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Staphylococcal Enterotoxins G And I Elicit Long-Term Anti-Tumor Responses In Humanized HLA-DQ8αβ Transgenic Mice

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STAPHYLOCOCCAL ENTEROTOXINS G AND I ELICIT LONG-TERM ANTI-TUMOR RESPONSES IN HLA-DQ8αβ TRANSGENIC MICE

by

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This dissertation, submitted by Peter Knopick in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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Peter Knopick
5-14-18

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To my sister.

Shannon Devine Pringle

1971-2017

ABSTRACT

Immunotherapies have evolved into a collection of tools used clinically in the treatment of several pathologies. *Staphylococcal* enterotoxin G and I are potent T cell activators that drive multifaceted immune responses and have been implicated in antitumor responses. The addition of SEG and SEI as useful immunotherapies could be underappreciated as mice do not respond as humans would to superantigen stimulation. HLA-DQ8 mice with established B16-F10 tumors receiving 50μg each SEG and SEI 6 and 9 days post tumor implant resulted in 80% progression free survival >300 days. In comparison, allogeneic C57BL/6 mice did not benefit from SEG/SEI therapy. Remarkably, 100% of HLA-DQ8 mice survived rechallenge with $2.5x10^5$ live B16-F10 cells >200 days post implant. In addition, SEG/SEI boosted an irradiated cell vaccine response against LLC in HLA-DQ8 mice resulting in 100% protection. However, SEG/SEI did not provide the same benefit against established Lewis lung carcinoma. The data presented herein demonstrate SEG and SEI elicit antitumor responses against melanoma and support further investigation into clinical application.

CHAPTER I

INTRODUCTION

Staphylococcal Enterotoxins

Staphylococcal enterotoxins (SE) G and I are powerful immunostimulatory proteins produced by *Staphylococcus aureus*. In 1998, SEG and SEI were characterized from different enterotoxigenic strains of *S. aureus* and were found to have superantigenic properties comparable to classical enterotoxins, like SEA and SEB [1]. Further investigation identified several other toxins in proximity to SEG and SEI within the enterotoxin gene cluster (*egc)* of *S. aureus'* pathogenicity island 3 (SaPI3) [2,3]. The crystal structures, bound to major histocompatibility complex (MHC), have also been elucidated for both SEG and SEI [4,5]. Several enterotoxins identified in the gene cluster have been shown to activate large numbers of T cells and are classified as superantigens [6-9]. Superantigens are molecules present in nearly all aspects of cellular biology; bacteria make superantigens, viruses, found endogenously in animals and suggested in plants. Superantigens are classified as molecules that elicit polyclonal cell activation in a large percentage of the cell population [10]. Superantigens were originally classified as only activating large number of T cells with B cell superantigens

documented later [11]. These non-glycosylated proteins interact outside of typical antigen processing and presentation pathways. In general, antigens are broken down proteins presented as small, ~10 amino acid, peptide lengths which are eloquently loaded onto the cell surface; taking place in 1 out of every 10,000 cells [12]. Superantigens can bypass cellular processing to directly bind to the cell surface, in turn activating >20% of cells leading to massive activation and cytokine release. Dysregulation as a result of SE activation can lead to a lethal cytokine storm. Enterotoxins can provoke emesis when ingested by humans and monkeys, often as a result of bacterial contamination of food and/or water. Mice nor rats have emetic responses to enterotoxins likely due to a lack of specific neurological architecture [13], yet still respond to enterotoxins with typical T cell activation and cytokine release.

Superantigens isolated from *Streptococcus* and *Staphylococcus* species have been extensively characterized [14-17], in addition to some viral superantigens (18). *Yersinia pseudotuberculosis* mitogen (YPM) is the only identified superantigen from Gram negative bacteria [19], suggesting superantigens are not advantageous for gram negative bacteria and are either genetically repressed or have been lost over time. Lipopolysaccharide (LPS) from gram negative bacteria has been shown to dramatically enhance the effects of superantigen stimulation [14]. Enterotoxins can activate T cells by binding to the variable beta region on the t cell receptror (TCR) and MHC. Superantigens can be categorized into groups based on MHC interactions that: (1) bind the TCR and MHC α-chain (SEB & SEG); (2) bind

TCR and MHC β-chain (SEI); or (3) crosslink MHC by binding to the α-chain and β-chain (SEA) [14, 20, 21, 28-34]. There is controversial evidence to support some superantigens can also bind with MHC Class I molecules, but this interaction has not been completely characterized [22]. SE specificity towards Vβ segments of the TCR have also been extensively characterized [23- 25]. Superantigen dissociation rates where SEA has been shown to stay at the cell surface longer than SEB [26,27]. Endogenously expresses superantigens in mice have been shown to selectively eliminate T cells based on V beta specificity; and like many superantigens, endogenously synthesized superantigens are thought to be derived from viral DNA [28].

T cells and Cancer

Lymphocytes, immune cells slightly larger than a red blood cell (RBC), have once again become major players in the current landscape of cancer therapy. There are two general types of lymphocytes in humans and mice, B and T lymphocytes, or B and T cells. SEG and SEI activate T cells through the help of MHC on antigen presenting cells (APCs), like B cells. In combination, T cells work synergistically with APCs and thus responsible for the pathology associated with superanitgens [29-35]. Historically B and T cells were classified based on the site of maturation and development. Nearly all lymphocytes originate from the bone marrow. Cells that migrate and later mature in the bone marrow are designated B cells; whereas cells migrating through the thymus for maturation are called T cells. Ironically, B cells were originally identified in the bursa of Fabricius, a lymphoid structure in

birds that atrophies after 6 months of age, not the bone marrow, a rare happenstance with immunological nomenclature. B cells from birds are similar to mammalian B cells yet are derived from entirely different structures, the bursa and bone marrow respectively [32].

The immune system is the collection of these cells, and many more, contributing to a delicate balance to recognize self vs non-self. Immune cells that detect abnormalities and homeostatic imbalance activate to purge anything deemed foreign. T cells, many expressing either $CD4^+$ and $CD8^+$, are responsible for surveying the host environment and eliminate cells that appear abnormal, either malignant or not. The inability for the immune system to recognize abnormal cells is a well-accepted mechanism to describe metastatic disease, like cancer. T cell mediated immunity in response to cancerous, abnormal cells deploy a mix of secreted proteins, like granzymes, perforin, interferon gamma (IFNy), and cell to cell contact killing [59-62]. Countless therapies have attempt to utilize T cells against cancer including the more recent advent of chimeric antigen receptor (CAR) T cells directed specifically at antigens on tumors [62]. These cells are then infused back into the patient through adoptive transfer. Even coupled with advancements in T cell therapy delivery [61], the reliability to which these adoptively transferred cells confer long term anti-tumor responses is yet unattainable.

Murine Models of Cancer

The B16 melanoma cell line originated from a spontaneous melanoma in C57BL/6J mice. The cell line was later manipulated by injecting cells intravenously into mice, sacrificing the mice 3 weeks later to harvest lungs and isolate lung metastases [115]. The pulmonary metastases were made into single cell suspensions, grown in vitro and reinjected into C57BL/6 mice. B16-F1 cells underwent the aforementioned process 1 time whereas B16-F10 cells where subject to 10 passages from mouse to culture dish [114-118]. B16-F10 cells where used for this study due to the highly metastatic potential of these cells compared to similar models.

Lewis lung carcinoma (LLC) cells were originally derived from a spontaneous arising tumor from C57BL/6 mice [119, 120]. LLC has been found to be a consistent, reproducible model to study non-small cell lung cancer (NSCLC) in mice and has been used in several preclinical research leading to approved therapies in humans. LLC and B16 cells are both epithelial in appearance, however, LLC cells grow loosely adherent to the flask whereas B16 cells strictly adhere. Of note, both cell lines will form large tumors which will overtake the omental tissue when injected intraperitoneally. LLC cells have also been observed to invade the mesentery as well as omental tissue (laboratory observation). The omental tissue in mice differs to humans in that it does not extend downward over the intestines. Murine omental tissue is also smaller, connected to the stomach, pancreas and spleen. Harvesting the omentum should be done using the method

described in Kahn et al. which uses a flotation technique to isolate the omental tissue from the surrounding organs [102]. The omentum has been shown to contain immune aggregates, sometimes referred to as milky spots because of their fatty white appearance, along with fat and blood vessels. Preferential attachment to the omentum has been suggested to result from several factors: the available blood supply and VCAM expression on mesothelial cells which also secreted VEGF-A [104].

Historical Perspective: Immunotherapies

The treatment of malignant disease has been meticulously documented throughout many generations of human evolution where early recordings of the disease in the Edwin Smith Papyrus date back to around 3000 BC [51-58]. The many documented accounts throughout history illustrate an evolution of human responses tied to an emotional and physical relationship with disease. Often described are crude, yet sophisticated, techniques of excising disease, sometimes tumors, through surgical techniques; many of which, though refined, are still recognizable in practice. Swiss physician Paracelsus is often credited for laying the foundation for chemotherapy and regarded as a founding father of toxicology. Paracelsus' introduction to his self-described "poisonous compounds", including mercury, zinc, lead and more as treatments for internal disease eventually lead to his death in 1541 [55].

The ensuing centuries led to more and more observations that seemingly advanced understanding of malignant disease. Physicians and scientists would continue to make and mold hypothesis to endless, often reckless means. Jean Astruc, an early 1700s French physician, carried out experiments to identify differences in the acidity of cooked meat in relation to bile, by taste [57]. He noted no difference in taste between cooked beef or cancerous breast. For years, the battle between cancer and scientist will continue to claim countless lives and shape the course of human history. German Emperor Frederick III, ruling for 99 days until his death in 1888 from laryngeal cancer was examined by several physicians, including Rudolf Virchow, colloquially referred to as "the pope of medicine" [55]. Virchow's career, highlighted in numerous publications, which are not completely referenced here, include; linking cancer to the outgrowth of specific cells, specifically differentiating between splenic, myeloid appearance, and lymphatic, lymphocytic appearance, cells. Virchow is also one of several scientists credited in discovering leukemia, what he called leukämie [57]. Virchow, who eventually died of a broken leg sustained while jumping from a moving streetcar at age 80, was a staunch opponent to germ theory and was skeptical of the experiments demonstrating bacteria as a causative agent of disease[58].

Spontaneous remission of an inoperable neck sarcoma was observed by William Coley who later injected patients with preparations of bacteria and bacterial byproducts, known as "Coley's Toxins" [63]. His and others attempts at

controlling various malignancies stem from clinical observations in spontaneous regression of the diseases. Specifically, Coley administered Streptococcus isolations he procured from other patients, grown in culture, and administered to patients at the site of diseases in order to elicit erysipelas [64-66]. It was noted that the heat, or inflammation, generated was crucial in a durable response. These pioneering, yet rudimentary in practice, paved the way for years of research into toxin-based therapies. Coley, often credited as the Father of immunotherapy, the treatment of 30 (of 270) cases were outlined in a review published posthumously by his daughter, Helen Coley Nauts [64]. Attempts to refine as isolate compounds from these preparations is outside the scope of this discussion, suffice to say that many are still working on it at the time of writing this. One such component that has been shown to contribute towards controlling malignant cell outgrowth are superantigens [71-77].

The 1990s brought the race for tumor associated antigens (TAAs). In 1988, Steven Rosenberg, now current Head of the National Institute of Health's Tumor Immunology Section, and colleagues isolated tumor infiltrating lymphocytes (TILs), expanded them in vivo via IL-2, and infused them back into the patients [book]. Although this did not yield substantial responses, this was the first time T-cells were specifically used as a therapy, a paradigm shift in the way we think about treating cancer. Several labs over the next 30 years have been perfecting the use of T-cells as therapies [60, 74].

Enterotoxins and Cancer

Over the past decade, numerous immunotherapies have provided some respite for patients with a variety of cancer types [67]. Though still in its infancy, immunotherapy has yet to live up to expectations in providing consistent progression free survival, let alone complete regression. The first publication of monoclonal antibody use in clinical trials in 2002 targeted CTLA4 expressed on T cells [152]. It took until 2011 for Ipilimumab (Yervoy® under Bristol-Myers Squib) to be approved for the treatment of melanoma in the United Sates[153]. Cancer vaccine strategies have been largely unsuccessful with one approved therapy, Sipuleucel-T (Provenge® under Dendreon) in 2010, for hormone-refractory prostate cancer [154]. Although one can hypothesize revisiting this as a future waste of time, results have not been released (NCT01832870) from 9 patients receiving Sipuleucel-T and Ipilimumab. Nonetheless, combinations of different immunotherapies, specifically CTLA-4 and PD-1 antagonists, have proven beneficial when used together [154].

Several, largely unsuccessful, therapies have attempted to use SEs in to treat various cancers. Several of these studies were hindered by the presence of neutralizing antibodies to classical superantigens found in >80% of sampled human sera [68-70]. For decades, China has used Gaojusheng, an SEC based therapy, for the treatment of cancer [67]. Exciting work by Dr. David Terman, and others, have demonstrated the therapeutic efficacy in using superantigens for the enterotoxin gene cluster as in the treatment of pleural effusion in patients with

non-small cell lung cancer (NSCLC) [71]. In addition to neutralizing antibodies, SE therapy has been hindered by toxicity associated with increased proinflammatory mediators, like TNFα [21, 68-70]. Reducing adverse toxicity associated with SEs has included using monoclonal antibodies against CTLA-4 [77, 151], adjusting dosages and time administered, or adding compounds such as Δ9Tetrahydrocannabinol [76]. Kominsky et al. used SEA and SEB in the treatment of B16 melanoma and produced long term survival in C57BL/6 mice suggested an IFNγ mediated response that requires both $CD4^+$ and $CD8^+$ T cells [78].

The remainder of this document outline a series of experiments I performed using SEG and SEI in the treatment of B16 melanoma and Lewis lung carcinoma. By no means are these experiments comprehensive to understanding the immune responses elicited by SEG and SEI nor is the precise mechanism that underlies the phenomena presented herein. In that context, the following chapter outlines methods and information required, including tips and tricks observed along the way, for someone repeat these experiments. The results demonstrate a clear antitumor response that is benefited by SEG and SEI.

CHAPTER II

METHODS

Ethics

All research performed, including animal and tissue collection, was conducted in accordance with the Animal Welfare Act and with the approval of the University of North Dakota's Institutional Animal Care and Usage Committee (IACUC).

Mice

Mice were bred and maintained in pathogen-free conditions within the Center for Biological Research at the University of North Dakota. A laboratory inbred colony of C57BL/6 mice and human leukocyte antigen (HLA) transgenic mice were used for experiments. Transgenic mice include: HLA-DQ8 (DQA*0301/DQB*0302), originally a gift from Dr. Chella David (Mayo Clinic, Rochester, MN) and HLA-DR3 (DRA*0301/DRB*0301), gifted from Dr. Malak Kotb (University of North Dakota, Grand Forks, ND). The generation of these mice were described previously [84-86]. Similar to DR3 mice, DQ8 mice were generated on $H2^b$ haplotype mice lacking H2A (20) and inherent H2E expression, thus making them devoid of murine class II. Specific gene insert was confirmed via polymerase chain reaction (PCR) and subsequent protein expression was confirmed via flow cytometry using anti-DR (Clone Tu39,

BioLegend) and anti-DQ (Clone SK10, eBioscience) for HLA-DR3 and HLA-DQ8 confirmation, respectively **Table 1**.

Enterotoxins

Purified, recombinant SEG and SEI were obtained from Aldeveron (Fargo, ND). SEA, SEB and SEI were obtained from Toxin Technology (Sarasota, FL). All reagents were kept at 4°C or -20°C and subject to only 1 freeze-thaw cycle. The sequences used for SEG SEI manufacturing were originally elucidated by Munson et al. [1]. The protein sequence for SEI:

MQGDIGVGNLR_NFYTKHDYIDLKGVTDK_NLPIANQLEFSTGTNDL
ISESNNWDEISKFKGKK_LDIFGIDYNGPCKSKYM_YGGATLSGQYLNSAR
KIPINLWVNGKHKTISTDK_IATNKKLVTAQEIDVKL_RRYLQEEYNIYGHN
NTGKGKEYGYKSKFYSGFNN_GKVLFHLNNEKSFSYDL_FYTGDGLPVSFL
KIYEDNKIIESEKFHLDVEISY_VDSN

The protein sequence for SEG:

MQPDPKLDEL_NKVSDYKNNKGTMGNVM NLYTSPPVEGRGVINSR
QFLSHD LIFPIEYKSY NEVKTELENT ELANNYKDKK 81 VDIFGVPYFY
TCIIPKSEPD INQNFGGCCM YGGLTFNSSE 121 NERDKLITVQ
VTIDNRQSLG FTITTNKNMV TIQELDYKAR 161 HWLTKEKKLY
EFDGSAFESG YIKFTEKNNT SFWFDLFPKK 201 ELVPFVPYKF
LNIYGDNKVV DSKSIKMEVF LNTH

Antibody	Clone	Company
HLA-DQ	SK10	Invitrogen
HLA-DR,DP,DQ	Tü39	BioLegend
H-2K^{b}	AF6-88.5	BioLegend
I-A/I-E	M5/114.15.2	BioLegend
CD3	17A2	TONBO Biosciences
CD4	RM4-5	BioLegend
CD8	53-6.7	TONBO Biosciences
CXCR3	CXCR3-173	BioLegend
CTLA-4	UC10-4F10-11	TONBO Biosciences
PD-1	J43.1	TONBO Biosciences
TLR2	T2.5	BioLegend
TLR4	SA15-21	BioLegend
CD44	IM7	BioLegend
CD62L	MEL-14	BioLegend
KLRG1	2F1	TONBO Biosciences
CD25	PC61	TONBO Biosciences
FOXP3	MF23	TONBO Biosciences
CD103	2E7	BioLegend
CD39	Duha59	BioLegend
T-bet	4B10	BioLegend
IFNγ	XMG1.2	BioLegend
Perforin	S16009A	BioLegend
Granzyme B	QA16A02	BioLegend
Proliferation Dye		eBioscience
CD16/32	93	BioLegend
Annexin V		BioLegend
Propidium Iodide		TONBO Biosciences
Live/Dead		TONBO Biosciences

Table 1: Antibodies used for flow cytometry experiments.

Cell Culture

Tumor Cell Lines

B16-F10 (CRL-6475^{TM}) murine melanoma cells and Lewis lung carcinoma (LLC) (CRL-6475^{TM}) were obtained from American Type Culture Collection (ATCC) and maintained according to manufacturer recommendations in complete Dulbecco's Modified Eagle's Medium ((DMEM) Gibco) containing 10% heat inactivated fetal bovine serum (FBS) (Atlanta Biologicals), 50 IU/ml Penicillin and 50 μg/ml Streptomycin (MP Biologicals), and HEPES. Cells were maintained humid at 37°C with 5% CO_2. Frozen 1 ml aliquot stocks were made by freezing ~1x10^6 cells in complete DMEM supplemented with 50% vol/vol sterile dimethyl sulfoxide (DMSO). Cells were maintained in liquid nitrogen vapor phase.

Of note, tissue culture flasks and other plastics used for cellular assays remained consistent throughout these experiments (CytoOne and Falcon). It has been demonstrated that different surfaces of tissue culture plastics can alter phenotypes of adherent cells [92, 93]. In addition, breast implants with specific textured surfaces were found to increase the risk of breast cancer in 82% of patients receiving the implant [94, 95]. The World Health Organization (WHO) provisionally defined the cancer in 2016 as breast implant-associated anaplastic large cell lymphoma (BIA-ALCL) [96].

Primary Cell Isolation

Peritoneal lavage cells were isolated by sacrificing mice, injecting 5 ml HBSS (Gibco) into the peritoneal cavity, massaging for 10 seconds and collecting 4 ml of fluid. It is important to take note of injection as not to perforate or injection into the intestines. Cell yield will vary substantially without consistency in technique but will average ~1 million total cells. ACK lysis was used as needed while maintaining consistency. Cells were filtered through a 70 μm cell strainer before counting and use.

Splenocytes were isolated by passing spleens through a 70 μm cell strainer (Falcon) with bottom plunger of 5ml or 10ml syringe. Cells were washed with DMEM, ACK lysed, washed and strained again before counting. Cell yield will depend but should average 50-100 million total cells in naïve mice.
Inguinal lymph nodes were isolated and placed in 1ml HBSS. Nodes should sink when removed without major fat contamination. Both lymph nodes were mashed through a 70 μm cell strainer (Falcon) with bottom plunger of 5ml or 10ml syringe. Cells were washed with DMEM and strained again before counting. Cell yield is varied but average 0.5-1 million total cells per lymph node in naïve mice.

Omental tissue was isolated and washed by transferring through several wells containing HBSS. The tissue was placed in 1 ml media in 24 well plates and cells were allowed to disassociate freely into the media over 48 hours.

Tumor Implant

Tumors were implanted intraperitoneally resulting in solid tumors at the omentum ultimately leading to death of the animal.

Prior to implant, cells were grown in T75 tissue culture flasks and harvested for use at < 80% confluency. Cells were rinsed with HBSS prior to addition of trypsin w/EDTA (Gibco) for ~5 minutes. cDMEM was used to neutralize trypsin and cells were washed 2 times with HBSS. Cells were resuspended at $1x10^5$ or $2.5x10^6$ cells/ml in HBSS w/ca^+mg^+. The stock was aliquoted into .5ml tubes for individual dosages to insure consistent tumor burden among mice.

Of note, LLC cells typically present as a mix of suspension and adherent cells. For these studies, only the adherent cells were used for implant. This should not affect overall results as this technique was deployed in vitro and mixed populations continued throughout each subculture.

Just before injection, after cell aspiration, wipe the needle with 70% ethanol to reduce subcutaneous tumors devolving at the injection site outside the peritoneal cavity.

Vaccination Protocol

Mice were injected with 1 x 10^6 irradiated (15,000 rads) B16-F10 melanoma cells IP in 100µl HBSS on day -13. Mice received 100µl injections IP of SEG (50µg) + SEI (50µg) on days -7 and -3. Mice were challenged day 0 as described above. Mice were continuously evaluated and sacrificed when moribund.

Flow Cytometry

Cell Staining

Cells were washed with HBSS, stained with Ghost Dye for viability (TONBO), FC blocked and stained for extracellular antigens via standard protocols. Cells were fixed and permeabilized using Foxp3 staining buffer kit (TONBO) for intracellular cytokine and transcription factor analysis. See **Table 1** for list of antibodies used in this manuscript. Fluorescence minus one (FMO) and single stained controls were used for gating and compensation. Gating strategies are indicated within each experiment. In general, doublets and cell debris were excluded with only Ghost Dye negative cells being used for analysis. Samples were analyzed using a BD LSRII or Symphony A3 flow cytometer in the North Dakota Flow Cytometry and Cell Sorting (ND FCCS) Core. Data was analyzed using FlowJo software.

Proliferation Assay

T cell proliferation was evaluated using carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies) or Cell Proliferation Dye eFluor 450 (eBiosciences) dye. In short, splenocytes were stained with proliferation dye as described previously (25). After which, $2x10^5$ cells/well were seeded in 96-well round-bottom tissue culture plates (Becton Dickinson) in cDMEM and stimulated with Concanavalin A (1μg/ml) (Sigma Aldrich) or indicated superantigens for 72 hrs (37°C, 5% CO_2 and humidity) in 200μl total volume. After 3 days, cells were processed for flow cytometry.

Cytotoxicity Assay

Cytotoxicity was measured by annexin V and propidium iodide (PI) (TONBO) staining. Briefly, B16-F10 cells were seeded 2 hours prior to coculture with lymphocytes at indicated ratios. CD4 and CD8 cells were isolated from C57BL/6 and HLA-DQ8 splenocytes using EasySep™ CD4 and CD8 negative selection kits (STEMCELL Technologies) in accordance with manufacturer specifications. All cells were counted using a hemocytometer, cell viability was determined via trypan blue exclusion (>90% viabke) and purity was assessed via flow cytometry. Cells were cocultured for 4 hours (37°C, 5% CO_2 and humidity) and stained with annexin V and PI using BioLegend's annexin V binding buffer according to manufactures recommendations and analyzed immediately via flow cytometry. Cells were gated to exclude doublets and debris **Figure 1**.

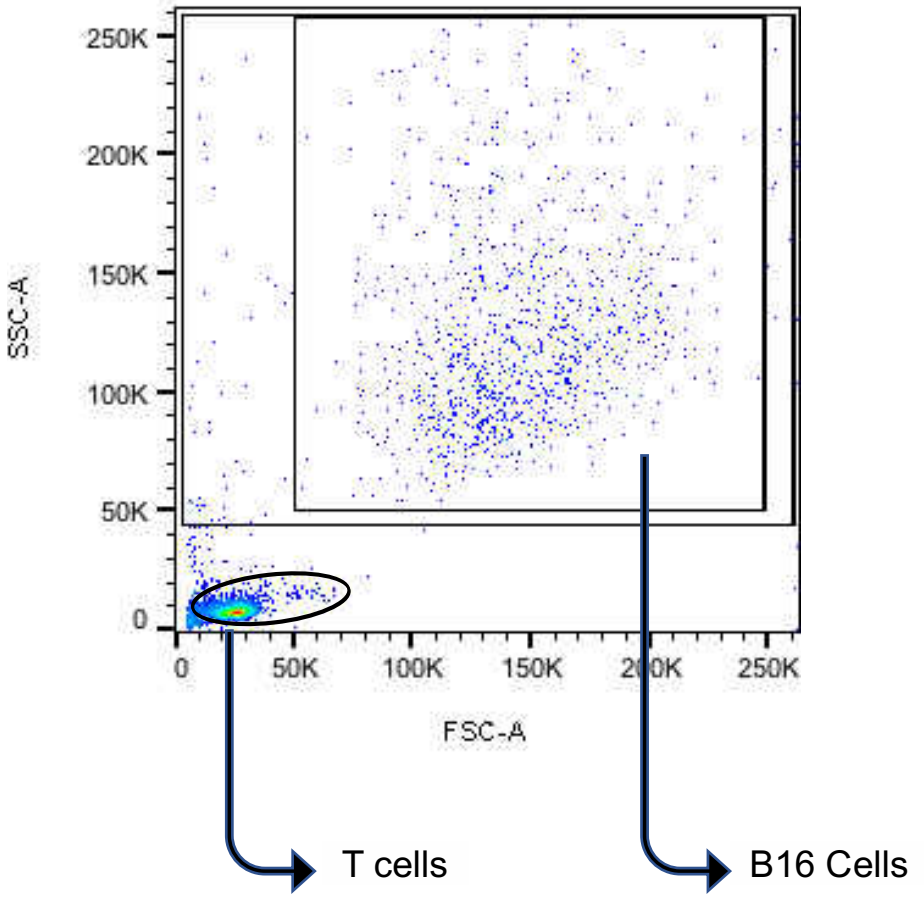


Figure 1 . Gating strategy for flow cytometry-based cytotoxicity assays.

T cells were cocultured with B16-F10 cells in 96 well flat bottom plates. Specific T cell responses and B16 responses can be distinguished based on separation via FSC vs SSC. Cytotoxicity was measured on B16 cells for annexin V and propidium iodide staining.

Cytokines and Chemokines

Blood was collected from mice in EDTA tubes via submandibular venipuncture. Plasma cytokine and chemokine concentrations were measured using BioLegend's LEGENDplex™ kits according to manufacturer recommendations. Samples were processed via flow cytometry.

Peripheral Blood Smear and Cytospin Staining

Blood smears were made from blood collected in ethylenediaminetetraacetic acid (EDTA). Body fluids and single cell suspensions were resuspend in cDMEM or HBSS w/10% serum to maintain cell integrity. Cytospins were spun for 3 mins at 80 x g. Smears and cytospins were stained with Hema 3™ Wright-Geimsa (Fischer Scientific). Several references provide reference valves for common laboratory mice (122). Several common morphologies exist between human and murine immune cells and therefore human morphological references can provide guidance.

Thymidine Incorporation Assay

C57BL/6 and HLA-DQ8 splenocytes ($2x10^5$ cells/well) were seeded in 96-well round-bottom tissue culture plates (Becton Dickinson) in complete RPMI. Splenocytes were cultured 72 hrs (37°C, 5% CO_2 and humidity) in 200µl total volume with medium alone, various concentrations of SEA, SEB, SEG, and SEI (0.001–1000 ng/ml) and with Concanavalin A (1.25µg/ml) (Sigma Aldrich). At

68 hrs incubation, cells were pulsed with 1 μCi/well [^{3}H] thymidine (Perkin Elmer); radioactivity was measured 4 hours later as described previously [98].

Statistical Analysis

One-way analysis of variance with Bonferroni's posttest and student's t test were performed where indicated. Kaplan Meier curves and Mantel-Cox Test were used to evaluate survival data. Statistical analysis was performed using GraphPad Prism software version 7.0a (La Jolla, CA).

