Final progress report

OTKA K 116340

Improving prognosis of acute GVHD by combining microbiome analysis and immunogenomics in a large-scale survey of the host's response to bone marrow transplantation (2016-2020)

Aim: The aim of this project was to identify novel prognostic and diagnostic markers heralding the rise of acute graft-versus-host disease (aGvHD), a frequent and often fatal adverse event of allogeneic hematopoietic stem cell transplantation (aHSCT). The project focused on markers capable of discriminating between cutaneous and gastrointestinal GvHD, the two major manifestations of the disease, as their exact prediction remains an unmet need, although they have marked differences in terms of prognosis, patient survival, and adequate therapy required.

Design: The project consisted of three major sub-projects, as follows; **Project A)** an analysis of pathogenic cytotoxic T cells homing to the skin and gut, the two key target organs of aGvHD, **Project B)** an investigation of perturbations of the plasma proteome during the development of aGvHD, and **Project C)** a study of aberrant changes affecting the gut microbiota, a major factor contributing to the initiation of the disease. Identification of novel biomarkers was attempted applying a hypothesis-free approach, i.e. -omics-based screening methods followed by marker validation and functional annotation.

Research framework: The project was carried out as an international clinical collaboration established between the Dept. of Genetics, Cell- and Immunobiology, Semmelweis University, Budapest, Hungary, the Dept. of Hematology and Stem Cell Transplantation of the South-Pest Hospital Centre (former St. Istvan and Saint Laszlo Hospital), Budapest, Hungary, and the Sidra Medical and Research Center, Doha, Qatar.

Results:

Clinical sample collection: Setting up and performing clinical sample collection, archival, biobanking, sample transfer between partners, and sample processing were all successfully completed. A total of 138 patients have been enrolled in the study, providing 561 peripheral blood samples and 503 fecal samples between 2016 and 2019.

Results obtained from Project A) Analysis of cytotoxic T cell homing during skin and gut damage caused by acute GvHD. This project was started to identify novel homing-markers of T cells causing GvHD in the hope that these may be used as novel markers of skin and gut GvHD.

- We set up multiparametric FACS-sorting of skin-homing (Ly/Singlet/Live/CD8β+/CLA+), gut-homing (Ly/Singlet/Live/CD8β+/integrinβ7+), and reference (Ly/Singlet/Live/CD8β+/CLA-/integrinβ7-) cytotoxic T lymphocytes (CTLs) retrieved from aHSCT patients developing no GvHD, skin aGvHD, gut aGvHD, or both manifestations simultaneously, and compared them by global transcriptome profiling to identify homing-related markers of clinical relevance.
- We observed that skin-homing CTLs of patients displayed a >16-fold overexpression of peptidase inhibitor 16 (PI16), a member of the CAP family, the biological functions of which are largely unknown.
- We confirmed these results at the protein level, and provided evidence that it is strictly restricted to T cells implying that it may bear functional relevance to the key effector cells of GvHD
- Deep phenotyping disclosed that PI16 is expressed by non-naïve, circulating, memory-like, skin-homing, resting CTLs displaying a CD8b+/CLA+/CD45RO+/CD127+/CD25+/CD69-/GzmB- phenotype.
- Further, we found that PI16, being originally described as a secreted plasma protein, is produced as a GPI-anchored plasma membrane-bound protein by skin-homing CD8+ T cells.
- Regulation of PI16 was found to be atypical, independent of the regulation of other skin homing markers (CLA, CCR10), an unaffected by known inducers and inhibitors of the canonical skin-homing program of CD8+ T cells (RA, calcitriol, etc).

