

HIGH AFFINITY ANTITUMOR HLA CLASS I-RESTRICTED TCRs THAT CAN BE STAINED WITH AN HLA/PEPTIDE MONOMER COMPLEX POSSESS BROAD CROSS-REACTIVITY

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TCR gene therapy is technically feasible and a promising treatment modality for cancer immunotherapy. Safety and efficacy largely depend on the selection of a TCR that induces minimal toxicity while possessing sufficient antitumor reactivity. Many, if not all, TCRs possess unwanted cross-reactivity in addition to desired antitumor reactivity. Interestingly, some TCRs exhibit chain centricity, where recognition of MHC/peptide complexes is dominated by one of the TCR hemi-chains. The shared TCR α gene, clone SIG35 α , recognizes HLA-A*02:01(A2)/MART1₂₇₋₃₅ when paired with various clonotypic TCR β counter-chains. Last year, we had demonstrated that, regardless of their HLA-A2 positivity, a substantial subset of peripheral CD8⁺ T cells transduced with SIG35 α gained reactivity for A2/MART1₂₇₋₃₅. Interestingly, when transduced with SIG35 α , peripheral CD4⁺ T cells, from both A2⁺ and A2⁻ donors, also recognized A2/MART1₂₇₋₃₅ and expanded in an antigen-specific manner upon stimulation. The generated A2/MART1₂₇₋₃₅-specific T cells used various TRBV genes with a high proportion encoding TRBV2, 5-1, or 27, and the CDR3 β sequences were highly diverse. Fourteen clonotypic TCR β chains were randomly chosen and individually reconstituted along with SIG35 α on human TCR $\alpha\beta$ -deficient T cells that do or do not express the CD8 coreceptor. These TCR transfectants possessed high structural and functional avidities. Surprisingly, four out of the 14 CD8⁺ TCR transfectants were clearly stained by an A2/MART1₂₇₋₃₅ monomer complex and two of the four were stained even in the absence of CD8. Importantly, these two highly avid TCR transfectants demonstrated broader cross-reactivity to A2-bound human-derived self-peptides homologous to the A2/MART1₂₇₋₃₅ peptide. These results suggest that affinity-matured and/or thymically unselected antitumor TCRs should be carefully selected for use in TCR gene therapy to avoid the unpredictable risk of TCR cross-reactivity.

REGULATORY INNATE LYMPHOID CELLS SUPPRESS ANTI-TUMOUR T CELLS

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Anti-tumour T cells are subject to multiple mechanisms of negative regulation. Recent findings that innate lymphoid cells (ILCs), including natural killer (NK) cells, regulate adaptive T cell responses led us to examine their regulatory potential in the context of cancer. We have identified a novel ILC subset that regulates the activity of tumour-infiltrating lymphocytes (TIL). Our approach allowed us to isolate human regulatory ILCs from high-grade serous ovarian tumours, define their suppressive capacity *in vitro*, and perform a comprehensive analysis of their phenotype and function. Notably, the presence of regulatory ILCs in TIL cultures correlated with impaired T cell expansion and a striking reduction in the time to disease recurrence in patients. Functional studies revealed that regulatory ILCs suppressed both CD4⁺ and CD8⁺ TIL expansion and cytokine production. ILCs with regulatory potential could be distinguished phenotypically from conventional NK cells and other ILCs, suggesting they may constitute a novel innate lymphocyte population. These studies demonstrate a previously unidentified cell population regulates tumour-associated T cells.

A PHASE II STUDY (NCT01883323) EVALUATING THE INFUSION OF AUTOLOGOUS TUMOR-INFILTRATING LYMPHOCYTES (TIL) AND LOW-DOSE INTERLEUKIN-2 (IL-2) THERAPY FOLLOWING NON-MYELOABLATIVE LYMPHODEPLETION USING CYCLOPHOSPHAMIDE AND FLUDARABINE IN PATIENTS WITH METASTATIC MELANOMA

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The first clinical trial of adoptive cell therapy using tumor-infiltrating lymphocytes (TILs) in Canada is currently accruing at the Princess Margaret Cancer Centre. The trial is a single arm phase II trial in metastatic melanoma, designed to assess safety and response. TILs are expanded *ex vivo* in an initial culture step, and then further expanded in a “rapid expansion protocol” (REP). On day 14 of the REP, the TILs are harvested for infusion. Patients first receive preparative lymphodepletion with cyclophosphamide (60mg/kg for 2 days) and fludarabine (25mg/m² for 5 days). This is followed by TIL infusion (1x10¹⁰ – 1.6x10¹¹ cells intravenously) and a moderate dose interleukin-2 (IL-2) regimen (125,000 IU/kg subcutaneous injection, daily for 2 weeks, with 2 days rest between weeks). This dose of IL-2 is lower than what is commonly used in current TIL protocols, with the aim of reducing the significant treatment-limiting toxicity associated with high-dose IL-2 therapy. The trial follows a two-stage Simon design with a maximum target of 12 evaluable patients. To date, eight patients have received investigational treatment. Cell doses ranged from 5.5x10¹⁰ – 1.6x10¹¹. Response data is pending for the two of the treated patients. For the other patients, the best overall response (RECIST v1.1) was a partial response in two patients and stable disease in four patients. TCR Vβ repertoire analysis of the TIL infusion products suggests that CD8⁺ TILs tended to be more oligoclonal than CD4⁺ TILs. Immune monitoring demonstrates peripheral oligoclonal expansion select TCR Vβ family subtypes in some patients and indicates that putative regulatory T cells did not markedly expand in peripheral blood following moderate dose IL-2 therapy. Our experience shows that TIL-based trials are feasible at our centre. Accrual will continue and data on safety, clinical responses and immune monitoring will be collected.

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PODOCALYXIN AS A PROGNOSTIC MARKER AND TARGET FOR MONOCLONAL ANTIBODY THERAPY IN CARCINOMA

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In kidney podocytes and other tissues, podocalyxin acts as an anti-adhesive surface protein to promote the formation of lumens and reduce intra- and inter- cellular adhesion. Podocalyxin is normally expressed on vascular endothelia, a sub-set of neurons and hematopoietic stem cells and, at much lower levels, on epithelial cells specialized in forming ducts and tubes in several tissues including breast, kidney and pancreas. However, in neoplastic disease, podocalyxin is highly expressed by tumor cells in some highly aggressive adenocarcinomas of the breast, pancreas, kidney, colon and ovary. Podocalyxin tumor expression invariably correlates with poor outcomes (overall survival and disease-free survival) and, in the case of colon cancer, identifies patients that would benefit the most from adjuvant chemotherapy. These findings indicate that podocalyxin is an important marker of prognosis and may also facilitate treatment decisions in the clinic.

To determine if podocalyxin, beyond acting as a prognostic marker, has a functional role in tumor progression and metastasis we silenced podocalyxin expression in MDA-MB-231 cells; a basal-like breast cancer cell-line that expresses high levels of endogenous podocalyxin and displays aggressive tumor growth and metastasis in xenografted mouse models. We found that deletion of podocalyxin attenuated both the growth of primary tumors and formation of distant metastases. Next, we developed a novel monoclonal antibody (PODOC1) against tumor-expressed human podocalyxin. We found that PODOC1 delays MDA-MB-231 Matrigel invasion *in vitro*. Moreover, systemic treatment of tumor-bearing mice with PODOC1 inhibits both primary tumor development and metastatic progression. These results suggest that targeting podocalyxin expression is a selective therapeutic approach for breast cancer. Importantly, because podocalyxin expression is associated with metastases in many other tumor types, podocalyxin-targeted therapies may be a widely applicable in the treatment of carcinomas.

RECEPTOR FOR LACTATE DEHYDROGENASE V IS A NOVEL THERAPEUTIC TARGET FOR GLIOBLASTOMA

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In order to develop effective immunotherapies for cancer it is important to understand the crosstalk between the tumor and the tumor-supportive immune cells in the microenvironment. In patients with aggressive brain tumors, such as the Grade IV glioblastoma (GBM), myeloid cell accumulation supports local immunosuppression, angiogenesis, and chemoresistance. We have recently shown that extracellular, GBM-derived lactate dehydrogenase 5 (LDH5) influences gene expression in myeloid cells to generate a natural killer cell-suppressive phenotype. Although extracellular LDH5 is not known to have a role in cell signaling, we hypothesized that LDH5 found in the highly necrotic tumor, mediates crosstalk between tumor cells and myeloid cells, influencing the functions of both populations. We found that macrophages, monocytes, and GBM cells all internalize LDH5 in a manner consistent with receptor mediated endocytosis, a function that can be blocked with a monoclonal antibody against LDH5. We found that LDH5 alters the metabolic phenotype of macrophages by increasing ATP production, lactate export, and glycolytic rate following oligomycin administration. Monocytes treated for 24 hours with LDH5 have decreased levels of intracellular reactive oxygen species and a depolarized mitochondrial membrane potential with no change in viability. GBM tumor cells treated with LDH5 also exhibit depolarized mitochondria, without changes in mitochondrial mass or cell viability. Interestingly, under hypoxic conditions, exposure of these cells to LDH5 causes increased levels of intracellular GTP, while under normoxic conditions, GTP is reduced by LDH5. This suggests that LDH5 helps to tolerize tumor and immune cells to an environment with extensive necrosis and reduced oxygen, both hallmarks of GBM. Our data indicate a receptor for LDH5 that could act as a therapeutic target by altering metabolic functions of tumor cells and associated immunosuppressive myeloid cells in the tumor microenvironment.

MUTATION REACTIVE AUTOLOGOUS T CELLS FOR THE TREATMENT OF PANCREATIC CANCERS

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Advances in Adoptive Cell Therapy (ACT) - which involves isolation of natural tumor infiltrating T cells (TILs), *ex vivo* activation and expansion, and then re-delivery to the patient – has proven successful for some patients. While the antigens mediating TIL reactivity remain largely unknown, examples of T cells that respond to single amino acid changes originating from somatic tumor mutations have been described. These types of neoantigens, when originating from common recurrent tumor mutations, could be broadly useful ACT targets. KRAS codon 12 mutations are the most common recurrent point mutations in human cancers and account for more than 90% of all pancreatic ductal adenocarcinomas (PDACs). Given the predominance of these KRAS mutations, and their potential immunogenicity from previous studies, we carried out an analysis of The Cancer Genome Atlas (TCGA) data to explore the frequency of predicted immunogenic mutant KRAS epitopes and their HLA restriction patterns. We identified HLA allele A*02:01 as the most common restricted allele for the predominant KRAS aa 5-14 epitopes.

Previous studies have tested MHC presentation of synthetic mutant KRAS peptides, however there has been no demonstration of the natural presentation of mutant KRAS peptides. To model KRAS mutant epitope presentation, we used PANC-1 cells, which are hemizygous for the KRAS G12D mutation and are HLA-A*02:01 positive, were examined using weak acid elution and Mass Spectroscopy-Multiple Reaction Monitoring (MS-MRM). We identified approximately 1201 copies of the mutant G12D peptide (KLVVVGADGV) per cell, compared to 10839 copies of the wild-type peptide (KLVVVGAGGV).

We propose to activate and expand T cells from PDAC patient peripheral blood, and isolate mutation-reactive cytolytic T-cell clones for subsequent ACT, as a secondary therapy after primary treatment, to prevent recurrence. Our approach leverages the mutation specificity of PDACs and the convenience of peripheral blood as a source of diverse, viable and non-exhausted cytolytic T cells.

HUMAN REGULATORY T CELL ISOLATION IN 55 MINUTES USING EASYSEP™ RELEASABLE RAPIDSFERES™

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The preferred marker for identifying regulatory T cells (Tregs) is FOXP3 but its intracellular localization currently precludes it from being used for isolating viable cells. Human FOXP3⁺ Tregs are characterized as CD4⁺ T cells expressing high levels of CD25 and low levels of CD127. We have developed a novel immunomagnetic approach for the isolation of magnetic-particle-free human Tregs from PBMCs in as little as 55 minutes. This offers a vast improvement in speed over other immunomagnetic Treg isolation approaches, which typically require more than 2 hours. The standard approach for isolating Tregs combines CD4⁺ T cell negative enrichment followed by CD25 positive selection. By contrast, our novel approach is to first perform the CD25 positive selection step with EasySep™ Releasable RapidSpheres™, a new type of magnetic particle that can be rapidly released from the isolated cells with a no-incubation, no-wash protocol performed at room temperature. Subsequent depletion of CD127^{high} and non-CD4⁺ T cells is achieved by negative enrichment with EasySep™ Dextran RapidSpheres™. Starting with 5x10⁷ PBMCs, a purity of 87.6% ± 3.1% and recovery of 2.7x10⁵ ± 8.2x10⁴ Tregs (CD4⁺CD127^{low}CD25⁺FOXP3⁺) can be achieved (*n*=6). EasySep™ isolated Tregs can be expanded *ex vivo* using our novel ImmunoCult™ CD3/CD28/CD2 T cell Activation and Expansion Supplement and ImmunoCult™-XF, a serum- and xeno-free culture medium. Taken together, this is the first EasySep™ kit to incorporate the EasySep™ Releasable RapidSpheres™ and it exemplifies the potential of this technology for use in rapidly isolating functional, magnetic particle-free cell populations possessing complex cell surface phenotypes.

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"RE-STIMULATED" TUMOR-INFILTRATING LYMPHOCYTES AND LOW-DOSE INTERLEUKIN-2 THERAPY IN PATIENTS WITH PLATINUM RESISTANT HIGH GRADE SEROUS OVARIAN, FALLOPIAN TUBE, OR PRIMARY PERITONEAL CANCER (NCT01883297)

There is increasing evidence that epithelial ovarian carcinoma is immunogenic and may respond to immunotherapy. Adoptive cell therapy with autologous tumor-infiltrating lymphocytes (TILs) has shown remarkable clinical activity in early phase trials in the setting of metastatic melanoma. We have developed a clinical trial based on the adoptive transfer of TILs for ovarian cancer. Our preclinical work focused on evaluating TIL expansion from ovarian tumors, and also on developing a novel *in vitro* method of enhancing TIL function prior to adoptive transfer. In this method, expanded TILs are re-stimulated *in vitro* for one day with anti-CD3 mAb, IL-2 and autologous dendritic cells matured with TNF, IL-1 β and IL-6. The "Re-stimulated TILs" are then harvested for infusion.

