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TARGETING EWING SARCOMA (ES), OSTEOSARCOMA (OS) AND NEUROBLASTOMA (NB) WITH ANTI-MCAM CHIMERIC ANTIGEN RECEPTOR (CAR) MODIFIED NATURAL KILLER (NK) CELLS

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Keywords: NK, CAR, pediatric solid tumors.

Background & Aim: Background Pediatric patients with metastatic ES, OS and NB have a dismal average 5-year survival (<25%). Novel therapeutic approaches are desperately needed (Nayyar 2019). The melanoma cell adhesion molecule (MCAM) is highly expressed in pediatric solid tumors and constitutes a novel target for immunotherapy (Orentas, 2012). We previously demonstrated the anti-tumor efficacy of anti-CD20 CAR NK cells against Burkitt lymphoma in a humanized xenograft mouse model (Chu 2015). NKTR-255 is an investigational IL-15Rα-dependent, polymer-conjugated, recombinant human IL-15 agonist that retains the full spectrum of IL-15 biological activity, including expansion of NK cells (Miyazaki 2021, Robinson 2021). Aims Here we developed an anti-MCAM CAR NK cell and investigated its in vitro and in vivo efficacy alone or in combination with NKTR-255 (provided by Nektar Therapeutics) in promoting NK cell cytotoxicity against ES, OS and NB.

Methods, Results & Conclusion: Method Peripheral blood mononuclear cells (PBMCs) were expanded into NK cells ex vivo using K562-mbI21–41BBL feeder cells in the presence of IL-2. Anti-MCAM CAR NK cells were generated by non-viral electroporation of anti-MCAM CAR mRNA into expanded NK cells. Results We found a significantly increased cytotoxicity of anti-MCAM CAR NK cells compared to mock NK cells against ES, OS and NB cells (Fig 1A). The enhanced cytotoxicity of the anti-MCAM CAR NK cell is due to specific targeting of MCAM, because CRISPR/Cas9 mediated MCAM knockout diminished the sensitivity of tumor cells to anti-MCAM CAR NK compared to mock NK cells. Furthermore, the combination of NKTR-255 significantly increased the cytotoxic activity of anti-MCAM CAR NK cells (Fig 1B). In vivo studies, we found that anti-MCAM CAR NK cells significantly decreased tumor growth (Fig 1C) and prolonged animal survival compared to vehicle and mock NK cells in a NB xenograft mouse model, but not in an ES patient derived xenograft (PDX) model. Furthermore, using an orthotopic xenograft mouse model of ES, we found that anti-MCAM CAR NK therapy significantly reduced lung metastasis (42% vs 14%, p<0.05) and prolonged animal survival (0% vs 50%, p<0.05), and NKTR-255 further enhanced these anti-tumor effects of anti-MCAM CAR NK cells (0% lung metastasis and 71% survival) (Fig 1D). Conclusion Our findings demonstrated efficacy of anti-MCAM CAR NK cells alone and/or in combination with NKTR-255 against malignant pediatric solid tumors in vitro and in vivo. Supported in part by U54 CA232561.

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NKG2A SUPPRESSION ENHANCES THE FUNCTION OF EX VIVO PM21 PARTICLE-EXPANDED NATURAL KILLER CELLS

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Keywords: NK cells, Adoptive Cell Therapy, NKG2A.

Background & Aim: One mechanism employed by tumor cells to evade immunosurveillance is through induction of the surface expression of unconventional HLA ligands that agonize inhibitory receptors on immune cells. Specifically on some tumors, HLA-E is either expressed or its expression is known to be induced by IFNγ and is indicative of resistance to immunotherapy. HLA-E agonizes the CD94/NKG2A inhibitory complex on NK cells and some T cells to lessen their cytotoxicity, potentially decreasing the efficacy of cellular therapy with these immune cells. The CD94/NKG2A inhibitory complex is in balance with CD94/NKG2C stimulatory complex. HLA-E binds both of these complexes and the cytolytic activity of NK cells is influenced by the relative ratios of CD94 complexed to NKG2A or NKG2C. Thus, altering the ratio to increase NKG2C will increase NK cell cytotoxicity.