- Expression was maintained even after T cell entry into the skin, however, it was promptly discontinued upon re-activation. Disappearance of PI16 was not dependent on any known forms of GPI anchor cleavage-mediated shedding, but on rapid cessation of gene transcription upon T cell activation.
- Unfortunately though, we found that PI16 expression is neither related to, nor affected by cutaneous aGvHD, ultimately rendering this novel skin-homing T cell marker irrelevant in diagnostic or prognostic terms.
- Nevertheless, as this observation is relevant to T cell homing, several attempts were made to better characterize PI16's functions. It could be confirmed that PI16, acting in the nanomolar range, is a partial inhibitor of cathepsin-K, an inflammatory protease involved in skin immune homeostasis.
- Research is currently ongoing to identify any proteins that could either interact or form protein complexes with PI16 in the plasma membrane of skin-homing CD8+ T cells, in the hope that such data may provide better insight into the biological functions of this elusive protein.

Results of the above project have been published in: **Lupsa N$**, **Érsek B$**, Horváth A, Bencsik A, Lajkó E, Silló P, Oszvald Á, Wiener Z, **Reményi P, Mikala G**, **Masszi T**, Buzás EI, **Pós Z#&**: Skin-homing CD8+ T cells preferentially express GPI-anchored peptidase inhibitor 16, an inhibitor of cathepsin K., EUROPEAN JOURNAL OF IMMUNOLOGY 48: (12) pp. 1944-1957. 2018 (**bold**: OTKA K 116340 grant participant $:First author **#:** last author &: corresponding author)

Additional results obtained from Project a) Setting up a transgenic, minor histocompatibility antigen mismatch-based murine aGvHD model. This project was started to gain insight into CD8+ T cells' actions within GvHD-affected tissues of a murine host. The rationale behind such experiments is that isolation of CD8+ T cells from the gut tissue of affected human patients challenging, as resection of larger gut biopsies providing sufficient material is contraindicated in full-fledged gastrointestinal aGvHD.

- Grant-supported personnel and technical knowledge obtained from the studies above was utilized to facilitate the completion of this project, already running at the time of grant approval. We were hopeful that a murine disease model could serve as a useful complementation of the proposed human studies, considering that several tissue-level CD8+ T cell analyses cannot be performed in humans.
- Our intention was to develop a novel, clean experimental aGVHD model in that, unlike other conventional mouse GvHD models, there is only one, well-characterized target antigen for T cell inducing GvHD, it is presented in all target organs, and all grafted T cells recognize it in the same way, thereby allowing for proper comparison of CD8+ T cell responses in different target tissues, such as the gut and the skin.
- We developed a novel mouse aGvHD model (Act-mOVA/OT-I) in that transgenic TCR-carrying CD8+ T cells recognize the chicken ovalbumin$^{257-264}$ (SIINFEKL) antigen epitope, ubiquitously expressed in virtually all tissues under the control of CMV ie enhancer/chicken beta-actin promoter, the epitope being presented for CD8+ T cells in the context of a single MHC I allele (H2-K^{b}).
- We confirmed that the model developed a CD8+ T-cell dependent, 100% lethal aGvHD in the murine host within a matter of days, making the system highly practical for experimental use
- We confirmed that CD8+ T cells became activated by the cognate miHA (SIINFEKL) of the host, clonally expanded as assessed by TRC assays, gained effector functions and produced GzmB, became capable of releasing this cytotoxic effector molecule, and successfully infiltrated the target organs of GvHD, infiltration peaking at the day of the demise of the host.
- We showed that the model developed tissue damage recapitulating human gastrointestinal and hepatic GvHD, and even GvHD-related lymphocytic bronchitis, which is a rare, but clinically relevant event in human aGvHD. However, rather unexpectedly, the model did not develop cutaneous aGvHD. Based on indirect evidence we suspect that this may have been caused either by the fact that in this model, membrane-bound ovalbumin is expressed as a fusion protein, the structure of which may affect antigen presentation, and/or by the fact that the number of adequate APCs presenting this particular antigen to CD8+ T cells in the skin is rather low compared to other tissues.
- The model allowed us to gain insight into tissue-level differences between CD8+ T cells. We were able to demonstrate that gut-infiltrating CD8+ T cells have a surprisingly low number in spite of the massive organ

damage caused which is related to several genes hinting at a slower cell-cycle in this particular tissue CTL subset. Gene set enrichment analysis and false discovery rate-corrected transcriptome profiling confirmed that this is because graft CD8+ T cells promoting local tissue damage in the gut are less responsive to IL-2 mediated growth signaling, display lower expression of many G1-phase cyclins, and may be more prone to FAS-mediated apoptosis, than CTLs in other tissues. This observation is consistent with a terminally differentiated, highly active killer CTL phenotype and contributes to the understanding of the reasons of the heavy tissue damage caused by CTLs to the gut of GvHD patients, as compared to the relatively mild tissue damage caused by them to other tissues displaying the same mismatched major or minor antigens.