CHAPTER III

RESULTS

Characterization of Staphylococcal Enterotoxin G and I

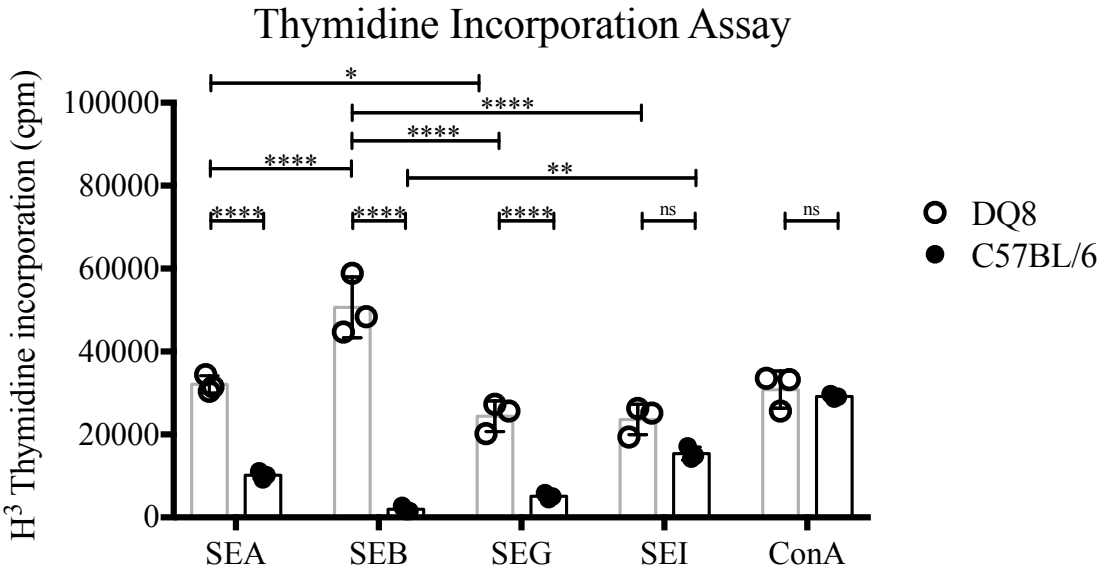
SEG and SEI have been shown to cause robust T cell proliferation and with massive cytokine release in human T cell cultures [4, 5, 73]. Murine T cell responses vary in response to SE stimulation. HLA-DQ and C57BL/6 splenocytes were used to measure activity of SEG and SEI compared to classical superantigens, SEA and SEB using a standard 3 day thymidine incorporation assay **Figure 2A.** Splenocytes were isolated from 6-8 week old mice, stained with cell proliferation dye and stimulated with SEA, SEB, SEG, SEI (all 1μg/ml), using concanavalin A (1μg/ml and media alone as controls. As demonstrated previously, both $CD4^+$ and $CD8^+$ cells respond to SE stimulation and stimulated to a greater extent in HLA transgenic mice compared to C57BL/6 **Figure 2B**. These data confirm previous findings of enterotoxin potency and support using HLA transgenic mice to more accurately investigate the efficacy of SEG/SEI therapy.

SE stimulation, including SEG and SEI, has been associated with increased levels of interferon gamma (IFNγ) both in vivo and in vitro. To identify which cells are responsible for this production, splenocytes were isolated and stimulated with SEG, SEI and SEG+SEI for 3 days. After which, cells were blocked with

brefeldin A and monosen (BioLegend), permeabilized and stained for IFNγ **Figure3**. These data reveal CD8+ T cells from both C57BL/6 mice and HLA-DQ8 mice produce IFNγ and $CD4^+$ T cells from DQ8 mice produce more IFNγ than C57BL/6.

Immune signals provoked by SE stimulation are often met with inhibitory immune signals that balance the immune response. PD-1 and CTLA-4, two such inhibitory molecules produced on T cells in response to activation, expression was evaluated on $CD4^+$ and $CD8^+$ T cells in response to SE stimulation (all 1μg/ml) **Figure 4**. $CD4^+$ and $CD8^+$ T cells were gated on CXCR3 expression, a chemokine receptor upregulated on activated Th1-like cells [126-130]. Collectively, these data support the potential for using immune checkpoint inhibitors, antibodies against PD-1 and CTLA-4, to enhance SEG/SEI activated, $CXCR3^+$ T cells.

A


Thymidine Incorporation Assay
H3 Thymidine incorporation (cpm)
100000
80000
60000
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*
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ns
ns
SEA
SEB
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ConA
DQ8
C57BL/6

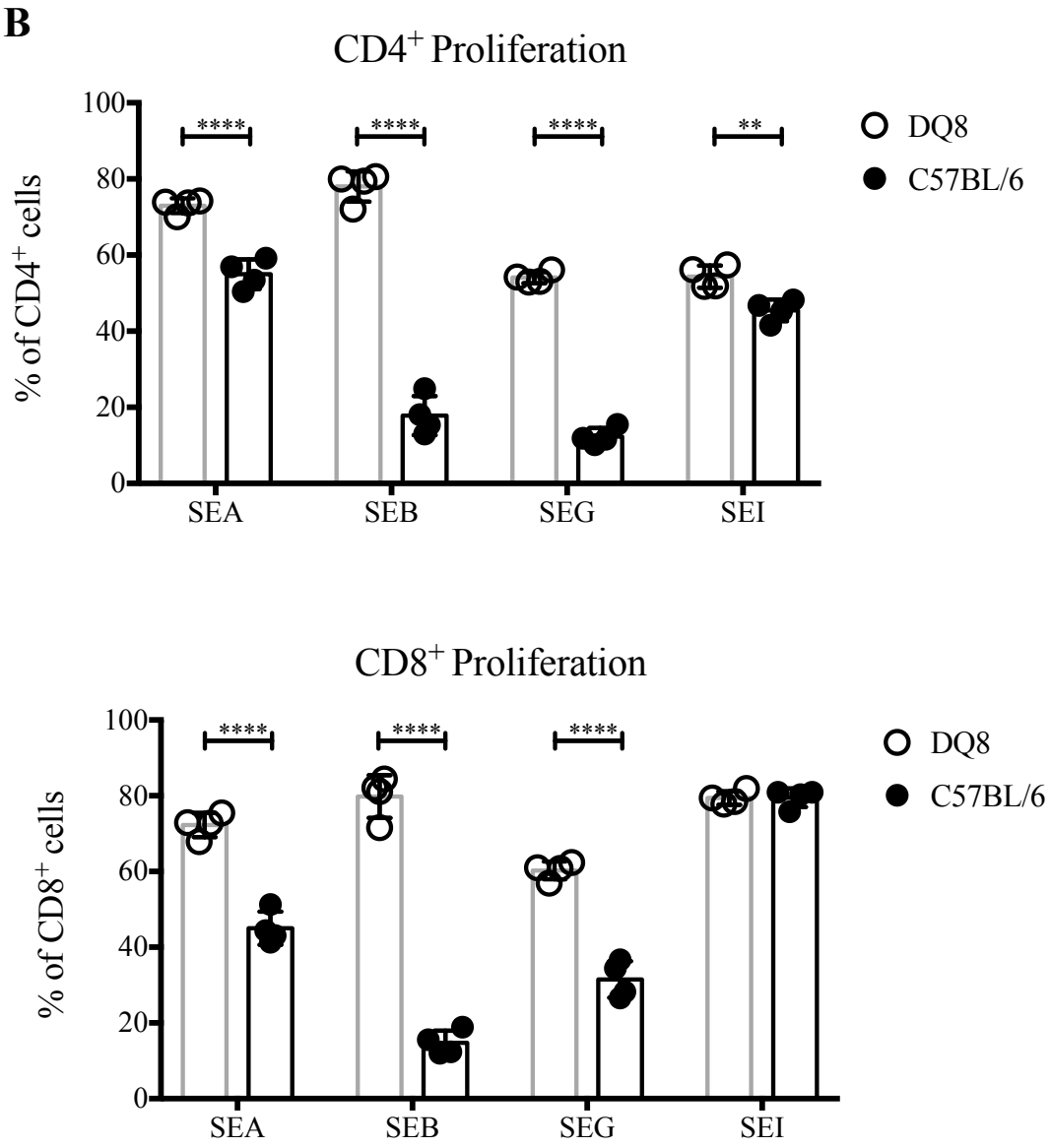
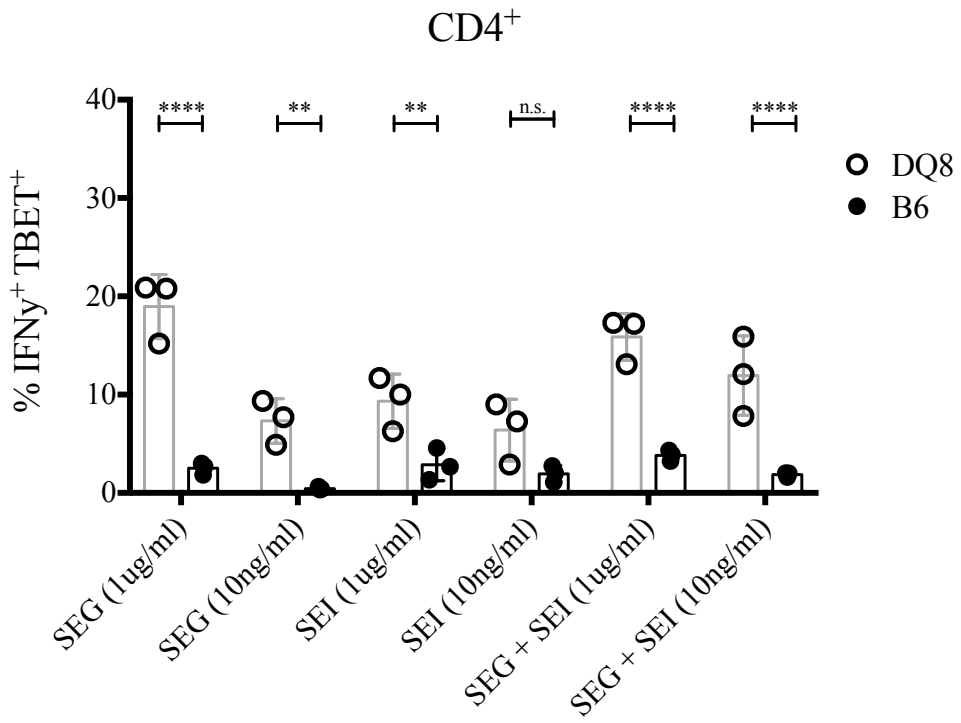


Figure 2 . DQ8 mice respond strongly to staphylococcal enterotoxins G & I.

Splenocytes isolated from 6-8 wk old DQ8 mice exhibited stronger mitogenicity to SEs (1ug/ml) compared to C57BL/6 mice in typical thymidine incorporation assay (A). Specific CD4+ and CD8+ T cell proliferation was measured by dye dilution via flow cytometry and reported as % proliferation (B). n=3-5. Graphed mean with SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.001.

A



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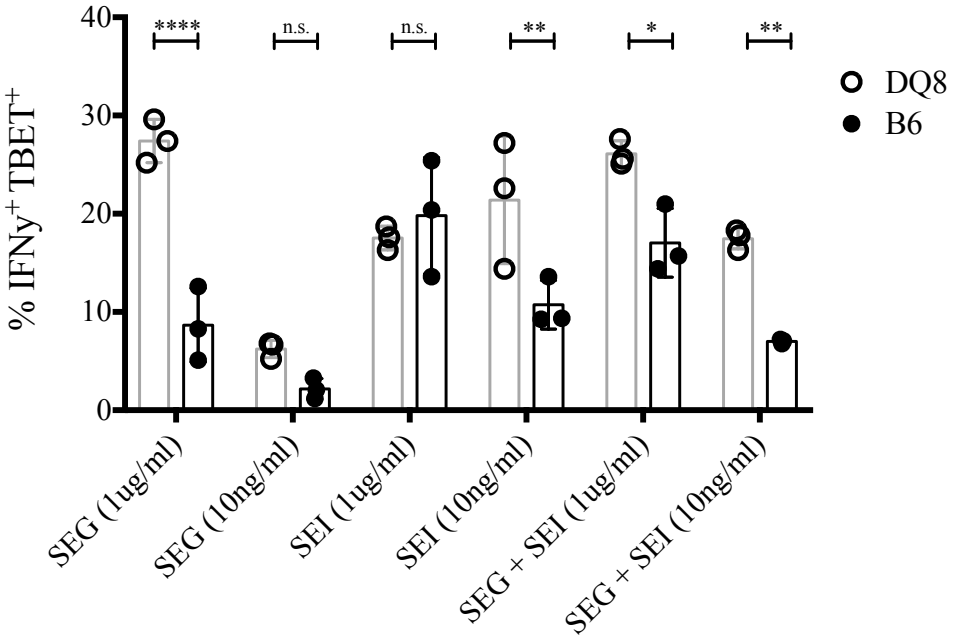

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C

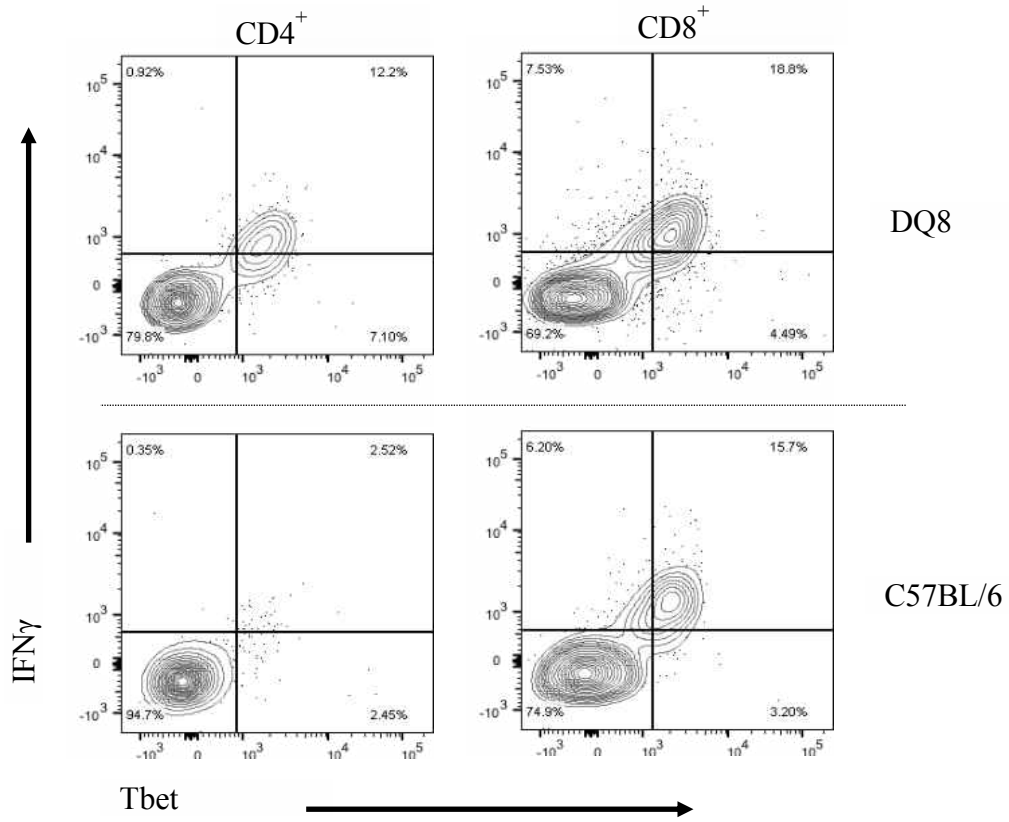
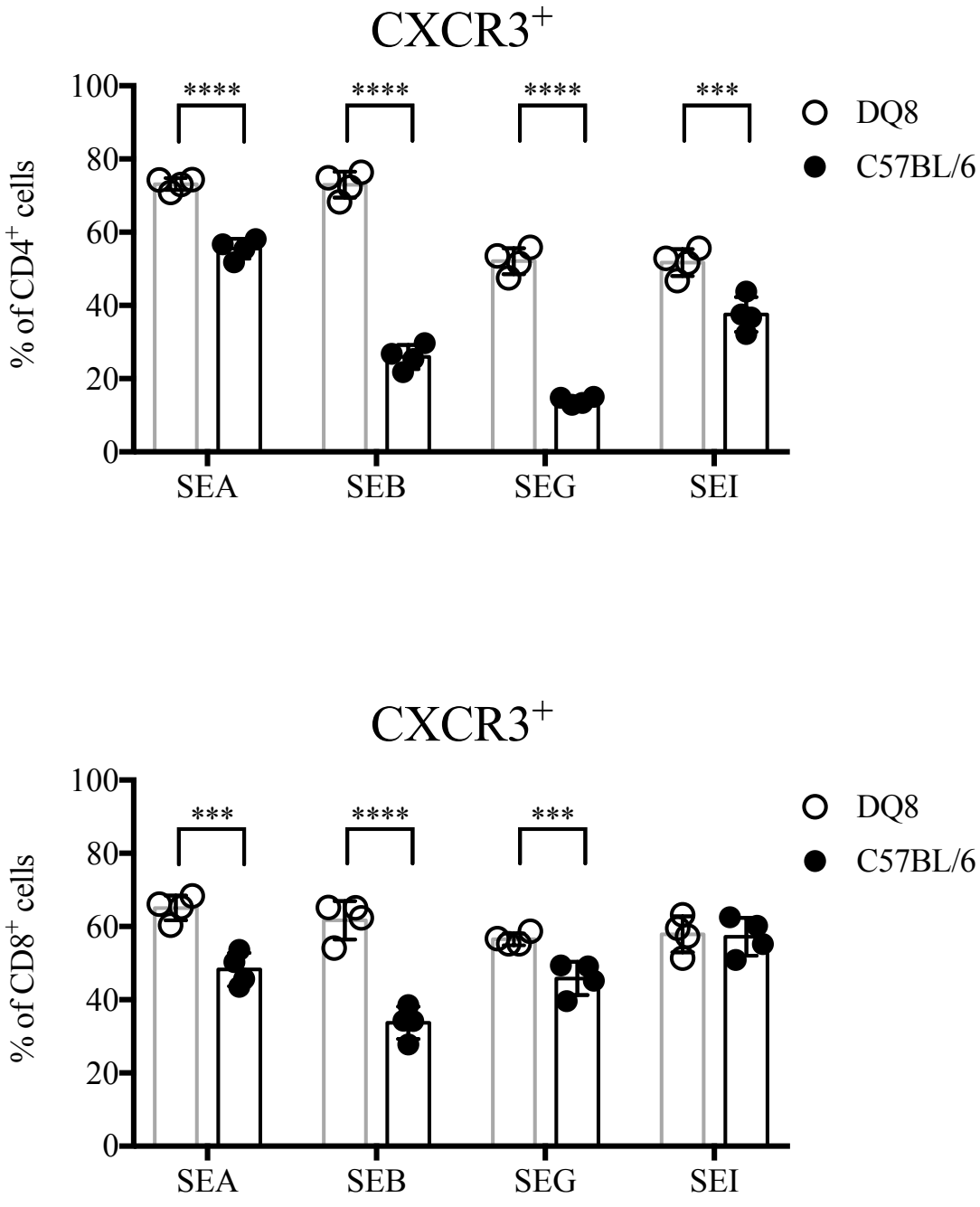


Figure 3. SEG and SEI elicit Type 1 effector CD4+ and CD8+ T cells.

Splenocytes were stimulated for 3 days with indicated SE concentrations where CD4+ (A) and CD8+ (B) cells were evaluated for intracellular IFNy and Tbet production. Representative flow data of SEG+SEI (10ng/ml) (C). n=3-5. Graphed mean with SD. *p<0.5, **p<0.01, ***p<0.001, ****p<0.001

A


CXCR3+
% of CD4+ cells
100
80
60
40
20
0
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SEA
SEB
SEG
SEI
DQ8
C57BL/6
CXCR3+
% of CD8+ cells
100
80
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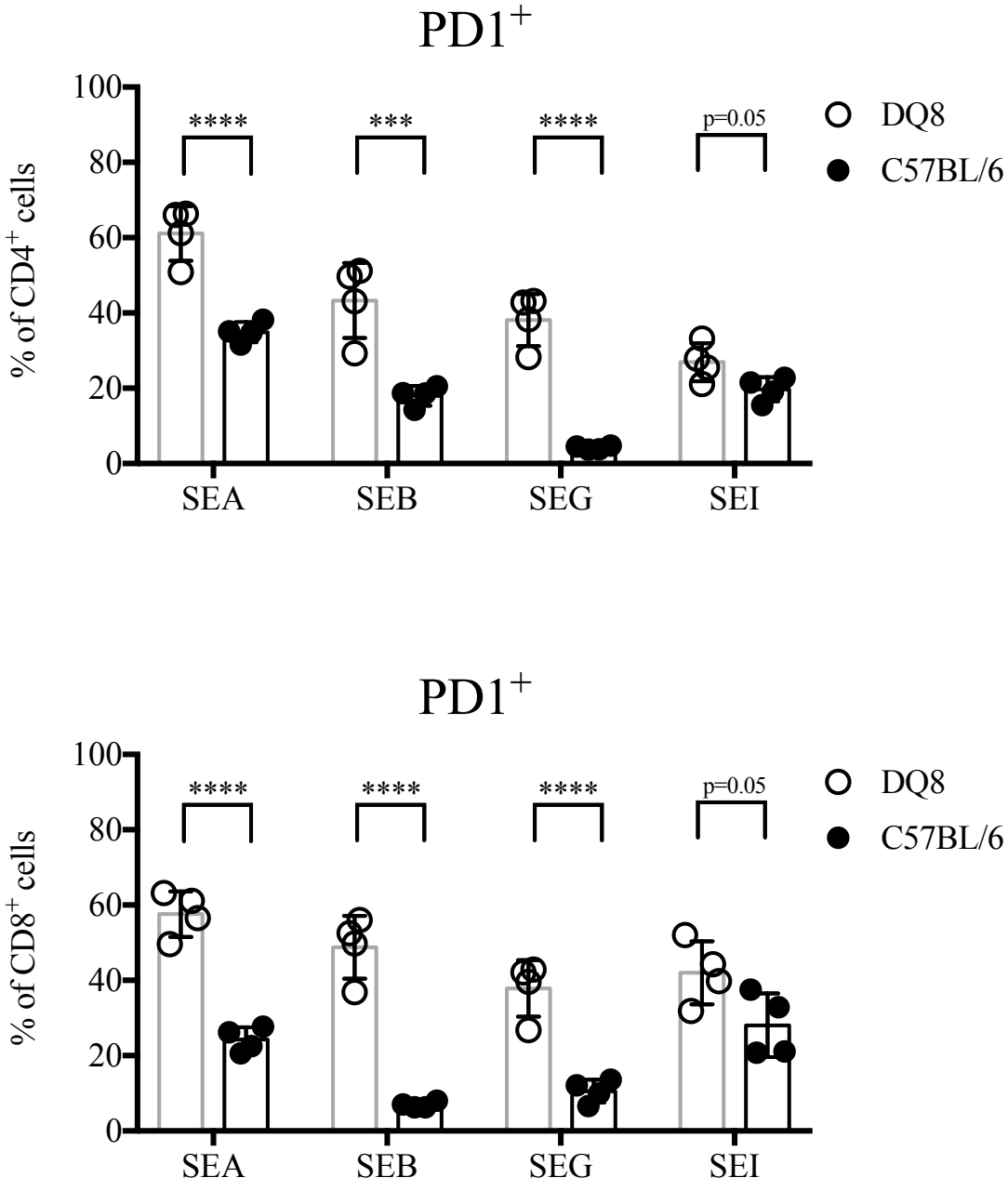
SEA
SEB
SEG
SEI
DQ8
C57BL/6

Continued…

B


PD1+
% of CD4+ cells
100
80
60
40
20
0
****
***
****
p=0.05
SEA
SEB
SEG
SEI
DQ8
C57BL/6
PD1+
% of CD8+ cells
100
80
60
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20
0
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p=0.05
SEA
SEB
SEG
SEI
DQ8
C57BL/6

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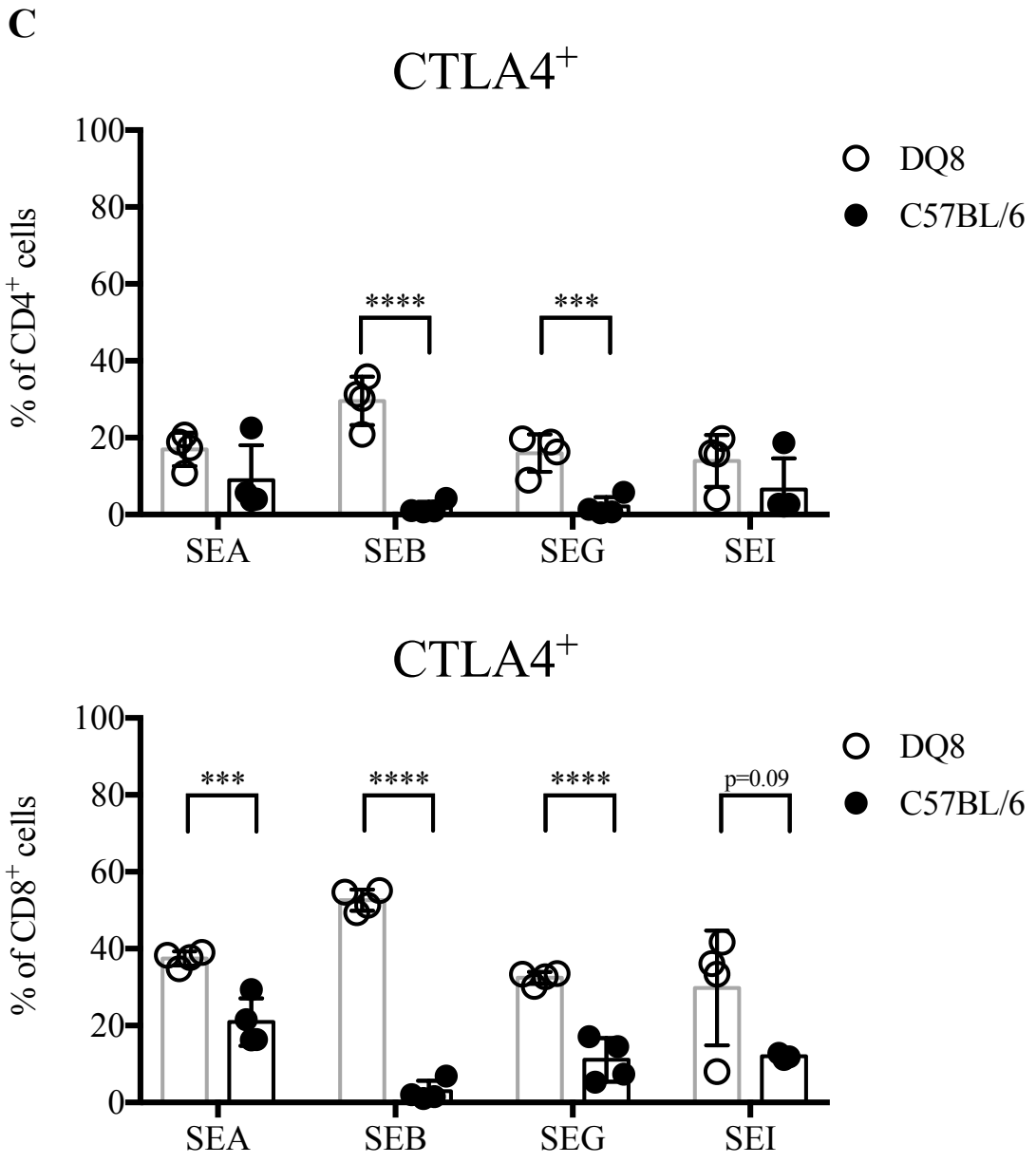


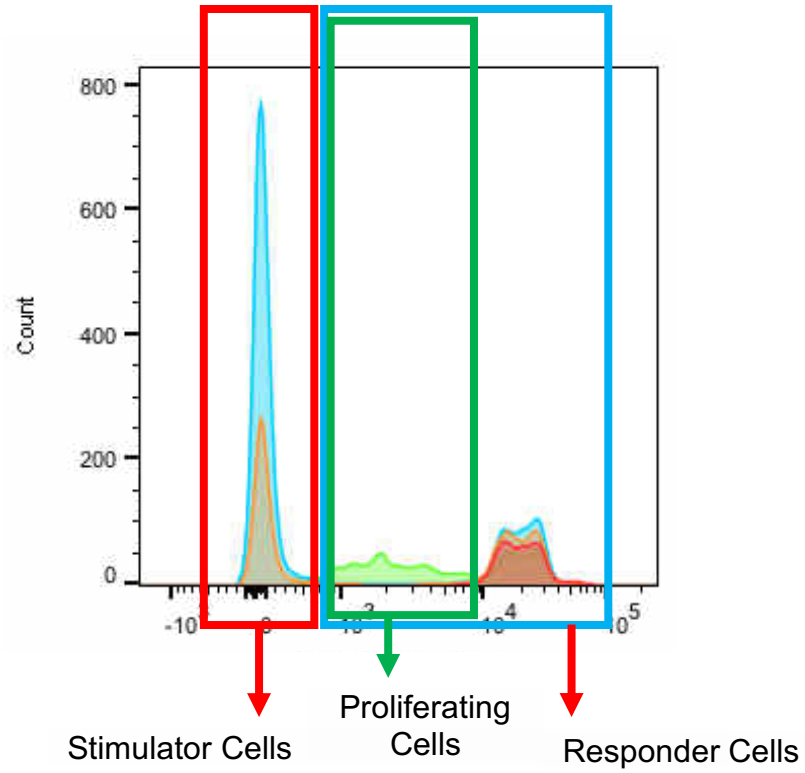
Figure 4. Activated CD4+ and CD8+ T cells express PD-1 and CTLA-4.

