An Antitumor Antibody Response to Attenuate Metastatic Melanoma in a Murine Model

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Introduction

Immunotherapy has the potential to transform cancer treatment by harnessing the immune response to disease. Metastatic melanoma has extensive disease burden and requires additional therapies for potential treatment. Previously, a common area of research was harnessing the immunological response of graft versus host disease (GVHD) against both hematological and solid tumor cancers. This is typically accomplished by extensive immunosuppression in patients and transplanting bone marrow from a donor to elicit a graft versus leukemia effect (1,2). A refinement in this technique would target the B cell pathway. The B cell pathway has the potential to mutate and increase target cancer cell affinity which would like lead to enhanced CTL response. This may not be possible in immunocompromised murine or human models of disease where there is an intrinsic defect or deficiency in CD4(+) T-cells. A murine model of disease showed that B cell activation did not occur with exogenous CD4(+) T-cell transplantation with a CD4(+) T-cell deficient recipient (3). Targeting the B cell pathway requires use of the indirect pathway of allorecognition utilizing the MHC Class II (4). Previous work indicates transplantation of donor CD4 T Cells can cause development of germinal center (GC) alloantibody response via the indirect pathway (5). Stimulation of the GC reaction leads to development of B cells via affinity maturation and development of memory B cells and plasma cells (6). Stimulation via adoptive transfer of CD4(+) T-cells may result in increased B Cell and germinal center development resulting in a potential antitumor response. Recently, Win et al showed the development of chronic graft rejection, autoantibody development, and germinal center development in a murine model of allogenic heart transplant with adoptive transfer of CD4(+) T-cells from an MHC Class II mismatched donor (7).

However, the humoral immune response typically shows tolerance to endogenous tumors. In order to develop a humoral response to cancer, self-tolerance must be overcome. It has been shown that down regulated CD8(+) T-reg activity in a murine model is associated with enhanced tumor control (8). In addition, CD4(+) T-cell activity has been associated with antitumor activity in virus induced sarcomas (9), leukemias (10), and solid tumors (11). In addition adoptive transfer of tumor-specific CD4(+) Th17 cells (12) and adoptive transfer of CD4(+) Th17 cells (13) showed antitumor activity in murine B16 melanoma (14). According to comprehensive analysis of current evidence CD4(+) antitumor activity is mediated by complex interactions of TH1, TH2, CTL, TH17, and TFH cells (15).

This experimental model focuses on promoting the humoral immune response. It has been suggested that human allograft still has varying expressions of MHC class II and thus mismatch (16). This is similar to the murine model of transplantation used in this experiment. In MHC Class I mismatched models, the prominent effect is cellular rejection and not humoral (17). The MHC Class II mismatched model would avoid this response, while developing a potential sustained alloantibody response against the tumor (18). CD4(+) T-cells transplanted into recipient mice would avoid detection by NK cells by expressing compatible MHC Class I and prolonging the potential of alloantibody generation and affinity maturation (19). This murine model of melanoma aims to examine if the development of autoantibodies extends to the tumor antigen and results in improved tumor control.

Results

Two independent experiments showed statistically significant reduction of pulmonary metastasis compared to control (n=10, mean met count; 14.6 vs. 32.2, p=0.02) and (n=20, mean met count; 13.5 vs. 28.8, p=0.01) at day 10 post adoptive cell transfer of either $4x10^6$ or $5x10^5$ cells. There was also statistically significant reduction of pulmonary metastasis compared to autologous cell transfer control (n=20, mean met count; 13.5 vs. 27.9, p=0.008) when $5x10^5$ cells were used. The level of serum anti-OVA antibodies was increased in both experiments. However, only the experiment using $5x10^5$ cells showed the increase to be statistically significant when compared to no cell transfer control (n=20, mean conc. ng/mL, 15.25 vs. 2.78, p=0.0003) and autologous cell transfer control (n=20, mean conc. ng/mL, 15.25 vs. 0.05, p<0.0001). Consistent with this observation was the finding that the frequency of secondary follicles was statistically significantly increased compared to autologous cell transfer control (n=20, mean % frequency of secondary follicles/total follicles; 69% vs. 14%, p<0.0001). In contrast, pulmonary metastasis was identical in all arms when analyzed at day 20 post adoptive cell transfer.