Results of the above project have been published in: **Érsek B$, Lupsa N$,** Pócza P, Tóth A, Horváth A, Molnár V, Bagita B, Bencsik A, Hegyesi H, Matolcsy A, Buzás EI, **Pós Z#&**: Unique patterns of CD8+ T-cell-mediated organ damage in the Act-mOVA/OT-I model of acute graft-versus-host disease., CELLULAR AND MOLECULAR LIFE SCIENCES 73: (20) pp. 3935-3947., 2016 (**bold**: OTKA K 116340 grant participant $:First author **#:** last author &: corresponding author)

Additional results obtained from Project a) Linking the appearance of CD8+ T cell-B cell couplets in secondary lymphatics to gastrointestinal GVHD. As part of the planned collaboration with the Sidra Medical and Research Center, we were able to exploit the above transgenic murine aGVHD model to confirm that a curious interaction takes place between CD8+ T cells and B lymphocytes during the development of gut GvHD, within the affected tissues.

- Previous research performed at Sidra MRC identified extremely rare, rather unusual CD8+ T cell-B cell interactions occurring in the blood, resulting in the formation of CTL-B cell couplets, i.e. temporary cell-cell contacts remaining stable for several minutes to hours. It was shown that the interaction was not an analytical or sample processing artefact, involved live B cells and CTLs in a strict 1:1 ratio, was independent of antigen presentation and/or recognition, was CD27/CD70 dependent, resulted in functional re-programming of the CTL, and led to subsequent chemokine release from the B cell (CXCL9/MIG, CXCL10/IP-10 and CXCL11/ITAC). Interestingly, this interaction became more prominent in aHSCT patients suffering aGvHD.
- In our Act-mOVA/OT-I murine aGvHD model we were able to show that the tissue compartment that this interaction becomes the most frequent in, is in fact not the blood, but rather the Peyer's patches in the inflamed gut tissue during the development of gut aGvHD. Congenial labeling confirmed that this phenomenon involved graft T and B cells and not residual host lymphocytes.
- Further studies disclosed that CD8+ T cells involved were mostly terminally differentiated antigen-experienced effector CTLs (Temra-like CTLs) that, however, did not kill their interacting B cell partners, even if they were fully capable of killing, as B cells artificially loaded with cognate antigen of the CTL were promptly eliminated in couplets.
- Live imaging and cell tracking provided evidence that the interaction involved repositioning of CD9 and CD8 containing lipid rafts, could not be inhibited by blockade of MHC or CD8 either, and resulted in Ca2+ influx into both partners, indicative of ongoing intracellular signaling
- B cells participating in couplet formation became apoptosis-prone CD95hi cells after detachment form the CTL, while CTLs are subject of a temporary translocation of B-cell membrane proteins to the CTL cell surface, an extremely intriguing observation

Results of this project were presented at: Kizhakayil D$, Sathappan A, **Pos Z, Lupsa N,** Raynaud CM, Gentilcore G, Al-Aghbar MA, Maccalli C, Grivel JC, van Panhuys N, Deola S**#**: B-T cell interactions in Graft-versus-Host Disease, 46th Annual Meeting of the EBMT, 2020 (**bold**: OTKA K 116340 grant participant $:First author **#:** last author $: corresponding author)

Results obtained from Project b) Investigation of perturbations of the plasma proteome during the development of aGvHD. This sub-project sought to identify so far unknown tissue-derived but circulating blood proteins that may be of clinical value in the prognosis or diagnosis of cutaneous and gastrointestinal aGvHD. We assumed that proteins with

an expression restricted to either of the two key aGvHD target organs (i.e. skin and gut), affected by cellular stress, compromised barrier function, or cytotoxic destruction by CTL killing, could be utilized for such purposes.