SEG and SEI (1ug/ml) demonstrate increased CXCR3 expression (A), along with PD-1 (B) and CTLA4 (C) expression on DQ8 T cells . All cells were gated on Singlets>Live cells>cells>CD3. n=3-5. Graphed mean with SD. *p<0.5, **p<0.01, ***p<0.001, ****p<0.001

Peritoneal Tumor Models

Cells injected into the peritoneal cavity have been demonstrated to associate with specific locations within the cavity with several fat deposits have been extensively characterized in throughout the peritoneum. DQ8 mice were originally created using multiple strains of mice under the same MHC allotype, $H2^b$. It is reasonable to hypothesis tumor models using cells originating from C57BL/6 mice, MHC allotype $H2^b$, would implant in HLA mice. Mixed lymphocyte reactions using mitomycin C treated C57BL/6 splenocytes were cocultured with DQ8 splenocytes to measure proliferation **Figure 5**. These data do not indicate DQ8 T cells proliferate in response to mitomycin C or irradiated (data not shown) C57BL/6 splenocytes.

To this end, several tumor cell lines were investigated for potential use. Murine colon cancer cell line MC38 was implanted ($2.5x10^5$ cells IP) in various strains of 6-8 week old male mice **Figure 6**. In general, HLA mice survived longer than allogeneic C57BL/6 mice; however, using tumors models with LD_{50} resolving at day 50 would be time consuming. The BALB/c derived mammary tumor cell line 4T1, $H2^d$ allotype, was implanted IP in 6-8 week old female mice to confirm allotype specificity in HLA transgenic mice **Figure 7**.



	SampleID	Proliferation :: Freq. of Parent
	Unstimulated	3.27%
	MLR Low S:R ratio	4.51%
	MLR High S:R ratio	3.20%
	SEB	53.9%

Figure 5 . DQ8 lymphocytes to not proliferate when cocultured with C57BL/6 splenocytes.

Mitomycin C Treated C57BL/6 Splenocytes (Stimulator Cells, red) were cocultured with DQ8 splenocytes (Responder Cells, blue) for 3 days. $CD3^+$ T cell proliferation (green) was measured by dye dilution via flow cytometry and reported as % proliferation. SEB was used as a positive proliferative control.

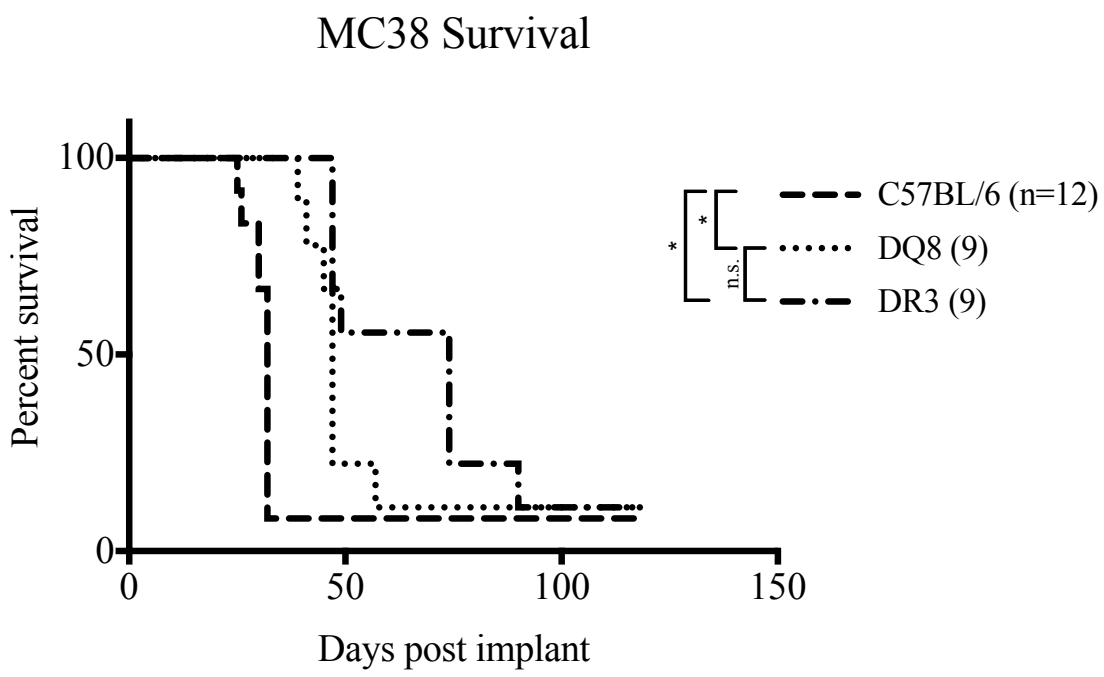


Figure 6 . MC38 survival in HLA transgenic mice.

2.5 x 10^5 live MC38 cells were implanted into the peritoneal cavity, suspended in 100ul HBSS, of 6-8 week old male DQ8, DR3 and C57BL/6 mice. Kaplan Meier curves with Log-rank test shown.

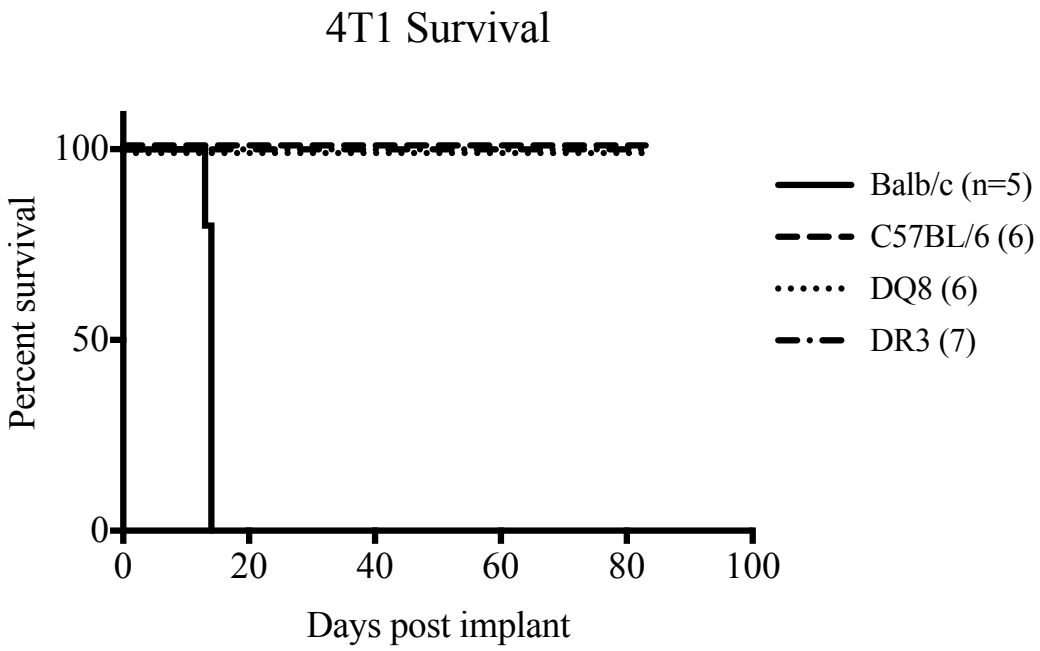


Figure 7. 4T1 survival in HLA transgenic mice.

2.5 x 10^5 live MC38 cells were implanted into the peritoneal cavity, suspended in 100ul HBSS, of 6-8 week old female DQ8, DR3, BALB/c and C57BL/6 mice. Kaplan Meier curves with Log-rank test shown.

In this study, B16-F10 murine melanoma, a classic tumor model used widely used to elucidate antigen specific T cell responses, and Lewis lung carcinoma (LLC) cells, the premier model for NSCLC, were chosen. Cells, and treatment, were administered intraperitoneally making sure to wipe the needle with 70% alcohol to prevent subcutaneous implant. B16 cells were found to preferentially attach to the omental tissue with minimal seeding throughout the mesentery **Figure 8**. LLC cells also attach to omental tissue but appear to favor the mesentery later post implant.

Omental tissue has long been identified as an important site for immune responses in the peritoneum. Several studies have characterized immune cells within the omentum from lymphoid follicles, often referred to as milky spots (sorn). Therefore, C57BL/6 mice and HLA transgenic mouse omentum was evaluated using a similar technique outlined in [102]. In **Figure 9**, omental tissue was harvest, washed with HBSS and allowed to float in cDMEM for 2 days; after which cells were analyzed via flow cytometry. These data compare previously reported T cell percentages in omental tissue [105] and show no differences in T cell composition among mice tested. However, $CD4^+$ and $CD8^+$ T cell populations are altered in DQ8 mice.

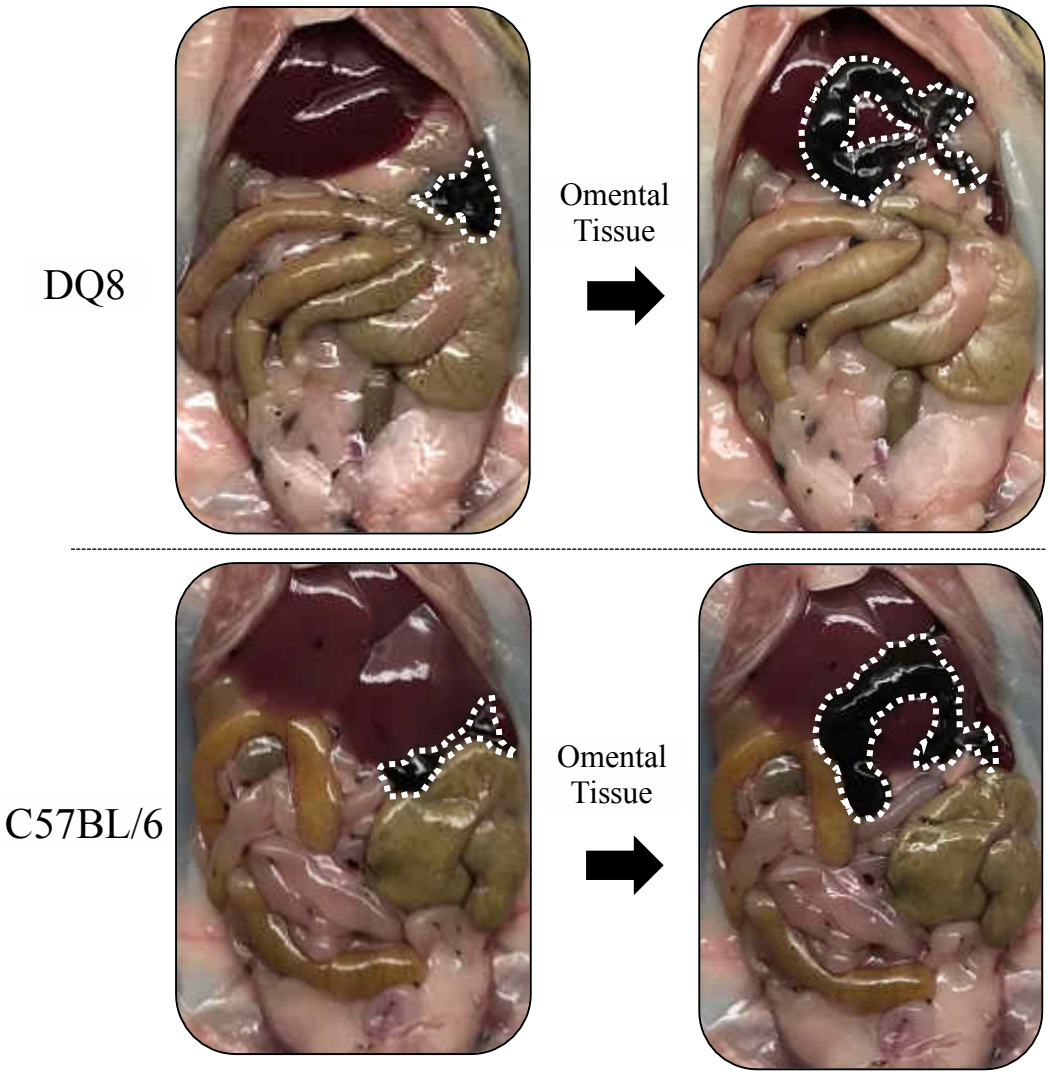


Figure 8 . B16-F10 cells establish at the omentum by day 6 post implant.

2.5 x 10^5 live B16-F10 cells were implanted into the peritoneal cavity, suspended in 100ul HBSS, of 6-8 week old female DQ8 and C57BL/6 mice. Images were taken 6 days post implant with representative mouse shown. Undisturbed peritoneal cavity **(left)** and omental tissue exhumed **(right)** show similar tumor mass (**dotted white line)**.

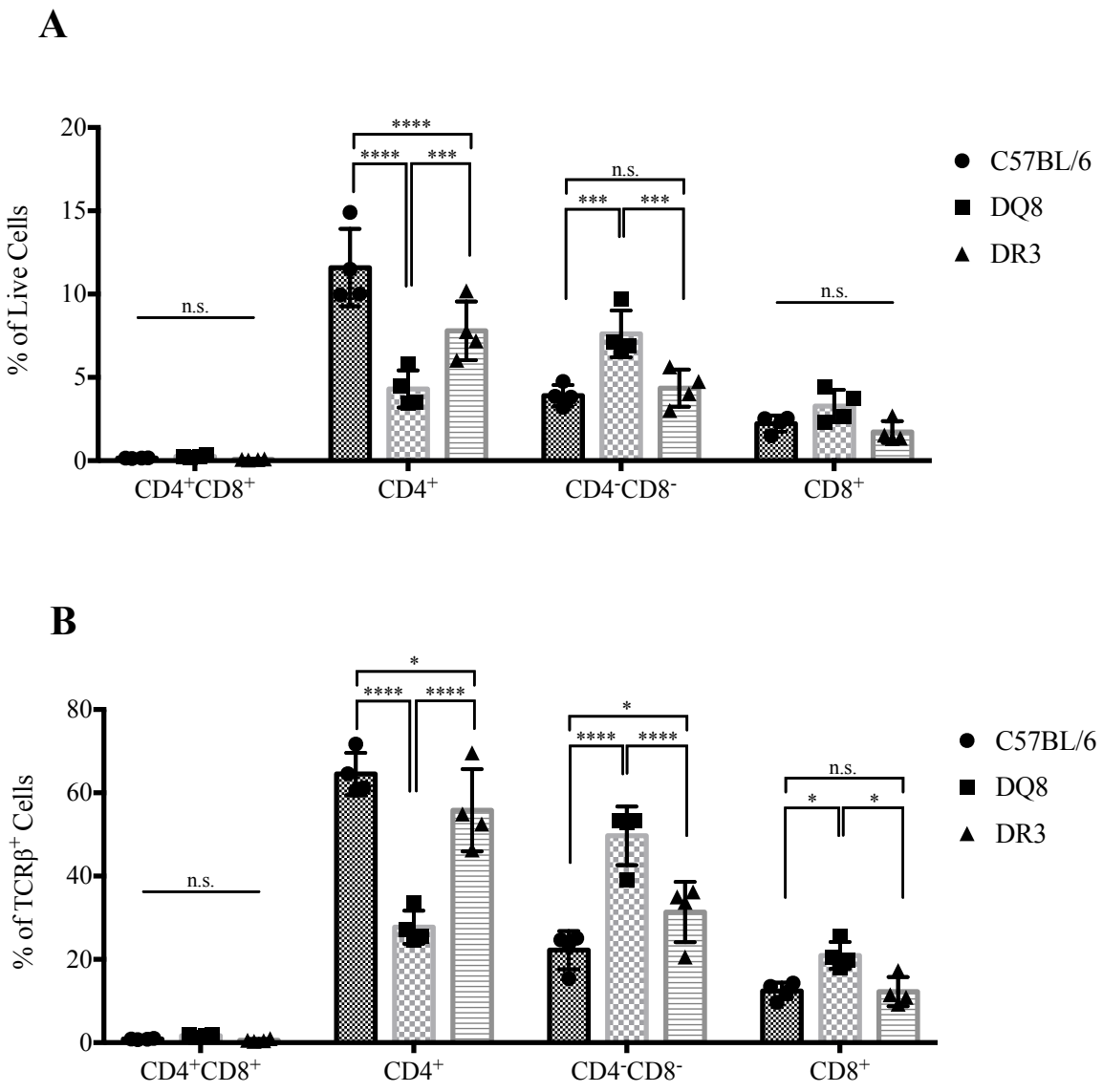


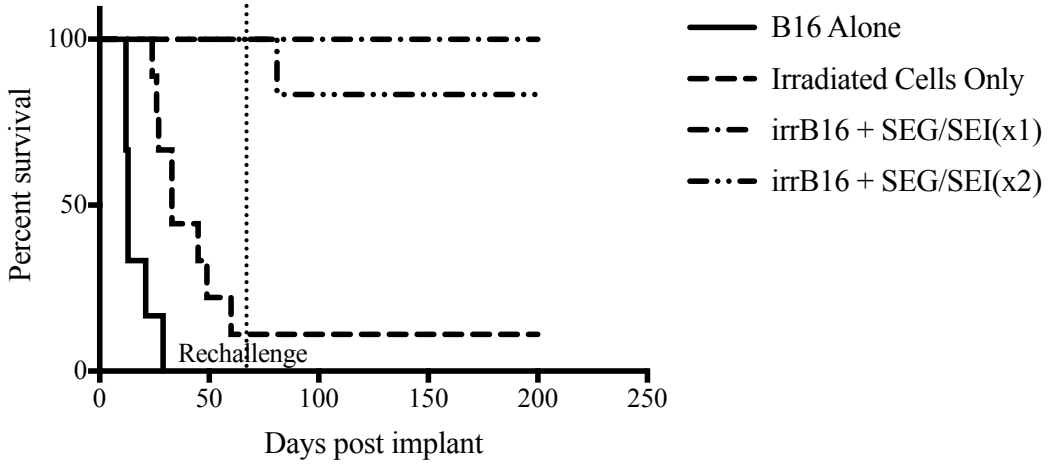
Figure 9 . Omental T cell characterization.

Omental tissue was placed in 1ml media and incubated for 2 days. Cells (including adherent cells collected w/trypsin dissociation) were analyzed after exclusion of doublets, debris and dead cells. T cells reported as % live cells **(A)** and % TCRβ+ **(B)**. Graphed mean with SD. *p<0.5, **p<0.01, ***p<0.001, ****p<0.001

SEG/SEI in Treatment of B16 Melanoma

The therapeutic efficacy of enterotoxins was demonstrated previously by Kominsky et. al. [78] using SEA and SEB in a vaccine model for B16 melanoma in C57BL/6 mice. In this study, 60% of mice receiving $1x10^6$ irradiated (15,000 rads) 13 days before tumor challenge survived >140 days when boosted with SEA+SEB (25μg each) prior to tumor challenge. Unfortunately, these findings have been difficult to translate into human therapies due to neutralizing antibodies against them in patients with prior exposure. To that end, SEG and SEI were used following the same protocol in Kominsky et. al. **Figure 10.** Comparatively, SEG + SEI provided minimal benefit in C57BL/6 mice when rechallenged ($2.5x10^5$ cells) >60 days post initial implant **10B**. Interestingly, ~10% of DQ8 **10A** mice responded well to B16 challenge when receiving just the irradiated cells alone compared to DR3 **Figure 10C.** Moreover, SEG/SEI boosted the vaccine response in DQ8 mice where 3/3 mice survived initial tumor challenge with either 1 single day -7, or 2 treatments before initial implant. Upon rechallenge, 1/6 mice died along with age matched naïve controls (data not shown). Not surprisingly, SEG/SEI did not provide appreciable benefit for C57BL/6 mice in the B16 vaccine model. Additionally, these data support using DQ8 over C57BL/6 mice to study the therapeutic potential of SEG and SEI.

A



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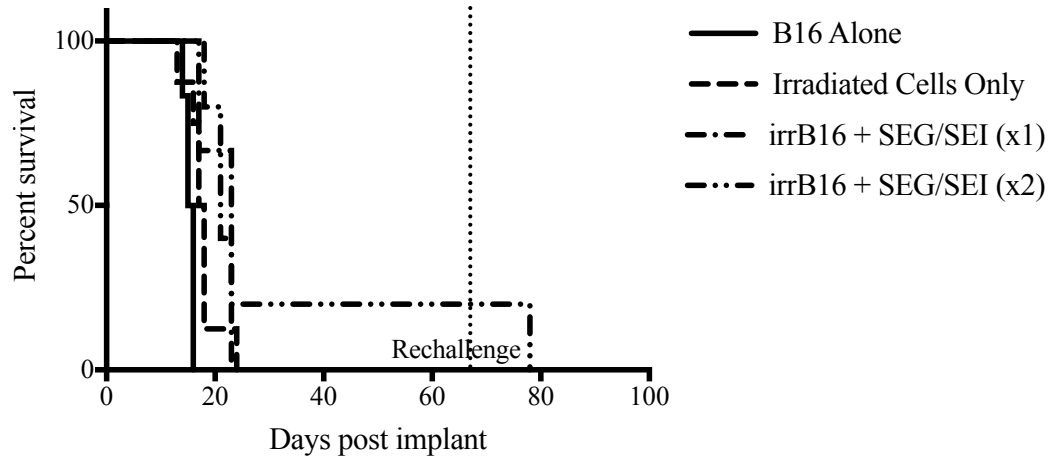

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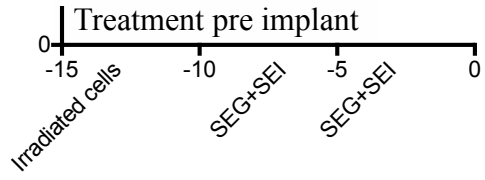

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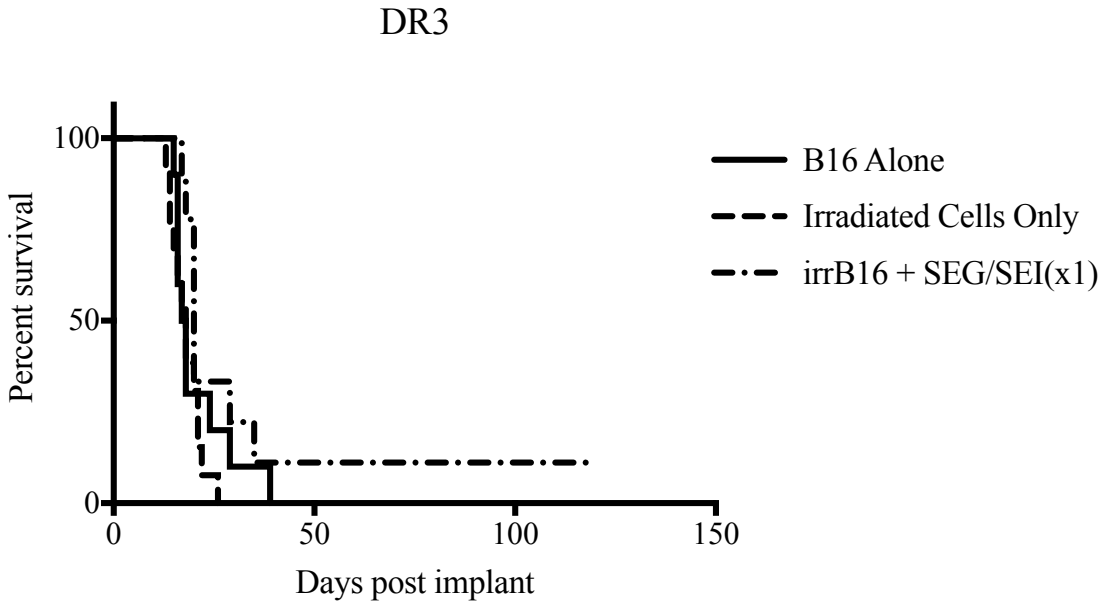
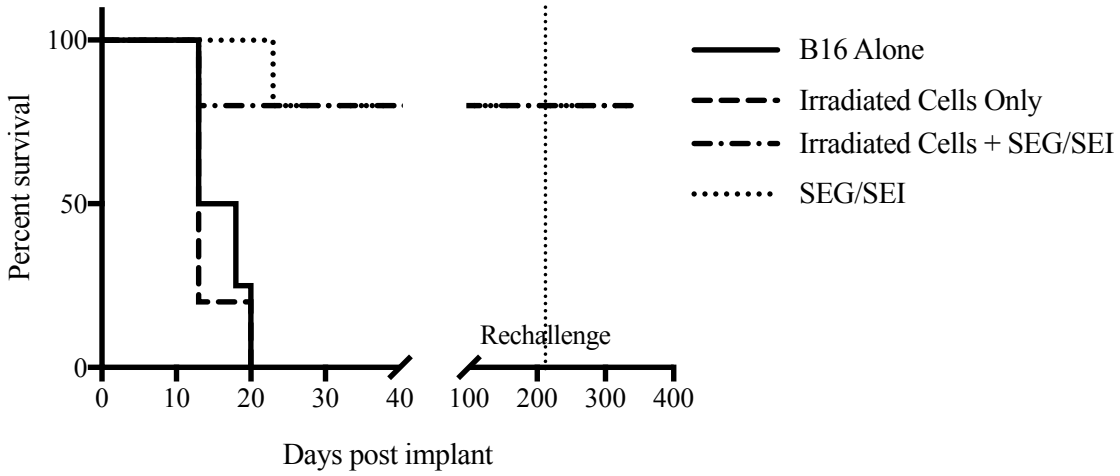


Figure 10. SEG and SEI promote long term survival in B16-F10 vaccinated DQ8 mice.

DQ8 **(A),** C57BL/6 **(B)** and DR3 **(D)** 6-8 week old female mice were injected with $1x10^6$ irradiated (15,000 rads) B16-F10 cells IP day -13 **(C)**. Treated mice were injected with 50ug each SEG+SEI day -7 and -3. SEG/SEI(1x) groups received SEG+SEI on day -7 only; whereas SEG/SEI(2x) groups received both doses of SEG/SEI. DQ8 and C57BL/6 mice surviving >60 days were rechallenged with $2.5x10^5$ live B16-F10 cells intraperitoneally. Age matched controls died as expected (data not shown). Kaplan Meier curves with Log-rank test shown. n=3-10.

Vaccine strategies for various cancers using animal models provide useful information immunologically yet provide minimal translational benefit when applied clinically. The treatment of an established solid tumor is more clinically beneficial and was investigated using **Figure 8** as an established B16 tumor in DQ8 mice **Figure 11**. Treatment of C57BL/6 established tumors did not provide meaning benefit even when using a lower initial tumor implant, 10,000 cell IP **Figure 11C**. This representative experiment shows long term survival in ~80% of DQ8 mice rechallenged >200 days post initial cell implant. Based on the results of vaccination experiments, it was hypothesized that irradiated cells could provide longer term benefit to prevent reoccurrence. In this experiment, using irradiated cells 4 days post implant did not provide apparent benefit with or without SEG/SEI. Considering laboratory observations over these experiments, 2 injections of SEG/SEI (50μg) were provided better overall therapeutic efficacy. That said, injections of SEG/SEI proved toxic in some C57BL/6 mice and nearly all DR3 mice. DR3 mice did not benefit from a single SEG/SEI in an established B16 model (data not shown); although, lower amounts of SEG/SEI could provide benefit in DR3 mice but were not evaluated by this researcher.