Experiment 1

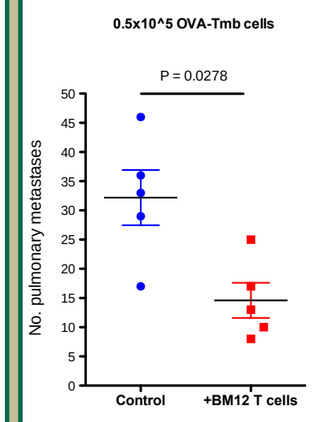

Figure 1: (n=10) Pulmonary metastasis count with adoptive transfer of $4x10^6$ CD4(+) T-cells in C57BL/6 mouse from bm12 donor transfected with $0.5x10^5$ OVA-Tmb (B16 melanoma) cells. There was a statistically significant difference in number of pulmonary metastases at Day 10.

Experiment 2

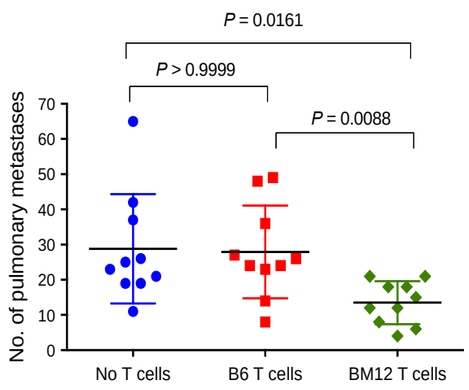

Figure 2: (n=30) Pulmonary metastasis count with adoptive transfer of $5x10^6$ CD4(+) T-cells in C57BL/6 mouse from bm12 donor transfected with $0.5x10^5$ OVA-Tmb (B16 melanoma) cells compared to autologous and no cell transfer controls. There was a statistically significant difference in number of pulmonary metastases at Day 10.

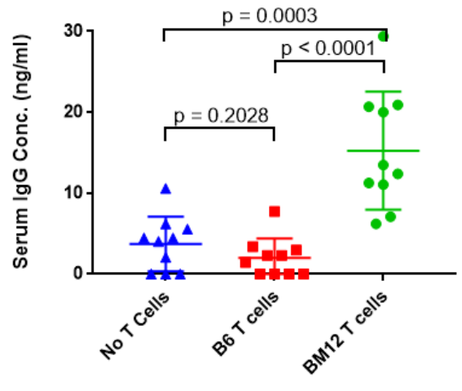

Figure 3: (n=30) Serum IgG concentration in ng/mL with adoptive transfer of $5x10^6$ CD4(+) T-cells in C57BL/6 mouse from bm12 donor transfected with $0.5x10^5$ OVA-Tmb (B16 melanoma) cells compared to autologous and no cell transfer controls. There was a statistically significant difference in serum IgG concentration at Day 10.

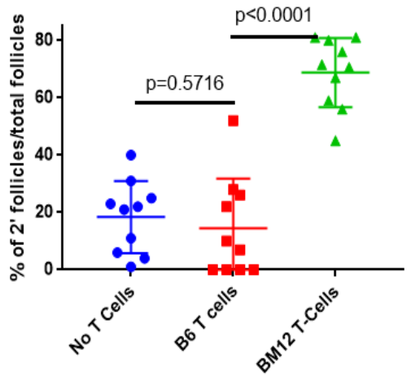

Figure 4: (n=30) GC development with adoptive transfer of $5x10^6$ CD4(+) T-cells in C57BL/6 mouse from bm12 donor transfected with $0.5x10^5$ OVA-Tmb (B16 melanoma) cells compared to autologous and no cell transfer controls. There was a statistically significant difference in percentage of GCs/total follicles at Day 10.