- Based on *in silico* data-mining of public RNA- and protein-expression databases we selected plasma proteins that a) were produced in a tissue-restricted manner, i.e. exclusively by the skin or the small intestine, b) were either actively secreted or known to leak into the blood plasma c) were present in the blood in amounts detectable by ELISA d) had robust and sensitive ELISA assays available d) had a reasonable chance that their production, release, leakage, or plasma availability was affected by local tissue damage occurring in aGvHD.
- We selected I-FABP, occludin and cytokeratin-20 as novel marker candidates for gut aGVHD, and cytokeratin-15 as a marker candidate for skin GvHD. Next, we compared them with the currently known best diagnostic plasma markers of skin and gut aGvhD (elafin and Reg3a, respectively), and other published markers of GvHD not capable of discriminating between skin and gut GvHD (sIL2RA, sTNFRI), both in terms of their diagnostic, and their prognostic value.
- Diagnostic value was assessed by comparing plasma levels of the markers in aHSCT patients developing no GvHD, cutaneous GvHD, gastrointestinal GvHD, and both modalities simultaneously, at the time of diagnosis (n=10 each, 40 patients, 40 samples total), by ANOVA and ROC curve analysis.
- Prognostic value was determined by following plasma levels of the above markers in 521 blood samples collected from 98 aHSCT patients in 6 time points before and after aHSCT. Out of these, 449 samples of 81 patients were eligible for analysis. Analysis was done using mixed-model ANOVA and multiple logistic regression to incorporate known factors modifying disease outcome, such as age, gender, applied conditioning regimen, GvHD prophylaxis, therapy, donor-recipient gender match, MHC match, etc.
- Reference diagnostic markers, such as elafin and Reg3a could be linked to skin ($p=0.0274$, ROC AUC=0.7083) and gut GvHD ($p<0.0001$, ROC AUC=0.8263), respectively, confirming that patient groups were selected correctly, and behaved in line with literature data. Also, known risk factors of GvHD. e.g. male gender, multiple MHC mismatches, aggressive conditioning. etc. were found to affect disease outcome as expected.
- As for the novel markers, levels of occludin and cytokeratin 15 were not affected by GvHD development and hence were excluded from further studies. However, plasma I-FABP was found to profoundly diminished in all aGvHD groups at the time of diagnosis ($p=0.0315$) compared to healthy controls, and this marker showed considerable sensitivity and specificity, as well (ROC AUC=0.7586). In addition, cytokeratin-20 showed gradual decrease in patient samples, peaking in patients with the best possible outcome (no GvHD) and reaching lowest levels in the worst outcome group (both skin and gut aGvhD developed). Unfortunately though, the dynamic range of this latter marker was very narrow, and this fact did not allow discrimination between healthy aHSCT patients and mild GvHD cases, only between healthy patients and severe cases ($p=0.0260$, ROC AUC=0.7465).
- Next, novel diagnostic markers (I-FABP, CK20) and established diagnostic controls (elafin, Reg3a) were analyzed to test their prognostic value as well. I-FABP, elafin, and Reg3a were unable to predict the development of GvHD; neither the disease in general, nor its tissue-specific manifestations in particular. This observation may be disappointing, but in case of elafin and Reg3a, it is in line with published data; exact prediction of GvHD based on plasma markers remains an elusive goal.

This study could not be completed in a timely manner, because the human blood CK20 ELISA assay used, provided by Sigma-Aldrich (RAB1410), has been discontinued in the middle of the project without advance notice. We are testing possible alternatives, among others the development of an in-house made CK20 assay based on Sigma's antibodies.