The clinical trial is a first-in-human, phase I, single centre study with the primary endpoints of feasibility and safety. Secondary endpoints are clinical and immunological responses. The trial design is a standard 3+3 dose escalation, with the following three dose levels: 3×10^7 , 1×10^8 then 3×10^8 Re-stimulated TILs. The investigational treatment involves preparative lymphodepletion with cyclophosphamide (30mg/kg/day x 2 days), followed by intravenous infusion of Re-stimulated TILs. Interleukin-2 therapy commences thereafter (125,000 IU/kg/day subcutaneous injection for ≤ 10 doses). With the aim of reducing acute IL-2-related toxicities, our protocol uses a lower dose regimen of IL-2 therapy compared to that used in the landmark TIL protocol developed at the National Cancer Institute. Eligible patients must have measurable platinum-resistant disease, ECOG ≤ 1 and adequate organ function. Radiological response by RECIST v1.1 and immune-related Response Criteria is performed 4 weeks post TIL infusion and 3 monthly thereafter. Initial immune monitoring will focus on tracking the phenotype and persistence of TILs after transfer.

TUMOR INFILTRATING PLASMA CELLS ARE ASSOCIATED WITH TERTIARY LYMPHOID STRUCTURES AND PROTECTIVE IMMUNITY IN HUMAN OVARIAN CANCER

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Rationale: Tumor-infiltrating plasma cells (PCs) and their associated autoantibodies are present in ovarian and other cancers, but their contribution to tumor immunity remains poorly understood. In autoimmunity, PCs contribute to antigen spreading to CD4+ and CD8+ T cells, leading to exacerbated tissue destruction. Based on this, we reasoned that PCs might enhance tumor-infiltrating T cell (TIL) responses in cancer.

Hypothesis: Tumor-infiltrating PCs are associated with cytotoxic T cell responses and patient survival in ovarian cancer.

Methods: We used multicolor immunohistochemistry, flow cytometry, and gene expression analysis to study immune responses in three cohorts of high-grade serous ovarian cancer (HGSC) patients.

Results: Approximately 20% of HGSC cases contained PC infiltrates, which constituted up to 90% of cells in tumor stroma. PC infiltrates were associated with tertiary lymphoid structures (TLS), which exhibited germinal centres and other hallmark features of lymph nodes. Tumors containing PC infiltrates had significantly increased densities of CD8+ TIL and were associated with favorable prognosis. Analysis of gene expression data from The Cancer Genome Atlas revealed a two-gene plasma cell signature associated with prolonged survival and expression of genes involved in cytotoxic immune responses, lymphocyte recruitment, PC survival, and TLS formation. This signature was also associated with expression of a greater number of cancer-testis (CT) antigens.

Conclusions: Tumor-infiltrating PCs are associated with increased CD8+ TIL, cytotoxic gene signatures, and CT antigen expression in HGSC. This suggests PCs may serve to amplify cytotoxic T cell responses. We are currently cloning IgG from single sorted PCs to identify their target antigens and to determine whether these are also recognized by CD4+ and CD8+ TIL. A better understanding of tumor-infiltrating PCs and their autoantibody targets may lead to new strategies to enhance CD4+ and CD8+ TIL responses.

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CHARACTERIZATION OF ACTIVATED TUMOR-INFILTRATING B LYMPHOCYTES

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Tumor-infiltrating B-lymphocytes (TIL-B), expressing CD19⁺ or CD20⁺, have been identified in several cancers. Although their presence frequently correlates with a positive clinical outcome, their heterogeneity and function are not well characterized.

Aim: To characterize the differentiation and activation status of TIL-B in lung and kidney tumors, and define their function.

Methods: PBMCs and TILs were isolated from same patients of lung or kidney cancer, and then stained for B cell differentiation and activation markers using flow cytometry (e.g. CD19, CD20, CD24, CD25, CD38, CD69, CD80, CD86, IgD) (n=5-10). In addition, B cells of cancer patients were *in vitro* cultured in simulating conditions (e.g. with sCD40L/IL-4) (n=5-7).

Results: Most circulating B cells were IgD⁺ cells, including 5% transitional memory CD24⁺⁺ CD38⁺⁺ cells, while lung and kidney TIL-B were equally further differentiated, being IgD⁻ (60-70%) (p<0.05). In addition, more CD69⁺ and CD86⁺ cells, and fewer CD95⁺ expression is observed amongst IgD⁻ TIL-B than circulating PBMCs (p<0.05), indicating the presence of recently activated B cells in the tumor. Increased activation is also supported by the presence of cytokine-producing cells and by the modulated expression of chemokine receptors (CXCR4, CCR6 and CCR10). In addition, we observed an enhanced expression of CD25, CD69, CD80 and CD86 (p<0.05), as activation markers, and the production of cytokines like TNF on *in vitro* cultured B cells.

Conclusions: B cells infiltrating tumors are more differentiated and activated when compared to circulating B cells in the same patient. Tumor-infiltrating B cells can be activated and expanded *in vitro*. Understanding the tumor-infiltrating B cells and standardizing method to expand competent B cells would promote the development of adoptive cell therapies against cancer.

A VERSATILE HIGH-THROUGHPUT MICROFLUIDIC PLATFORM FOR ANTIBODY DISCOVERY FROM NATURAL IMMUNE REPERTOIRES

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The large majority of monoclonal antibody (mAb) therapeutics have been generated and isolated from natural immune systems, typically using murine hybridomas. As the “low-hanging” fruits of antibody discovery targets have become saturated, next-generation antibody discovery approaches hold high potential for finding improved mAbs with optimal properties, for addressing targets that have proven difficult or intractable by traditional methods, and for harnessing novel sources of antibody diversity.

We have developed an antibody discovery platform based on microfluidic selection of mAbs directly from single immune cells. Antibody-secreting cells (ASCs) from patients or immunized animals are loaded and compartmentalized into microfluidic devices containing arrays of nanoliter-volume chambers, enabling the detection of antibody secretion from single cells within minutes. Integrated microfluidics allow for the programmed exchange of reagents to implement a wide array of selection assays including bead-based binding measurements, as well as cell-based assays of binding or function. A fully automated instrument enables the identification and recovery of selected ASCs based on fluorescent imaging and analysis. Recovered cells are then analyzed for high-efficiency recovery (>75%) of paired heavy and light chain variable region sequences using a next-generation sequencing approach. This platform allows for the multi-parameter antibody selection from any species, with throughput of over 1,000,000 cells per run and recovery of over 100 paired sequences in approximately 7 days.

We will present preliminary data in the isolation of antibodies against ion channel (Nav1.7) and GPCR (CXCR4) targets in mice and rabbits. We will further demonstrate our platform in the isolation of fully human mAbs from blood samples, including antibodies cross-reacting with different influenza hemagglutinin types, and targeting virulence factors from a bacterial pathogen. In the later we will show that very deep screening (~1,000,000 ASCs) allows for the detection and isolation of ultra-rare antigen-specific mAbs from healthy human patients with no detectable titers.

IFN γ -armed oncolytic virus for cancer treatment

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Oncolytic viruses are known to stimulate the immune system but the extent by which they revert tumor-induced immune suppression is not well characterized. By specifically replicating in tumor cells, they are believed to trigger immune activation through the release of tumor-specific antigens in the context of viral pathogen associated molecular patterns to allow for simultaneous TLR co-stimulation. In this study, we used 4T1 mammary adenocarcinoma and CT26 colon carcinoma murine tumor models to demonstrate the general activation of immune cells by treatment with vesicular stomatitis virus. We observed a rapid and sustained upregulation of the pro-inflammatory cytokines IFN γ , IL-6 and TNF α in the blood following treatment. Also, flow cytometry analysis showed a greater activation of dendritic cells, natural killer cells and T cells in the blood and the spleen of VSV-treated animals. Moreover, an ELISPOT assay allowed us to demonstrate the presence of an increased number of IFN γ -secreting splenocytes following virus treatment. As a further means to activate immune responses, an oncolytic virus was engineered to encode the pro-inflammatory cytokine IFN γ . This virus demonstrated greater activation of dendritic cells and secretion of proinflammatory cytokines. The VSV-IFN γ virus treatment slowed tumor growth, minimized lung tumors and prolonged survival in both tumor models. Taken together, these results show the great potential of oncolytic viruses as immune stimulators to generate a tumor-specific immune response as well as their potential in targeted gene therapy by expression of beneficial genes specifically within the tumor.

PD-1 EXPRESSION CORRELATES WITH FAVORABLE PROGNOSIS IN HIGH- GRADE SEROUS OVARIAN CANCER AND IS SELECTIVELY EXPRESSED ON INTRAEPITHELIAL (CD103⁺) TUMOR-INFILTRATING CD8 T CELLS

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Rationale: PD-1 is an important immunoregulatory molecule in cancer, but the mechanisms that regulate its expression *in vivo* are poorly understood. We recently reported that $\alpha_E(CD103)\beta_7$ specifically demarcates 'intraepithelial' CD8⁺ tumor-infiltrating lymphocytes (ep-CD8 TIL) in ovarian cancer. $\alpha_E(CD103)\beta_7$ is a TGF- β -regulated integrin that mediates retention of lymphocytes in peripheral tissues by binding to E-cadherin expressed on epithelial cells. We hypothesized that $\alpha_E(CD103)\beta_7$ -mediated retention of ep-CD8 TIL within tumor epithelium might also lead to PD-1 expression by facilitating chronic exposure to tumor antigen.

Methods: PD-1⁺ TIL were enumerated in a large cohort of ovarian tumors (N = 489) with known CD103⁺ TIL content. Multi-parametric immunohistochemistry and flow cytometry were used to assess the intratumoral location and functional phenotype of TIL in prospectively collected specimens.

Results: PD-1⁺ cells were present in 38.5% of high-grade serous carcinomas (HGSC) but were less prevalent in other histological subtypes. PD-1 expression strongly associated with increased survival in HGSC (hazard ratio=0.4864; *P*=0.0007). PD-1 expression was largely restricted to the CD3 T cell compartment and was expressed on a variable proportion of both CD4 and CD8 T cells. There was a high degree of PD-1 and CD103 co-expression within the CD8 TIL compartment, and PD-1⁺CD103⁺ CD8 TIL were preferentially localized to intraepithelial regions of the tumor. PD-1⁺CD103⁺ CD8 TIL appeared quiescent when assessed directly *ex vivo*, yet were capable of robust cytokine production after stimulation *in vitro* with PMA/ionomycin. Moreover, they showed negligible expression of additional exhaustion-associated markers including TIM-3, CTLA-4 and LAG-3.

Conclusion: As hypothesized, the vast majority of CD8⁺CD103⁺ TIL express PD-1, consistent with prolonged exposure to tumor antigens. Nonetheless, they remain functionally competent and prognostically favorable, indicating they are not terminally exhausted. We speculate that, after standard treatment, PD-1⁺CD103⁺ CD8 TIL might regain anti-tumor activity in vivo, an effect that could potentially be augmented by immune modulation.

EVALUATING THE IMMUNOGENICITY OF THE OVARIAN CANCER MUTANOME

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A hallmark of cancer is the accumulation of mutations that allow cells to proliferate uncontrollably. A small proportion of tumor mutations can give rise to mutant peptides bound to MHC I or II, enabling recognition by host T cells. While mutations are a significant source of tumor antigens in cancers with high mutation loads, such as melanoma, far less is known about cancers with average mutation loads, such as ovarian cancer (mean 12.9 versus 1.7 coding mutations/megabase, respectively). Moreover, most studies to date have evaluated pre-existing T cell responses to mutations. Far less is known about mutation-specific vaccine-induced T cell responses. **We hypothesized that mutation-specific peptide vaccines can elicit therapeutic T cell responses towards ovarian cancer.** To test this concept, ID8-G7 mouse ovarian tumor cells underwent exome and RNA sequencing. We identified 114 somatic point mutations, 44 of which were non-synonymous and transcribed. Expressed mutations were ranked according to predicted MHC class I binding scores (NetMHCpan). 29-mer peptides encompassing the 21 top-ranked mutations were individually co-injected with poly(I:C) into naive mice. 15/21 mutated peptides elicited peptide-specific CD4+ and/or CD8+ T cell responses. For 9 peptides, T cell responses were specific for the mutated (versus wild type) peptide. However, none of the T cell lines recognized ID8-G7 tumor cells by ELISPOT. Furthermore, neither prophylactic nor therapeutic vaccination with mutated peptides extended survival of ID8-G7 tumor-bearing mice. We conclude that none of 21 evaluated mutations gave rise to naturally processed MHC class I or II epitopes to confer tumor cell recognition by CD4+ or CD8+ T cells. Using the epitope prediction parameters from this study as a guide, we are currently analyzing data from The Cancer Genome Atlas to determine the average number of potentially targetable mutations in human ovarian cancer with the goal of developing mutation-specific immunotherapies for this disease.

RAPID EXPANSION OF FUNCTIONAL HUMAN T CELLS USING A NOVEL SERUM-FREE AND XENO-FREE CULTURE MEDIUM

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The adoptive transfer of functionally active, genetically modified T cells encoding receptors for tumor antigens is a promising treatment strategy in cancer immunotherapy. To produce an adequate number of these T cells for therapeutic efficacy, expansion in vitro is necessary. Traditionally, T cells are activated and expanded in media that contain human serum to promote cell growth and viability. However, because serum contains many uncharacterized components and possible infectious agents, a defined, serum-free medium is preferable.

