characterize EBV-specific T cells, have excluded this population. Interestingly, our findings suggest that the differentiation model of Seder et al. [10] can be extended to CD8+ T cells in which TNF-α single or TNF-α/IL-2 double memory T cells exhibited, likewise, the highest CCR7 expression. In the CD8+ T cell compartment, triple cytokine and IFN-γ single-producing cells had a

significantly higher CCR7 expression than IFN-γ/TNF-α producers. Moreover, the enhanced frequencies of cytotoxic, multifunctional EBV-specific T cells with an intermediate differentiation memory phenotype found in BM, are of great interest for adoptive immunotherapy in EBV disease. However, in our CMV study and based on the CCR7 expression levels, triple cytokine producers were predominantly TEM. Others have also shown that upon expansion, TCM cells transiently develop a TEM phenotype but, unlike TEM cells, retain a higher proliferative potential and a long-term persistence *in vivo* [11]. Thus, the CCR7- CMV-specific T cells observed in BM may represent a subset of memory T cells with an increased proliferative capacity and the ability to achieve or retain a multicytokine profile.

In conclusion, based on cytokine profiles and differentiation status, we revealed the existence of different EBV- and CMV-specific memory T cell subsets at distinct differentiation stages. It is essential to validate the biological relevance of the distinct cell types and responses described. We believe that from the CMV and EBV studies we have established a solid read-out to screen immune memory to these viruses that can be easily applied to the follow-up of post-transplant diseases related with EBV reactivation or CMV severe infections in the same group of patients. In addition, the developed panel for the TLR/NK and APN studies was further adapted to subsequently extend the analysis to the immunological effect of APN on NK cells [12]. Flowcytometry has also provided to our group the tools to establish the relationship between this adipocyte-derived cytokine, TLR-4 and cardiac inflammation and injury [13] and to describe the effect of TLR1 gene polymorphism in NK and T cells functions, relating them to Helicobacter pylori-induced gastric disease [14]. In addition, we have established a panel to study the immunomodulatory effects of a neoadjuvant treatment with rIL-21 administered subcutaneously in patients with stage III melanoma. We analyzed the influence of rIL-21, a pleiotropic cytokine, on monocytes, NK cells, T cells subsets, Tregs, Th17, and IL-13-producing type-2 T cells simultaneously, in order to correlate it with the observed anti-tumor effect.

4.5 Flowcytometry - a technical challenge

Frequently, scientific questions are easily identified but a major barrier relies on finding an appropriate technical approach to reach answers. Due to our lab research field, we have a special interest in cytometric approaches which demand a continuous update on the advances extensively revised in a recent paper [15]. The opportunity of multiple analyzes comes together with the need to

address several pitfalls. For reliable flowcytometry measurements, a proper calibration of the equipment and strict quality control are required for a correct comparison between experiments performed at different time points and using the same setting. For this reason, cytometer compensation was applied using BD Comp beads and a compensation algorithm from DIVA software that allows reduction of the laser interference and filters fine variations due to external factors such as temperature or after laser replacement. The importance of an optimized panel design cannot be overemphasized and is a lengthy and labour-intensive process. There is a need to distinguish between the markers required for the study and those that could be interesting but are not necessary. Another challenge is to appropriately choose fluorochromes based in the conjugation of dim markers on the brightest channels and markers with on/off expression on the dimmest channels, in order to minimize the spreading of fluorescence. The laborious work of antibody titration and the choice of an appropriate gating strategy and exclusion markers are essential to avoid artefacts in staining patterns. For example, by the use of forward scatter versus side scatter profile one can delimit distinct cell populations based on their relative dimension, doublets can be distinguished from single cell events based on their area- versus height-forward scatter profile and live/dead cells are discriminated by staining with an amine reactive dye to avoid unspecific binding. Additionally, to better define the analyzed population it is necessary to stain several other lineage markers besides the ones expressed by the cells under study, which will facilitate the exclusion of potential confounding populations that unspecifically bind to antibodies relevant in the experiment. As an example, in the CD107a degranulation assay to analyze T cell cytotoxicity, we have used a CD19 and CD14 dump channel, with a dramatic background reduction. Likewise, the inclusion of CD16 with the same fluorochrome allowed the exclusion of NK T cells exhibiting increased cytotoxic properties, which would not be identified by staining only with the CD3 T cell marker. The choice of negative and positive controls is crucial in avoiding artefacts while characterizing rare cells populations, especially depending on the cytokine production profiles. For instance, positive events counted in response to incubation with DMSO were subtracted from the response obtained by specific peptide stimulation as this solvent by itself elicits T cells responses. As for the HLA-A2-influenza-peptide tetramer staining, an HIV-tetramer was the control, while for responses to HSV-1 viral lysate, uninfected Vero cells were used. A positive control can also help to evaluate if the experimental protocol preserved cell functionality and confirm the efficacy of the staining and measuring steps. For this reason, when analysing memory populations, Staphylococcal enterotoxin