A



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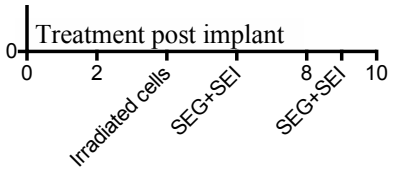

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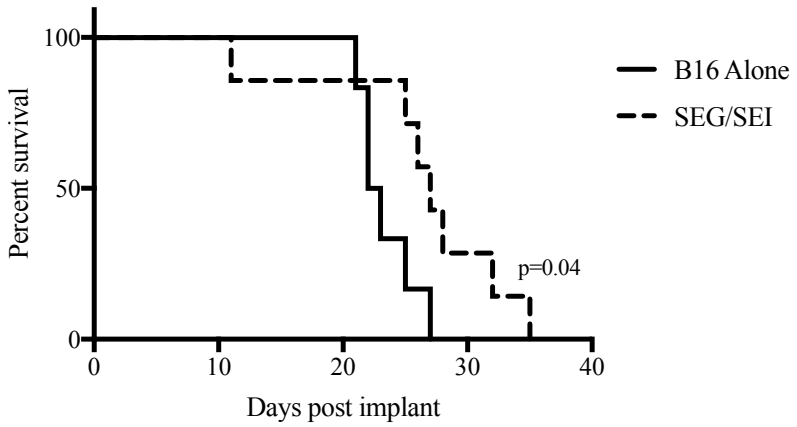

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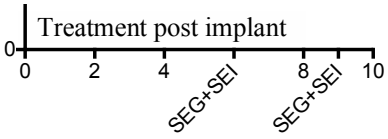

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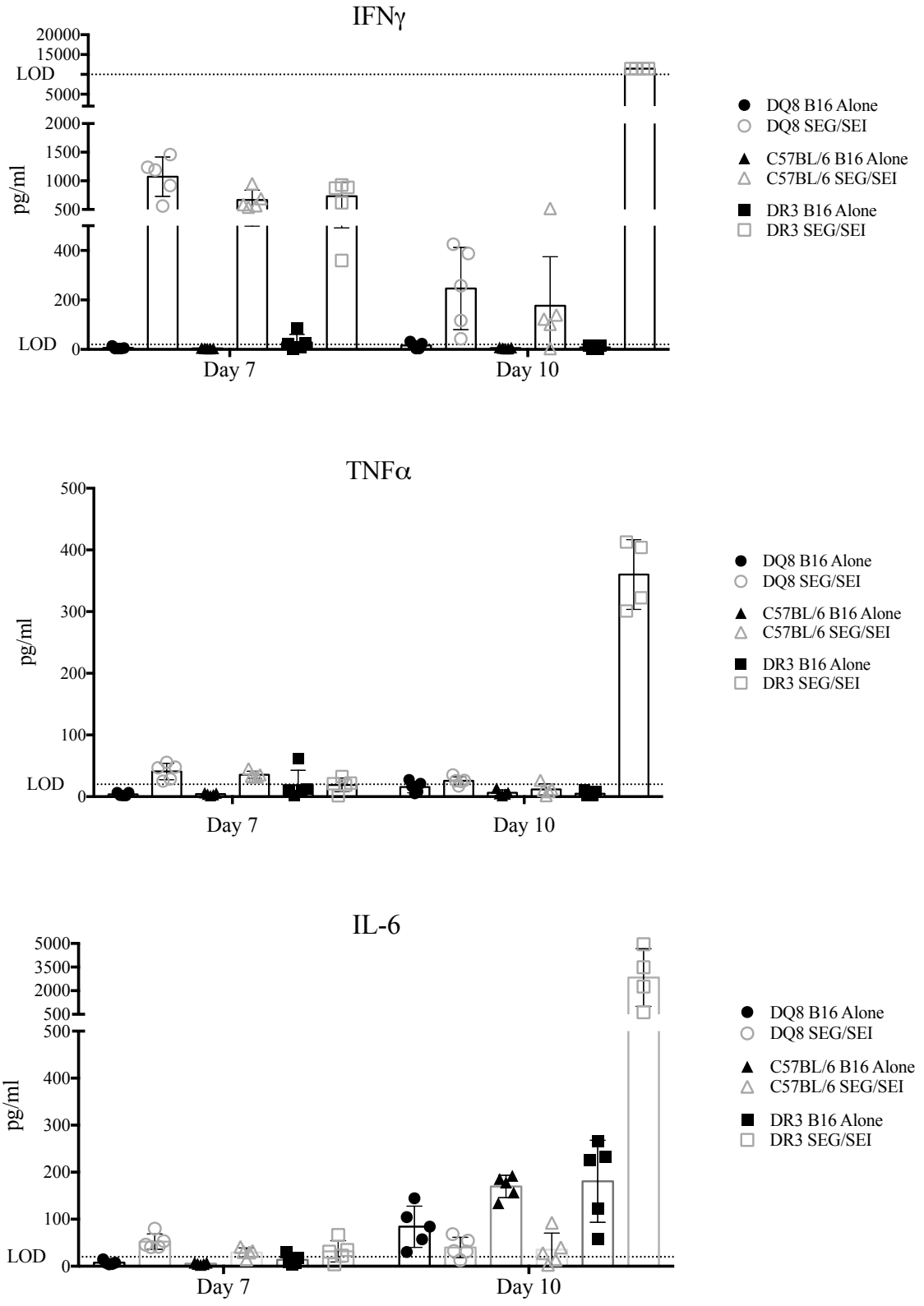
Figure 11 . SEG and SEI promote long term survival in DQ8 mice with established B16-F10 tumors.

Mice were implanted with $2.5x10^5$ **(A)** or $1x10^4$ **(B)** live B16-F10 cells intraperitoneally day 0 and followed the treatment protocols **(C)** and **(D)**, respectively. Mice surviving >200 days were rechallenged with $2.5x10^5$ live B16-F10 cells intraperitoneally **(A)**. Age matched controls died as previously demonstrated (data not shown). n=4-10. Kaplan Meier curves with Log-rank test shown.

Characterization of the SEG/SEI Anti-B16 Response

Plasma cytokines **Figure 12** and chemokines **Figure 13** were measured ~24 hours after each SEG/SEI injection measured using BioLengend's LEGENDplex™ using BD FACSymphony™ flow cytometer. Standard curves were evaluated for appropriate error considered appropriate for all parameters given. The data can be interpreted collectively and demonstrate a powerful IFNγ response in all mice tested and levels are not as elevated 24 hours after the second injection. This decreased level could be a result of inhibitory molecules on the T cells after SE stimulation, **Figure 4**; or the IFNγ response peaks before 24 hours after the second SEG/SEI injection. Eluded to in earlier with B16 survival, DR3 mice demonstrate a toxic inflammatory response, a cytokine storm, demonstrated by increased TNFα, IL-6 and IL-10 after the second SEG/SEI injection. Numerous CC and CXC chemokines are increased in response to SEG/SEI, namely IFNγ associated chemokines. Again, taken collectively, chemokine responses can be grouped into Th1, Th2 and Treg response for example [124, 125]. DR3 mice mirror the inflammatory response seen in aforementioned cytokine levels; like CCL11, and evidence of regulatory type responses from CCL20 and IL-10. A recent review nicely summarizes the vast implications of chemokines in relation to cancer immunotherapies [125]. These data, when taken at face value, provide insight into the nature of the systemic immune response from SEG/SEI and clearly show increased IFNγ plasma concentrations regardless of MHC. Although these data prove SEG/SEI is toxic to DR3 mice, there is no clear difference in DQ8 vs C57BL6 cytokine or chemokine levels.


IFNγ
20000
15000
LOD
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2000
1500
1000
500
400
200
LOD
0
pg/ml
Day 7
Day 10
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DQ8 SEG/SEI
C57BL/6 B16 Alone
C57BL/6 SEG/SEI
DR3 B16 Alone
DR3 SEG/SEI
TNFα
500
400
300
200
100
LOD
0
pg/ml
Day 7
Day 10
DQ8 B16 Alone
DQ8 SEG/SEI
C57BL/6 B16 Alone
C57BL/6 SEG/SEI
DR3 B16 Alone
DR3 SEG/SEI
IL-6
5000
3500
2000
500
500
400
300
200
100
LOD
0
pg/ml
Day 7
Day 10
DQ8 B16 Alone
DQ8 SEG/SEI
C57BL/6 B16 Alone
C57BL/6 SEG/SEI
DR3 B16 Alone
DR3 SEG/SEI

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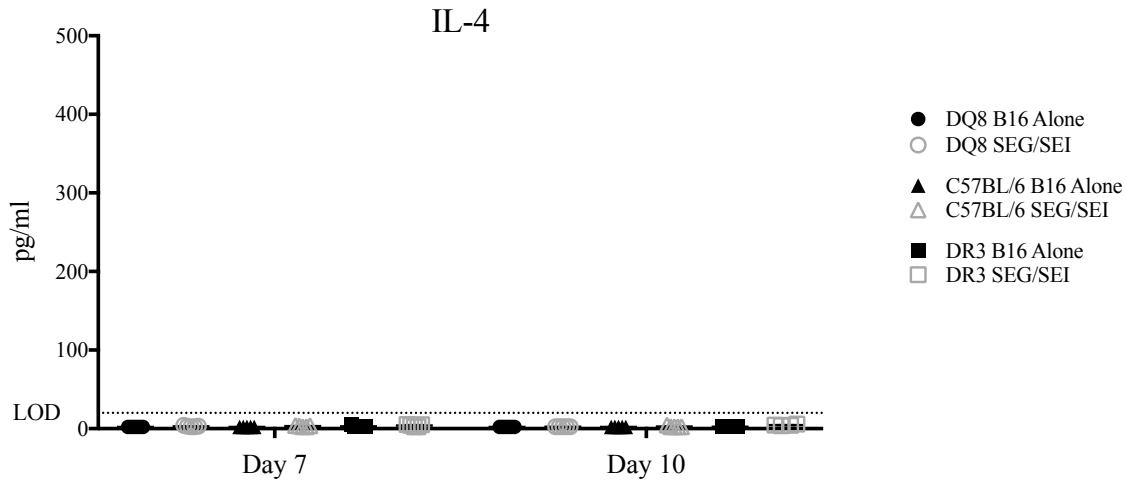
IL-4
500
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100
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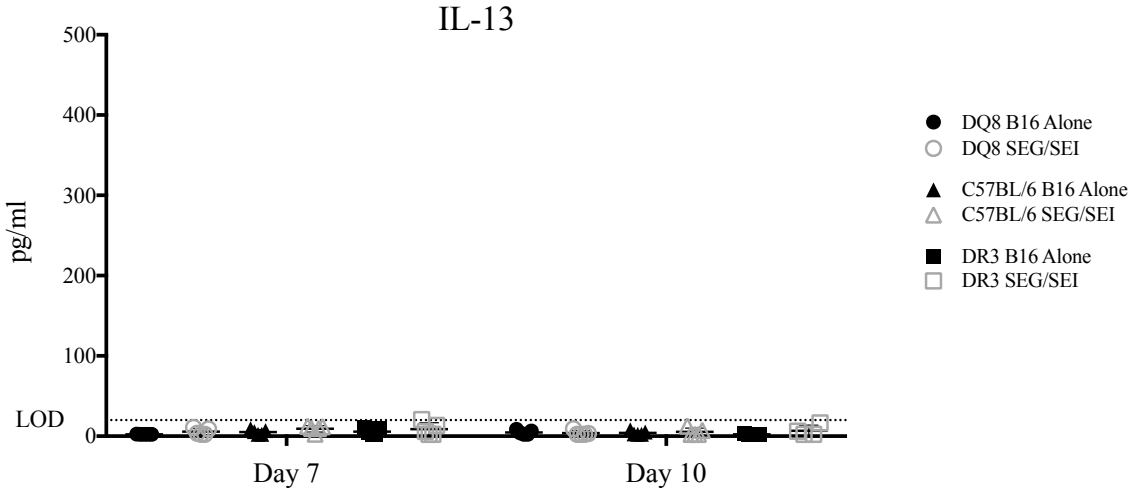
pg/ml
LOD
Day 7
Day 10
DQ8 B16 Alone
DQ8 SEG/SEI
C57BL/6 B16 Alone
C57BL/6 SEG/SEI
DR3 B16 Alone
DR3 SEG/SEI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pg/ml
LOD
Day 7
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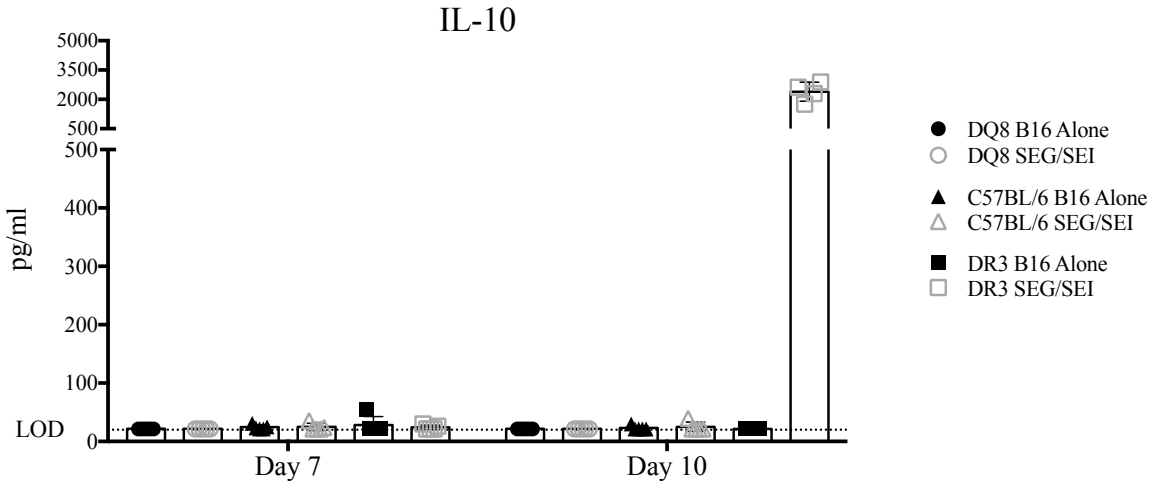
DQ8 B16 Alone
DQ8 SEG/SEI
C57BL/6 B16 Alone
C57BL/6 SEG/SEI
DR3 B16 Alone
DR3 SEG/SEI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pg/ml
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Day 7
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DQ8 B16 Alone
DQ8 SEG/SEI
C57BL/6 B16 Alone
C57BL/6 SEG/SEI
DR3 B16 Alone
DR3 SEG/SEI

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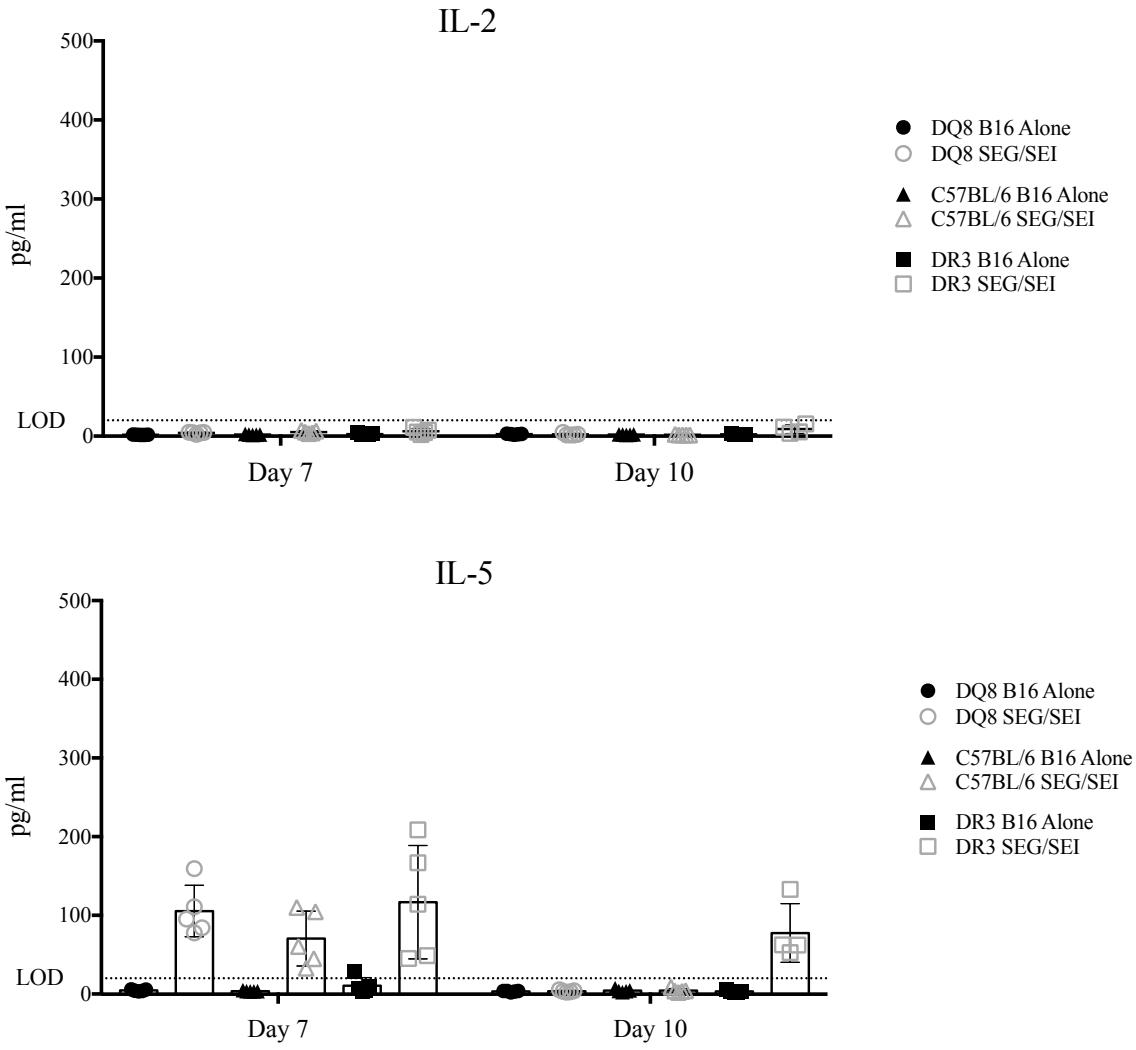
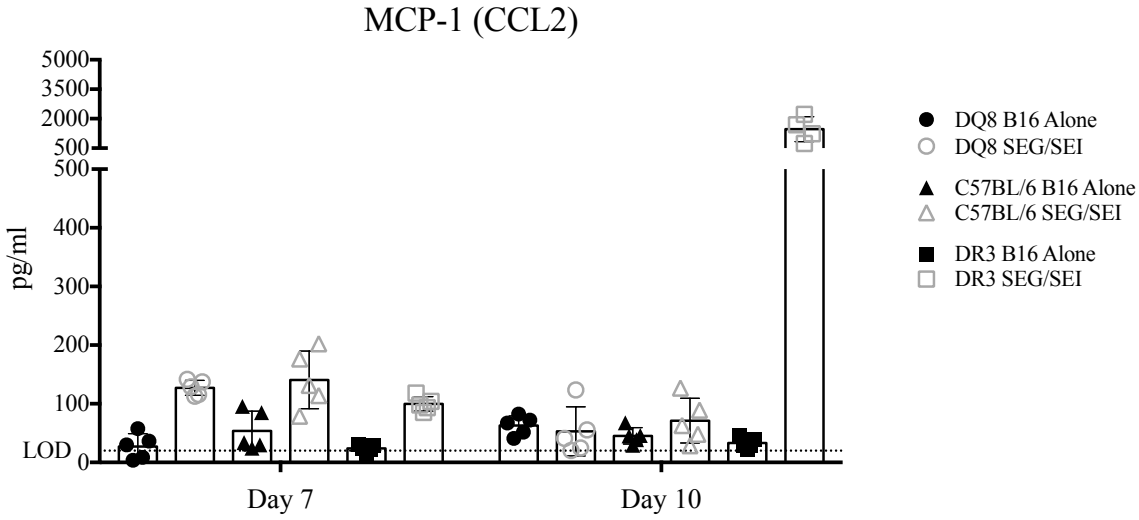
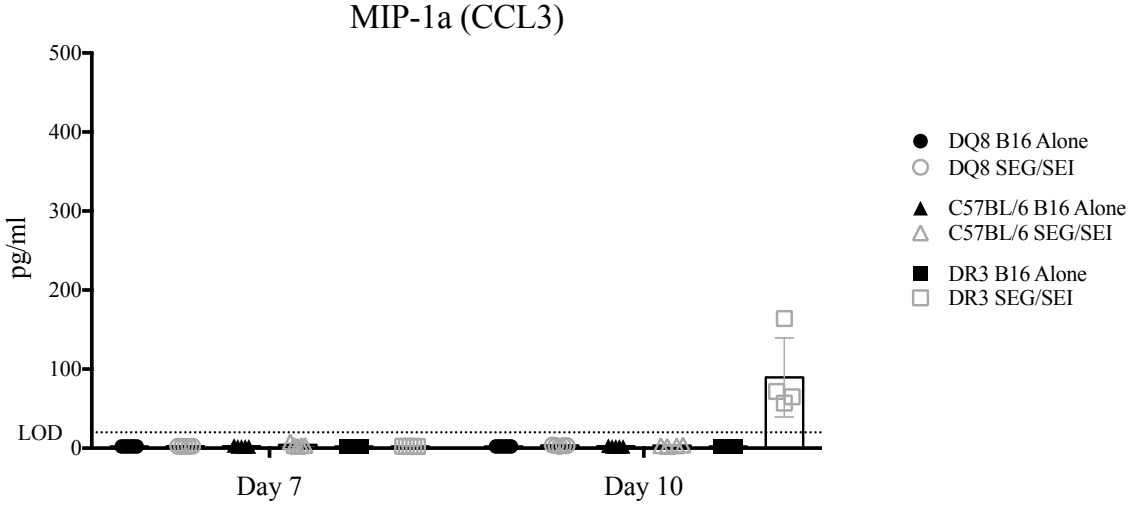
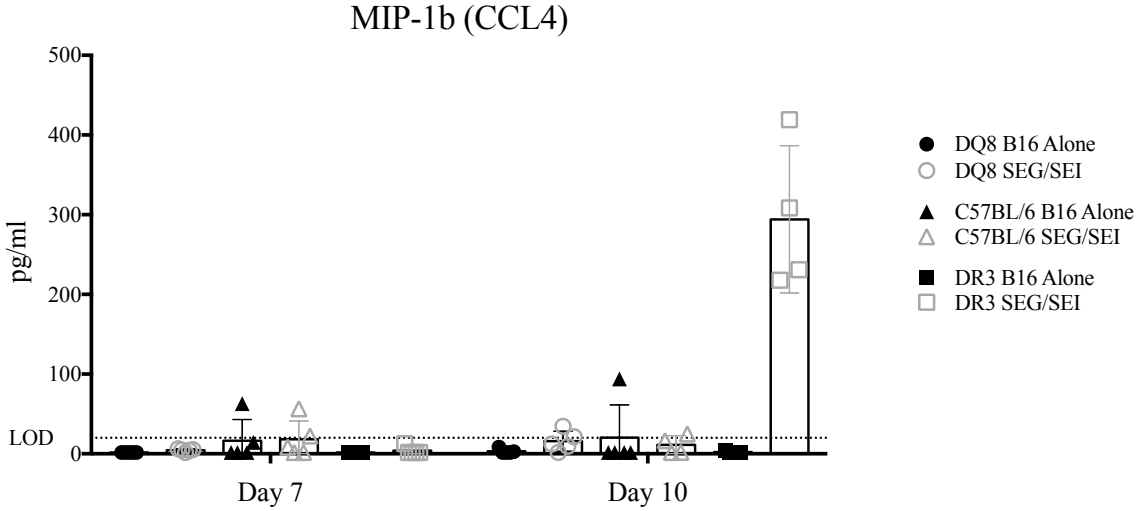


Figure 12 . Plasma cytokine concentrations 24 hours post SEG/SEI in mice with established B16-F10 melanoma.

All mice were implanted with $2.5x10^5$ live B16-F10 cells intraperitoneally. Blood was collected from the submandibular vein in EDTA tubes 24 hours after each SEG/SEI administration . All plasma samples were frozen and run together using BioLegend's LEGENDplex Mouse Th cytokine panel. All DR3 mice died 24 hours post second SE injection. n=4-5.

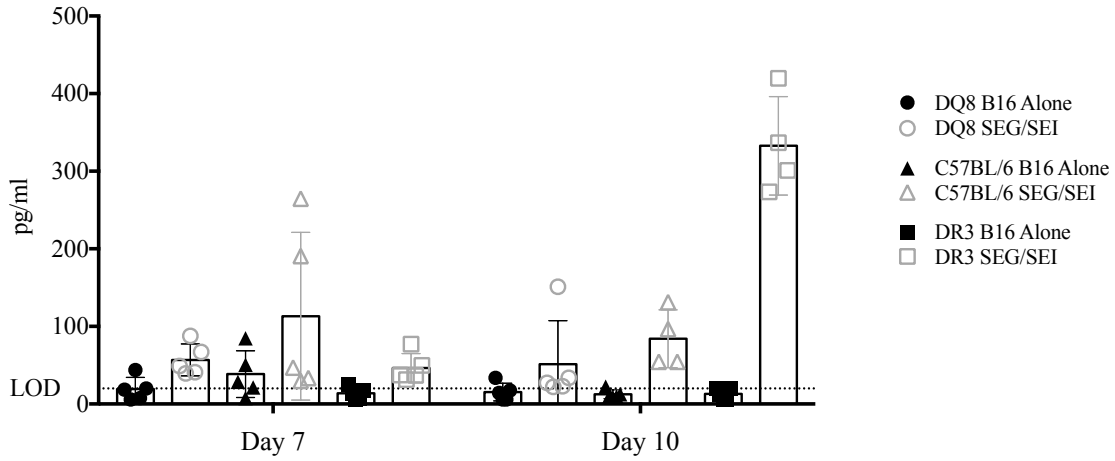

MCP-1 (CCL2)
5000
3500
2000
500
500
400
300
200
100
LOD
0
pg/ml
Day 7
Day 10
DQ8 B16 Alone
DQ8 SEG/SEI
C57BL/6 B16 Alone
C57BL/6 SEG/SEI
DR3 B16 Alone
DR3 SEG/SEI


MIP-1a (CCL3)
500
400
300
200
100
LOD
0
pg/ml
Day 7
Day 10
DQ8 B16 Alone
DQ8 SEG/SEI
C57BL/6 B16 Alone
C57BL/6 SEG/SEI
DR3 B16 Alone
DR3 SEG/SEI

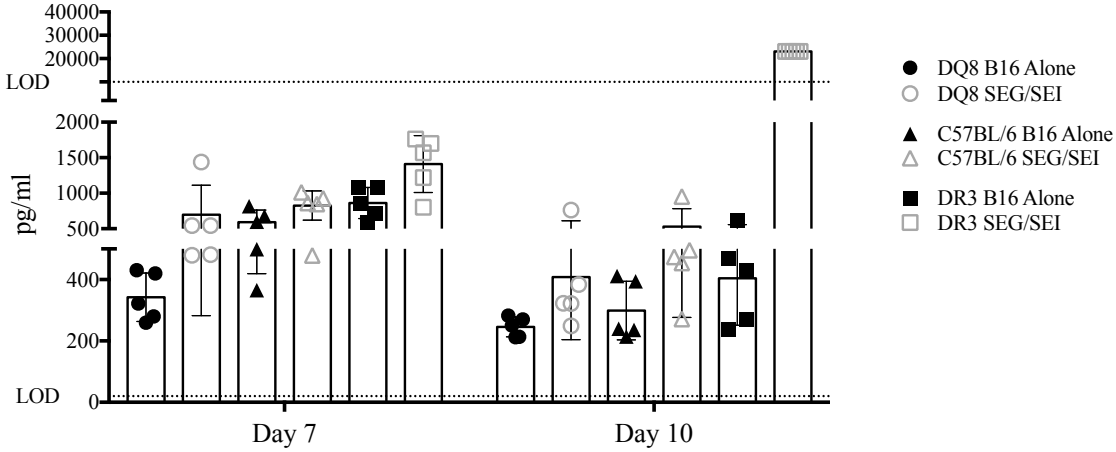

MIP-1b (CCL4)
500
400
300
200
100
LOD
0
pg/ml
Day 7
Day 10
DQ8 B16 Alone
DQ8 SEG/SEI
C57BL/6 B16 Alone
C57BL/6 SEG/SEI
DR3 B16 Alone
DR3 SEG/SEI

Continued…

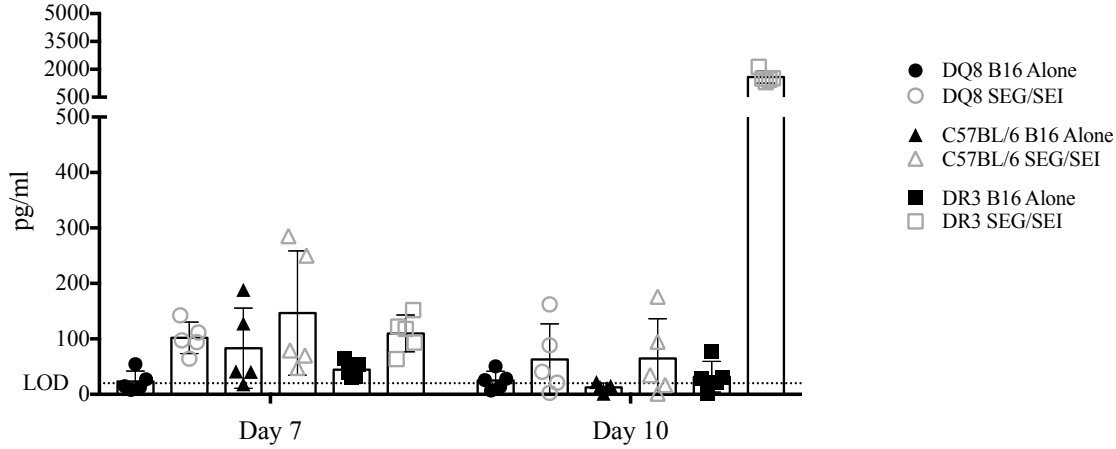
RANTES (CCL5)