We have developed a novel serum-free and xeno-free T cell expansion medium called ImmunoCult-XF that allows for the rapid expansion of activated T cells. T cells (5×10^4 cells/mL) immunomagnetically isolated from peripheral blood were activated using a soluble CD2/CD3/CD28 activation reagent and expanded in ImmunoCult-XF supplemented with IL-2 (10 ng/mL) for 21 days, with re-activation every 6-8 days. The expansion of viable CD3⁺ cells averaged 2,700-fold after 21 days in ImmunoCult-XF (n=12). This level of expansion was similar to that in X-VIVO 15 medium supplemented with 5% human serum (2,400-fold; n=8), and significantly higher than those in all other serum-free media tested (8-160 times higher; p<0.05; n=6). After activation and expansion, the T cells converted to a CD45RA⁻CD45RO⁺ memory phenotype (38±5% on day 0 versus 100±0.02% on day 21; n=4), while the frequencies of CD4⁺CD8⁻ and CD4⁻CD8⁺ T cells remained relatively unchanged (CD4⁺CD8⁻: 57±13% on day 0 versus 62±18% on day 21; CD4⁻CD8⁺: 30±11% on day 0 versus 23±16% on day 21; n=11). Finally, the expanded T cells were functional as demonstrated by intracellular IL-4 and IFN-gamma expression (45±16% IFN-gamma⁺ cells and 28±15% IL-4⁺ cells; n=6) 4 hours after stimulation with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 µg/mL). Taken together, these results demonstrate that large numbers of functional T cells can be generated in vitro under a completely serum-free culture condition.

DEVELOPMENT OF A CELL-BASED IL-12 CANCER IMMUNOTHERAPY USING A GENETICALLY ENGINEERED MOUSE MODEL OF BREAST CANCER

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Interleukin-12 (IL-12) is a pro-inflammatory cytokine that plays a major role in the immune response. IL-12 causes increases in interferon-gamma (IFN- γ) production, leads to a Th1 differentiation bias and cell-mediated immunity. Due to its many potent pro-inflammatory effects, the use of IL-12 for cancer immunotherapy continues to be the subject of clinical interest. Many of the IL-12 mediated therapies developed in the past failed clinical trials due to toxicity associated with high systemic IFN- γ . To reduce systemic levels of IL-12 we are using autologous cancer cells to deliver high levels of IL-12 at the local site where the immune system meets the cancer cell. Upon injection, these IL-12 transduced host tumour cells cause sustained immune mediated rejection, even to challenges with the original non-transduced parent tumour. The effectiveness of this model has thus far been demonstrated in murine leukaemias and various solid tumour models. Current work in our lab looks at the development of a transgenic model of breast cancer, which will be used to demonstrate that IL-12 cancer immunotherapy can be expanded to clinically relevant models. Our objective is to develop a clinically relevant model of cell-based IL-12 cytokine immunotherapy using the MMTV-PyMT breast cancer model

It has been demonstrated that primary MMTV-PyMT breast cancer cells can be maintained in culture and enriched for an epithelial tumour forming population. From this enriched population, clones of non-transduced and IL-12 transduced cell lines have been generated. Subcutaneous injection of non-transduced PyMT cells results in tumour development, while injection of IL-12-transduced PyMT cells results in tumour clearance.

CD4⁺ T CELL PLASTICITY ENGENDERS ROBUST IMMUNITY IN RESPONSE TO IL-12 CYTOKINE THERAPY

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Inciting the cellular arm of adaptive immunity has been the fundamental goal of cancer immunotherapy strategies, specifically focusing on inducing tumour antigen-specific responses by CD8⁺ cytotoxic T lymphocytes (CTL). However, there is an emerging appreciation that the cytotoxic function of CD4⁺ T cells can be effective in a clinical setting. Harnessing this potential will require an understanding of how such cells arise. In this study we use an IL12-transduced variant of the 70Z/3 leukaemia cell line, LV12.2, in a B6D2F1 (BDF₁) murine model system to reveal a novel cascade of cells and soluble factors that activate anti-cancer CD4⁺ killer cells. We show that natural killer T (NKT) cells play a pivotal role by activating dendritic cells (DC) in a contact-dependent manner; soluble products of this interaction, including MCP-1, propagate the activation signal culminating in the development of CD4⁺CTLs that directly mediate an anti-leukaemia response while also orchestrating a multi-pronged attack by other effector cells. A more complete picture of the conditions that induce such a robust response will allow us to capitalize on CD4⁺ T-cell plasticity for maximum therapeutic effect.

A NON-HUMAN PRIMATE MODEL OF CYTOKINE RELEASE SYNDROME (CRS): TOWARDS IMPROVED SAFETY OF CHIMERIC ANTIGEN RECEPTOR (CAR) T CELL THERAPY

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Adoptive T-cell therapy using Chimeric Antigen Receptor (CAR) T cells has produced encouraging results in clinical trials. In particular, CAR T cell therapy against CD19, a molecule commonly expressed in B cell malignancies, produced complete remissions in 90% of patients with refractory B-cell acute lymphocytic leukemia (ALL). However, this novel and powerful immunotherapy is not without significant side effects, including cytokine release syndrome (CRS), which occurs in the majority of patients. CRS manifests clinically when high levels of inflammatory cytokines are released and includes fever, hemodynamic instability as well as neurologic toxicity. Although CRS is usually a self-limited complication, severe CRS can develop in >25% of patients and can be life-threatening. Given the fact that CRS is associated with increased levels of inflammatory cytokines, the treatment of CRS requires immunosuppressive agents, which are antagonistic to and thus diminish the effectiveness of CAR T cells.

We aim to create a non-human primate model using *Macaca mulatta* that closely recapitulates CRS observed in patients using CD20 CAR. Once the CRS model is established, our goal is to modify CAR T cells by imparting immunosuppression resistance, thereby allowing the use of immunosuppressive agents in patients to mitigate the severity of CRS without compromising the anti-leukemia potential of the CAR T cells.

We investigated the rhesus B-lymphoblastoid cell lines (B-LCLs) for CD20 expression and found it to be similar to human LCLs. We further demonstrated *in vitro* activity of human and rhesus CD20 CAR T cells against rhesus B-LCLs in chromium release and cytokine release assays. We subsequently transduced rhesus T cells to a high efficiency (60-75%) to express either green fluorescence protein (GFP) or the CD20 CAR and have expanded them *ex vivo* to quantities sufficient for autologous infusion and have currently begun to test their effects *in vivo*.

RAPID ISOLATION OF HIGHLY PURE T, B, AND NK CELLS IN 8 MINUTES

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Studying the complex interaction between the immune system and cancer requires the isolation of highly pure cell populations, an often time-consuming step in the experimental workflow. To streamline this step, we have developed improved EasySep™ kits for the isolation of human T, B and natural killer (NK) cells in as little as 8 minutes. The improved kits offer the fastest method for isolating untouched human lymphocyte subsets from fresh or previously frozen peripheral blood mononuclear cells (PBMCs), or from leukapheresis samples, without the need to lyse red blood cells.

The column-free, immunomagnetic EasySep™ negative isolation procedure involves labelling and removing unwanted cells using bispecific antibody complexes, which crosslink cell surface antigens to magnetic particles, then separating the cells using a magnet. To achieve faster and simpler separations we have developed novel magnetic particles, EasySep™ Dextran RapidSpheres™, which provide rapid magnetic labelling and separation. The EasySep™ antibody cocktail is added, followed by a 5-minute incubation. The magnetic particles are then added, and the sample tube is placed immediately into a hand-held EasySep™ magnet. After a 3-minute separation, the desired cells are collected by simply pouring the sample into a new tube.

Our new kits offer a large improvement in speed without any compromise in the quality of the isolated cells: purities of $97.5 \pm 0.4\%$ (average of $n=10 \pm SD$) were achieved for $CD3^+$ T cells, $96.5 \pm 0.9\%$ ($n=10$) for $CD4^+$ T cells, $87.8 \pm 3.2\%$ ($n=10$) for $CD8^+$ T cells, $95.5 \pm 2.2\%$ ($n=18$) for B cells, $95.8 \pm 1.6\%$ ($n=18$) for naïve B cells, and $84.0 \pm 8.1\%$ ($n=66$) for NK cells. We additionally show that the isolated cells are functional; for example, they can be readily expanded *in vitro*. The new 8-minute EasySep™ cell isolation kits facilitate the preparation of highly purified untouched and functional cells.

CHITOSAN THERMOGELS FOR LOCAL T LYMPHOCYTE DELIVERY FOR CANCER IMMUNOTHERAPY

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Introduction:

The success of systemic adoptive T cell transfer lies in the capacity of the antigen-experienced cytotoxic T lymphocytes (cTL) to access and persist within the tumour microenvironment. The mimicking of tertiary lymphoid structures (TLS) that promote a protective immune response against cancer can be achieved using an injectable biocompatible matrix releasing anti-tumour proliferating cTL. Prime candidates for this application are liquid, chitosan-based, biocompatible thermogels which rapidly gelify at physiological temperatures. Therefore, we aimed to fine-tune an injectable chitosan-based thermogel formulation that would provide an environment permitting the three-dimensional (3D) proliferation and release of cTL whose activation state can be influenced by the surrounding conditions. We have developed a novel formulation that is cytocompatible and injectable, and that has ideal mechanical properties and porosity for T cell encapsulation and growth. With such promising characteristics, we hypothesize that the injection of these cTL loaded hydrogels into the tumour microenvironment will provide a means for a continuous delivery of cTL towards the reprogramming of inflammation mechanisms and the reduction of tumour burden.

Materials and Methods:

Novel T-cell cytocompatible chitosan thermogels were prepared using combinations of gelling agents. Their rheological properties, mechanical strengths, pH, osmolality, and morphology were evaluated. Three formulations were selected for human T cell encapsulation. Biocompatibility was assessed using live/dead staining and fluorescent microscopy. Thermogel- and supernatant-derived T cells and tumour infiltrating lymphocytes (TIL) were immunophenotyped over time using flow cytometry.

Results and Conclusion:

We have optimized thermogel formulations supporting the encapsulation of cTL *in vitro*. Flow cytometry and microscopy demonstrate which novel thermogel formulation is best suited for cell viability, proliferation, and escape over time, along with the maintenance of cTL cellular phenotype and an activation status that can be influenced by surrounding conditions. Our injectable 3D lymphocyte cultures may serve to complement current adoptive cell transfer immunotherapies.

TOLL-LIKE RECEPTOR LIGANDS DELAY ACUTE LYMPHOBLASTIC LEUKEMIA ONSET VIA DEPLETION OF PRE-LEUKEMIC CELLS

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Onset of pediatric acute lymphoblastic leukemia (ALL) depends on the long-term survival and evolution of a pre-leukemic cell population that arises during in-utero. Detection of chromosomal translocations indicates that about 1% of all newborns harbor early-occurring abnormal cells; however, only 1 in 100 of these infants will progress to leukemia. This reveals that progression to leukemia is not the inevitable fate of these early-occurring abnormal cells.

Infection has been postulated to play a role in the development of pediatric ALL. While epidemiologic studies have implicated exposure to infection as a protective factor, the impact of immune modulation during the pre-leukemic phase has not been reported. In this study, we use the B cell precursor (BCP) leukemia-prone Emu-RET transgenic mouse to investigate whether exposure to infection associated danger signals influences disease progression via changes in pre-leukemic cell survival.

Using splenocyte cultures from pre-leukemic mice, we observed significant depletion of pre-leukemic cells following incubation with a panel of ligands for infection-related pattern recognition receptors. The strongest activity was observed with ligands for Toll-like receptors 7/8 (R848), and 9 (CpG) with >75% reduction in pre-leukemic cell number (p<0.01).

We were able to determine that this mechanism works primarily through indirect pathways, and is mediated almost exclusively by soluble factors produced by responding immune effector cells.

Consistent with these in vitro results, we have previously shown that administration of CpG to leukemia-prone mice early in life reduced the size of the pre-leukemic cell population and significantly delayed disease onset (p<0.0001).

Our results are the first to demonstrate that exposure to infection-related danger signals alters leukemia progression through the reduction in pre-leukemic cell viability. These findings provide mechanistic support for the protective effects of early-life infections and suggest novel strategies to eliminate the early-occurring abnormal cells that can give rise to initial disease and relapse.

Our lab has developed a new approach focusing on Surgery for Mesothelioma After Radiation Therapy (SMART), with promising results in a phase I/II clinical trial. We believe that radiation is important to achieving activation of the immune system and may contribute to the benefits observed in patients.

Purpose: To develop a mouse model to analyze the immunogenic effect of Local Radiation Therapy (LRT) and its impact on immune cell recruitment and activation in the context of MPM.

Hypothesis: LRT administered to a tumor before surgical debulking is more immunogenic than surgery alone and contributes to tumor free survival.

Methods AE17 cells transfected with Ovalbumin (OVA) were injected subcutaneously into the flank of C57BL/6 mice and the tumor was radiated with a total dose of 15 Gy delivered in three 5 Gy fractions over 3 days. Mice were divided into three groups: 1) Surgery alone, 2) LRT before surgery, 3) Surgery before LRT. Mice without tumor recurrence were rechallenged in the opposite flank after 40 days. Blood, spleen and lymph nodes were analyzed by flow cytometry to evaluate proliferation and activation of anti-tumor specific CD8 T cells. Tumors were analyzed by immunohistochemistry.

Results: Mice receiving LRT before and after surgery had greater tumor free survival and higher cure rate than mice receiving only surgery. Cured mice that received radiation first rejected the tumor when rechallenged 5 out of 10 times. Activated anti-tumor specific CD8 T cells were more abundant in the group that received LRT prior to surgery, and a greater proportion of CD8 T cells were observed infiltrating the tumor.

Conclusions: LRT before surgical resection of a tumor induces the greatest proliferation and activation of specific anti-tumor CD8 T cells. These specific anti-tumor T cells may be responsible for rejection of the tumor after rechallenge and longer tumor free survival.

A NOVEL HIGH-THROUGHPUT SCREENING APPROACH FOR THE DETECTION OF CYTOTOXIC T-CELL RECEPTOR EPITOPES

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One of the largest remaining hurdles in current adoptive cancer immunotherapy protocols is the lack of knowledge regarding the antigenic determinants of a tumor that elicit tumor-infiltrating lymphocyte reactivity. Existing methods in use for T-cell antigen identification are limited in scalability and reliability and, therefore, require the biased selection of small candidate antigen panels to systematically pan for positive hits.