B was chosen as a positive control due to the capacity to stimulate antigen-specific T cells. For the measurement of antigen expression levels, the same fluorochrome-labeled isotype-matched monoclonal antibody is required to define negativity for this marker. Another important variable to control is the ideal time point to measure functional responses. In the *ex vivo* virus-specific T cell analysis, based on our previous studies, cytokine staining was performed after 18h, while "recall responses" and degranulation capacity from expanded cells could be already assessed after a restimulation period of 6h. In contrast, while analysing TLR-trigger NK responses a 24h stimulation period was more appropriate.

When multiple markers and hundreds of phenotypic combinations are available to explore, data analysis tools become invaluable. We now possess highly complex software to help in this task in a large extent. SPICE software is a precious tool to interpret results and was chosen for data analysis. It has the potential to perform Boolean gating, join categorical data and normalize for background biological controls, as required in our intracellular cytokine assays. Due to the complexity and large amount of data generated, the task of clearly exposing our results has been increasingly difficult. Software like Pestle helps in fulfilling this need.

In conclusion, the future of polychromatic flowcytometry development and application is likely to remain quite vibrant [15] and its potential to examine human immunity and disease will certainly improve with incredible resolution. Specifically, by the detection of cytokines and a sharp characterization of the producer cells we will be able to better define the role of distinct leukocyte subsets and their interplay in immune and inflammatory responses. These advances come together with rewarding scientific knowledge but also with the need of constant and careful updating.

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Eidesstattliche Versicherung

„Ich, Manuel Guerreiro, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Characterization of cytokine responses and memory cell subsets using a multicolor flowcytometry-based approach“, selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.

_____

Berlin, 19.02.2014 Manuel Nuno D. M. Guerreiro

Erklärung über den Anteil an den Publikationen

Publikation 1: Guerreiro M, Na IK, Letsch A, et al. **Human peripheral blood and bone marrow Epstein-Barr virus-specific T cell repertoire in latent infection reveals distinct memory T cell subsets**. Eur J Immunol. 2010 Jun;40(6):1566-76.

Anteil: Konzeption der Arbeit, Leitung und Durchführung der wesentlichen Experimente, Verfassen des Manuskripts

Publikation 2: Na IK, Letsch A, Guerreiro M, et al. **Human bone marrow as a source to generate CMV-specific CD4+ T cells with multifunctional capacity**. J. Immunother. 2009 Nov-Dec;32(9):907-13.

Anteil: Mitarbeit bei der Methodenentwicklung un durchführung der Experiment

Publikation 3: Wilk S, Scheibenbogen C, Bauer S, Jenke A, Rother M , Guerreiro M et al. **Adiponectin is a negative regulator of antigen-activated T cells.**Eur J Immunol. 2011 Aug;41(8):2323-32.

Anteil: Methodik zur Erarbeitung / Unterweisung von Mitautoren in etablierte Methoden

Publikation 4: Yang CA, Raftery MJ, Hamann L, Guerreiro M et al. **Association of TLR3-hyporesponsiveness and functional TLR3 L412F polymorphism with recurrent herpes labialis**. Hum Immunol.2012 Aug;73(8):844-51.

Anteil:Methodik zur Erarbeitung / Unterweisung von Mitautoren in etablierte Methoden

Publikation 5: Bretschneider I., Clemente M.J., Meisel C., Guerreiro M. et al. **Discrimination of T cell subsets and T cell receptor repertoire distribution.** Immunol. Res. **58**, 20-7.