Eotaxin (CCL11)



TARC (CCL17)



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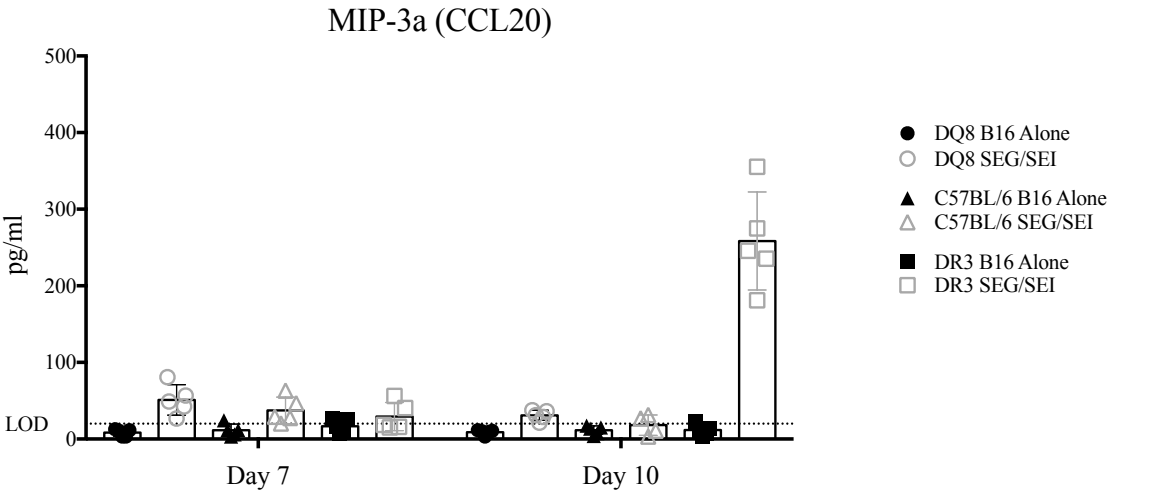
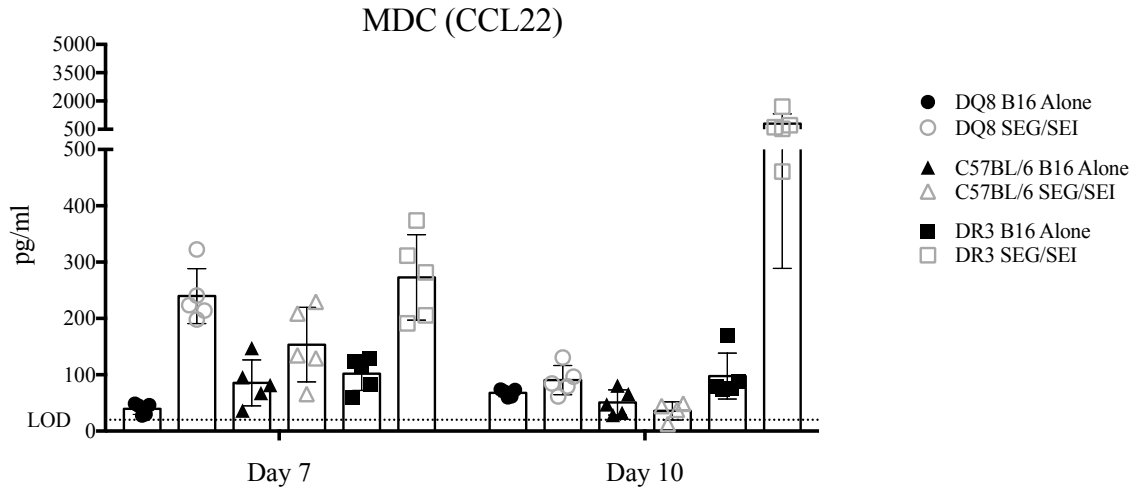
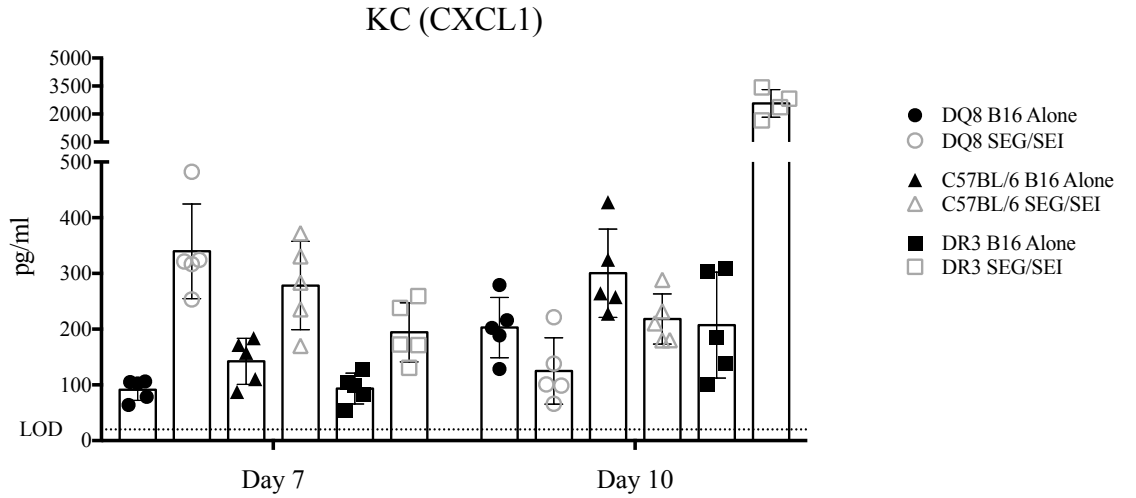
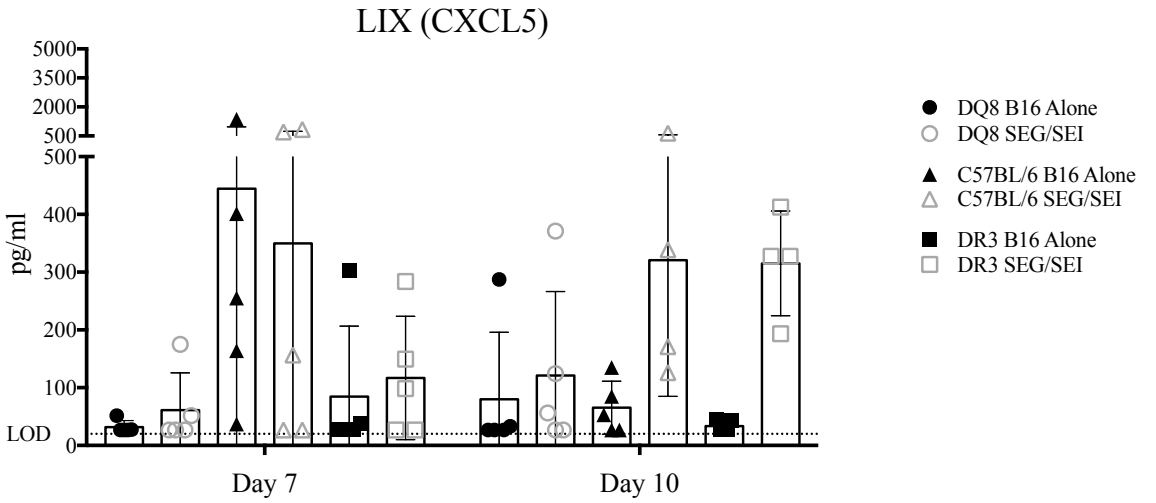
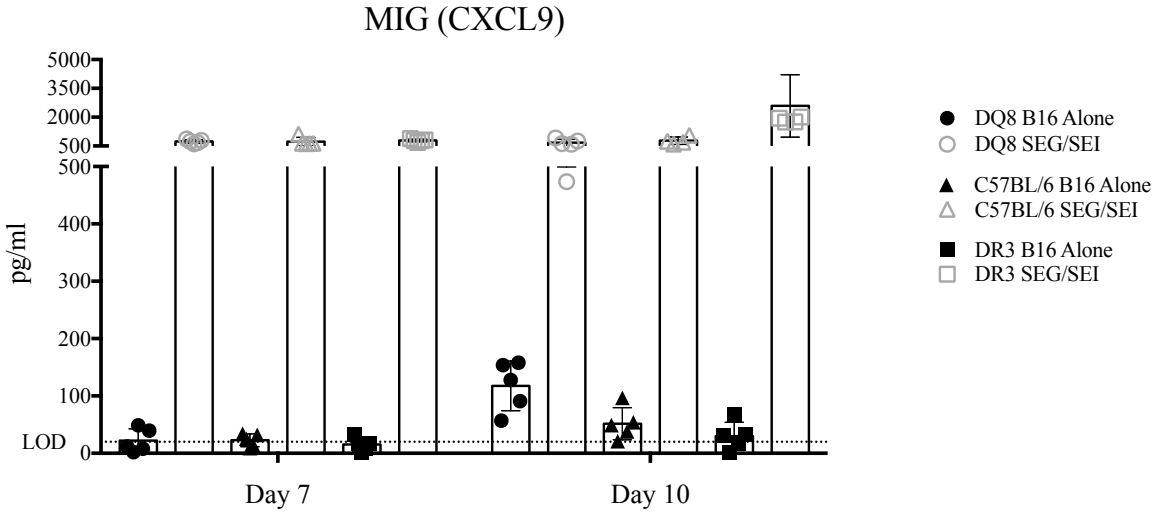

MIP-3a (CCL20)
500
400
300
200
100
LOD
0
pg/ml
Day 7
Day 10
DQ8 B16 Alone
DQ8 SEG/SEI
C57BL/6 B16 Alone
C57BL/6 SEG/SEI
DR3 B16 Alone
DR3 SEG/SEI

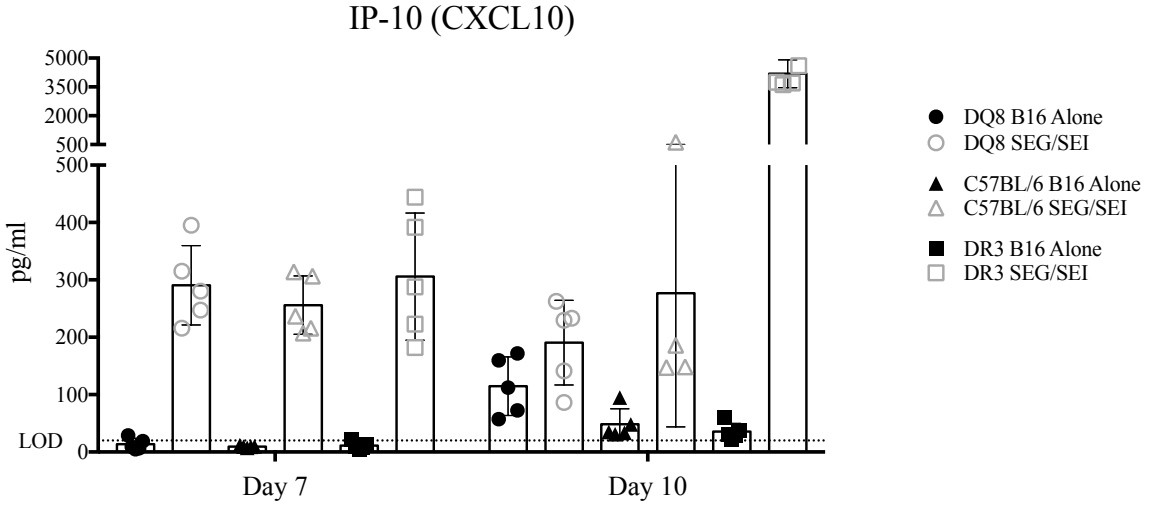

MDC (CCL22)
5000
3500
2000
500
500
400
300
200
100
LOD
0
pg/ml
Day 7
Day 10
DQ8 B16 Alone
DQ8 SEG/SEI
C57BL/6 B16 Alone
C57BL/6 SEG/SEI
DR3 B16 Alone
DR3 SEG/SEI


KC (CXCL1)
5000
3500
2000
500
500
400
300
200
100
LOD
0
pg/ml
Day 7
Day 10
DQ8 B16 Alone
DQ8 SEG/SEI
C57BL/6 B16 Alone
C57BL/6 SEG/SEI
DR3 B16 Alone
DR3 SEG/SEI

Continued…


LIX (CXCL5)
5000
3500
2000
500
500
400
300
200
100
0
LOD
pg/ml
Day 7
Day 10
DQ8 B16 Alone
DQ8 SEG/SEI
C57BL/6 B16 Alone
C57BL/6 SEG/SEI
DR3 B16 Alone
DR3 SEG/SEI


MIG (CXCL9)
5000
3500
2000
500
500
400
300
200
100
0
LOD
pg/ml
Day 7
Day 10
DQ8 B16 Alone
DQ8 SEG/SEI
C57BL/6 B16 Alone
C57BL/6 SEG/SEI
DR3 B16 Alone
DR3 SEG/SEI


IP-10 (CXCL10)
5000
3500
2000
500
500
400
300
200
100
0
LOD
pg/ml
Day 7
Day 10
DQ8 B16 Alone
DQ8 SEG/SEI
C57BL/6 B16 Alone
C57BL/6 SEG/SEI
DR3 B16 Alone
DR3 SEG/SEI

Continued…

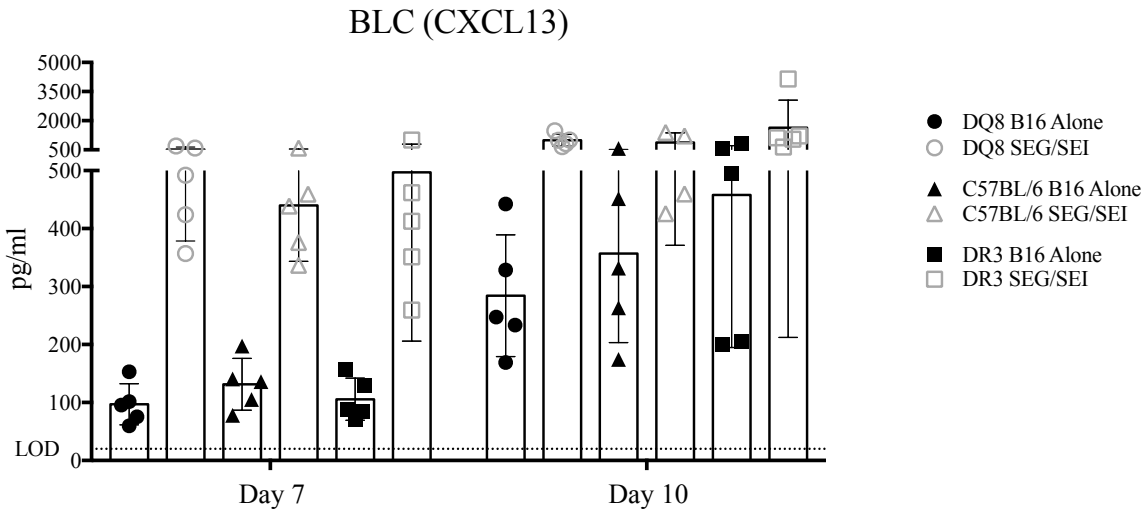


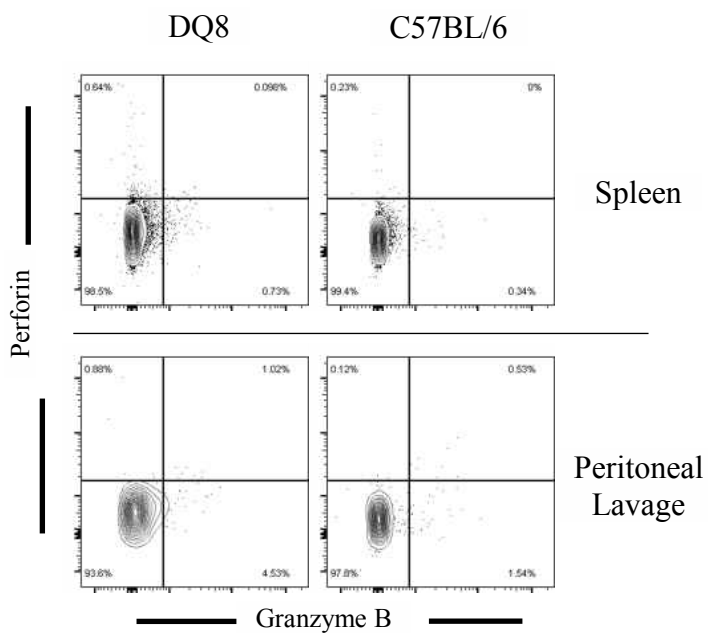
Figure 13. Plasma chemokine concentrations 24 hours post SEG/SEI in mice with established B16-F10 melanoma.

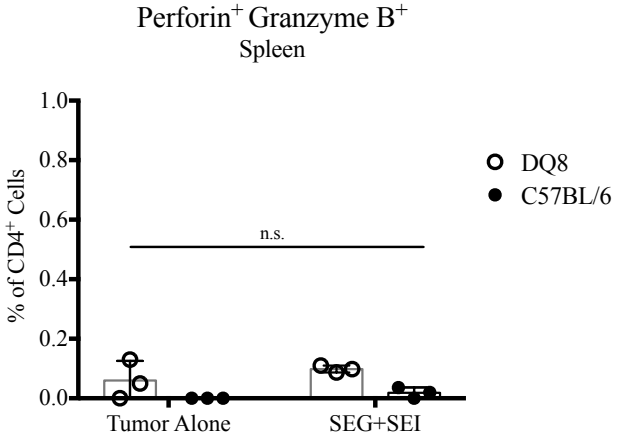
All mice were implanted with $2.5x10^5$ live B16-F10 cells intraperitoneally. Blood was collected from the submandibular vein in EDTA tubes 24 hours after each SEG/SEI administration . All plasma samples were frozen and run together using BioLegend's LEGENDplex Mouse Proinflammatory chemokine panel. All DR3 mice died 24 hours post second SE injection. n=4-5.

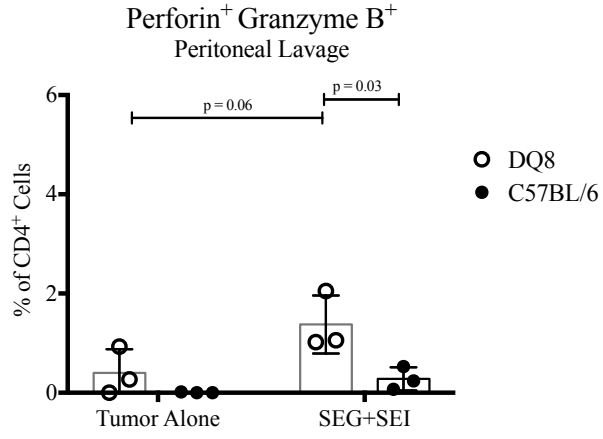
The data presented insofar demonstrate SEG/SEI elicit robust IFNγ responses but do not delineate antitumor response between DQ8 and C57BL6 mice. Cell specific differences in DQ8 and C57BL6 responses to B16 melanoma, including cell populations and effector molecules, were evaluated at different time points during treatment. In **Figure 14,** spleens and peritoneal lavage cells were collected 13 days post implant and stained for surface CD3, CD4 and CD8 expression, including intracellular perforin and granzyme B in $CD4^+$ **A** and $CD8^+$ **B** T cells. Flow cytometry analysis reveals that DQ8 mice produce more $CD8^+$ T cells that contain perforin and granzyme B in both the spleen and peritoneal cavity.

CD3, CD4, CD25 and CD39 expression including intracellular FoxP3 was evaluated similarly to investigate T regulatory cell (Treg) responses **Figure 14C**. CD39 was selected, out of many, to investigate suppressive functionality of Tregs by Treg cells converting ATP to adenosine [134-137]. It stands to reason that inadequate T cell responses are responsible for poor survival in C57BL/6 mice, however, regulatory mechanisms cannot be ruled out here and warrant further investigation.

A

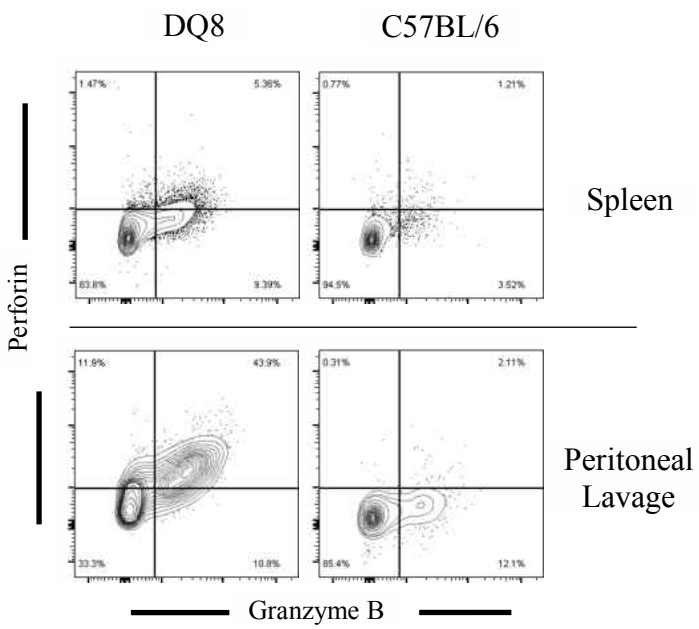

DQ8
C57BL/6
Spleen
Peritoneal Lavage
Perforin
Granzyme B
0.64%
0.098%
99.5%
0.73%
0.23%
0%
99.4%
0.34%
0.88%
1.02%
93.6%
4.53%
0.12%
0.53%
97.8%
1.54%

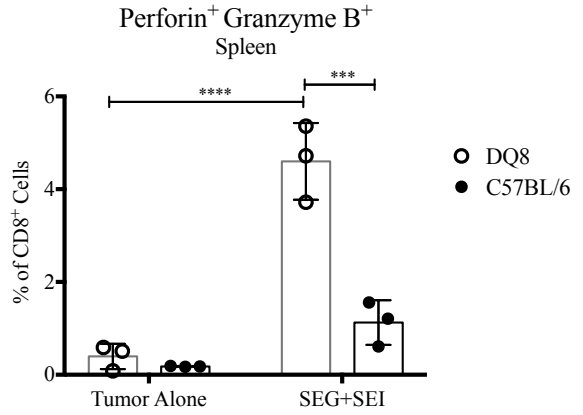

Perforin+ Granzyme B+
Spleen
% of CD4+ Cells
1.0
0.8
0.6
0.4
0.2
0.0
n.s.
Tumor Alone
SEG+SEI
DQ8
C57BL/6

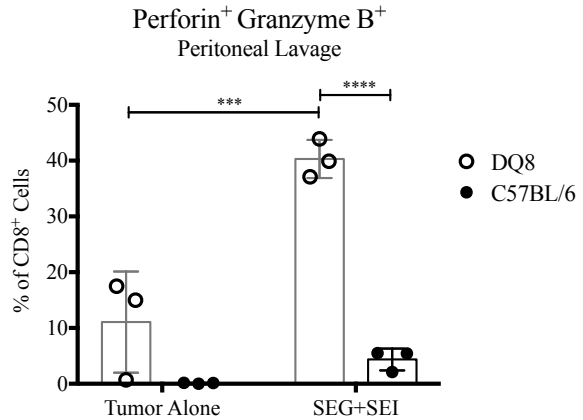

Perforin+ Granzyme B+
Peritoneal Lavage
% of CD4+ Cells
6
4
2
0
p = 0.06
p = 0.03
Tumor Alone
SEG+SEI
DQ8
C57BL/6

Continued…

B


DQ8
C57BL/6
Perforin
Granzyme B
Spleen
Peritoneal Lavage
1.47%
5.38%
63.8%
9.39%
0.77%
1.21%
94.5%
3.52%
11.9%
43.9%
33.3%
10.8%
0.31%
2.11%
85.4%
12.1%


Perforin+ Granzyme B+
Spleen
% of CD8+ Cells
****
***
Tumor Alone
SEG+SEI
DQ8
C57BL/6


Perforin+ Granzyme B+
Peritoneal Lavage
% of CD8+ Cells
***
****
Tumor Alone
SEG+SEI
DQ8
C57BL/6

Continued…

C

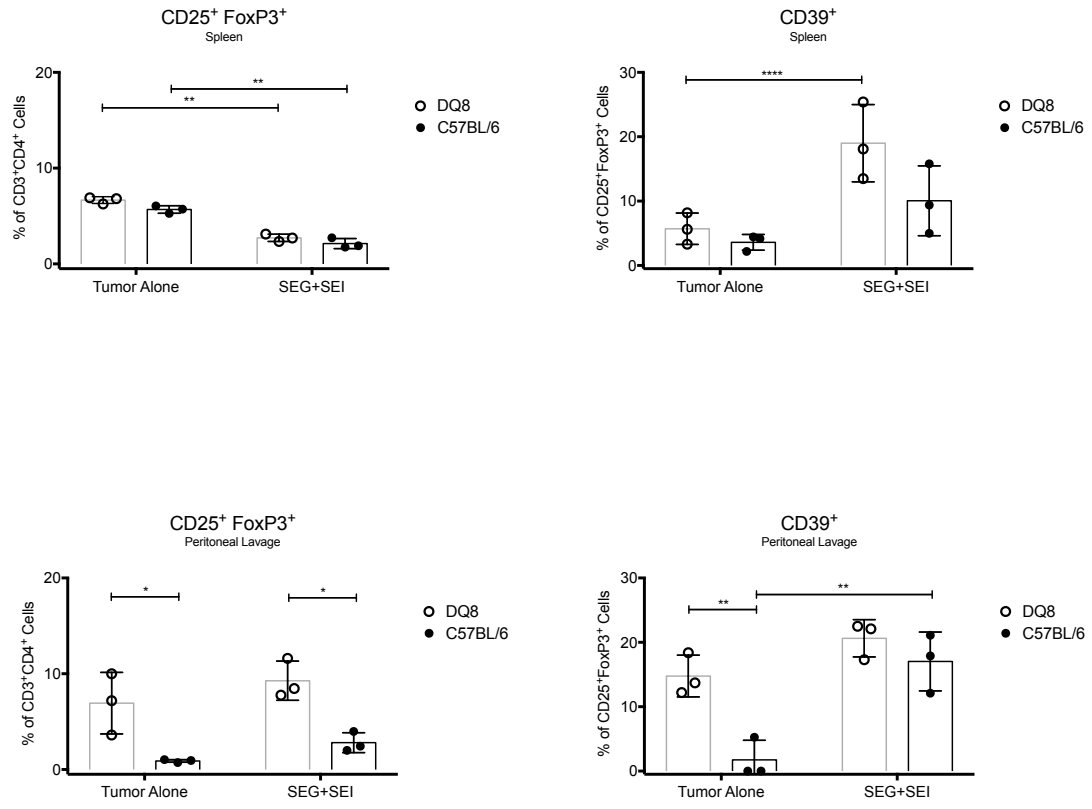
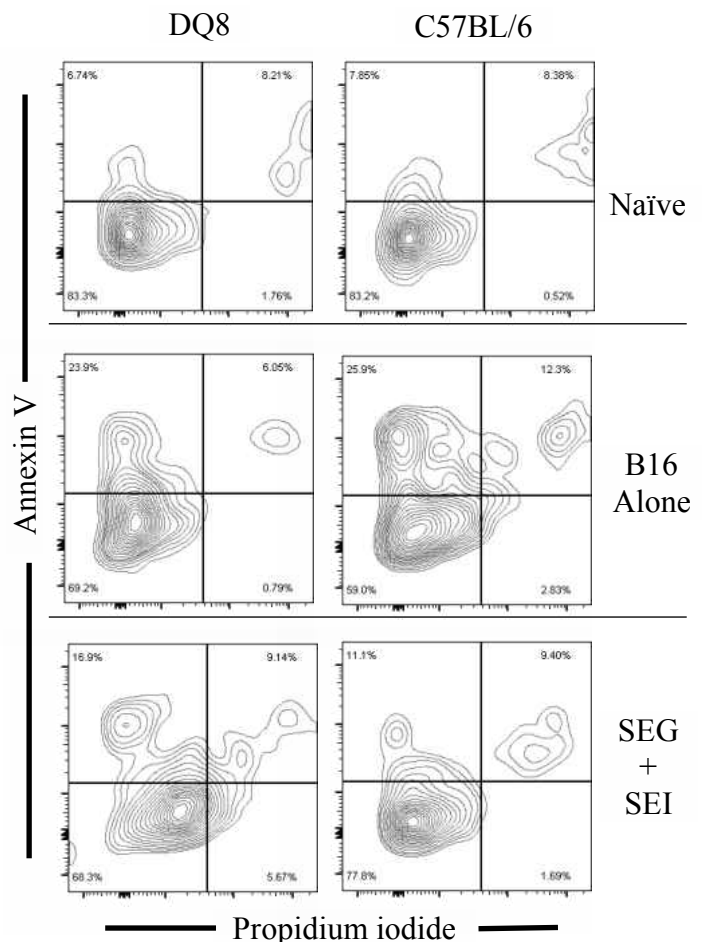


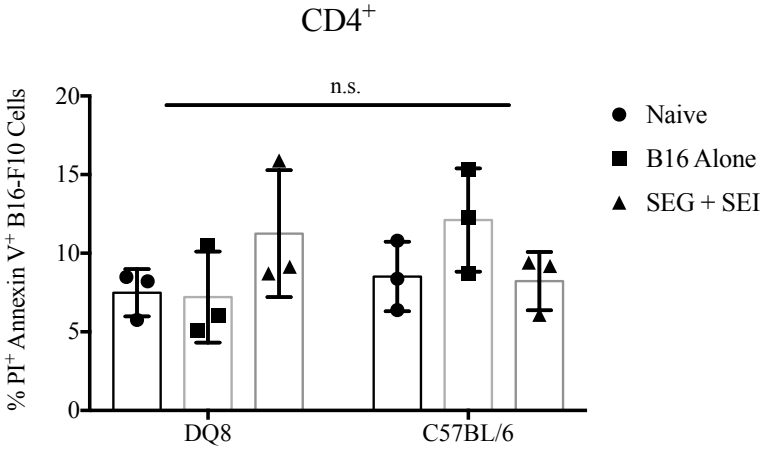
Figure 14 . HLA-DQ8 CD8$^+$ T cells produce perforin and granzyme B.