Methods

Animals
B6.H-2^{bm12} (bm12), C57BL/6 (H-2^b) (wild-type [WT B6]; mice were bred in-house. The bm12 mice are C57BL/6 that harbor a mutation in the H2-$Ab1^{bm12}$ allele resulting in a variant MCH class II allele. Animals were maintained in specific-pathogen-free conditions and all experiments approved by the United Kingdom Home Office under the Animal (Scientific Procedures) Act 1986.

Murine B16 Melanoma
To establish a murine model of melanoma, $0.5x10^5$ C57BL/6-derived B16 melanoma cells expressing OVA were injected via the tail vein into wild-type C57BL/6 mice. The melanin in B16 does not bleach like other pulmonary tissues and each tumor nodule can be visually counted to assess the tumor burden upon examination.

Adoptive Cell Transfer
CD4(+) T-cells were separated from donor bm12 mouse spleens using MACS separation with positive selection *(Miltenyi)*. 24h after inoculation with melanoma cells mice were injected with either $4x10^6$ or $5x10^5$ MHC class II mismatched CD4(+) T-cells from the bm12 mice. Controls included no injection and autologous cell transfer.

Quantification of Circulating Antibodies
Serum antibody responses were measured using an indirect ELISA specific for OVA IgG *(Chondrex Kit #3011).*

Histopathology and Immunohistology
Splenic GCs were identified by double-labeling 7 micrometer cryostat sections with rat anti-mouse B220 (clone RA3-6B2; BD Pharmingen, San Diego, CA) detected with Cy3-conjugated goat anti-rat IgG (clone 112-165-143; Jackson Immunoresearch Laboratories, West Grove, PA) and biotinylated rat anti-mouse GL-7 FITC. Sections were counterstained with 20% Harris' hematoxylin (Sigma-Aldrich, Poole, U.K.) and viewed using an IX81 microscope with a 320 0.70 UplanApo objective lens (Olympus, Tokyo, Japan). Images were photographed using an ORCA-ER digital camera (Hamamatsu Photonics, Hamamatsu City, Japan) and acquired with CellR 2.6 software (Olympus Soft Imaging Solutions, Munster, Germany). Numbers of GL-7 FITC GCs were expressed as a percentage of total B220+ lymphoid follicles. Two independent experiments were conducted consisting of either 5 or 10 mice per each control or treatment arm. Mice were sacrificed on either day 10 or day 20 post cell transfer and the effect on tumor burden was measured by counting pulmonary metastasis.

Immunohistochemistry

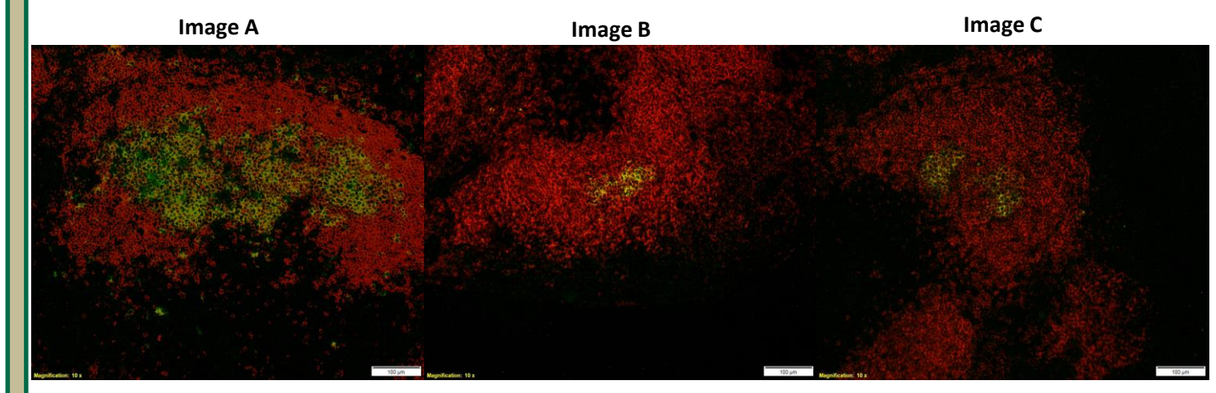

Figure 5: GC development with adoptive transfer of $5x10^6$ CD4(+) T-cells in C57BL/6 mouse from bm12 donor transfected with $0.5x10^5$ OVA-Tmb (B16 melanoma) cells [image A] compared to autologous [image B] and no cell transfer [image C] controls. B220 which indicates follicles is red and GL-7 which indicates GCs is green. These representative images show the extensive average GC development in the treatment group compared to controls.