Methods, Results & Conclusion: In this study NK cells ex vivo expanded with PM21-particle technology were shown to be highly cytotoxic and have elevated expression of NKG2A. These expanded NK cells also secrete high levels of IFNγ, inducing expression of HLA-E in tumor cells. To increase the cytotoxic potential of these ex vivo expanded NK cells, the potential to form CD94/NKG2A inhibitory complex was suppressed by either antibody blockade of NKG2A or deletion of the NKG2A gene by CRISPR/Cas9 editing (NKG2A KO PM21-NK cells). Cytotoxicity of NK cells against cancer cell lines stably expressing HLA-E was investigated by kinetic live-cell imaging either in monolayer or 3D spheroids. Multiple NK cell:target (NK:T) ratios were used to determine the effect of NKG2A blockade or down-regulation of NKG2A on NK cell cytotoxicity. Preliminary results show a 39% increase in cytotoxicity observed at 1:10 NK:T ratio at 96 h with anti-NKG2A over no antibody or isotype control and a 56% increase with NKG2A KO- PM21-NK cells over WT PM21 NK cells. Effects of NKG2A suppression on IFNγ and TNFα secretion and degranulation by NK cells will be presented as well. These data suggest that blockade of CD94/NKG2A complex can improve the function of ex vivo expanded NK cells and could provide an effector population with the potential for enhanced therapeutic efficacy.

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OFF-THE-SHELF PARTIAL HLA MATCHING SARS-COV-2 ANTIGEN SPECIFIC T CELL THERAPY: A NEW POSSIBILITY FOR COVID-19 TREATMENT

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We obtained from a single thymus, the main objective of this work is to be readily expanded from both COVID-19 and unexposed individuals and can therefore be manufactured as a biopharmaceutical product to be a rationale for restoring functional T cell immunity in COVID-19 patients by adoptive transfer of SARS-CoV-2 specific T cells. Our group has developed a new technology based on the transfer of regulatory T cells (Tregs) has acquired great interest in the treatment of diseases in which it is necessary to restore functional T cells immunity in COVID-19 patients. These results confirm the Go-Rex system can assess anti-tumor activity using the protocol developed by our group exhibit high viability (>90%) and high purity (>80%) in terms of CD25+FoxP3+ expression. ThyTreg have been observed to express low levels of immunogenicity markers (CD40L, CD80, CD86) by flow cytometry. Moreover, in vitro models of thyTreg co-culture with allogeneic peripheral blood mononuclear cells (PBMC) from healthy donors have been performed to determine if thyTreg generate an immunogenic response on PBMC, and ii) evaluate the capacity of thyTreg to suppress the proliferation of allogeneic PBMC. Even that the HLA disparity in the allogeneic co-cultures between thyTreg and PBMC was high (13 of the 21 typed pairs had HLA <4/12 concordance), thyTreg did not induce the expression of activation markers (CD25, CD69) nor the proliferation or the production of pro-inflammatory cytokines (IFN-g) by allogeneic PBMCs. Moreover, thyTreg greatly inhibit the proliferation of allogeneic CD4 and CD8 T cells, reaching levels of around 70% inhibition of proliferation at a 1:1 ratio. The results suggest that allogenic thyTreg are not immunogenic and are capable of exerting their suppressive function in an allogeneic context, indicating their possible off-the-shelf use as a treatment for transplant rejection, graft-versus-host disease, autoimmune diseases or the cytokine release syndrome characteristic of severe COVID-19 patients.

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**LONG-TERM CHARACTERIZATION OF T CELL PRODUCT INTERACTIONS USING IN VITRO 3D TUMOR MODELS AND THE GO-REX PLATFORM**

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**Keywords:** Co-Culture, T Cell, 3D.

**Background & Aim:** T cells are complex biologics that require multiple functions to exercise killing of target cells. T cells are able to detect chemokine gradients and migrate in the direction of high chemokine concentrations to arrive at tumor sites. Additionally, once at a tumor site, T cells need to navigate and infiltrate the physical environment surrounding and within tumors. Once T cells interact with and recognize cancer cells, they exercise their key function, killing. However, current in vitro assays are limited in their assessment of T cell functions: they assess each function independently and are restricted to short timeframes, thereby providing limited information about each parameter and hindering preclinical development of T cell products.

**Methods, Results & Conclusion:** Here we highlight the development of an in vitro G-Rex system by Wilson Wolf that can address critical biological properties of T cell products and provides a missing bridge between very short-term in vitro assays and long-term in vivo experiments. Named the Go-Rex, we found that this in vitro cell culture system, which has a gas permeable membrane at the base, allows for the non-disruptive, quantitative real-time assessment of the growth of genetically-engineered bioluminescent tumor models. Quantitative bioluminescence can be acquired using a common benchtop imager. We demonstrate that the growth of several different in vitro 3D cancer models can be optimized using the Go-Rex platform and assessed over the course of 2 weeks. Furthermore, to validate the benefit of the Go-Rex system, we tested the multi-tumor associated antigen (mTAA)-specific T cell therapy from Marker Therapeutics that is currently being explored in the clinic. We found that the Go-Rex system can assess anti-tumor activity using the protocol developed by our group exhibit high viability (>90%) and high purity (>80%) in terms of CD25+FoxP3+ expression. ThyTreg have been observed to express low levels of immunogenicity markers (CD40L, CD80, CD86) by flow cytometry. Moreover, in vitro models of thyTreg co-culture with allogeneic peripheral blood mononuclear cells (PBMC) from healthy donors have been performed to determine if thyTreg generate an immunogenic response on PBMC, and ii) evaluate the capacity of thyTreg to suppress the proliferation of allogeneic PBMC. Even that the HLA disparity in the allogeneic co-cultures between thyTreg and PBMC was high (13 of the 21 typed pairs had HLA <4/12 concordance), thyTreg did not induce the expression of activation markers (CD25, CD69) nor the proliferation or the production of pro-inflammatory cytokines (IFN-g) by allogeneic PBMCs. Moreover, thyTreg greatly inhibit the proliferation of allogeneic CD4 and CD8 T cells, reaching levels of around 70% inhibition of proliferation at a 1:1 ratio. The results suggest that allogenic thyTreg are not immunogenic and are capable of exerting their suppressive function in an allogeneic context, indicating their possible off-the-shelf use as a treatment for transplant rejection, graft-versus-host disease, autoimmune diseases or the cytokine release syndrome characteristic of severe COVID-19 patients.