We are developing and validating a novel methodology for identification of T-cell antigens capable of interrogating orders of magnitude more potential antigens than conventional methods. Our approach involves the use of a FRET active granzyme-B (GzmB)-cleavable reporter gene linked to a library of short peptide encoding sequences to be screened. This construct is delivered into seromatched antigen presenting cells (APC) by lentiviral gene transfer. Transduced target cells which receive a dose of GzmB from co-cultured CTL lines of interest can be detected via cleavage of the encoded reporter protein and isolated by FACS. The identity of recovered epitopes is then determined using next-generation sequencing. To our knowledge, this is the first system to employ target cells, rather than CTLs themselves, as a functional readout allowing for target cells to be pooled, screened, and recovered for direct identification of response-eliciting antigens.

Our results to date indicate that this sort-and-sequence approach is able to specifically distinguish the correctly targeted epitope out of a mixed cell population by recovering sequencing reads enriched with antigenic sequences relative to baseline. Ongoing experiments are being conducted to assess sensitivity by isolating sequences from endogenous cDNA libraries as well as signal-to-noise ratio by screening CTL against libraries of random background sequences seeded with positive epitope sequences.

T-CELL ANTIGEN COUPLER (TAC TECHNOLOGY)

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Engineering T cells with chimeric antigen receptors (CARs) is proving to be an effective method for directing T cells to attack tumors in an MHC-independent manner. Current generation CARs aim to recapitulate T cell signalling by incorporating modular functional components of the TCR and co-stimulatory molecules. Development of next generation CARs has relied upon trial and error evaluation of signalling domains. We sought to develop an alternate method to re-direct the T cell receptor which does not rely upon the incorporation of signalling domains into the chimeric receptor. To this end, we developed the T-cell Antigen coupler (TAC) technology. The membrane-anchored TAC redirects the TCR in the presence of tumor antigen. We also included components of the CD4 co-receptor to provide requisite Lck signaling upon ligation of the tumor antigen. Our prototype receptor was directed against the HER-2 proto-oncogene. We have determined that engineering peripheral blood T cells with this novel receptor (CD4-TAC) engenders tumor-antigen specific activation of numerous T cell functions, including cytokine production, degranulation and cytolysis – equivalent to, if not greater than, a 2nd generation CAR bearing the CD28 and CD3zeta signalling domains. Future iterations of the engineered T cells will include chimeric co-stimulatory receptors to enhance T cell functionality and reduce off target toxicity. This research was supported by the Canadian Institutes of Health Research and the Terry Fox Foundation.

EVALUATION OF SUNTINIB AND ONCOLYTIC RHABDOVIROTHERAPY IN A MOUSE TRIPLE NEGATIVE BREAST CANCER MODEL

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Oncolytic rhabdovirotherapy (ORV) efficacy is limited due to two factors—host anti-viral immunity and an immunosuppressive tumor microenvironment. Jha *et al*, 2013 showed that combining Su with ORV therapy improves viral productivity by inhibiting protein kinase R (PKR) activity. Literature also shows that Su has off target immunomodulatory effects on the host such as reducing the number of immunosuppressive myeloid derived suppressor cells (MDSCs) and improving anti-tumor CD8 T cell response. This led us to hypothesize that pre-treatment with Su will allow for better ORV efficacy in a ORV infection resistant model of breast cancer, 4T1. Indeed, pretreatment with Su followed by ORV therapy improved survival compared to ORV or Su monotherapy in the 4T1 model ($p < 0.001$). Mechanistically, Su improves ORV productivity (1-3 logs) in the tumor and reduces IFN- β in the tumor and serum *in vivo* but not *in vitro*. Based on this data, we hypothesized that Su improves viral productivity in the tumor in a tumor cell independent manner. To start investigating this, I enumerated professional IFN producing cells such as plasmacytoid dendritic cells (pDC) and macrophages ($M\Phi$) in the tumor and spleen. Thus far I have demonstrated that Su treatment reduces the number of splenic plasmacytoid dendritic cells ($CD11c^{lo/int} Gr1^+ B220^+$), MDSCs and increases the number of lymphocytes ($CD8^+$, $CD4^+$ T cells and B cells) in both the spleen and the tumor of 4T1 bearing animals. Future experiments will investigate the mechanism by which Su alters pDC number, function and characterize the effect of Su treatment on anti-tumor T cell function.

Abstract: CHIMERIC PROTEINS AS IMMUNE TARGETS IN PROSTATE CANCER

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Background: Cancer vaccines aim to elicit antigen-specific T cell responses against tumor antigens. Most prostate cancer vaccines to date target mis-expressed or over-expressed proteins; however, these proteins are often dispensable for the tumor, allowing for antigen escape, or have tolerance mechanisms in place that may curb induction of T cell immunity. Recent studies provide compelling evidence that tumor-specific mutations are a novel source of T cell targetable antigens (neoantigens). Metastatic Castration Resistant Prostate Cancers (mCRPC) contain several recurrently mutated fusion proteins that may serve as viable immune targets. The TMPRSS2:ERG fusion protein is found in a large proportion of mCRPC, is involved in several oncogenic pathways, and predicts poor overall survival; thus, this fusion is likely functionally important for tumor maintenance, progression, and metastasis.

Hypothesis: Gene fusions, such as TMPRSS2:ERG, generate chimeric amino acid sequences that are targetable by T cells.

Methods and Results: With this aim, we pulsed autologous dendritic cells with peptides corresponding to the TMPSS2:ERG type VI fusion site to activate and expand naïve fusion-specific T cells from peripheral blood of healthy donors. After two rounds of stimulation, expanded T cell cultures were assessed by interferon- γ ELISPOT for recognition of fusion peptides. T cell responses to two epitopes spanning the TMPRSS2:ERG fusion were confirmed in an HLA-A*02:01 healthy donor. These two peptides were predicted to bind HLA-A*02:01, which was confirmed by MHC stabilization assays. Currently, we are assessing whether these minimal peptides are naturally processed as well as whether antigen-specific T cell clones can lyse tumor cells that express the TMPRSS2:ERG type VI fusion protein.

Conclusions and Future Directions: Future studies will assess TMPRSS2:ERG positive mCRPC patients for the presence of pre-existing T cell responses to this fusion. Our findings to date have implications for the use of fusions as T cell targetable epitopes for therapeutic vaccination against fusion oncogenes in prostate cancer.

HMGB-1 RELEASE AND THE CD8 T CELL RESPONSE ELICITED BY RADIATION TREATMENT IN MALIGNANT PLEURAL MESOTHELIOMA

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Background: Malignant pleural mesothelioma (MPM) is a cancer of the lung lining that is difficult to treat and linked with the inhalation of asbestos fibers. Cytotoxic T lymphocytes circulate through the body and are able to kill tumor cells. However, an inhibitory tumor microenvironment renders T cells unable to inhibit tumor growth. Furthermore, MPM has few known tumor associated antigens; making it difficult to target interventions. Danger associated molecular pattern (DAMP) molecules offer a new avenue of treatment. DAMPs, released from cells undergoing cell death, are implicated in stimulating the antigen uptake, maturation, and antigen presentation of dendritic cells; crucial activators of cytotoxic T cells that mediate tumor killing. We are keen to observe the role of HMGB-1 in the context of radiation treatment for mesothelioma.

Purpose: To investigate the CD8+ T cell immune response elicited by HMGB-1 release and to correlate HMGB-1 released by MPM to survival.

Hypothesis: Radiation treatment of MPM results in tumor cell death and the subsequent release of HMGB-1, recruitment of CD8 T cells, and stimulation of tumor cell killing.

Methods: Transwell filters were used to assess migration towards recombinant HMGB-1 protein, *in vitro*. Tumor killing assay assessed the ability of recombinant HMGB-1 to promote MPM cell death by CD8 T cells. C57BL/6 mice received radiation, radiation and anti-HMGB-1 Ab or no treatment to MPM tumors that were established by MPM cell line subcutaneous injection.

Results: *In vitro*, HMGB-1 is able to stimulate CD8 T cell migration and MPM tumor cell line killing. Mice with radiated tumors displayed an increase in tumor CD8 T cell number and serum HMGB-1 levels. Anti-HMGB-1 Ab treatment decreased tumor infiltrating CD8 T cell numbers in radiated mice and resulted in lower survival than radiation alone.

Conclusion: HMGB-1 is crucial for the immunogenicity of radiation treatment in MPM.

SITE-SPECIFIC IMMUNOMODULATORS (SSIs): A NOVEL IMMUNOTHERAPY FOR CANCER

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Chronic inflammation that suppresses adaptive anti-tumour immunity and promotes neoplastic cell growth is a hallmark of cancer. Induction of acute-type inflammation can reverse tumour-induced immune suppression, leading to immune-mediated tumour regression. To mimic acute infection-type immune responses, Qu Biologics developed Site-Specific Immunomodulators (SSIs), a platform of immunotherapies derived from specific species of killed bacteria known to commonly cause infection in a particular organ or tissue. Repeated subcutaneous injection of SSI may provide an effective method for the induction of site-specific acute-type inflammation. In preclinical cancer models, SSIs stimulate the immune system and reverse dysfunction in the tumor microenvironment, enabling effective anti-cancer immune responses (see Kalyan *et al.*). In compassionate use protocols, 254 patients with advanced cancer were treated with one or more SSIs for up to 3.5 years. In retrospective analysis, patients with metastatic breast cancer receiving SSIs as part of their treatment (the largest patient group) had a 20 month longer median survival than those not treated with SSIs. In a case-matched study in all late-stage cancer patients, those receiving SSI as part of their treatment had a median survival advantage of 12 months compared to those not receiving SSI (n = 43 per group). While this experience comprises uncontrolled, unblinded observations, the data suggest that SSI may induce productive anti-tumour immunity. Qu's QBKPN SSI product, designed to induce a lung site-specific response, is currently being studied in a Phase 2a clinical trial in patients with non-small cell lung cancer, in collaboration with the BC Cancer Agency (Trial NCT02256852).

TUMOUR ASSOCIATED T CELLS FROM HIGH-GRADE SEROUS OVARIAN CARCINOMA PATIENTS RECOGNIZE THE CANCER TESTIS ANTIGEN LACTATE DEHYDROGENASE C

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Although the presence of intraepithelial CD8⁺ T cells is a positive predictive marker of survival in high grade serous carcinoma (HGSC), the paucity of known tumour-specific T cell targets is a notable barrier to the rational design of immunotherapies for this disease. In our cohort of HGSC cases, we found that the Cancer Testes (CT) antigen lactate dehydrogenase C (LDHC) is expressed in 75% of tumours. Therefore, we hypothesize that LDHC is a potential immunological target in HGSC, and that T cells that recognize LDHC can be activated and used for adoptive immunotherapy. We first sought to examine whether endogenous LDHC specific T cells were present in ascites of HGSC patients. Patients were prioritized based on expression of LDHC within their tumours by qPCR. CD8⁺ T cell cultures derived from each ascites sample were expanded using a standard Rapid Expansion Protocol. These cultures were screened for reactivity to a peptide library encompassing all possible epitopes of the LDHC protein via interferon- γ ELISpot. Reactive CD8⁺ T cells were sorted based on antigen-dependent 4-1BB upregulation, further expanded, and used to identify the minimal epitopes recognized. We identified five T cell clones that reacted to LDHC peptides. We are currently evaluating these clones' ability to differentiate between LDHC and its ubiquitously expressed isoform, Lactate Dehydrogenase A. Additionally, we are testing whether the LDHC minimal epitopes are naturally processed by antigen processing and presentation machinery of HGSC tumour cells.

At this time, we can conclude that there are T cells harboured within the patient repertoire which can recognize LDHC. An LDHC specific T cell response within the tumour environment provides strong rationale to develop T cell therapies targeting this antigen in HGSC.

AN EXPERIMENTAL APPROACH TO DISTINGUISHING THE PREDOMINANT MECHANISMS OF IMMUNE EVASION IN HIGH-GRADE SEROUS OVARIAN CANCER

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Background: There is a strong association between CD4+ and CD8+ tumor-infiltrating lymphocytes (TIL) and survival in high-grade serous carcinoma (HGSC). Further, TIL can recognize tumor-specific antigens, which raises the possibility they exert selective pressure on the evolving tumor. In response, tumors can evade immune pressure by two broad mechanisms: antigen loss or functional impairment/deletion of T cells. Recently, our lab reported anecdotal evidence of deletion of a tumor-reactive T-cell clone in HGSC, leading us to hypothesize this may be a common mechanism of immune evasion. We intend to test this hypothesis in a cohort of 20 HGSC cases by assessing the magnitude and clonal diversity of tumor-reactive TIL harvested at serial time points from HGSC patients undergoing standard treatment. Here we describe our first efforts to develop this experimental approach.

Methods: CD4+ and CD8+ TIL were expanded with high-dose IL-2 from an HGSC ascites sample and assessed by IFN- γ ELISPOT and flow cytometry for tumor recognition.

Preliminary results: Expanded CD4⁺ and CD8⁺ TIL showed robust expression of IFN- γ and up-regulation of CD137 upon stimulation with autologous tumor from a synchronous time point, indicating strong tumor recognition.

Next steps: Currently, we are expanding TIL from serial time points from this and other patients and assessing recognition of synchronous and asynchronous tumor samples. In addition, we are developing methods to track individual TIL clonotypes by high throughput TCR sequencing of FACS-purified CD137+ tumor-reactive subpopulations.

Significance: This study is designed to elucidate the predominant mechanisms of immune evasion in HGSC. Our results will help us design an optimal protocol for adoptive T cell therapy of recurrent disease.