Splenocytes and peritoneal lavage cells were evaluated 13 days post B16-F10 implant ($2.5x10^5$ cells IP) from DQ8 and C57BL/6 mice. Effector molecules perforin and granzyme B production in CD4$^+$ (A) and CD8$^+$ (B) T cells, along with T regulatory cells (C) via flow cytometry. . Groups presented were treated with 50ug each SEG & SEI on day 6 and 9 post implant. n=3. Graphed mean with SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.001

In order to specifically determine which cells are functionality cytotoxic, CD4+ and CD8+ T cells were isolated from spleens 13 days post B16 implant from treated and non-treated mice, including naïve controls. Specific T cells were cocultured with B16-F10 melanoma cells for 4 hours in vivo at 50:1 effector:target ratio **Figure 15**. Cytotoxicity was measured and reported as annexin V and propidium iodide double positive B16-F10 cells, gated to exclude T cells. Unfortunately, these data did not show meaningful differences in cytotoxicity after 4 hours coculture.



Gate: Singlets>B16-F10



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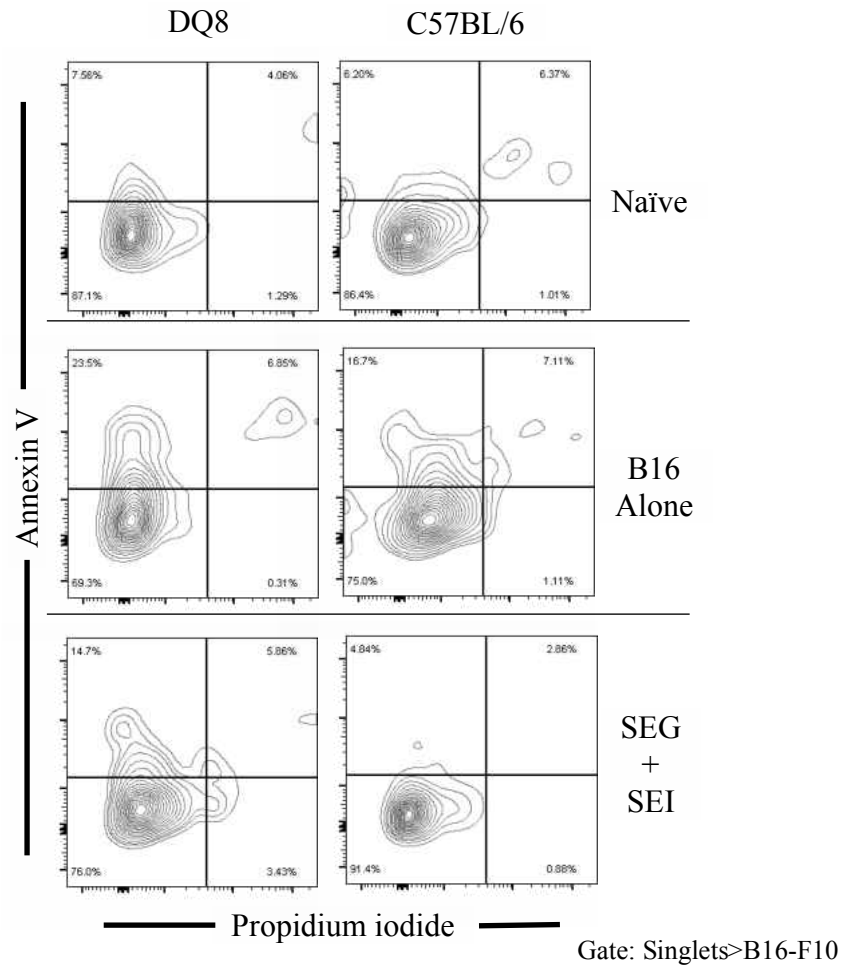
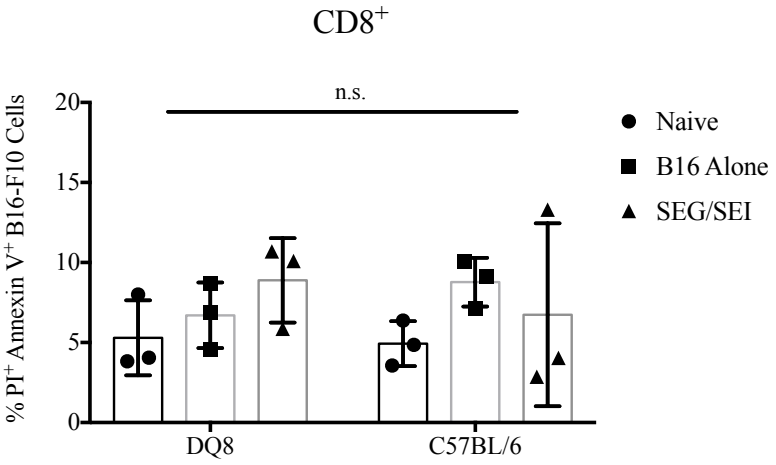

DQ8
C57BL/6
Naïve
B16
Alone
SEG
+
SEI
Annexin V
Propidium iodide
Gate: Singlets>B16-F10


CD8+
n.s.
% PI+ Annexin V+ B16-F10 Cells
20
15
10
5
0
DQ8
C57BL/6
Naive
B16 Alone
SEG/SEI

Continued…

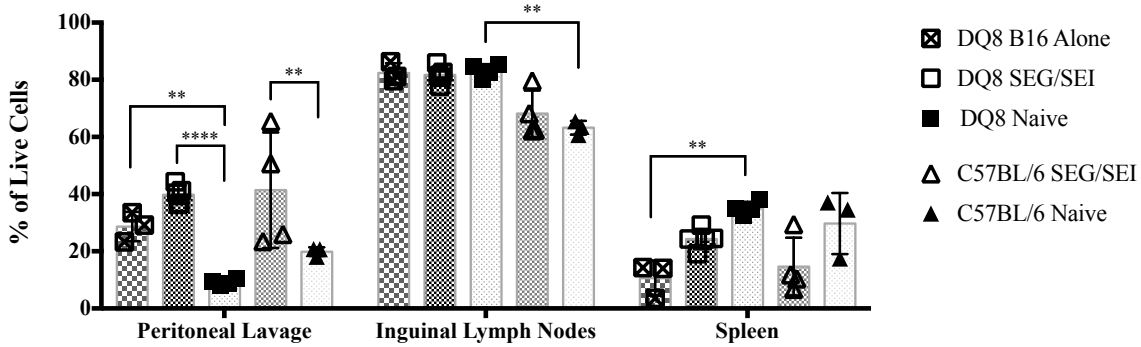
Figure 15. Day 13 splenocyte cytotoxicity against B16-F10.

$CD4^+$ **(A)** and $CD8^+$ **(B)** T cells isolated from splenocytes 13 days post B16-F10 implant ($2.5x10^5$ cells IP) from mice treated with 50ug each SEG & SEI on day 6 and 9 post implant, nontreated B16 alone and naïve controls. Cells were cocultured at a 50:1 (T cell:B16) ratio for 4hrs in 96 well flat bottom plates. Representative flow cytometry plots of annexin V and PI staining (Gate: Singlets>B16-F10 cells) shown and reported as % annexin V^+/PI^+ B16-F10 cells. n=3. *$p<0.05$.

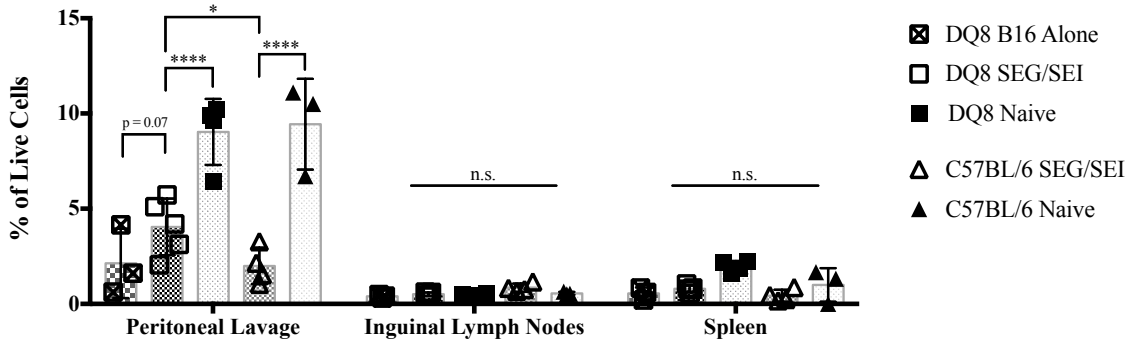
The presence of $CD8^+$ T cells that contain perforin and granzyme 13 days post tumor implant suggest a potential cell mediated response in DQ8 mice not present in C57BL/6 compatriots. Expanding on day 13 observations, day 15 lymphoid tissue was evaluated for memory responses generated from SEG/SEI compared to naïve controls **Figure 16**. B16 tumor alone groups for memory responses proved variable in results possibly due to the nature of the inherent immune response to B16. The B16 alone group was include for DQ8 mice for comparison. These data highlight the immune cell populations in the spleen, inguinal lymph nodes and the peritoneum. The percent $CD3^+$ population between naïve C57BL/6 and DQ8 were no different in spleens and peritoneum **Figure 16A** and were increased day 15 post implant in response to SEG/SEI treatment. Not unexpected, $CD49b^+$ NK cells were similarly increased with SEG/SEI treatment including $CD49b^+TCR\beta^+$ NKT cells. Further analysis finds the breakdown of $CD4^+/CD8^+$ populations differ between genotypes with DQ8 mice presenting lower $CD4^+/CD8^+$ ratios compared to C57BL/6 mice **Figure 16B,C**. Given that these mice have similar numbers of $CD3^+$ T cells, the differences between $CD4^+$ and $CD8^+$ memory cell phenotypes were explored.

A

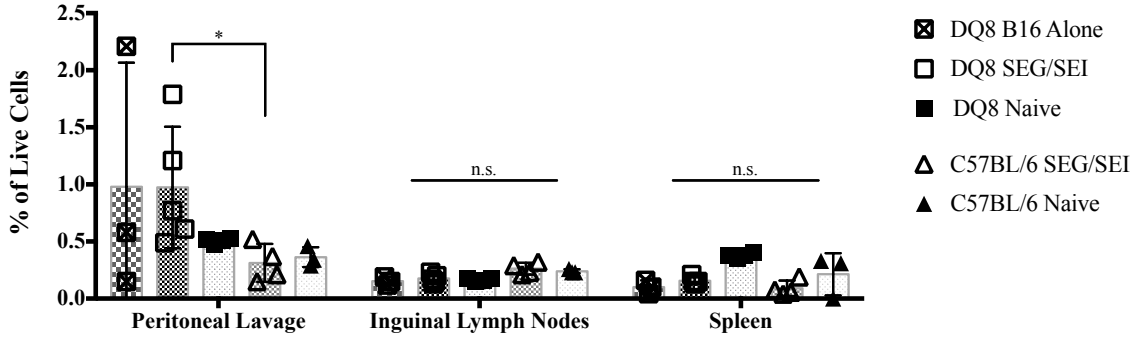
CD3$^+$



NK Cells

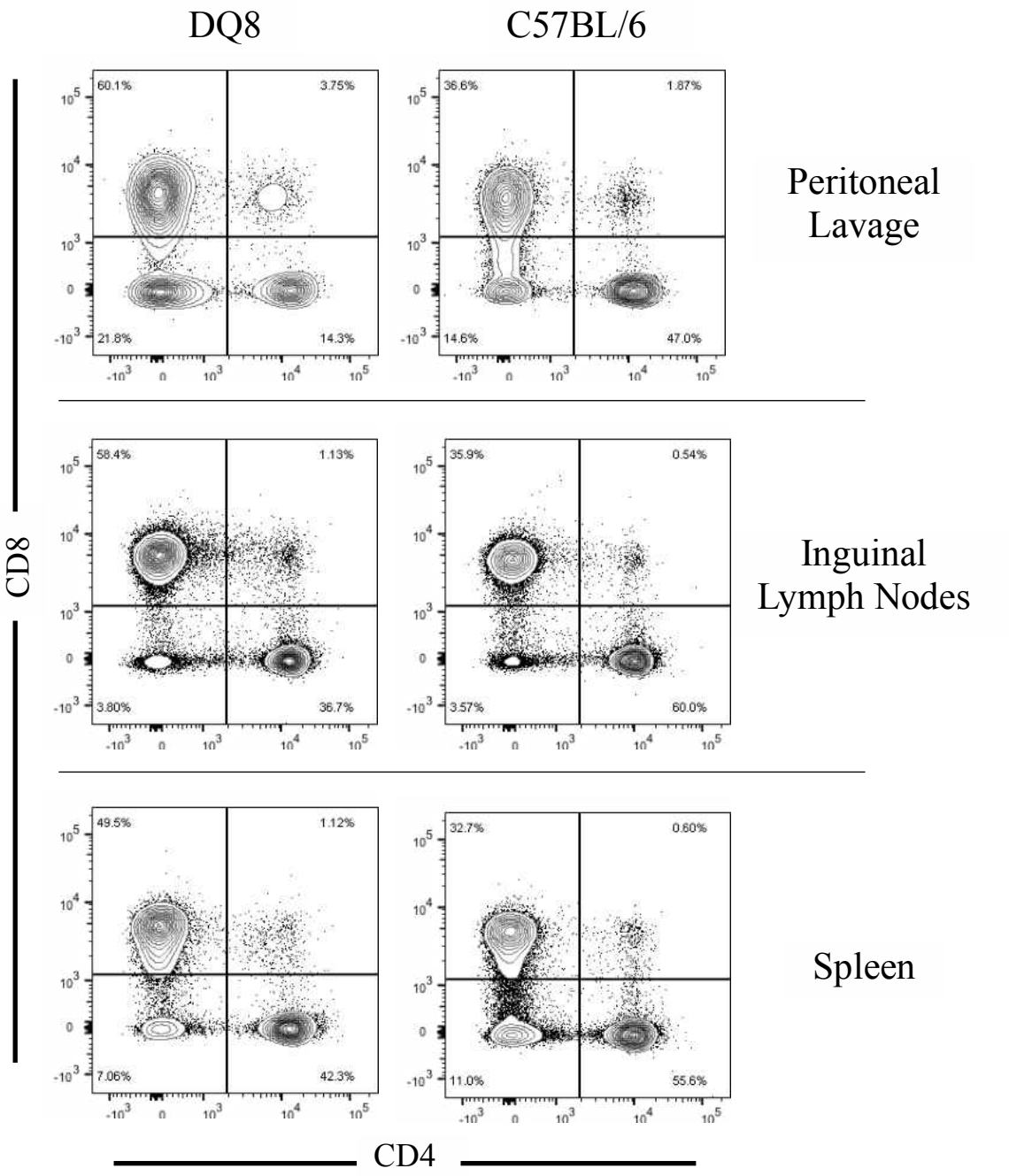


NKT Cells



Continued…

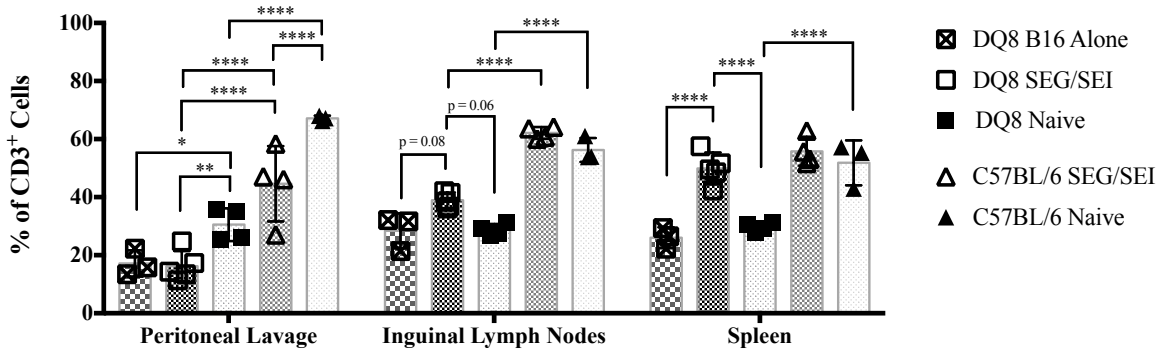
B


DQ8
C57BL/6
Peritoneal Lavage
Inguinal Lymph Nodes
Spleen
CD8
CD4
60.1%
3.75%
21.8%
14.3%
36.6%
1.87%
14.6%
47.0%
58.4%
1.13%
3.80%
36.7%
35.9%
0.54%
3.57%
60.0%
49.5%
1.12%
7.06%
42.3%
32.7%
0.60%
11.0%
55.6%

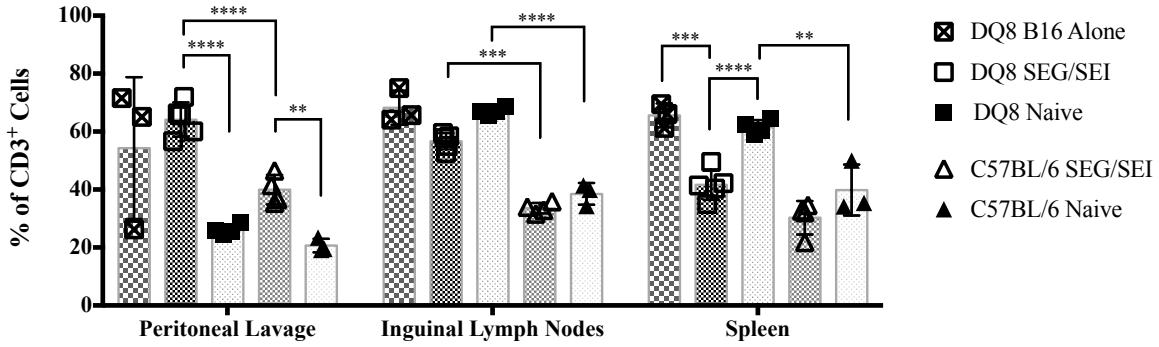
Continued…

C

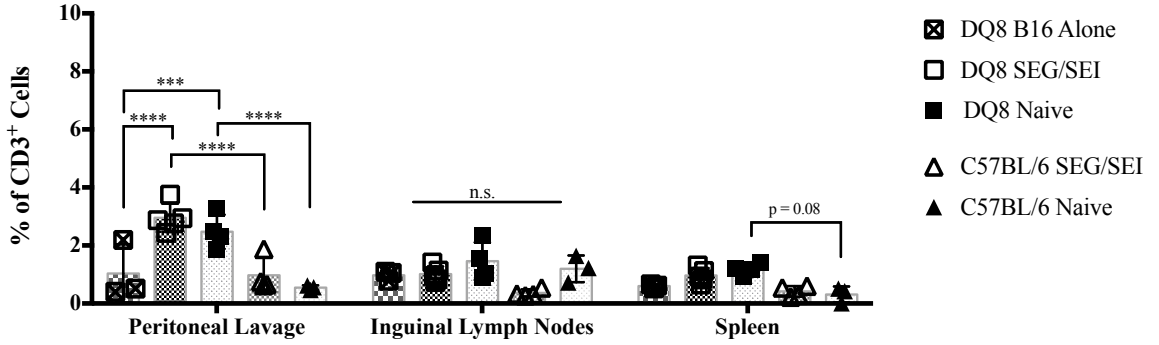
CD4$^+$



CD8$^+$



CD4$^+$CD8$^+$

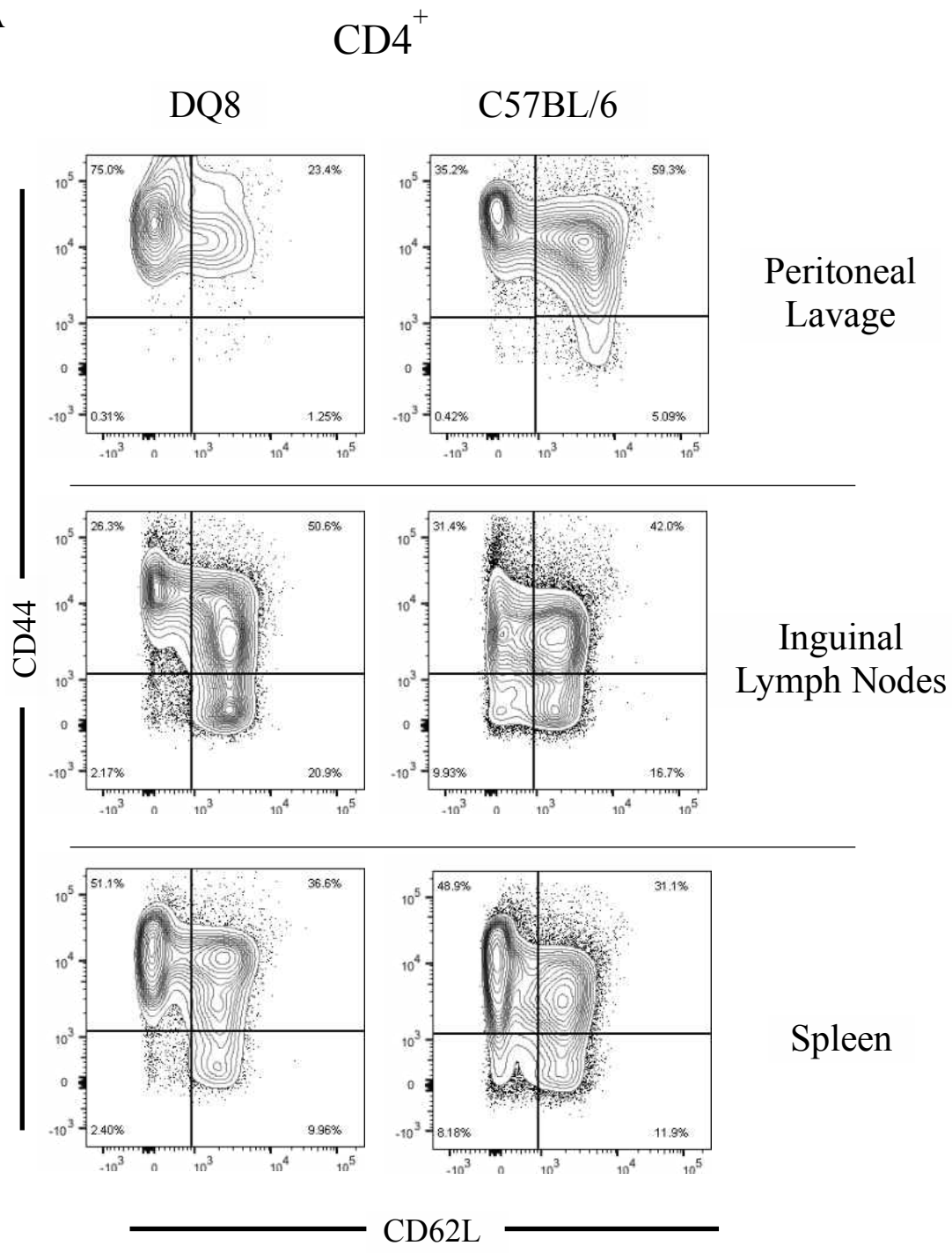


Continued…

Figure 16. $CD8^+$ T cells are increased in SEG/SEI treated HLA-DQ8 mice. DQ8 and C57BL/6 splenocytes, peritoneal lavage cells, and inguinal lymph nodes were evaluated 15 days post B16-F10 implant ($2.5x10^5$ cells IP) from SEG/SEI treated and naïve mice. (A) $CD3^+$ cells were increased in SEG/SEI treated groups, whereas $CD49b^+$ (NK) cells were decreased. (B,C) $CD4^+$ and $CD8^+$ T cells are reported as % live cells. Groups presented were treated with 50ug each SEG & SEI on day 6 and 9 post implant. n=3-5. Graphed mean with SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.001.

As mentioned previously, the durability of T cell therapies providing lasting immunity is limited. In that light, memory T cell populations were characterized using anti-CD44, -CD62L, -CD3, CD4, CD8 and KLRG1 and analyzed via flow cytometry **Figure 17**. Including KLRG1 was used to differentiate between effector and memory cells with effector cells expressing higher levels upon activation and decreased levels in memory cells [142-145]. Take together data provide a glimpse into the trafficking of immune cells in response to SEG/SEI. It would be easy to focus on the effector, $CD44^{+}CD62L^{-}$, populations, but the differences in naïve mice complicate the interpretation of these data. Of note, $CD8^{+}CD44^{+}CD62L^{+}KLRG1^{-}$ cells are increased in the inguinal lymph nodes and spleens of DQ8 mice compared to controls **Figure 17 B,F**. Differences could be a result of a delayed response in C57BL/6. $CD8^{+}CD44^{+}CD62L^{+}$ cells in the peritoneum of C57BL/6 are increased compared to DQ8 mice. Taking into consideration day 13 and 15 responses these data suggest SEG/SEI elicit a powerful, lasting CD8 response against B16 melanoma unseen in identically treated C57BL6 mice.