**Background & Aim:** Background. Immunological characteristics of COVID-19 show pathological hyperinflammation associated with lymphopenia and dysfunctional T cell responses. These features provide a rationale for restoring functional T cell immunity in COVID-19 patients by adoptive transfer of SARS-CoV-2 specific T cells. Our results show that SARS-CoV-2 specific T cells could be expanded in both COVID-19 recovered and unexposed groups. Immunophenotypes were similar in both groups showing CD4+ T cell dominance, but CD8+ and CD3+CD56+ T cells were also present. Antigen specificity was determined by ELISPOT, intracellular cytokine assay, and cytotoxicity assays. One out of 14 individuals who were previously unexposed to SARS-CoV-2 failed to show antigen specificity. Moreover, ex-vivo expanded SARS-CoV-2 specific T cells mainly consisted of central and effector memory subsets with reduced alloreactivity against HLA-unmatched cells suggesting the possibility for the development of third-party partial HLA-matching products. Conclusion. In conclusion, our findings show that SARS-CoV-2 specific T cell can be readily expanded from both COVID-19 and unexposed individuals and can therefore be manufactured as a biopharmaceutical product to treat severe COVID-19 patients.

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**ALLOGENEIC USE OF TREG CELLS OBTAINED FROM PEDIATRIC THYMIC TISSUE (THYTREG) AS A CELLULAR THERAPY TO SUPPRESS EXACERBATED IMMUNE RESPONSES**

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**Keywords:** Treg, Cell immunotherapy.

**Background & Aim:** Due to its immunomodulatory potential, therapy based on the transfer of regulatory T cells (Tregs) has acquired great interest in the treatment of diseases in which it is necessary to restore immune homeostasis. Until now, autologous Treg cell therapy has proven to be safe, but the employment of blood as the source of Treg presents several limitations in terms of Treg recovery and the quality of the employed Tregs. Our group has developed a new technology to produce massive amounts of GMP Treg derived from the pediatric thymic tissue discarded in pediatric cardiac surgeries (thyTreg) that could overcome the main obstacles. Indeed, we are employing thyTreg cells with success in a clinical trial as autologous cell therapy in transplanted children. Given the large amounts of thyTreg that can be obtained from a single thymus, the main objective of this work is to evaluate the immunogenicity of thyTreg and confirm that its immature phenotype makes possible the allogeneic use of this cellular therapy in order to treat a range of immune diseases and patients.

**Methods, Results & Conclusion:** The thyTreg obtained in the laboratory using the protocol developed by our group exhibit high viability (>90%) and high purity (>80%) in terms of CD25+FoxP3+ expression. ThyTreg have been observed to express low levels of immunogenicity markers (CD40L, CD80, CD86) by flow cytometry. Moreover, in vitro models of thyTreg co-culture with allogeneic peripheral blood mononuclear cells (PBMC) from healthy donors have been performed to i) determine if thyTreg generate an immunogenic response on PBMC, and ii) evaluate the capacity of thyTreg to suppress the proliferation of allogeneic PBMC. Even that the HLA disparity in the allogeneic co-cultures between thyTreg and PBMC was high (13 of the 21 typed pairs had HLA <4/12 concordance), thyTreg did not induce the expression of activation markers (CD25, CD69) nor the proliferation or the production of pro-inflammatory cytokines (IFN-g) by allogeneic PBMCs. Moreover, thyTreg greatly inhibit the proliferation of allogeneic CD4 and CD8 T cells, reaching levels of around 70% inhibition of proliferation at a 1:1 ratio. The results suggest that allogenic thyTreg are not immunogenic and are capable of exerting their suppressive function in an allogeneic context, indicating their possible off-the-shelf use as a treatment for transplant rejection, graft-versus-host disease, autoimmune diseases or the cytokine release syndrome characteristic of severe COVID-19 patients.