Synergistic effects of OSM and TNF α on estrogen receptor expression in breast cancer

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Currently endocrine therapy of breast cancer is the most important systemic treatment available for estrogen-receptor alpha (ER α)-positive breast cancer. Unfortunately, up to 50% of these patients will fail ER-targeted therapies due to either de novo or acquired resistance. It has recently been shown that inflammation derived cytokines may be a player in the development of more aggressive, therapy-resistant ER-positive breast cancers. Previous studies have focused predominantly on cytokines such as IL-6, IL-1 α , TNF α , and TGF β . Oncostatin M (OSM) is an IL-6 family cytokine that is produced at high levels by macrophages and other immune cell types. We have recently shown that OSM suppresses ER α in breast tumor cell lines and we have found correlations between OSM and low ER and reduced levels of ER regulated genes in breast tumors, suggesting that this may be a functional and clinically important pathway in-vivo. Also we have found that while OSM is the most effective, some other cytokines (TNF α , TGF β 1, TGF β 2, IL-1 β , IL-8) also influence ER expression, and synergistic effects between OSM and TNF α result in the most significant suppression. Additionally, our results suggest that these cytokine effects on ER are associated with parallel induction of the S100A7 gene in cell lines. S100A7 may serve as a biomarker of cytokine action. It also mediates some of the effects of OSM and promotes tumor progression through pro-survival and invasive pathways, and influences the pattern of inflammation through its chemotactic effects on inflammatory cells. Ongoing work seeks to confirm these observations in the in-vivo setting and establish a role for these effects in the resistance to endocrine therapy.

THERAPEUTIC EFFECT OF PD-1 BLOCKADE IN THE MBT-2 MURINE BLADDER CANCER MODEL.

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Background: Non-specific immunotherapy using Bacillus Calmette-Guerin (BCG) is currently the preferred treatment to prevent non-muscle-invasive bladder cancer (NMIBC) recurrence after surgery. However, it remains suboptimal as recurrences and progressions are respectively observed in 60% and 20% of cases. Among the most promising alternative approaches to BCG is the blockade of immune checkpoints. Using the MBT-2 murine bladder cancer model, we analyzed the phenotype of the tumor-infiltrating immune cells, and their expression of various immune checkpoints such as PD-1, CTLA-4, LAG-3, and TIM-3. We hypothesized that blocking PD-1/PD-L1 pathway would boost the anti-tumor response.

Methods: MBT-2 tumors were grown subcutaneously in CH3 mice. After tumor dissociation using a GentleMACS, tumor-infiltrating immune cells and their immune checkpoint expression was characterized by multicolor flow cytometry. PD1 blockade was performed by 4 i.p. injections of anti-PD-1 monoclonal antibody (mAb) in MBT-2-tumor-bearing mice.

Results: The analysis of MBT-2 tumors showed that their microenvironment is characterized by the presence of cells with a suppressor phenotype. Indeed, regulatory T cells (Treg) (CD3 ϵ +CD4+FOXP3+) represented \approx 23% of the CD4+ tumor-infiltrating-lymphocytes (TILs) (CD3 ϵ +CD4+) and about 29% of these TILs were Tr1 (CD3 ϵ +CD4+IL10+). Moreover, the majority CD4+ and CD8 α + TILs expressed PD-1 and TIM-3 but very few or no CTLA-4 or LAG-3 molecules. PD-1 pathway blockade in mice bearing MBT-2 tumors resulted in a drastic reduction of tumor growth and even the cure of 1 out of 6 mice.

Conclusions: These data indicate that in MBT-2 tumors, blocking the PD1/PD-L1 pathway could stimulate an effective anti-tumor immune response. This protection might be improved by combining PD1/PD-L1 blockade with the blockade of other immune checkpoint such as TIM-3 which is also highly express on MBT-2 tumor TILs. Finally, these results also suggest that combination of immune checkpoint inhibition with BCG therapy might result in an even more effective therapy against NMIBC.

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PROGNOSTIC VALUE OF CD1 α TUMOR-INFILTRATING DENDRITIC CELLS IN NON-MUSCLE INVASIVE BLADDER CANCER

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Background: Bladder tumors are 5th in incidence in Canada and occur as non-muscle-invasive bladder cancer (NMIBC) in 70% of cases. After transurethral resection they recur in 60% of cases and progress to muscle-invasive bladder cancer in 20%. It is essential to detect tumors most likely to recur or to progress to adjust treatment accordingly. Characterizing tumor-infiltrating immune cells is proposed as new way to classify cancers. Previous studies showed that tumor-infiltrating dendritic cells (TIDCs) were significantly associated to prognosis in NMIBC. We analysed TIDCs in bladder tumors to evaluate their contribution to the evolution of tumors.

Methodology: Formalin-fixed paraffin-embedded initial non-muscle invasive bladder tumors from 107 patients were analyzed. Immunohistochemistry staining was performed on 5 μ m thick sections using monoclonal antibodies to CD83, CD209, and CD1 α , identifying DCs at different levels of maturity. The density of TIDCs in tumor, papillary axis, and stromal areas, and in lymphoid aggregates was determined by two independent observers in a blinded manner.

Results: CD209+ immature TIDCs were the most abundant type of DCs observed in tumors, mostly in stromal areas. CD1 α + TIDCs cells on the other hand were present mostly in tumor areas while CD83+ mature TIDCs predominated in lymphoid aggregates. Although CD209+ and CD83+ tumor infiltration had no prognostic value, their level of infiltration was associated to tumor characteristics. CD209+ immature TIDCs were significantly more abundant in lymphoid aggregates of T1 than Ta tumors (p=0.04), while CD83+ mature TIDCs were more abundant in the papillary axis (p=0.028), the stroma (p=0.037), and in tumor areas (p=0.017). The density of CD1 α + cells in lymphoid aggregates was also higher in T1 tumors (p=0.013). Moreover, tumors with the highest density of CD1 α were more at risk of recurrence (HR=17.47, p=0.019).

Conclusion: The density of TIDCs in non-muscle invasive bladder tumors is associated with tumor characteristics and evolution.

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Investigating the role of autophagy in the breast tumour microenvironment and its implications in anti-tumour immunity

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The breast tumour microenvironment (TME) upregulates autophagy as a survival mechanism to combat cellular stress and fuel tumour growth. As such, therapies to target autophagy in breast cancer are currently in clinical trials. Not only does the TME play an important role in tumour initiation and response to therapy, but it also has implications in the immunological recognition of tumour cells. A limited number of studies have shown that autophagy can impact immunosurveillance and anti-tumour immunity, however little is known about how autophagy regulates immunological processes within the TME. We conducted preliminary studies to assess the role of autophagy in the host-tumour interaction. We found that mice deficient for the essential autophagy gene *Atg5* (*Atg5*^{-/-}) had a significant impairment in their ability to form syngeneic breast tumours. Moreover, tumours from *Atg5*^{-/-} knockout mice had increased CD8⁺ T cells and CD19⁺ B cells, as well as reduced CD4⁺ T cells. This is one of the first studies demonstrating that deletion of host autophagy not only impairs tumour growth, but it may also enhance the anti-tumour immune response to breast cancer. To test this, we will confirm the requirement of autophagy in eliciting an anti-tumour immune response. Moreover, we will examine the relationship between autophagy and tumour infiltrating lymphocytes and correlate them with treatment response and patient outcome in breast cancer. This study will not only allow us to gain further molecular insights into the tumour-host interaction in breast cancer, but it will also identify a potential new way to enhance the adaptive immune response to eradicate breast cancer more effectively

MELANOMA INDUCES, AND ADENOSINE SUPPRESSES, CXCR3-COGNATE CHEMOKINE PRODUCTION AND T CELL INFILTRATION OF LUNGS BEARING METASTATIC-LIKE DISEASE

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Melanoma-specific vaccines have minimal efficacy in patients with established disease but enhance survival when administered in the adjuvant setting. Therefore, we hypothesized that organs bearing metastatic-like melanoma may differentially produce T cell chemotactic proteins over the course of tumor development. Using an established model of metastatic-like melanoma in lungs, we assessed the production of specific cytokines and chemokines over a time-course of tumor growth, and we correlated chemokine production with chemokine receptor-specific T cell infiltration. CXCR3-cognate chemokines (CXCL9 and CXCL10) were significantly increased in lungs bearing minimal metastatic lesions, but chemokine was at or below basal levels in lungs with substantial disease. Chemokine production correlated with infiltration of the organ compartment by transferred CD8⁺ tumor antigen specific T cells in a CXCR3- and host IFN- γ -dependent manner. Adenosine signaling suppressed chemokine production and T cell infiltration in the advanced metastatic lesions, and suppression could be partially reversed by the adenosine receptor antagonist aminophylline. Collectively, our data demonstrate that CXCR3-cognate ligand expression is required for efficient T cell access of tumor-infiltrated lungs, and ligands are expressed in a temporally restricted pattern that is governed, in part, by adenosine. Thus, modulation of adenosine activity may impart therapeutic efficacy to immunogenic but clinically ineffective vaccines.

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PERSONALIZED CANCER IMMUNOTHERAPY THROUGH MINOR HISTOCOMPATIBILITY ANTIGEN TARGETING

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Allogenic hematopoietic cell transplantation (AHCT) can cure hematological malignancies refractory to cytotoxic therapy. The therapeutic potential of AHCT largely depends on the so-called graft-versus-leukemia (GVL) effect mediated by donor T cells recognizing mainly Minor histocompatibility antigens (MiHA) on the malignant cells. Our collaborators developed a method based on deep sequencing and high-throughput mass spectrometry to determine HLA*A0201 associated MiHA. Our objectives are to validate these novels MiHA and to develop a clinical grade-compliant method to reliably expand MiHA-specific CD8 T-cell lines.

To evaluate the immunogenicity of newly discovered MiHA, we adapted a previously published 10-day protocol based on immunomagnetic T-cell selection, peptide-loaded dendritic cells and cytokine-driven activation of antigen-specific T cells. We validated this approach using the MiHA HA-1 and generated a product composed of 65% CD8 T cells, 0.3% of which are multimer HLA-A2/HA-1 positive. Importantly, the IFN γ -ELISpot assay is sufficient to determine the antigen-specificity of the T cell line. Based on ELISpot assay, we show that two putative MiHA peptides are immunogenic. Examining the polyfunctionality by flow cytometry, we can estimate that at least 3.5% of CD8 T cells in the culture are antigen-specific for one of the two peptides newly identified.

Our clinical grade-compliant method to generate MiHA-specific T-cell lines hinges on a co-culture with peptide-pulsed dendritic cells and responder T cells, followed by an enrichment step using IFN γ capture and rapid expansion protocol. Our results using HA-1 as a model MiHA showed that IFN γ -secreting T cells enrichment after 3 dendritic cells stimulations followed by 12 days of rapid expansion led to a MiHA-specific T-cell product with no evident sign of culture-driven exhaustion.

Our results set the stage for a phase I clinical trial using HLA-A2 associated MiHA-specific T-cell lines for the treatment of high risk hematological malignancies.

T CELLS ENGINEERED WITH CHIMERIC ANTIGEN RECEPTORS TARGETING NKG2D LIGANDS DISPLAY LETHAL TOXICITY IN MICE

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T cells engineered to express a chimeric antigen receptor (CAR), called CAR-T cells, have shown significant promise as an adoptive transfer therapeutic for cancer. We have explored the use of CARs which target tumors via the NKG2D receptor; given that NKG2D ligands are overexpressed on stressed cells, such as tumor cells, they are an attractive therapeutic target. We evaluated the use of two unique NKG2D-based CAR constructs: i) a fusion of the full length NKG2D receptor and cytoplasmic CD3 ζ , and ii) the extracellular domain of NKG2D on the scaffold of a conventional second-generation CAR (intracellular CD28 and CD3 ζ domains). In addition, we combined the NKG2D-CD3 ζ fusion with co-expression of the adaptor protein DAP10 in order to enhance CAR surface expression. All three of these CAR constructs were expressed on murine T cells from BALB/c and C57BL/6 hosts and their functionality compared. *In vitro*, BALB/c derived CAR-T cells showed increased functionality and surface expression of the CARs, suggesting strain-specific differences exist when using NKG2D-based CARs. Upon adoptive transfer of the NKG2D-CAR-T cells into their respective syngeneic hosts, we observed evidence of rapid, severe toxicity resulting in morbidity and mortality. The severity of this toxicity paralleled *in vitro* CAR expression levels; NKG2D-based CARs with higher levels of cell surface expression showed exacerbated toxicity as did those in BALB/c hosts, supporting CAR configuration and strain specific differences. Pre-treatment of mice with a chemotherapeutic agent, cyclophosphamide, prior to adoptive transfer of NKG2D-CAR-T cells exacerbated toxicity, particularly in BALB/c mice. These data demonstrate that enhancing the surface expression of CARs may be hazardous when targeting ligands which are not tumor specific. In addition, these data demonstrate that NKG2D-based CARs have the potential to drive serious off-tumor toxicities and urge extreme caution be used in their continued development.

DECODING THE T CELL RESPONSE IN TUMOURS USING LARGE SCALE RNA-SEQ DATASETS

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T cell receptor (TCR) repertoires of tumours, typically derived from TCR-seq experiments, provide insights regarding the anti-tumour immune response. Many studies do not include this specialized type of data, instead performing more standard RNA-seq experiments. We wanted to determine if RNA-seq datasets could be used to observe a subset of the TCR repertoire present in tumours.

TCRs survey peptides presented on the surface of cells, and are able to detect those unique to a tumour. The highly variable CDR3 region of the TCR comes into closest contact with the MHC-presented peptide, defining the antigen specificity of the TCR. To find these CDR3 sequences in RNA-seq data, we selected the CDR3 annotation tool MiTCR due to its speed and sensitivity. RNA-seq data contains sequences from all transcripts being expressed, increasing the probability of errant CDR3 annotations. We performed a parameter space exploration to minimize false positives and maximize CDR3 recovery. We then characterized this recovery using *in silico* simulations of RNA-seq datasets. We determined that the most abundant TCRs in a sample are those detected by RNA-seq. To validate these observations, we performed deep RNA sequencing on three colorectal carcinoma samples for which TCR-seq data already exists.