Results obtained from Project c) A study of aberrant changes affecting the gut microbiota, a major factor contributing to the initiation of the disease. The primary objective of the third sub-project was the identification of any bacterial phyla, genera or strains the abundance of which may influence the risk, or predict the development of a GvHD. There is established consensus that the key initiating factor of aGvHD upon aHSCT is the cytotoxic conditioning regimen of aHSCT itself, a major stress factor that damages the gut barrier, induces local microbial dysbiosis,

inflammation and a cytokine storm allowing the maturation of APCs, and ultimately, the expansion of APC-induced T cells, most prominently CTLs.

- Composition of the microbiome was analyzed by 16S rDNA sequencing of a sample set consisting 503 fecal samples collected from 98 aHSCT patients in 6 time points before and after aHSCT. Out of these, 449 samples of 81 patients were eligible for analysis. Sequencing was done using the Illumina MiSeq platform followed by QIIME-based analysis. The biome information was later analyzed using several downstream modules, among which LEfSe modules were used to discover the microbial communities characterizing different biological conditions. Inter- and intra-sample richness and diversities were measured as alpha and beta diversity, respectively, using the R packages; finally, the random forest analysis was carried out to identify the probable microbial biomarkers that could differentiate the GvHD from non-GvHD condition.
- Results showed that the gut microbiome profile predominantly showcased Bacteroidetes (up to 94%), Firmicutes (up to 6%) and Proteobacteria (up to 8%) at the Phylum level. Further analysis revealed that the Bacteroidetes abundance fluctuated as the days progressed in GvHD patients with nearly 50% reduction on day 100 compared to the pre-treatment timepoint, comparatively the Non-GvHD patients showed a steady decrease from pre-treatment towards the 100th day. Non-GvHD patients when compared with the GvHD had a lesser number of Bacteroidetes at all timepoints.
- On the 100th day GvHD subjects had nearly 80% more Bacteroidetes than the non-GvHD patients. Firmicutes number steadily decreased in GvHD patients from pre-treatment towards the Day 100, whereas the Firmicutes numbers kept fluctuating in Non-GvHD subjects. Bacteroidetes/Firmicutes ratio was almost double in Pre-treatment stage of Non-GvHD compared to GvHD, but the ratio evened out and became almost similar on Day 100.
- GvHD patients displayed a sharp decrease in pathogenic Proteobacteria on day 7, 14 and 21. Non-GvHD patients showed a fluctuation in the case of Proteobacteria, but the numbers were low on the pre-treatment and day 7. In general, proteobacteria numbers were low in non-GvHD subjects compared to GvHD. In Non-GvHD the proteobacteria numbers gradually increased towards day 100 with a little bit of fluctuation between day 14 and 100. Epsilonbacteroeota is prominently found only in GvHD on Day 7 and Day 14.
- Interestingly phylum Epsilonbacteroeota constituted only the species Campylobacter concisus in GvHD. When the dead subjects were compared against the live subjects in GvHD and non-GvHD, Epsilonbacteroeota was found only in the dead subjects indicating the possibility of it being a marker to identify the severe GvHD cases. Random forest analysis on Day 7 and 14 revealed the Campylobacter as one of the biomarkers that can distinguish the GvHD from Non-GvHD. Random forest analysis also discovered Roseburia, Ruminiclostridium, and Escherichia Shigella as the key markers on Day 7 and Day 14 samples. Random forest analysis between the GvHD alive and GvHD subjects reiterated the significance of Campylobacter as a key marker apart from Escherichia Shigella indicating the deterioration of the immune system.
- LEfSe analysis of the subjects indicated that the common gut microbes like Ruminococcaceae, Lachnospiraceae, Agathobacter etc were abolished completely in the GvHD patients as the days progressed. In the case of Non-GvHD patients Negativibacillus, a newly identified gut-colon bacterium was found in the Pre allo-HSCT timepoint and Tyzzerella which is associated to CVD risk in the previous studies were found at 100+ timepoint. Further analysis was carried out using the different timepoints, which indicated a dynamically changing microbial population at different timepoints based on the GvHD status and other clinical conditions.
- Taken together, these observations demonstrate that the architecture of the microbiome is altered in GvHD patients and identifying these changes will contribute to the discovery of potential biomarkers. Further analysis is being carried out to confirm these potential early biomarkers that can help in an effective therapeutic regime.