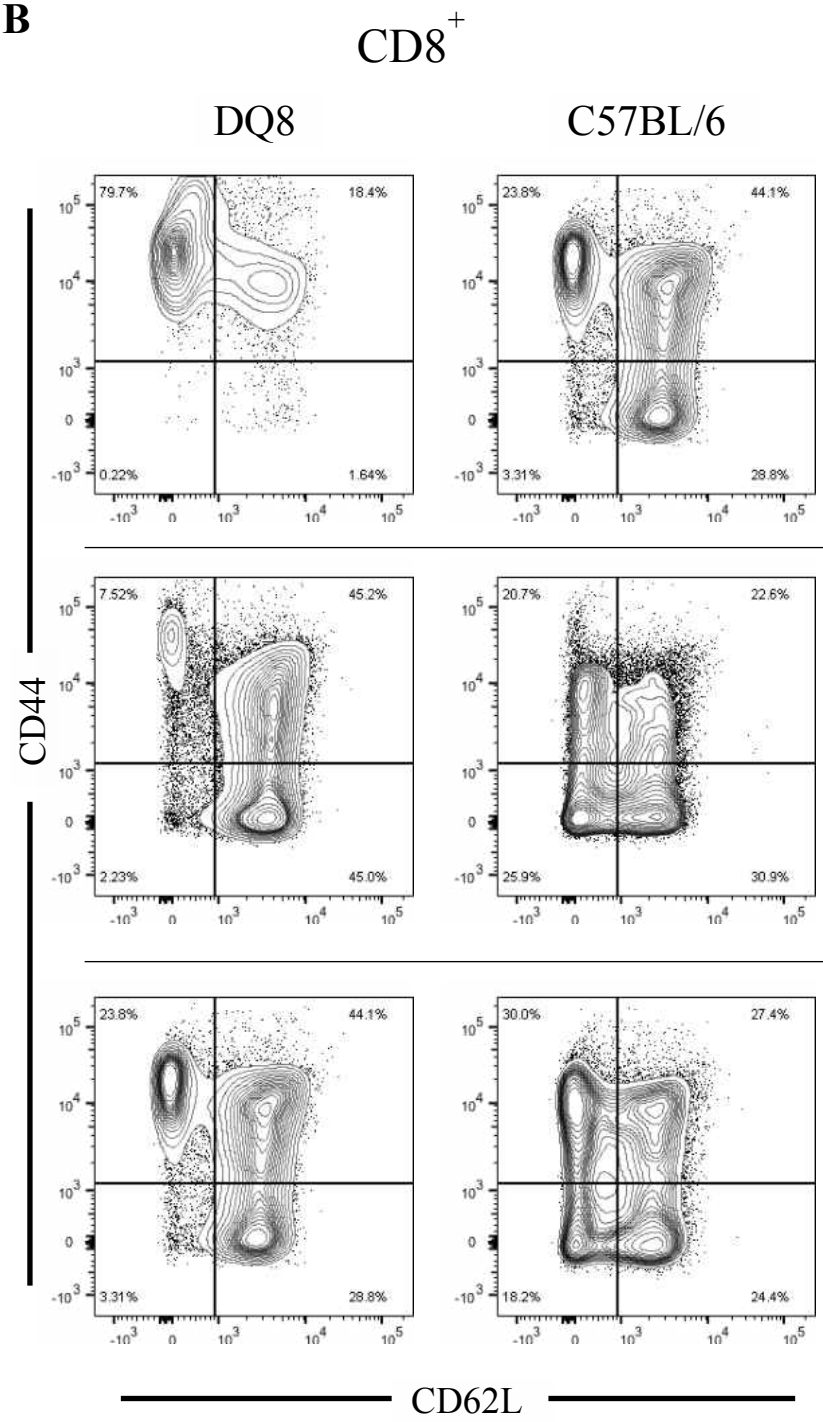
A



Gate: Singlets>Live>Cells>$CD3^+$> $CD4^+CD8^-$

Continued…

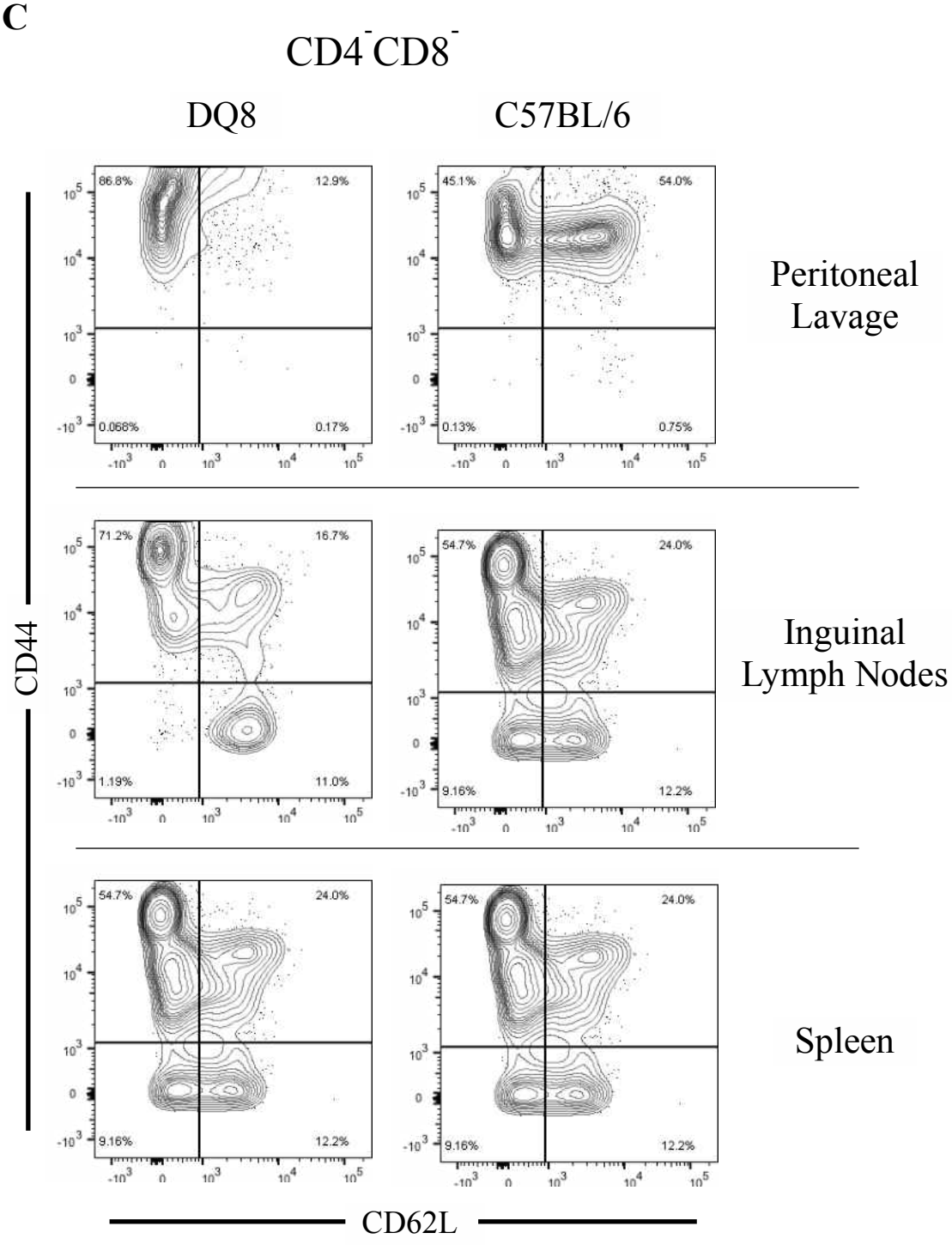
B



Gate: Singlets>Live>Cells>$CD3^{+}$> $CD4^{-}CD8^{+}$

Continued…

C



Gate: Singlets>Live>Cells>CD3^{+}> CD4^{-}CD8^{-}

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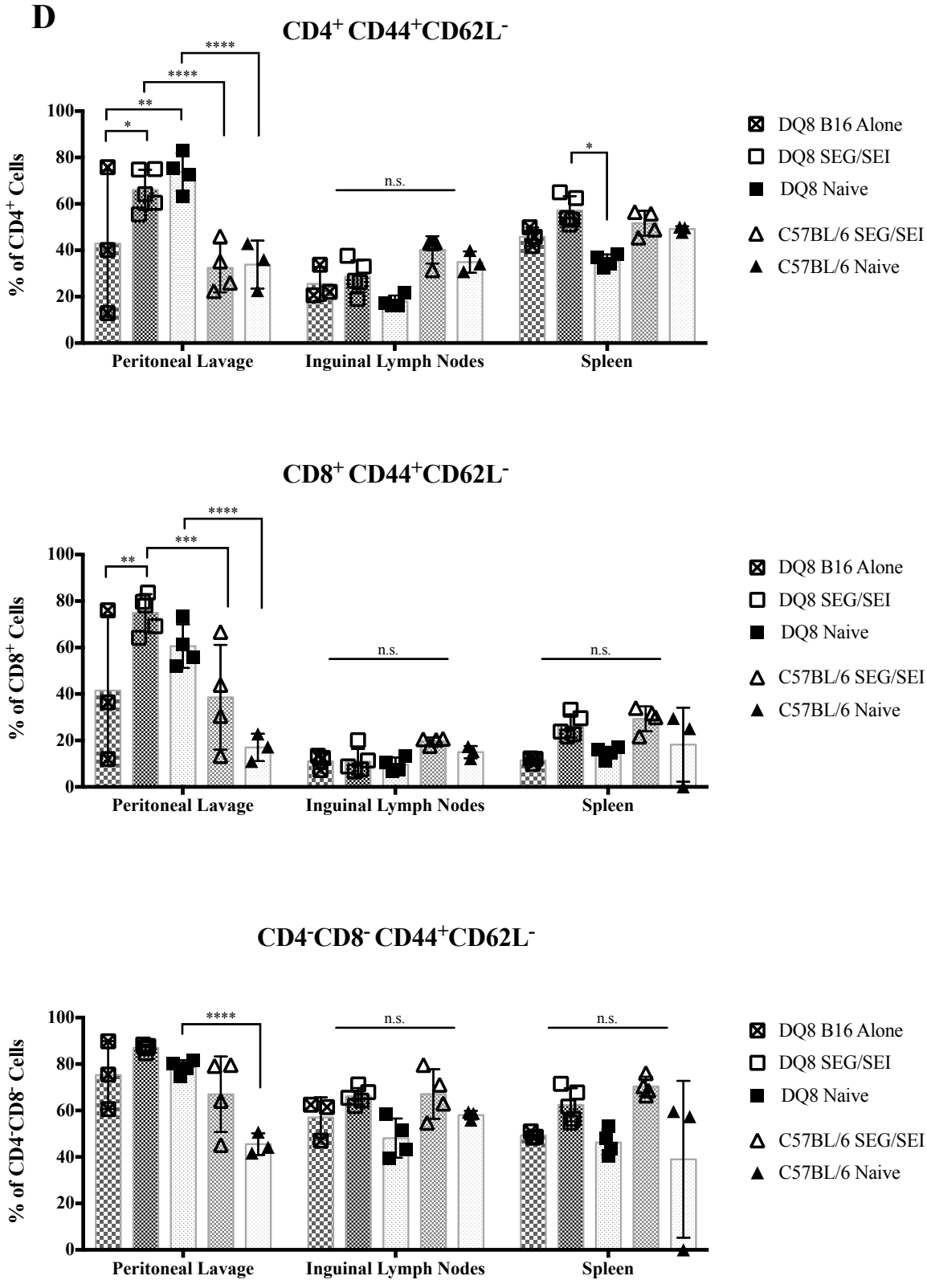
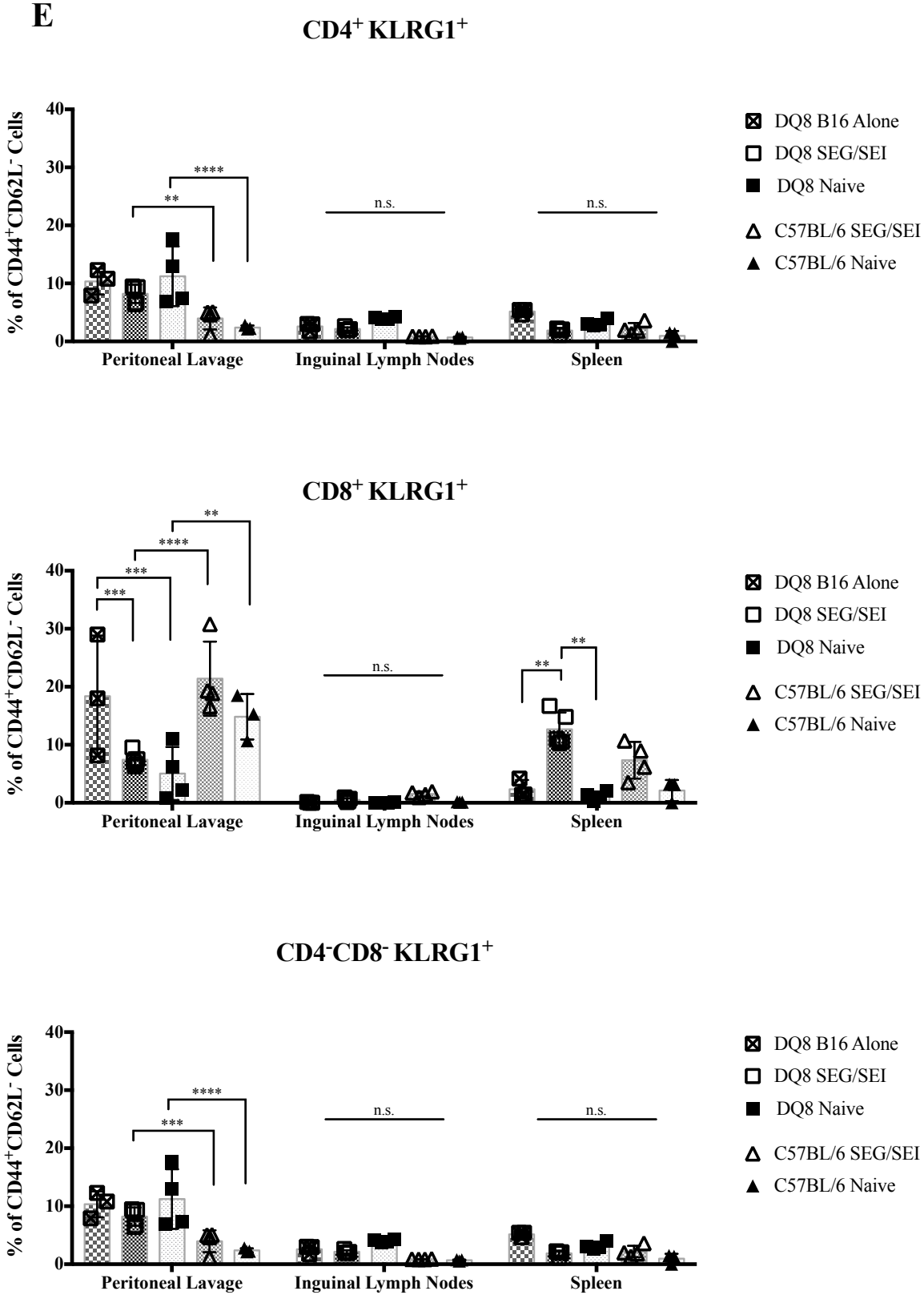
D


CD4+ CD44+CD62L-
% of CD4+ Cells
100
80
60
40
20
0
****
****
**
*
n.s.
*
Peritoneal Lavage
Inguinal Lymph Nodes
Spleen
DQ8 B16 Alone
DQ8 SEG/SEI
DQ8 Naive
C57BL/6 SEG/SEI
C57BL/6 Naive
CD8+ CD44+CD62L-
% of CD8+ Cells
100
80
60
40
20
0
****
***
**
n.s.
n.s.
Peritoneal Lavage
Inguinal Lymph Nodes
Spleen
DQ8 B16 Alone
DQ8 SEG/SEI
DQ8 Naive
C57BL/6 SEG/SEI
C57BL/6 Naive
CD4-CD8- CD44+CD62L-
% of CD4-CD8- Cells
100
80
60
40
20
0
****
n.s.
n.s.
Peritoneal Lavage
Inguinal Lymph Nodes
Spleen
DQ8 B16 Alone
DQ8 SEG/SEI
DQ8 Naive
C57BL/6 SEG/SEI
C57BL/6 Naive

Continued…

E


CD4+ KLRG1+
% of CD44+CD62L- Cells
40
30
20
10
0
**
****
n.s.
n.s.
Peritoneal Lavage
Inguinal Lymph Nodes
Spleen
DQ8 B16 Alone
DQ8 SEG/SEI
DQ8 Naive
C57BL/6 SEG/SEI
C57BL/6 Naive
CD8+ KLRG1+
% of CD44+CD62L- Cells
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n.s.
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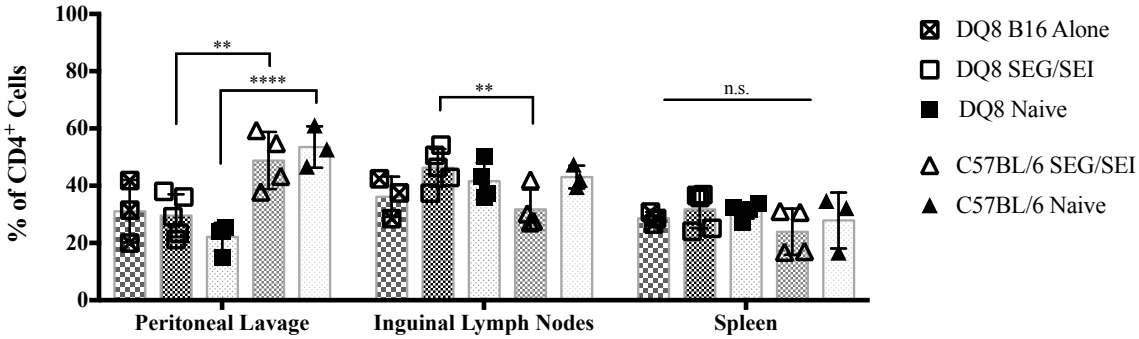
Peritoneal Lavage
Inguinal Lymph Nodes
Spleen
DQ8 B16 Alone
DQ8 SEG/SEI
DQ8 Naive
C57BL/6 SEG/SEI
C57BL/6 Naive
CD4-CD8- KLRG1+
% of CD44+CD62L- Cells
****
***
n.s.
n.s.
Peritoneal Lavage
Inguinal Lymph Nodes
Spleen
DQ8 B16 Alone
DQ8 SEG/SEI
DQ8 Naive
C57BL/6 SEG/SEI
C57BL/6 Naive

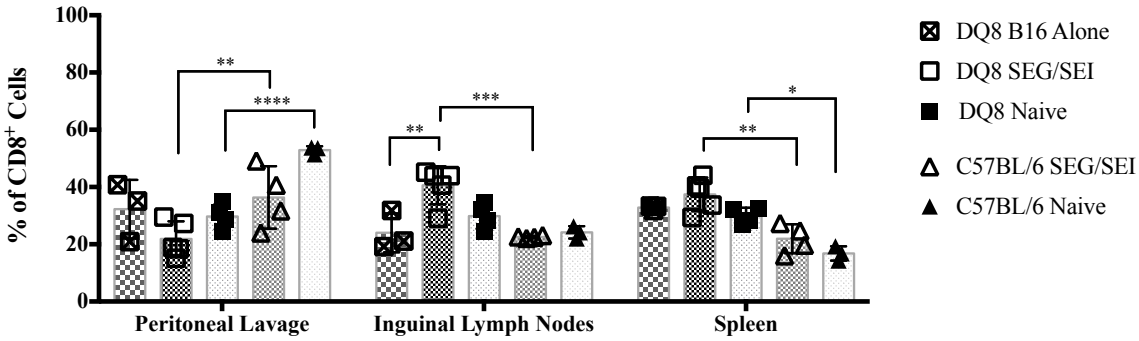
Continued…

F

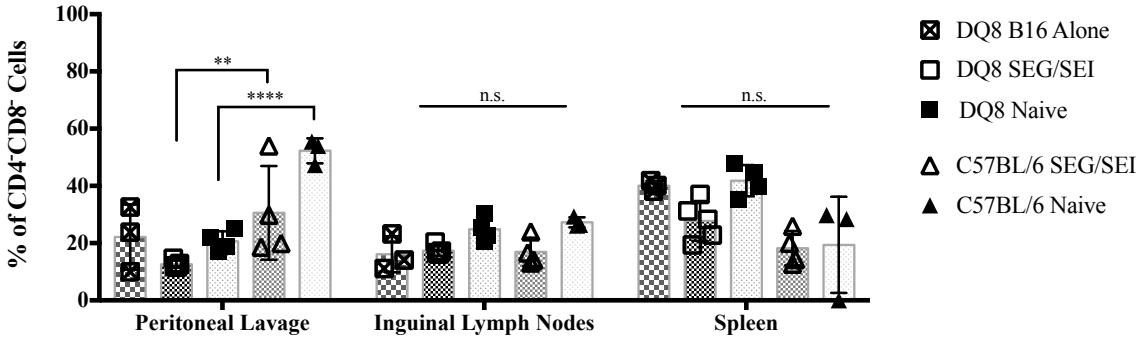
$CD4^+ CD44^+CD62L^+$



$CD8^+ CD44^+CD62L^+$



$CD4^- CD8^-$ $CD44^+CD62L^+$



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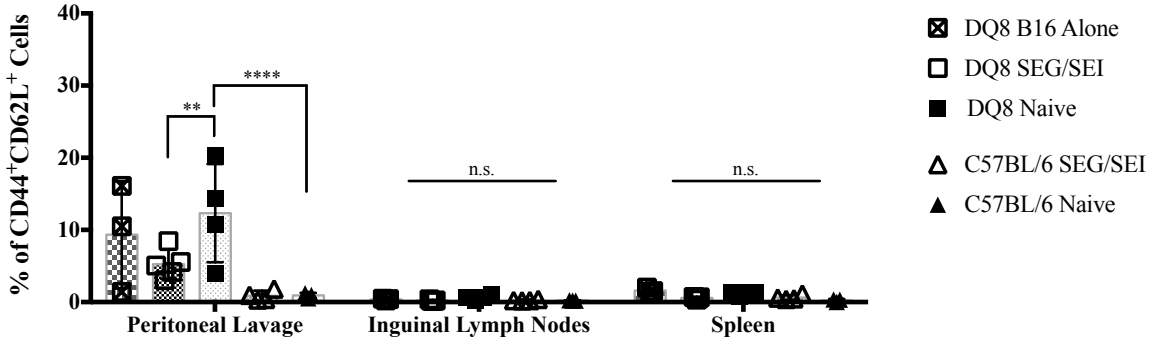
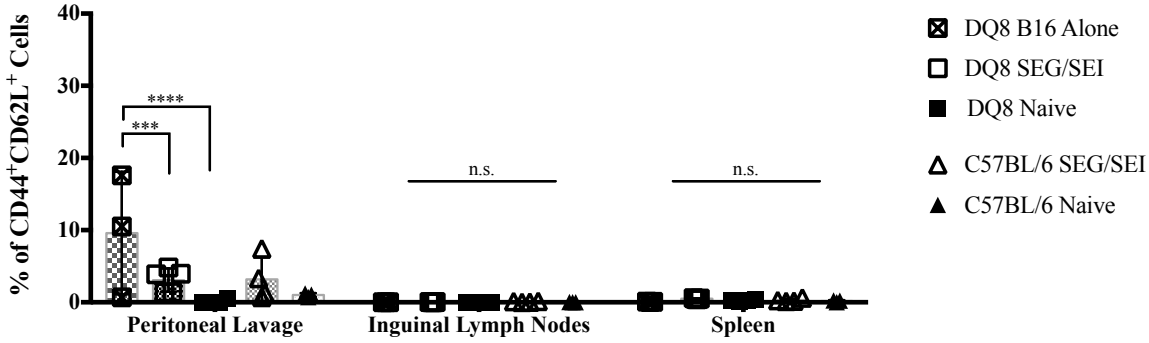
G

$CD4^+$ $KLRG1^+$



$CD8^+$ $KLRG1^+$



$CD4^-CD8^-$ $KLRG1^+$

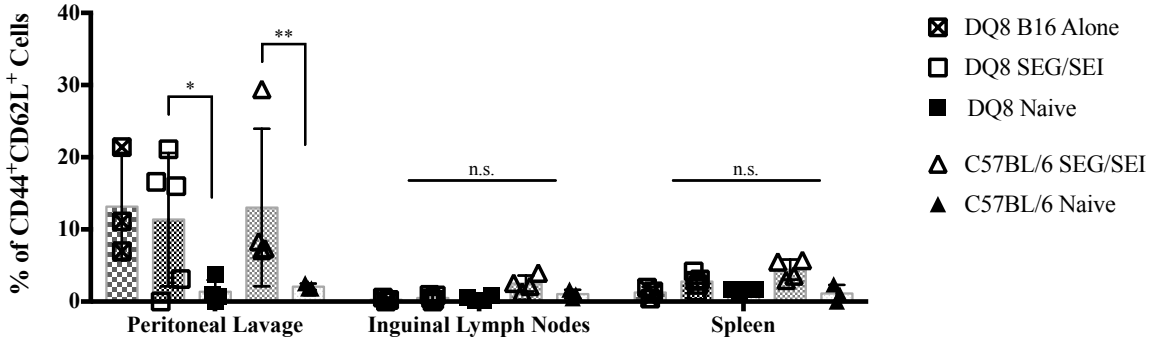


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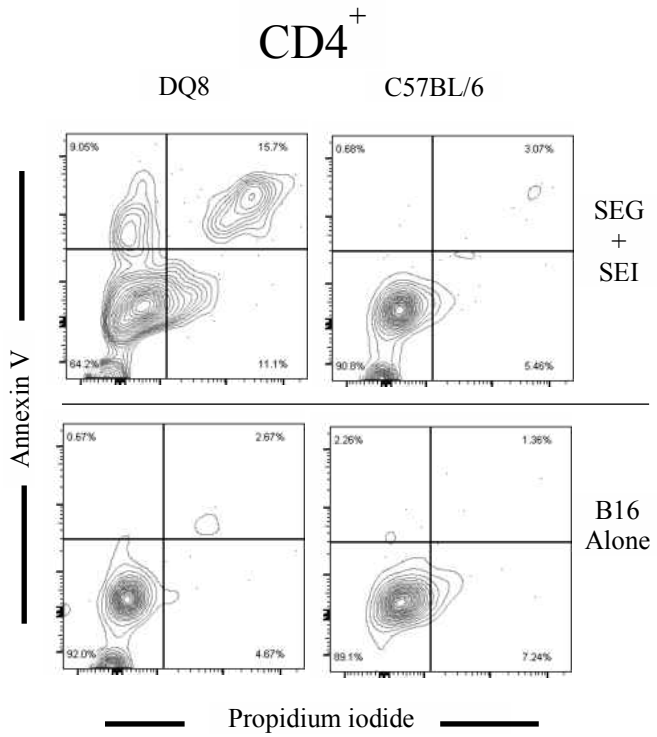
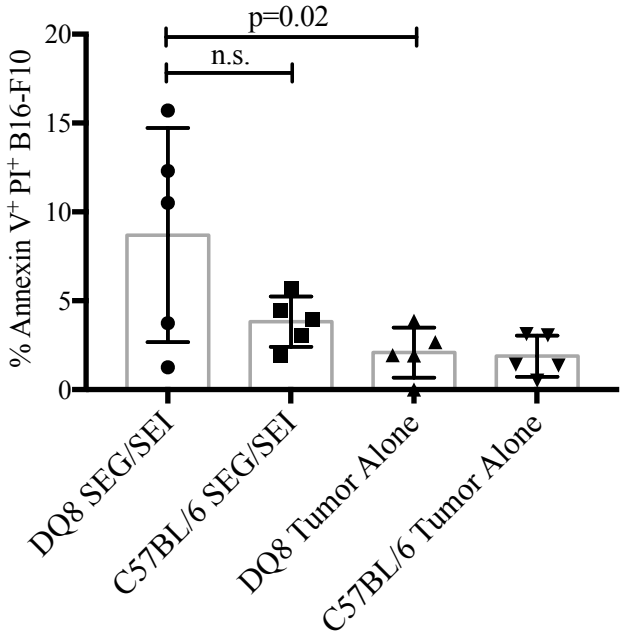
Figure 17. $CD44^{+}CD62L^{+}KLRG1^{-}CD8^{+}$ T memory cells are increased in SEG/SEI treated HLA-DQ8 mice.

DQ8 and C57BL/6 splenocytes, peritoneal lavage cells, and inguinal lymph nodes were evaluated 15 days post B16-F10 implant ($2.5x10^5$ cells IP) from SEG/SEI treated and naïve mice. Representative scatter plots of cells from mice treated day 6 and 9 post implant with SEG/SEI (A-C). CD44 and CD62L expression (D,F) along with KLRG1 (E,G) was used to characterized effector and memory CD4/CD8 T cells were gated to exclude doublets, debris and dead cells from analysis. n=3-5. Graphed mean with SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.001

A



Gate: Singlets>B16-F10



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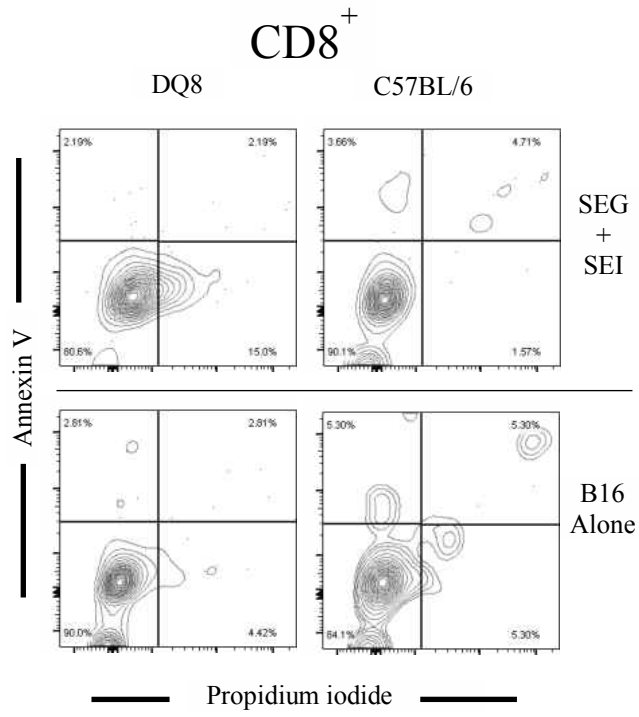
B



Gate: Singlets>B16-F10

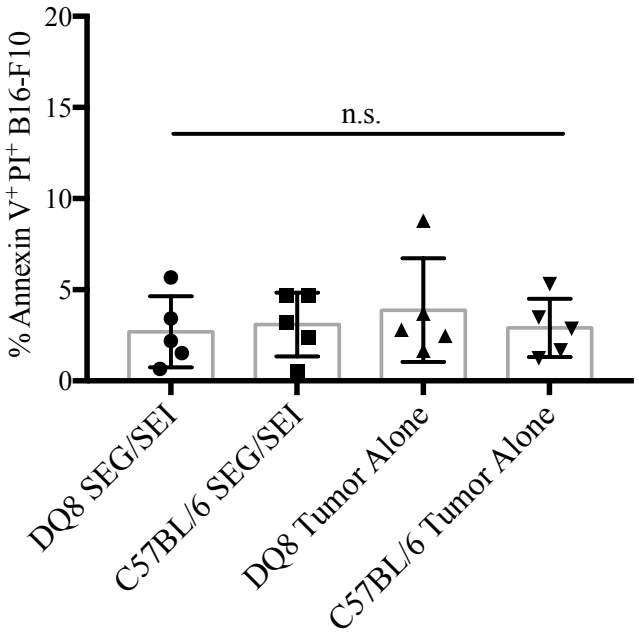


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Figure 18 . Day 15 peritoneal lavage cell cytotoxicity against B16-F10.