We ran our analysis pipeline on 1,842 RNA-seq samples from The Cancer Genome Atlas. We obtained 26 CDR3s per subject on average. Five percent of all CDR3s recovered were shared between at least two subjects, and greater than 80% of these were detected in previously published TCR-seq studies, likely indicating non-tumour-specific public TCRs. Work is continuing to associate patterns of CDR3 sequences with predicted tumour epitopes.

A LYMPHOCYTE BASED CELL-TO-CELL THERAPEUTIC DELIVERY SYSTEM

Daniel Woodsworth, Eric Yung, Robert Holt
Michael Smith Genome Sciences Centre

With their ability to sense and integrate a wide range of signals, and actuate context-dependent responses, engineered cell-based systems are promising next-generation therapeutics. Cytotoxic lymphocytes (CLs) are an ideal chassis for developing such systems for two reasons: (i) CLs possess a unique cell-to-cell molecular transfer system in the granzyme-perforin pathway; and (ii) T-cell receptors (TCRs), or chimeric antigen receptors (CARs), can endow CLs with an exquisite level of specificity in controlling activation of this pathway, and in targeting a cell population defined by its antigen profile.

We are developing a cell-to-cell therapeutic delivery system by engineering the granzyme-perforin pathway, which, unmodified, involves CL secretion of granzyme B (GzB) and perforin, followed by perforin facilitated GzB target cell entry and apoptosis induction. We are engineering CLs to transfer a GzB-payload fusion protein to targeted cells, where the GzB motif of the fusion protein acts as a chaperone to ensure appropriate packaging, trafficking and delivery to the target cell.

We first showed the feasibility of this approach by demonstrating that a GzB-tdTomato fusion protein is transferred from NK-92MI natural killer cells to target K562 cells. We are now developing this system as a novel cancer therapeutic for apoptosis resistant tumor cells, a major challenge in cancer therapy. A common resistance mechanism is overexpression of the inhibitor of apoptosis protein XIAP, which we have shown renders target cells resistant to NK lysis. We have constructed various GzB-toxin fusions, and functionally verified their toxicity in HeLa cells. We are now evaluating the efficacy of GzB-toxin mediated NK cell killing of K562 cells that overexpress XIAP.

Engineering the granzyme-perforin pathway represents a completely novel approach to molecular delivery, which, when combined with CAR or TCR targeting, could pave the way to a new class of cell-based therapeutics, capable of executing complex in vivo therapeutic activity.

DESIGN AND OPTIMIZATION OF MASS CYTOMETRY TO MEASURE IMMUNE RECONSTITUTION POST-HEMATOPOEITIC STEM CELL TRANSPLANT

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Hematopoietic stem cell transplant (HSCT) is an effective cancer immunotherapy treatment for patients with hematological malignancies. While HSCT is a curative treatment, patients are at high risk of developing graft-versus-host disease (GVHD). Understanding immune reconstitution post-HSCT is important for discerning the mechanisms controlling the desired graft-versus-leukemia effect versus GVHD, as well as understanding the mechanisms of action of common immunosuppressive therapies used in HSCT, such as anti-thymocyte globulin (ATG). Immune reconstitution is a complex process involving multiple cell types and subsets, making it challenging to measure in limiting samples from lymphopenic patients. We aimed to develop a new mass cytometry-based method to measure expression of up to 40 parameters measured simultaneously in one million PBMCs, creating a comprehensive immune reconstitution panel that would not be possible using conventional fluorescence flow cytometry. Because reconstitution of regulatory T cells (Tregs) has a major impact on transplant outcomes, we first focused on developing a novel protocol that enables simultaneous detection of FOXP3, the Treg lineage-defining transcription factor, cell surface markers and cytokines on cryopreserved PBMCs. We first tested the conventional protocol to detect FOXP3, which is optimized for fluorescence flow cytometry, and found that it failed to detect FOXP3 by mass cytometry. We therefore tested a variety of different methods to fix and permeabilize cells, and then created a new protocol that allows robust and sensitive detection of FOXP3. This new protocol was also more sensitive than commercially-available protocols optimized for mass cytometry. We also compared different anti-FOXP3 mAbs and found that the 236A/E7 clone was the most sensitive. With this optimized method, we are now able to measure expression of FOXP3 together with 36 other proteins and experiments to measure how immune reconstitution is affected by ATG in patients who have undergone a hematopoietic stem cell transplant.

Research funded by a grant from the Leukemia and Lymphoma Society of Canada
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Role of Notch Signaling in anti-Tumor-Associated Antigen T Cell Activation with Artificial Antigen Presenting Cells

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Adoptive cell transfer of ex vivo-expanded tumor infiltrating lymphocytes (TILs) is currently an effective tool in the treatment of metastatic melanoma. While promising, it is not a widely applicable immunotherapy due to limitations in proliferative potential and access to TILs and endogenous antigen presenting cells (APCs) that are required for TIL expansion. Unlike patient-derived natural APCs, such as dendritic cells, “artificial” APCs (aAPCs) are a readily accessible and easily manipulated cell source that has been shown to support priming and activation of tumor-associated antigen (TAA)-specific CD8⁺ cytotoxic T lymphocytes (CTLs). Here we addressed whether Notch signaling would influence the expansion and lead to enhanced cytotoxic function of TAA-specific CTLs obtained from human naïve peripheral blood CD8⁺ CTLs. K562 erythroleukemia-derived cells (aAPCs) expressing costimulatory ligands and presenting a TAA peptide (Mart-1) were modified to express Delta-like-4 (DLL4), a member of the family of ligands for Notch receptors. We hypothesize that provision of Notch signaling during priming and early activation will generate an increased pool of Mart-1- specific CTLs with enhanced effector function. Our preliminary results using DLL4⁺ aAPCs point to an important role for adopting Notch-driven CTL expansion strategies, and earlier polyclonal stimulations suggest the effect of DLL4 may manifest at the effector cell stage. Others have shown that provision of DLL4 to mouse CD4⁺ T lymphocytes during priming generates lymphocytes with greater anti-tumor activity, thus induction of this phenomenon in lymphocytes capable of inducing direct lysis of tumor cells, such as CTLs, is an attractive therapeutic avenue. An important goal of our work is to address the current practical limitations in cancer immunotherapy and generate a translatable strategy to render all patients eligible for immunotherapy.

CONSTRUCTION AND USE OF DARPIN LIBRARY FOR THE DISCOVERY OF TUMOR SPECIFIC ANTIGEN BINDER PROTEINS.

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Adoptive transfer of tumor-specific T cells is proving to be an effective strategy for treating established tumors. In order to extend the specificity of T lymphocytes beyond their MHC-peptide complexes, a strategy has been developed that allows redirecting cytotoxic T cells to tumor cell surface antigens. One method of generating these cells is accomplished through engineering bulk T cell populations to express chimeric antigen receptors (CARs) which are specific for tumor antigens. This strategy combines an antigen-binding moiety, most commonly a single chain antibody, together with an activating immune receptor. However, single chain antibody typically shows low thermal stability and tends to denature or aggregate under the conditions of practical uses, thus limiting their biomedical and biotechnological applications. Tandem repeat proteins represent an alternative to single chain antibodies as a binding moiety within CAR. ~20 classes of such proteins are found in all forms of life where they participate in multiple protein-protein interactions. They are characterized by well defined topology and stability. Using a consensus designed ankyrin repeat protein (DARPin) with specificity for the tumor-associated antigen HER-2, we have generated anti-HER-2 DARPin CARs for expression on murine or human CD8⁺ T lymphocytes. Our murine anti-HER-2 DARPin CAR is expressed on the surface of murine CD8⁺ T cells, is functionally activated (production of IFN- γ and TNF- α) upon stimulation with HER-2, and induces specific killing of HER-2⁺ tumor cells. We constructed a new designed ankyrin repeat protein (DARPin) library expressed in filamentous bacteriophage display system in a purpose to select binding proteins for known tumor-specific antigens and to discover new tumor-specific targets expressed on tumor cell surfaces. Currently the library is being screened with BCMA (B-cell maturation antigen) and Multiple Myeloma cell line.

Combination of Oncolytic Reovirus and Dendritic Cell Therapy Improve Immunotherapeutic Strategies for Breast Cancer

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Breast cancer is the most common cancer affecting Canadian women and accounts for nearly 30% of all newly diagnosed cancer cases. Despite a large choice of treatments it is still difficult to predict the outcome of breast cancer and the response to treatment. Thus, better treatment strategies are needed especially in patients with poor prognosis. Oncolytic viruses such as reovirus (RV) are non-pathogenic viruses, which specifically target and lyse cancer cells due to genetic abnormalities, with no effect on normal cells. Recently, RV is used in human phase III clinical trials against different histological malignancies. The challenge of these trials is the elicitation of anti-viral immune response, which results in viral clearance. We hypothesized that RV, in addition to its direct oncolytic effects can be used as an immune adjuvant for both augmentation of immune effector mediated tumour regression and immunosurveillance against breast cancer recurrence. To address this effects, murine dendritic cells (DC) from Balb/c were propagated and pulsed with EMT6 cells infected with RV to examine whether functional immune effectors could be generated in vitro. In addition, in vivo studies were done using a syngenic murine breast cancer model (EMT6/Balb/c). We have demonstrated that RV infected breast cancer cells is an efficient priming agent for DCs. Moreover, using in vitro LDH cytotoxicity assays these DCs are capable of anti-tumour activity. In order to prove that these effectors had activity in vivo we immunized mice with either s.c injection of EMT6/RV (SC group) or i.v injection of DCs pulsed by EMT6/RV (IV group). The SC group had significantly reduced tumor recurrence compared to control group. Interestingly IV group were completely protected from tumour recurrence. In turn, we also identified that IV group had the highest percentage of CD4+ and CD8+ T cells with higher cytotoxic activity and T cell proliferation to tumour antigen compared to the other groups. To the best of our knowledge, the idea of combining RV, and DC have never been tried in treatment of cancer. Taken together, these data will provide new treatment strategies with resultant improved efficacy and safety of our breast cancer patients to be translated into phase I/II clinical trials.

PROGNOSTIC BENEFIT OF TUMOR-INFILTRATING LYMPHOCYTES IN PATIENTS WITH HIGH GRADE SEROUS OVARIAN CANCER IS DEPENDENT ON TREATMENT REGIMEN, SURGICAL OUTCOME AND T CELL DIFFERENTIATION

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Tumor-infiltrating lymphocytes (TIL) are associated with a better prognosis in high grade serous ovarian cancer (HGSC). However, it remains largely unknown how this prognostic benefit of TIL relates to current standard treatment of surgical resection and (neo-)adjuvant chemotherapy. To address this outstanding issue, we compared TIL infiltration in a unique cohort of advanced stage HGSC patients primarily treated with either surgery or neo-adjuvant chemotherapy.

Tissue Microarray (TMA) slides containing samples of 171 patients were analyzed for CD8+ T cell infiltration by immunohistochemistry. CD8+ T cell subsets in freshly isolated TIL were characterized by flow cytometry based on differentiation, activation and exhaustion markers. Relevant T cell subset markers were validated using immunohistochemistry (TMA) and immunofluorescence (full slides).

A prognostic benefit for patients with high intratumoral CD8+ TIL was observed if primary surgery had resulted in a complete cytoreduction (no visible tumor present). By contrast, optimal (<1 cm of remaining tumor) or incomplete cytoreduction fully abrogated the prognostic effect of CD8+ TIL. Subsequent analysis of primary TIL by flow cytometry and immunofluorescence identified CD27 as a key marker for a naïve-like, yet antigen-experienced and potentially tumor-reactive CD8+ TIL subset. In line with this, CD27+ TIL remained associated with an improved prognosis even in incompletely-cytoreduced patients. Finally, neither CD8+ nor CD27+ cell infiltration was of a prognostic benefit in patients treated with neo-adjuvant chemotherapy.

Our findings indicate the treatment regimen, surgical result and the differentiation of TIL should all be taken into account when studying immune factors in HGSC or, by extension, selecting patients for immunotherapy trials.

CCR5⁺ REGULATORY T CELLS INDUCED BY METASTATIC BREAST CARCINOMAS MIGRATE TOWARD A GRADIENT OF CCL8 AND ACCUMULATE IN THE PRIMARY TUMOUR AND METASTATIC LUNG TISSUE

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Despite their role in the regulation of immunity toward innocuous antigens, regulatory T cells (Tregs) may contribute to tumour growth and metastasis by suppressing T cell-mediated anti-tumor cytotoxicity. Expression of CCR5 by CD4⁺CD25⁺Foxp3⁺ Tregs is important for their intratumoral migration and immune-suppressive function. Disruption of CCR5 signalling through antagonism of the CCR5 receptor or its ligands has been found to decrease the growth and metastasis of melanoma, pancreatic and colon cancers in pre-clinical immunocompetent models. The objectives of this study are to determine if CCR5⁺ Tregs are recruited to primary breast tumours and metastatic sites, and to examine their role in the development of metastatic foci. We have found that the metastatic mammary carcinomas 4T1 and 4T07 induce the accumulation of CCR5⁺ Tregs in the primary tumour and metastatic lungs of mice. CCR5⁻ Tregs were observed in non-metastatic 67NR primary tumours, although Tregs were not elevated in the lungs of 67NR tumour-bearing mice. The production of CCL8, an endogenous ligand of CCR5, was increased in the primary tumour and lungs of 4T1 and 4T07 tumour-bearing mice, and our preliminary data suggest that CCL8 may be secreted by macrophages *in vivo*. Tregs migrated toward CCL8 *ex vivo*, and this migration was inhibited by the CCR5 antagonist, Maraviroc, without impacting Treg viability. We are currently investigating whether treatment of tumour-bearing mice with Maraviroc decreases the accumulation of CCR5⁺ Tregs *in vivo* and the formation of lung nodules in mice bearing metastatic mammary carcinomas. The inhibition of CCR5 represents a viable therapeutic strategy to prevent Treg homing to sites of tumour growth without systemically depleting them, thereby avoiding the off-target effects of global Treg depletion such as autoimmunity. Ultimately, we hope to advance the development of targeted, immune-based therapeutics for the treatment of metastatic breast cancer.