Conclusions

These preliminary results show antitumor activity of adoptive cell transfer of MHC class mismatched CD4(+) T-cells. The finding of reduced numbers of pulmonary metastases correlates with increased serum anti-OVA IgG and increased germinal center development in the spleen. Interestingly, using an 8-fold higher number of MHC class mismatched CD4(+) T-cells did not result in a statistically significant increase of serum OVA specific IgG.

Future Work

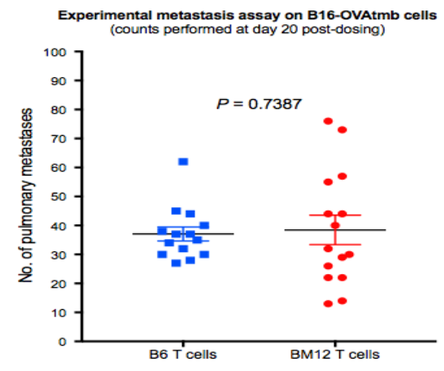

Figure 6: (n=30) Pulmonary metastasis count with adoptive transfer of $5x10^6$ CD4(+) T-cells in C57BL/6 mouse from bm12 donor transfected with $0.5x10^5$ OVA-Tmb (B16 melanoma) cells. There was no significant difference in number of pulmonary metastases at Day 20.

Additional research is required to further characterize this anti-tumor antibody response induced by the adoptive cell transfer of MHC class mismatched CD4(+) T-cells. For this purpose experiments are currently underway using a four-fold higher tumor burden.

References

1. Giralt S, Estey E, Albitar M, van Besien K, Rondón G, Anderlini P, et al. Engraftment of allogeneic hematopoietic progenitor cells with purine analog-containing chemotherapy: harnessing graft-versus-leukemia without myeloablative therapy. Blood. 1997;89(12):4531–6.
2. Porter DL, Roth MS, McGarigle C, Ferrara J, Antin JH. Induction of Graft-versus-Host Disease as Immunotherapy for Relapsed Chronic Myeloid Leukemia. N Engl J Med. 1994 Jan 13;330(2):100–6.
3. Choudhury A, Maldonado MA, Cohen PL, Eisenberg RA. The role of host CD4 T cells in the pathogenesis of the chronic graft-versus-host model of systemic lupus erythematosus. J Immunol. 2005;174(12):7600–9.
4. Ali JM, Bolton EM, Bradley JA, Pettigrew GJ. Allorecognition pathways in transplant rejection and tolerance. Transplantation. 2013;96(8):681–8.
5. Conlon TM, Saeb-Parsy K, Cole JL, Motallebzadeh R, Qureshi MS, Rehakova S, et al. Germinal center alloantibody responses are mediated exclusively by indirect-pathway CD4 T follicular helper cells. J Immunol. 2012;188(6):2643–52.

5 of 19 shown, additional references available upon request

Acknowledgements

Research was conducted in the Department of Surgery, University of Cambridge School of Clinical Medicine, Addenbrooke's Hospital, Cambridge, UK and the Sanger Institute, Wellcome Trust, Wellcome Genome Campus, Cambridge, UK. This research was completed at the University of Cambridge School of Clinical Medicine, Department of Surgery, Addenbrooke's Hospital. Additional support of the study was from the Sanger Institute, Cambridge, UK. The cross-functional team included transplant surgeons, immunologists, research scientists of other disciplines, laboratory staff, and administrative support. MUA received funding from the SELECT MD, USF Morsani College of Medicine, Tampa, FL.