Anteil: Methodik zur Erarbeitung / Unterweisung von Mitautoren in etablierte Methoden

Publikation 1

Na I.K., Letsch A., Guerreiro M., Bauer S., Noack I., Geginat J., Reinke P., Loesch M., Kienapfel H., Thiel E., Volk H.D. and Scheibenbogen C. (2009) *Human bone marrow as a source to generate CMV-specific CD4+ T cells with multifunctional capacity*. J. Immunother. **32**, 907-913. Impact Factor: 3.463

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Publikation 2

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Publikation 3

Wilk S., Scheibenbogen C., Bauer S., Jenke A., Rother M., Guerreiro M., Kudernatsch R., Goerner N., Poller W., Elligsen-Merkel D., Utku N., Magrane J., Volk H.D., and Skurk C. (2011) *Adiponectin is a negative regulator of antigen-activated T cells*. Eur. J. Immunol. **41**, 2323-2332. Impact Factor: 4.97

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Publikation 4

Yang C.A., Raftery M.J., Hamann L., Guerreiro M., Grütz G., Haase D., Unterwalder N., Schönrich G., Schumann R.R., Volk H.D. and Scheibenbogen C. (2012) *Association of TLR3-hyporesponsiveness and functional TLR3 L412F polymorphism with recurrent herpes labialis.* Hum. Immunol. **73**, 844-851. Impact Factor: 2.298

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Publikation 5

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Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Komplette Publikationsliste

Aggressive mature NK cell neoplasms: report on a series of 12 European patients with emphasis on flow cytometry based immunophenotype and DNA content of the neoplastic NK cells; M. Lima, A. Spinola, S. Fonseca, A.H. Santos, J. Rodrigues, L. Oliveira, M.L. Queiros, M. Goncalves, C. Lau, MA. Teixeira, C. Goncalves, C. Marques, M. Guerreiro, M. Cunha, F. Principe, J. Coutinho
(Leuk Lymphoma. 2014, Epub ahead of print)

What determines therapeutic choices for elderly patients with DLBCL? – Clinical findings of a multicenter study in Portugal; R. Alvarez, S. Esteves, S. Chacim, J. Carda, A. Mota, M. Guerreiro, I. Barbosa, F. Moita, A. Teixeira, J. Coutinho, F. Príncipe, JM. Mariz, M. José, MG. Silva
(Clin Lymphoma Myeloma Leuk. 2014, Epub ahead of print)

Discrimination of T cell subsets and T cell receptor repertoire distribution; I. Bretschneider, M.J.Clemente, C. Meisel, M. Guerreiro, M. Streitz, W. Hopfenmüller, J.P. Maciejewski, MW. Wlodarski, HD Volk
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Association of TLR3-hyporesponsiveness and functional TLR3 L412F polymorphism with recurrent herpes labialis; C.A. Yang, M J. Raftery, L. Hamann, M. Guerreiro, G. Grütz, D. Haase, N. Unterwalder, G. Schönrich, R.R. Schumann, H.D. Volk, C. Scheibenbogen;
(Hum Immunol. 2012, 73:844-51)

Adiponectin is a negative regulator of antigen-activated T cells; C. Scheibenbogen, S. Wilk, S. Bauer, A. Jenke, M. Rother, M. Guerreiro, R. Kundernatsch, N. Goerner, W. Poller, D. Elligsen-Merkel, N. Utku, H.D. Volk, C. Skurk
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Human peripheral blood and bone marrow EBV-specific T cell repertoire in latent infection reveals distinct memory T cell subsets; M. Guerreiro, I. Na, A. Letsch, D. Haase, S. Bauer, C. Meisel, A. Roemhild, P. Reinke, H.D. Volk, C. Scheibenbogen
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Human bone marrow as a source to generate CMV-specific CD4+ T cells with multifunctional capacity; I. Na, A. Letsch, M. Guerreiro, I. Noack, J. Geginat, P. Reinke; M. Loesch, H. Kienapfel, H.D. Volk, C. Scheibenbogen
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