GAMMA DELTA T CELLS AND NODAL IN THE BREAST TUMOUR MICROENVIRONMENT

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Gamma delta T cells (GDTc) kill transformed cells, and increased circulating GDTc levels correlate with improved outcome in cancer patients. However, among a panel of various tumor infiltrating lymphocytes (TIL), GDTc were deemed the most significant independent factor predicting negative clinical outcome in human breast cancer. We hypothesize that GDTc become functionally altered by Nodal, an embryonic morphogen secreted by breast tumour cells in the hypoxic tumour microenvironment and implicated in aggressive disease. We have identified GDTc TIL in serial sections of breast cancer tissue in which Nodal is also expressed. Under hypoxic compared to normoxic conditions, GDTc viability and cell density increase, as does expression of activating receptor CD56, the gamma delta TCR, HLA-I and CD95; conversely, the inhibitory receptor CD94 is down-regulated. Thus GDTc can survive and proliferate in a hypoxic environment, and are armed with increased activating receptors implicated in cytotoxicity. Blood-derived *in vitro* expanded primary human GDTc kill breast cancer cell lines; however, Nodal-expressing breast cancer cells (231shC) resist GDTc killing compared to those in which Nodal has been silenced (231shN). Thus, Nodal expressed by breast tumour cells appears to suppress GDTc cytotoxicity. Preliminary results from chick chorioallantoic membrane assays suggest greater infiltration of GDTc into 231shN compared to 231shC tumours. Surprisingly, GDTc themselves appear to express Nodal and this expression is induced by hypoxia. Stimulation with recombinant human Nodal results in the tyrosine phosphorylation of a ~50 kDa protein that we aim to identify. We now plan to employ additional cell lines as well as examine GDTc therapy and TIL in xenograft mouse models of breast cancer. Understanding the dynamic interplay between Nodal and GDTc infiltration in breast cancer lesions will be of utmost importance to develop safe and effective GDTc immunotherapies.

COMBINED CHEMOTHERAPY AND NATURAL KILLER T CELL IMMUNOTHERAPY ENHANCES PROTECTION FROM BREAST CANCER METASTASIS.

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Breast cancer is the most common cancer in Canadian women and the second leading cause of cancer related deaths. Given that most mammary tumours are surgically resectable and over 90% of breast cancer-associated deaths are due to metastasis, new therapeutic strategies targeting metastasis are required. Natural killer T (NKT) cells are a rare population of immune cells that have been shown to limit primary tumor growth and target distant metastatic disease in various animal models. We have shown that NKT cell activation improves survival in a model of post-surgical metastatic breast cancer. We are now expanding this work to determine whether NKT cell activation can be combined with chemotherapies to improve outcomes. In our model, 4T1 mammary carcinoma cells were injected into the mammary fatpad of syngeneic BALB/c mice. Tumours were resected at day 12, and mice were treated with cyclophosphamide or gemcitabine. On day 17, NKT cells were activated by transfer of dendritic cells loaded with the glycolipid antigen α -GalCer. Chemotherapeutics did not affect NKT cell activation as measured by serum IFN γ levels. Treatment with cyclophosphamide, gemcitabine, or α -GalCer-loaded dendritic cells alone reduced metastasis and prolonged survival. Combined treatments significantly enhanced survival. NKT cell activation decreased the frequency and immunosuppressive function of myeloid derived suppressor cells (MDSCs). Treatments resulted in enhanced tumor specific immunity as surviving mice exhibited slower tumour growth following secondary tumour challenge. This work provides a clear rationale for combining chemotherapy with NKT cell immunotherapy to target metastatic disease.

EX VIVO ANALYSIS OF T CELL CLONE-SPECIFIC PERSISTENCE OF INFUSED CAR T CELLS BY HIGH-THROUGHPUT TCR SEQUENCING

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Recombinant chimeric antigen receptors (CAR) have been used to redirect T cell specificity to the B cell lineage antigen, CD19. Our laboratory initiated a phase I/II clinical trial of therapy of relapsed and refractory CD19-positive B cell malignancies using a defined formulation of CD8⁺ and CD4⁺ CD19-specific CAR-modified T (CAR-T) cells. We found that robust *in vivo* proliferation of CD19 CAR-T cells during an effector phase after CAR-T cell infusion was noted in patients who attained remission, and their subsequent persistence was important for prevention of relapse. However, the factors that dictate the outcomes of CAR-T cell therapy have not been fully defined.

In this study, we aimed to understand the behaviour of specific clones of CD8⁺ and CD4⁺ CAR-T cells by tracking TCR clonotypes in CAR-T cells in the infused product and in recipients at distinct times after infusion. The lentiviral construct used to engineer T cells in this clinical trial incorporates a non-functional truncated epidermal growth factor receptor (EGFR) that serves as a marker of transgene expression and allows isolation of CAR-T cells from treated patients blood by fluorescence-activated sorting after labeling with a biotinylated anti-EGFR antibody (cetuximab). We used high throughput sequencing of the TRBV genes from sorted EGFR⁺ CAR-T cells to obtain an extensive characterisation of the clonal composition and diversity of sorted CAR-T cells from 7 treated patients with acute lymphoblastic leukemia (ALL). These studies will allow us to track the expansion and contraction of clonally related CAR-T cells after infusion.

TOWARD PERSONALIZED LYMPHOMA IMMUNOTHERAPY: IDENTIFICATION OF COMMON DRIVER MUTATIONS RECOGNIZED BY PATIENT CD8⁺ T CELLS

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Background: A fundamental challenge in the era of next-generation sequencing (NGS) is to design effective, personalized treatments based on the mutational profiles of tumours. Many newly discovered cancer mutations are difficult to target pharmacologically; however, T cell-based therapies may provide a valuable alternative owing to the exquisite sensitivity and specificity of antigen recognition. **Methods and Results:** We assessed the immunogenicity of common driver mutations in follicular lymphoma (FL), an immunologically sensitive yet currently incurable disease. By applying NGS to 10 frequently mutated genes, we identified mutations in 43/53 samples. CD8⁺ T cells specific for *bona fide* mutant epitopes were identified in 23% of patients. Responding T cells were present at low precursor frequencies, suggesting that therapeutic intervention would be required to achieve clinically effective T cell responses against these mutations. **Conclusions:** Our results support the concept of using NGS to design individualized immunotherapies targeting common driver mutations in FL and other malignancies. **Funding:** This research was supported by the Canadian Cancer Society and the BC Cancer Foundation.

ANTI-CANCER EFFICACY IN A PRECLINICAL MODEL OF LUNG CANCER USING QBKPN SITE-SPECIFIC IMMUNOMODULATION

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The immune system is armed with the intrinsic capacity of recognizing and eliminating cells that have undergone malignant transformation. The observation that an intricate relationship exists between immune activation and cancer dates back to the 1700's, when spontaneous tumor remission was observed in some patients experiencing acute microbial infections. Building upon this history, Qu Biologics has discovered that repeated subcutaneous injection of an immunotherapy derived from specific species of killed bacteria known to commonly cause infection in a particular organ or tissue may provide an effective method for the treatment of cancers growing in that organ/body site. We hypothesize Qu's proprietary platform of immunotherapies, called Site-Specific Immunomodulators (SSI), stimulate the body's immune system to reverse the immune suppression and dysfunction in the tumor microenvironment, enabling effective anti-cancer immune responses. To test this hypothesis, we evaluated tumor growth and survival in preclinical lung cancer models.

Repeated subcutaneous administration of Qu's lung specific SSI, QBKPN, significantly reduced tumor burden at day 16 post-inoculation ($p < 0.0001$), improving median survival by 10 days ($p < 0.005$). Similar results were obtained using the B16 model, an aggressive and poorly-immunogenic melanoma cell line growing as metastatic-like lesions in the lungs, demonstrating the site-specific anticancer efficacy is independent of cancer type. Anticancer efficacy was associated with site-specific infiltration of newly recruited monocytes and neutrophils to the lung and mobilization of antigen presenting cells in the lung-draining lymph node. These data complement our compassionate use clinical experience with SSI and provide strong evidence that Qu's SSI platform may be an innovative cancer immunotherapy approach for reconstituting effective immunosurveillance in the tumour microenvironment and improving therapeutic outcomes for cancer patients. QBKPN is currently being studied in a Phase 2a clinical trial in patients with non-small cell lung cancer, in collaboration with the BC Cancer Agency (Trial NCT02256852).

TRANSDUCTION OF NK-92 CELLS WITH A HIGH AFFINITY VARIANT OF THE F_C RECEPTOR TO ENHANCE ANTIBODY DEPENDENT CELLULAR CYTOTOXICITY

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Natural Killer (NK) cells are part of the innate immune system. They play a key role in the body's immunosurveillance and clearance of cells that have undergone malignant transformations. This has led to the investigation of various adoptive NK cell and NK cell line-based immunotherapies. Adoptive immunotherapy with primary NK cells has obstacles including limited ex vivo expansion and variations between patients. Using NK-derived cell lines bypasses these obstacles and thus has become attractive clinically. NK-92 is a human NK-derived cell line that is currently being tested in a phase I trial at Princess Margaret Cancer Centre.

One way to enhance current NK immunotherapies may be to genetically engineer them to express a factor that targets and activates them against cancer cells. One such molecule is the F_C receptor: CD16. Although primary NK cells already express CD16, NK-92 cells lack this polypeptide and kill cancer cells through other mechanisms. CD16 mediates antibody dependent cellular cytotoxicity (ADCC) by binding to the F_C portion of antibodies that are bound to tumor-associated antigens on the surface of cancer cells. Thus, adding CD16 onto NK-92 cells should enhance their specificity and cytotoxicity in the presence of cancer-specific antibodies by giving them another effective killing mechanism.

A high affinity variant, CD16a.F176V was subcloned into a lentivector (LV) construct. High-titer LV was packaged and used to effectively transduce NK-92 cells. The transduction has been shown to be stable. *In vitro* testing is currently underway to ascertain whether CD16a.F176V+ NK-92 cells demonstrate enhanced ADCC and if this makes them a superior immunotherapy effector over the NK-92 cell line currently in clinical trials. In our future work, we will test transductions of other receptors and other factors that might enhance the anticancer potential of the NK-92 (and similar) cell lines.

IMMUNOTHERAPEUTIC TARGETING OF AML WITH A NOVEL CD123 CAR

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Chimeric antigen receptors (CARs) are engineered receptors transduced into immune effector cells that combine the antigen binding abilities of an antibody with the cytotoxic potential of T cells. CARs are made up of an antigen recognition domain derived from a monoclonal antibody, linked through hinge and transmembrane domains to a costimulatory domain and a CD3 ζ intracellular signaling domain. The result is a high-specificity receptor targeted against a specific tumour associated antigen (TAA).

Recently, CARs have moved to clinical trials with some dramatic successes, especially in the case of the well-studied CD19 CAR. CD19, however, is only expressed in a certain subset of malignancies. Thus, current work is focused on identifying TAAs on a range of other malignancies that may similarly be targetable by CAR therapy.

CD123 is a TAA that is known to be expressed on AML cells. This antigen plays a role in proliferation and apoptotic resistance, and is associated with poor prognosis in patients. While it is upregulated on AML cells, it demonstrates much lower expression levels on normal cells in the myeloid progenitor subpopulation, thus CD123 is a viable target for a CAR immunotherapy targeting AML.

Novel anti-CD123 antibodies were isolated and sequenced by the Medin lab. The V_L and V_H chains were assembled in-frame (in both orientations) into a full CAR sequence. This construct was then subcloned into a lentiviral backbone to facilitate expression in immune effector cells. *In vitro* testing is currently underway to confirm binding specificity and cytotoxic potential of the resulting CAR-expressing effector cells. In our future work, these CD123-directed CAR effector cells will be tested *in vivo* using mouse models of AML. Finally, as we are building a GMP Vector Facility in Toronto to generate lentivectors for clinical trials, these constructs will be fast-tracked into production/pre-clinical testing should experimental results warrant.

B CELL RECRUITING ONCOLYTIC VIROTHERAPY (B-ROV): A NOVEL APPROACH TO INDUCE TERTIARY LYMPHOID STRUCTURES FOR ENHANCED IMMUNITY TO BREAST CANCER.

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Rationale: Tertiary lymphoid structures (TLS) are associated with robust antitumor immune responses and favorable prognosis in breast cancer and other malignancies. TLS are highly organized, resemble secondary lymphoid organs and are thought to be sites where antitumor immune responses are initiated and amplified. TLS formation is induced by the CXCL-13, a chemoattractant for B cells. We hypothesized that using a recombinant oncolytic virus (OV) to force CXCL13 expression within tumor tissue would result in the formation of TLS and increased anti-tumour immunity.

Methods: A mCXCL13 transgene was engineered into a tumour-selective strain of VSV (VSV-CXCL13). Mice bearing orthotopic NOP23 mammary tumours were treated with PBS, VSV-CXCL13, or VSV encoding an irrelevant transgene (VSV-GFP). Tumour growth was monitored and lymphocytic infiltrates were assessed by IHC.

Results: T cell and B cell infiltrates were negligible in PBS-treated animals. Both strains of VSV induced robust infiltration of tumours by T cells within 10 days of treatment. As predicted by our hypothesis, VSV-CXCL13-treated tumours showed a >4 fold increase in the number of tumour-infiltrating B cells compared to VSV-GFP treated tumours. This was accompanied by multiple lymphoid aggregates composed primarily of B cells. Median survival was 33 days for PBS-treated animals and 37 days for VSV-GFP-treated animals (n.s.). By contrast, VSV-CXCL13 treatment resulted in a median survival of 56 days (p = 0.0136).

Conclusion and future directions: An oncolytic virus encoding CXCL-13 induced B cell infiltrates and aggregates in mammary tumors, and this was associated with improved tumour control. We are currently performing time-course experiments to assess whether VSV-CXCL13-induced lymphoid aggregates ultimately evolve into bona fide TLS. We are also determining whether the recruitment of B cells following VSV-CXCL13 treatment results in the production of tumour-reactive antibodies and more potent anti-tumour T cell responses through enhanced antigen-presentation.