Results of this project were presented at: Bangarusamy DK$, Lakshmanan AP, Deola S, Kizhakayil D, **Pos Z, Lupsa N, Terranegra A#**: Identification of potential microbiome markers for aGvHD early diagnosis, 46th Annual Meeting of the EBMT, 2020 (**bold**: OTKA K 116340 grant participant $:First author **#:** last author $: corresponding author).

Other research activities supported in part by OTKA K 116340:

In the final section we summarize results obtained from a research project in that, in addition to the above, technical know-how obtained by studying CD8+ T cells in tissues affected by GvHD, free storage capacities and excess analytical time available on research instrumentation used for OTKA K 116340, has been utilized.

- As part of OTKA NN 114460, personnel participating in the present grant, affiliated with the Dept. of Genetics, Cell- and Immunobiology, Semmelweis University, Budapest, Hungary, established collaboration with the Department of Dermatology, Venereology and Dermatooncology, Semmelweis University, Budapest, Hungary
- The objective of this collaboration was to study CD8+ T cells' interactions with tissue fibroblasts (MAFs) present in the stroma of human melanoma tumors.
- It was considered feasible, and also deemed necessary by both collaborating parties to not only combine their research expertise, but also use free instrument time (for sample processing) and excess storage capacities made available by OTKA K 116340 (for biobanking), and also know-how and experimental protocols tested or developed for the purposes of OTKA K 116340 in OTKA NN 114460, as well (tested methodologies for isolation of CD8+ T cells from skin biopsies, GzmB ELISPOT, and medium-throughput 2D protein profiling).
- These studies disclosed that MAFs not only suppress NK cells, as published by others earlier, but are potent suppressors of CD8+ T cell activity in the tissue environment of a melanoma tumor, too.
- We found that MAFs induced selective impairment of several components of the molecular machinery required for proper CD8+T cell activation (NFkB, ERK), maturation (CD69), exertion of CTL functions (GzmB) and also killing, resulting in an anergic T cell phenotype.
- Data were obtained suggesting that these defects are the consequences of compromised intracellular signaling taking place in MAF-exposed CTLs. Data showed skewed intracellular signaling favoring Ag recognition while suppressing cellular perception of co-stimulation, which is a textbook case of aberrant signaling leading to T cell anergy.
- In addition to the above, MAF displayed significantly larger numbers of some negative T cell co-stimulatory molecules (VISTA and HVEM) than healthy normal fibroblasts, and CTL also altered their costimulatory receptor profile in a way that may give way to MAF pressure (TIGIT and BTLA, BTLA being a receptor of HVEM)
- Screening studies were carried out to identify the mechanism of MAF-mediated suppression. It became clear that the effect is not related to MAF TGFβ-1, IL-6, or PGE2 secretion or MAF IDO activity either.
- However, MAF showed unusually high arginase activity. This was of importance because arginase is a known suppressor of T cells, acting via local arginine depletion; L-arginine bioavailability being a rate-limiting parameter in T cell activation and clonal expansion. Of note, arginase activity measured in MAF was so high that it was sufficient to induce NOS uncoupling.
- By generating engineered MAF displaying increased arginase activity, and comparing them with MAF exposed to a potent and selective small molecular arginase inhibitor, we were able to demonstrate that the up-regulation of VISTA and HVEM on CTLs is a dose-dependent reaction to MAF arginase activity. That is, the phenomena described above were indeed direct consequences of MAF's upregulated arginase activity.

Results of the above project have been published in: $**Érsek B**, $Silló P, Ugur C, Molnár V, Bencsik A, Mayer B, Mezey E, Kárpáti S, **Pós Z#&**, Németh K**#**: Melanoma-associated fibroblasts impair CD8+ T cell function and modify expression of immune checkpoint regulators via increased arginase activity, CELLULAR AND MOLECULAR LIFE SCIENCES 2020: p. Epub ahead of print, 2020 (**bold**: OTKA K 116340 grant participant $:First author **#:** last author &: corresponding author).