INVESTIGATING EPIGENETIC REGULATION OF T CELL EXHAUSTION

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T cell exhaustion occurs during persistent viral infections and in many cancers and is characterized by an enduring disability of cytotoxic T cells to kill infected or malignant cells. Being the target of many emerging cancer immunotherapies, the accurate identification of exhausted T cells in cancers is of high importance. The current approach is based on cell surface marker expression but cannot provide the required accuracy, since many markers on exhausted T cells are shared with well-functioning effector T cells. We are currently performing a whole-genome DNA methylation screening to identify epigenetic marks that are unique to exhausted T cells. This epigenetic “exhausted T cell signature” will be used to develop a highly sensitive technique to accurately determine the frequency of exhausted T cells in unseparated tumor specimens from ovarian cancer patients. Moreover, in-depth analyses on the regulation and function of novel genes that display a distinct epigenetic profile in exhausted T cells will be performed to further our current understanding on the establishment and imprinting of exhaustion.

DMSO REPRESSES INFLAMMATORY CYTOKINE PRODUCTION FROM HUMAN BLOOD CELLS AND REDUCES AUTOIMMUNE ARTHRITIS

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Dimethyl sulfoxide (DMSO) is currently being used by many people, both topically and orally, to treat inflammatory conditions as well as cancer. However, little is known about the efficacy, safety and mechanism of action of DMSO in humans. We therefore examined the effects of this polar solvent on whole human blood, stimulated or not, *ex vivo*, with E. coli. We found that, between 1 and 2%, DMSO markedly inhibited cytokine production without affecting cell viability. However, at greater than 5%, significant death of leukocytes was observed, suggesting that DMSO has only a narrow window of efficacy. The two *in vivo* generated metabolites of DMSO, dimethyl sulfide (DMS) and dimethylsulfone (DMSO₂), required higher concentrations than DMSO to be anti-inflammatory. Mechanism of action studies using purified human monocytes revealed that DMSO's anti-inflammatory properties were due, at least in part, to inhibition of the ERK1/2, p38, PI3K and JNK pathways. *In vivo* mouse model studies revealed that topical administration of DMSO did not reduce the growth rate of subcutaneously implanted B16 melanoma cells, even though it did act *in vitro* to increase their differentiation. Importantly, however, topical administration of DMSO significantly ameliorated KBxN-induced arthritis in mice and this was associated with lower levels of inflammatory cytokines in the joints. Taken together, our studies suggest that DMSO may be useful for some inflammatory conditions but care must be taken to avoid toxic effects.

NOVEL PD-1 BLOCKADE BIOASSAY TO ASSESS THERAPEUTIC ANTIBODIES IN PD-1 AND PD-L1 IMMUNOTHERAPY PROGRAMS

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Programmed death receptor-1 (PD-1) and its ligand (PD-L1) are among the few important immunotherapy targets for cancer. Current PD1 assays measure cell proliferation or cytokine production in primary T cells which are tedious, have high assay variation and small assay window. To enable quantitative potency measurement for key anti-PD-1 drugs in the market or in clinical trials such as pembrolizumab and nivolumab, as well as anti-PD-L1 drugs in clinical trials such as MPDL3280A and BMS-936559, here we report the development of a robust bioluminescent cell-based PD1 blockade bioassay. For this, we built a PD-1 effector cells in Jurkat cells which stably express human PD-1 and a NFAT-RE-luciferase reporter, and a PD-L1 positive artificial Antigen Presenting Cells (PD-L1⁺ aAPC) in CHO-K1 cells which stably express PD-1 ligand, PD-L1. Once these two cell types were co-cultivated, transcriptional activation of NFAT pathway in PD-1 effector cells, mediated by binding of TCR complex with PD-L1⁺ aAPC, is significantly suppressed by PD-1/PD-L1 engagement. This inhibition can then be specifically reversed by co-incubation of PD-1 or PD-L1 blocking antibodies in dose-dependent manner, but not by the antibody for other immune checkpoint receptors such as anti-CTLA4 ipilimumab. We further developed both PD1 effector cells and PD-L1⁺ aAPC in Thaw-and-Use format so the cells can be plated for assay without the need of cell culture. The resultant PD1 assay using Thaw-and-Use cells brings the benefit of convenience, low day-to-day variation, and easy lab-to-lab assay transfer. We demonstrate the assay is able to measure relative potency for antibody biologics, and also can detect potency changes for stressed antibody samples. In summary, the reporter-based PD1 blockade assay provides a valuable tool for both drug screening and characterization in early drug discovery, and lot release and stability study in drug manufacture for therapeutic antibody drug candidates in PD-1 and PD-L1 immunotherapy programs.

TOLL-LIKE RECEPTOR-BASED THERAPY FOR CHILDHOOD ACUTE LEUKEMIA

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Acute lymphoblastic leukemia (ALL) is the most common malignancy diagnosed in children. Despite advances in the treatment, relapsed ALL remain an ongoing clinical challenge. Conventional chemotherapy is often insufficient to provide long-term cures in these challenging patients. Previously, we reported the ability of immunostimulatory DNA (CpG ODN) treatment both to enhance the immunogenicity of ALL blasts and to stimulate significant anti-ALL immune activity resulting in T cell-mediated protection against leukemia progression. To further explore the potential for toll-like receptor signaling to amplify immune responses against ALL, we investigated the ability of ligands for TLR2/1 (Pam3), TLR3 (poly I:C), and TLR7/8 (R848) to induce anti-ALL activity in comparison to CpG ODN.

Stimulation with Pam3 up-regulated CD40 expression on primary ALL cells, which were harvested from Emu-RET transgenic mice with spontaneous ALL, even higher than with CpG ODN stimulation *in vitro* ($p < 0.0001$). Furthermore, Pam3 induced the strongest lymphocyte-mediated cytotoxicity against ALL cells in a dose-dependent manner. In contrast to its *in vitro* efficacy, our syngeneic adoptive transfer studies showed that Pam3 treatment fail to inhibit the expansion of ALL cells providing no survival benefits. Both CpG ODN and poly I:C treatments significantly depleted the ALL cells in the recipients by day 21 post- leukemia challenge; however, only CpG ODN treatment conferred survival advantages. These results suggest that although its ability to initiate anti-ALL activity, the protective effect of poly I:C may be transient due to lack of T cell-mediated immune responses that is required for durable control over leukemia progression. Taken together, CpG ODN shows the most promising TLR-based therapy to eradicate residual disease after chemotherapy. Work is ongoing to understand the underlying mechanism of CpG ODN-induced anti-ALL immune responses by identifying key immune mediators.

COMBINING AN ANTI-HER2 MAB WITH TLR-3 AND -9 AGONISTS

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Introduction. Trastuzumab (HerceptinTM) is a humanized monoclonal antibody targeting HER2/ErbB-2. This receptor is overexpressed in approximately 30% of breast cancer, hence being a good target for therapy. Trastuzumab is approved since 1998 and has considerably improved the standard care of breast cancer, but unfortunately 15-20% of patients eventually relapse and develop a metastatic disease. Trastuzumab inhibits tumor cell proliferation and promotes anti-tumor immunity through mechanisms such as antibody-dependent cellular cytotoxicity (ADCC). Studies have shown that both innate and adaptive immune responses are important for trastuzumab activity. In mice, anti-ErbB2 mAb therapy requires IFN- γ -producing T CD8+ cells, type I IFN responses and MyD88-dependent Toll-like receptor (TLR) signaling. PolyI:C and CpG are well-known agonists of TLR-3 and TLR-9, respectively, and as such are potent inducers of type I IFN, IFN- γ , and IP-10 resulting in activation of NK cells and T cells. TLR-3 and TLR-9 agonists have also been shown to enhance ADCC and the recruitment of tumor-specific T cells.

Hypothesis. Administration of the TLR agonists CpG and polyI:C will synergistically act with the anti-ErbB2 mAb to improve the antitumor immune responses against ErbB2+ breast cancer.

Results. In two mouse models of ErbB2+ breast cancer, we demonstrated that combined administration of anti-ErbB2 mAb and CpG/polyI:C resulted in synergistic anti-tumor effects. Treatment of well-established ErbB2+ breast tumors with the combination regimen resulted in complete responses in the majority of mice, in contrast to single agent treatments. Mice were also protected against a re-challenge of tumor cells and had more effective CTL. Depletion assays revealed that NK cells are major mediators compared to CD8 T cells and pDC. Furthermore, qPCR have shown a dramatic upregulation of IFN- γ , TNF- α , IP-10 and IL-12 within the tumors treated with the combination of antibodies and TLR agonists.

DONOR *KIR3DL1* AND *HLA-B* SUBTYPE COMBINATIONS PREDICT ACUTE MYELOGENOUS LEUKEMIA CONTROL AFTER HLA-COMPATIBLE HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Diverse interactions between subtypes of the natural killer (NK) receptor *KIR3DL1* and *HLA-B* vary NK reactivity and HIV control. We hypothesized that these interactions could impact outcomes in allogeneic hematopoietic cell transplantation (HCT), where donor NK reactivity controls leukemic relapse. In 1328 patients with acute myelogenous (AML) undergoing HCT, subtype combinations of donor *KIR3DL1* and *HLA-B* predictive of weak or non-interaction were associated with lower relapse and overall mortality compared to strongly interacting pairs (relapse: HR=0.72, 95% CI: 0.57-0.90, p=0.004; mortality: HR=0.84, 95% CI: 0.72-0.98, p=0.030). Notably, these outcomes are especially prominent among recipients exhibiting *HLA-C1*⁺ and *C2*⁺ (relapse: HR 0.52, 95% CI: 0.37-0.73, p<0.001; mortality: HR 0.72, 95% CI: 0.57-0.91, p=0.006), and additive to the known beneficial combination of *KIR2DS1* + *HLA-C1* (relapse: HR=0.60, 95% CI 0.44-0.82, p=0.001; mortality: HR 0.77, 95% CI=0.63-0.95, p=0.01 compared with donor-patient pairs lacking beneficial *KIR3DL1* and *KIR2DS1* configurations).

HLA expression by AML cell lines and primary blasts is variable, upregulated by a pro-inflammatory environment, and capable of inhibiting NK cells. We find that NK cytotoxicity against *KIR* ligand-matched target cells corresponds with the predicted capacity of *KIR3DL1*:*HLA-B* subtype combinations for relapse control following HCT, suggesting that the NK cells' sensitivity to inhibition interferes with its anti-leukemic activity. Indeed, the intracellular retention of common *KIR3DL1*-null variants precludes inhibition of NK cells by *HLA-B*, but not their responsiveness against AML cells, demonstrating that inhibitory sensitivity and NK alloreactivity can be dissociated. Collectively, these findings reveal that sensitivity to inhibition by *HLA* present on leukemia cells is titrated by allele subtype combinations and a critical factor determining NK alloreactivity in HCT. Consideration of *KIR3DL1* and *HLA-B* subtypes in stem cell donor selection is highly feasible and may facilitate better disease control by minimizing NK inhibition in patients with AML undergoing allogeneic HCT.

Title: Interleukin-33 and Innate Lymphocytes are Immune Defense Prognosticators in Recurrent Cancer (by *Iryna Saranchova*)

Abstract

Type 2 innate lymphoid cells (ILC2s) potentiate adaptive immune responses and are the first responders to the pro-inflammatory “alarmin” interleukin 33 (IL-33) in the tissue microenvironment. However, their role in cancer progression is not defined. We demonstrate that metastatic carcinomas express reduced levels of IL-33 compared to syngeneic primary tumours. Metastatic tumours engineered to overexpress IL-33 have significantly reduced growth rates and result in a decreased frequency of circulating metastatic tumour cells. ILC2s mediate this process as metastatic tumours have significantly increased growth rates in mice genetically lacking ILC2s. Parallel studies in humans demonstrate that low tumour expression of IL-33 is a prognosticator associated with reoccurrence of prostate cancer 3.4 years more rapidly than IL-33 high-expressing tumours. These observations indicate that IL-33 can modify metastatic spread of the disease and demonstrate the role of ILC2s in cancer immune surveillance via a retinoid-related orphan receptor alpha (RORα)-IL-33-ILC2 axis.

WORKFLOW SOLUTIONS FOR HUMAN T CELL ISOLATION AND EXPANSION: EASYSEP™ T CELL ISOLATION WITH RELEASABLE RAPIDSPHERES™ AND IMMUNOCULT™ T CELL ACTIVATION AND EXPANSION SUPPLEMENT

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Adoptive cell cancer immunotherapy using patient derived, genetically modified T cells has demonstrated unprecedented success in early clinical trials. Currently, these revolutionary treatment approaches rely on several key steps including the isolation, genetic modification and expansion of patient derived T cells. Specialized reagents are required for each of these critical steps and must be seamlessly integrated into any workflow. We have developed novel reagents for the rapid isolation of highly purified human T cells from leukopheresis samples by a column-free immunomagnetic cell isolation method using EasySep™ Releaseable RapidSpheres™, a new type of magnetic particle that can be rapidly released from the isolated cells with a no-incubation, no-wash protocol performed at room temperature. Following their isolation, T cells are genetically modified to express a chimeric antigen receptor and must be expanded to therapeutically relevant numbers. To this end, we have developed an enabling technology using soluble monoclonal antibody complexes for the efficient activation and expansion of human T cells that can be incorporated into any T cell manufacturing workflow. Benefits of our novel ImmunoCult™ supplement include robust T cell expansion and the supplement is stable for at least two years at 2-8°C. Furthermore, the ImmunoCult™ supplement provides a gentle activation stimulus resulting in high viability of expanded T cells without skewing the ratio of CD4+ and CD8+ T cells and has been optimized for use with our new serum- and xeno-free ImmunoCult™-XF T Cell Expansion Medium. Taken together, we have developed a streamlined workflow solution for the isolation, activation and expansion of human T cells in serum-free culture conditions to enable this exciting new field. STEMCELL Technologies manufactures products under an ISO 13485 medical device quality management system and is actively pursuing higher compliant manufacturing of key reagents for cellular therapy applications.