$CD4^+$ **(A)** and $CD8^+$ **(B)** T cells isolated from peritoneal lavages of SEG/SEI treated and non-treated mice 15 days post implant. Cells were cocultured at a 10:1 (T cell:B16) ratio for 4hrs in 96 well flat bottom plates. Representative flow cytometry plots of annexin V and PI staining (Gate: Singlets>B16-F10 cells) shown and reported as % annexin V^+/PI^+ B16-F10 cells. n=5. *$p<0.05$.

SEG/SEI in Treatment of Lewis Lung Carcinoma

Pushing the success demonstrated by introducing SEG/SEI in the treatment of melanoma, LLC cells were used to evaluate a tumor with a different immune contexture. LLC cells were deployed similar to the peritoneal model used to assess SEG/SEI against B16 melanoma **Figure 19**. Survival of mice with B16 or LLC were similar albeit 250,000 LLC cells at implant prove more lethal in DQ8 mice compared to 10,000 cells at implant **Figure 20**.

SEG/SEI therapy proved ineffective in treating an established LLC tumor using the same treatment protocol which demonstrated robust anti-B16 responses **Figure 21**. It would be reasonable to think that lowering the tumor burden to 10,000 cells at implant would provide some benefit. Nope **Figure 22**. Irradiated LLC cells were used to provide antigen for an antitumor response which would in turn be enhanced with SEG/SEI. This provided no benefit to the mice. The only hypothesis supported throughout these experiments is that LLC tumors have a different immune contexture.

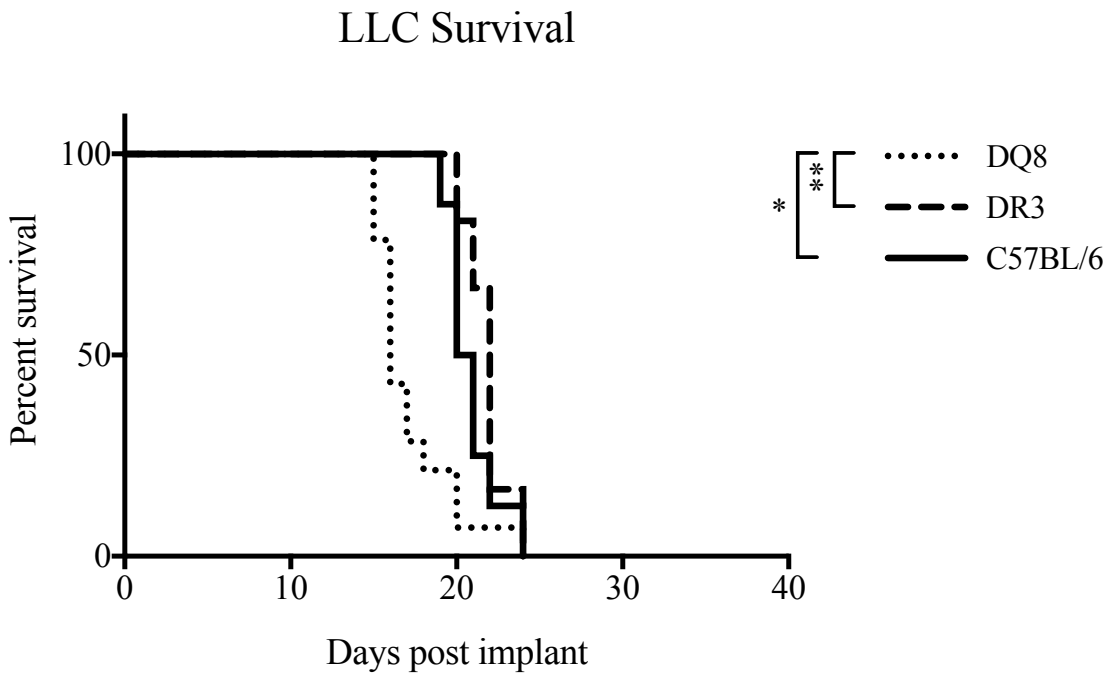


Figure 19. Lewis lung carcinoma implanted intraperitoneally is more lethal in HLA-DQ8 mice compared to allogeneic C57BL/6.

Syngeneic C57BL/6 6-8 w.o. female mice were implanted with $2.5x10^5$ live LLC cells IP along with DQ8 and DR3 mice. Kaplan Meier survival curves shown with Log-rank test corrected for multiple comparisons. n=6-14. *p=0.05, **p=0.01, ***p=0.001.

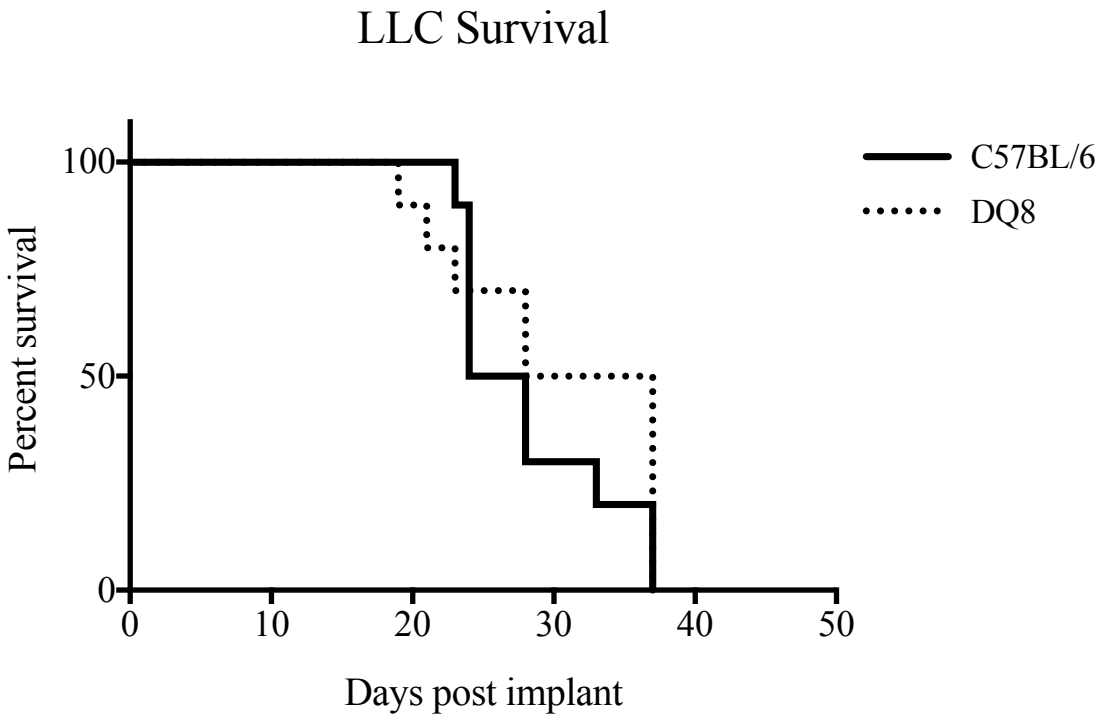


Figure 20. Mice implanted intraperitoneally with 10,000 LLC cells.

6-8 w.o. male mice were implanted with $1x10^4$ live LLC cells IP. Kaplan Meier survival curves shown. n=10.

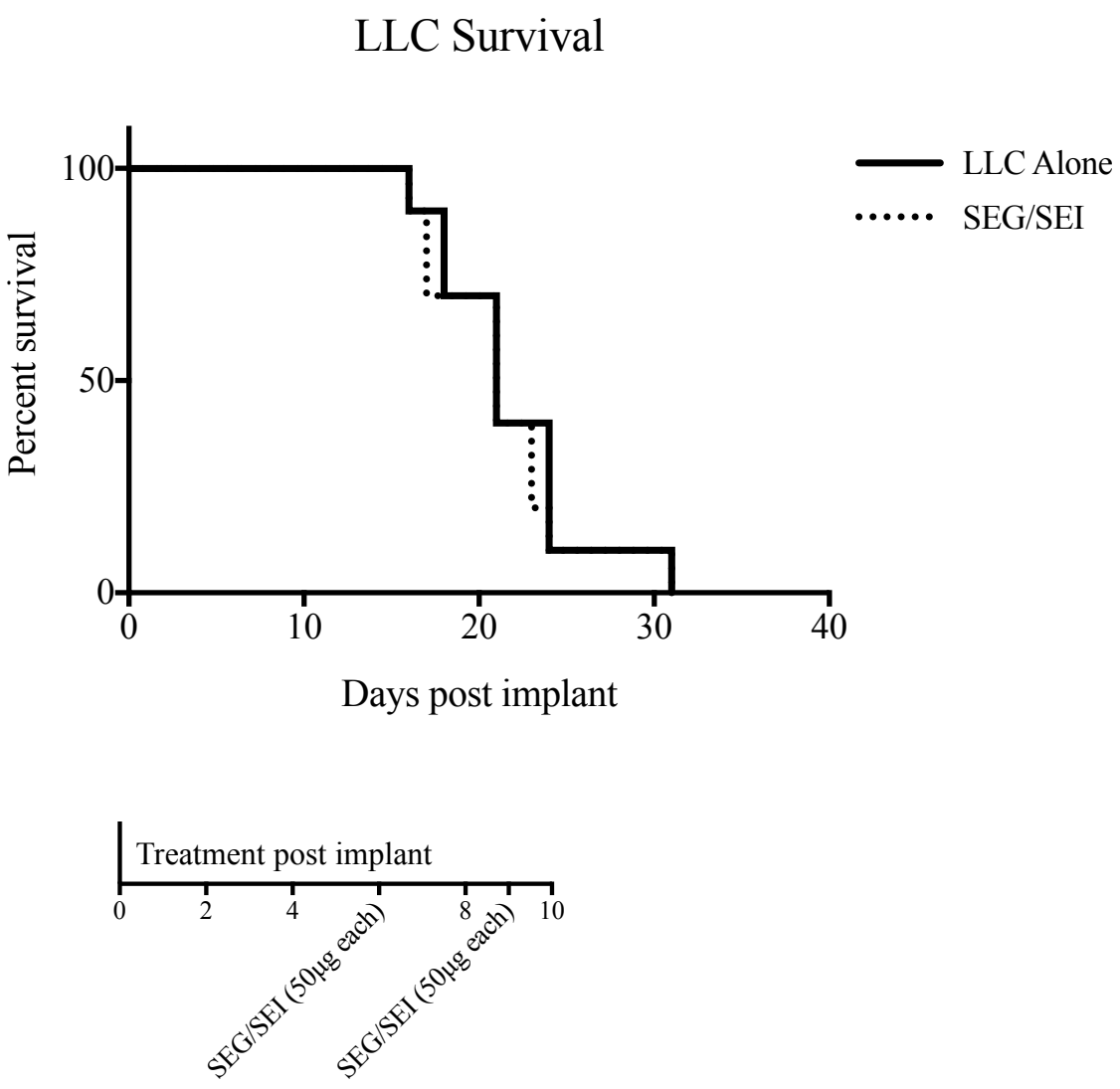


Figure 21. SEG/SEI does not elicit antitumor responses in DQ8 mice with an established LLC tumor.

DQ8 6-8 w.o. female mice were implanted with $2.5x10^5$ live LLC cells IP. Treated mice received 50µg SEG and 50µg SEI IP day 6 and 9 post implant. Kaplan Meier survival curves shown. n=10.

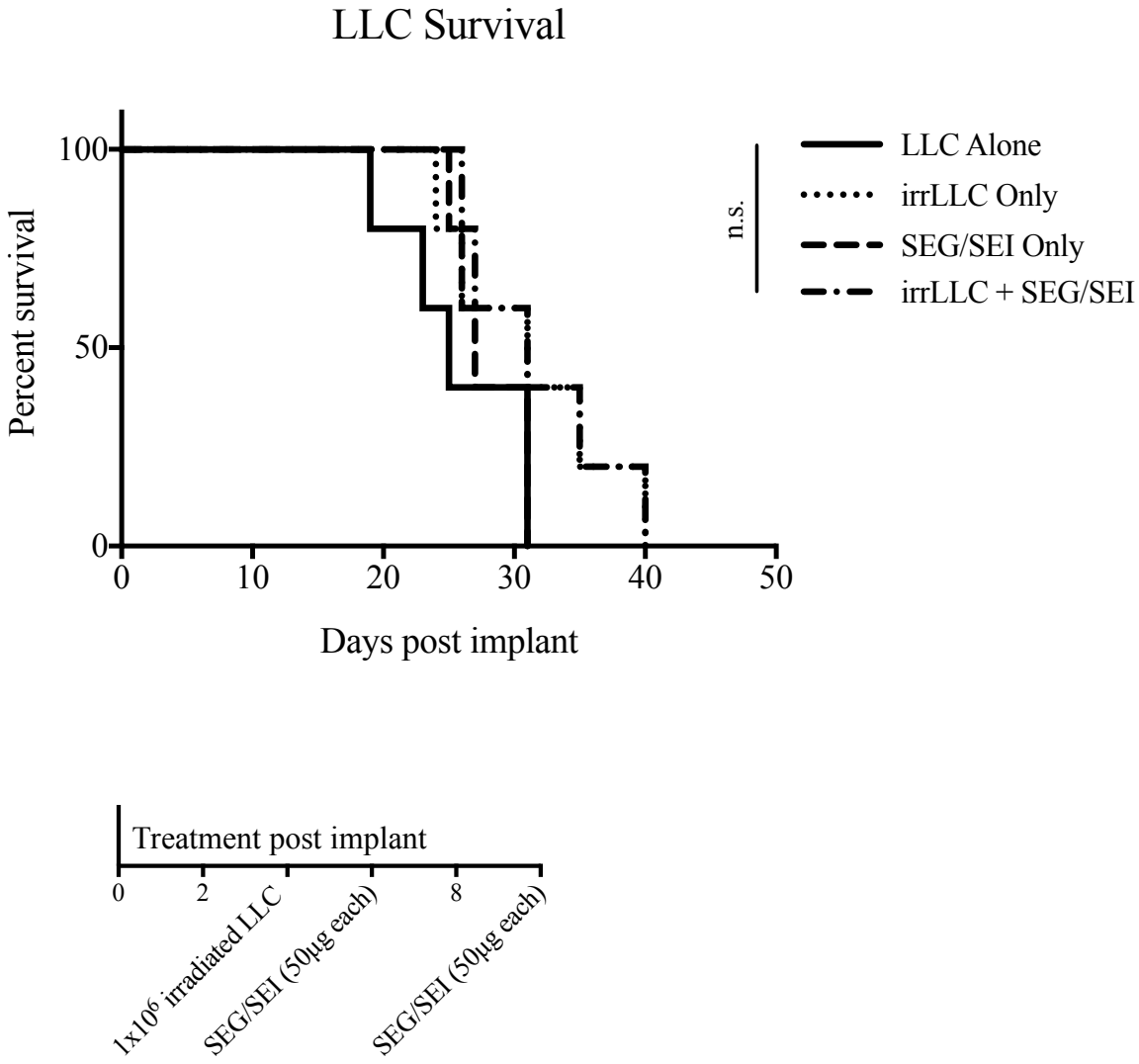


Figure 22. Initial LLC implant dosage does not affect SEG/SEI treatment outcome.

DQ8 mice implanted with $1x10^4$ live LLC cells received $1x10^6$ irradiated (15,000 rads) LLC cells, 50µg each SEG/SEI, both, or no treatment. Kaplan Meier survival curves shown. n=5.

There have been several immunotherapies recently approved that are used to augment the immune response. In fact, inflammation was critical and deemed necessary in Coley's descriptions of his treatments provoking erysipelas [63-66]. To that end, toll like receptor (TLR) agonists were included in the treatment protocol of LLC **Figure 23**. These data show a deleterious effect when used synergistically with SEG/SEI. This could simply be corrected with dosage titrations, but this researcher hypothesizes the use of TLR agonists will not favorably shape the immune environment of the LLC tumor.

Recently, several antibodies have been evaluated and approved that target inhibitory molecules, like PD-1 and PD-L1 on the T and LLC cells, respectively [150-156]. SE stimulation has been shown to increase inhibitory signals on T cells **Figure 4**. Therefore, anti-PD1 and anti-PDL1 were incorporated into the treatment protocol for LLC tumors **Figure 24.** Anti-PDL1 therapy was given 6 and 10 days post implant and conferred enhanced survival in DQ8 mice when given without SEG/SEI **Figure 24A**. Utilizing anti-PD-1 did prolong survival in DQ8 mice similar to using anti-PDL1. DQ8 mice receiving anti-PD-1 along with SEG/SEI on days 4 and 8 post implant increased survival in 10% of mice but did not protect upon rechallenge with LLC. The collection of LLC results using various immunotherapeutic tools again supports a highly inflammatory LLC tumor environment in the peritoneum of DQ8 mice. Although beneficial in B16 melanoma, the immune contexture of LLC tumors will require further investigation into unleashing T cell mediated tumor killing.

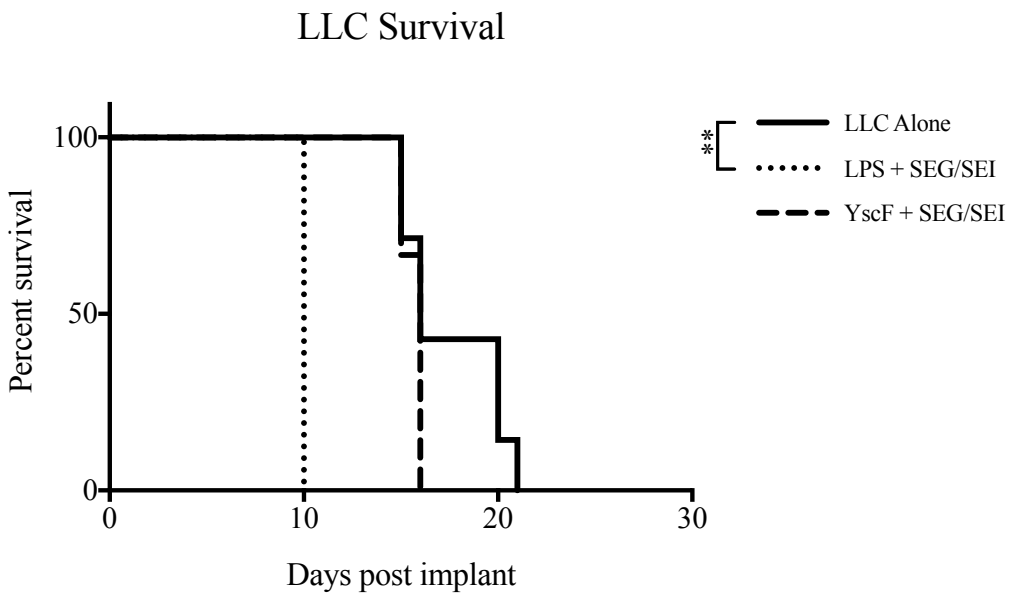
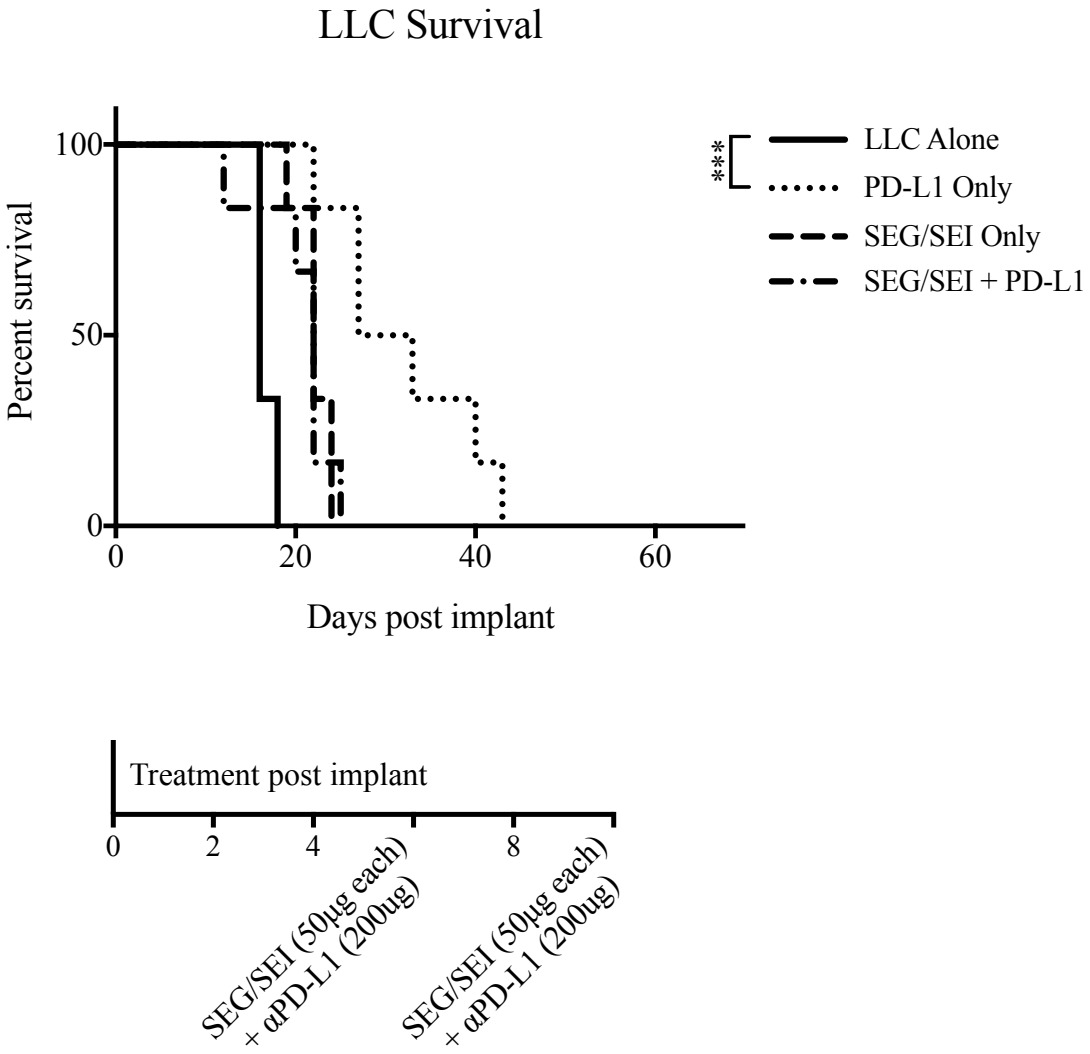


Figure 23. TLR agonists exacerbate SEG/SEI therapy.

6-8 w.o. female mice were implanted with $2.5x10^5$ live LLC cells IP. 20µg LPS (Sigma L6143) or 40µg YscF (Alvine) were combined with 50µg each SEG/SEI (France) just before injecting IP day 6. Treatment mice received 50µg each SEG/SEI day 9. . Kaplan Meier survival curves shown with Log-rank test. n=3/experimental, 6/controls. *p=0.05, **p=0.01, ***p=0.001

A


LLC Survival
Percent survival
100
50
0
0
20
40
60
Days post implant
***
LLC Alone
PD-L1 Only
SEG/SEI Only
SEG/SEI + PD-L1
Treatment post implant
0
2
4
8
SEG/SEI (50µg each) + αPD-L1 (200ug)
SEG/SEI (50µg each) + αPD-L1 (200ug)

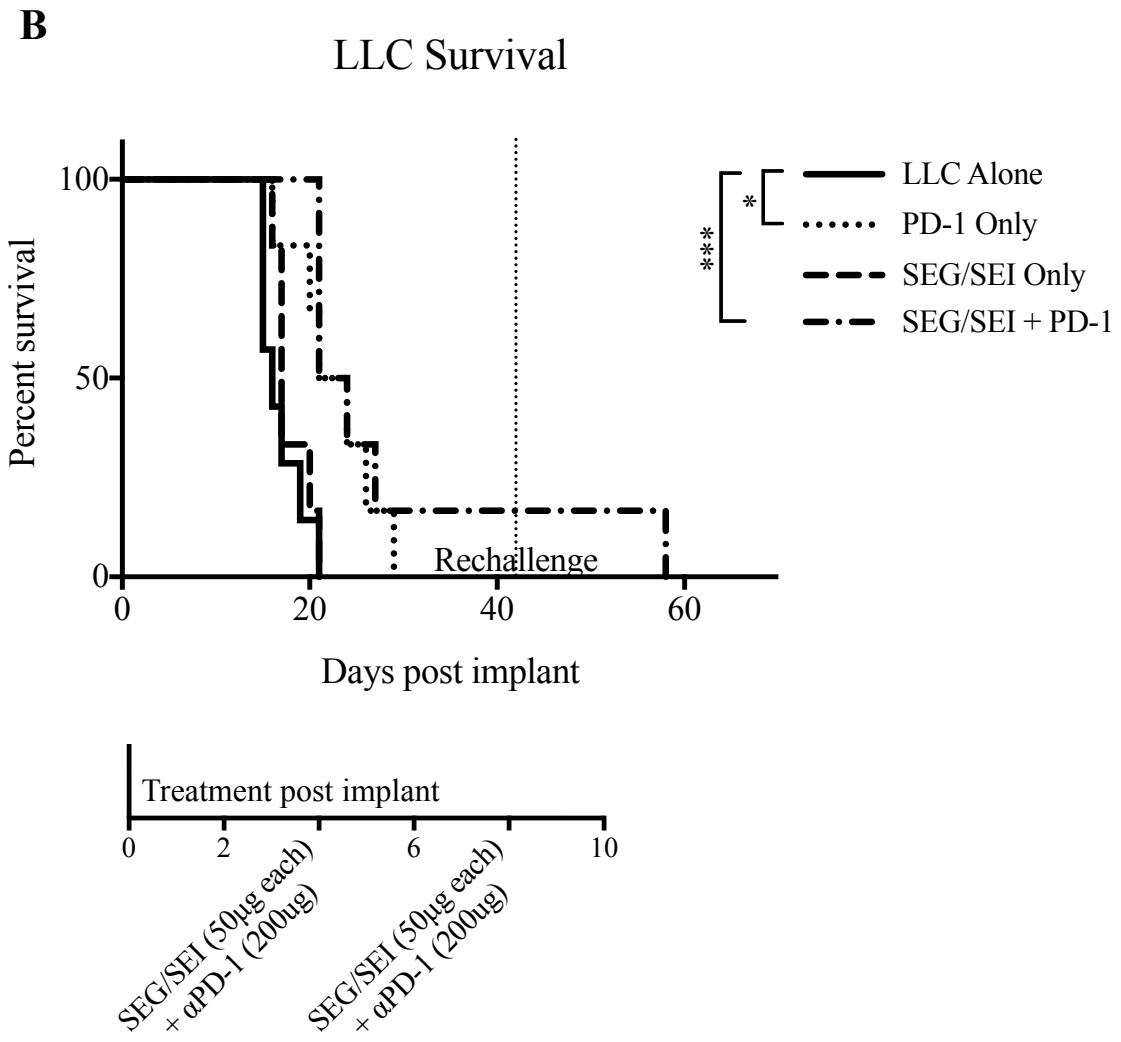


Figure 24. Including anti-PD-1 and anti-PD-L1 therapy to established LLC treatment protocol prolongs survival in DQ8 mice.

DQ8 mice implanted with $2.5x10^5$ live LLC cells received 50µg each SEG/SEI with 200µg anti-PD-L1 (A) or anti-PD-1 (B) at days indicated. Mice were rechallenged with $2.5x10^5$ live LLC cells and subsequently died with controls (data not shown). All injections done IP in <200µl total volume in 6-8 w.o. female mice. Kaplan Meier survival curves shown with Log-rank test corrected for multiple comparisons. n=6. *p=0.05, **p=0.01, ***p=0.001.

CHAPTER IV

DISCUSSION

It is necessary to take into consideration the history into current understanding of the immune system and the factors that can influence the interpretation of the data collected from studies in vitro or vivo. Mouse models have advanced to support grafting human cells creating humanized mice to study cancer. The experiments shown here use HLA transgenic mice that express a single cell surface receptor from the human immune system, DQ8. HLA transgenic mice do allow for some meaningful data into the role of receptor based specificity that can drive an autoimmune response. Autoimmune responses are characterized as an inappropriate reaction against host, self-antigen. Experiments that aim to elucidate a similar relationship in cancer have been underway for years. The search for tumor associated antigens (TAAs) peaked in the 1990s and has recently evolved into the search for neoantigens pioneered by Dr. Stephen Rosenberg [60]. Recently, another therapy using SEG/SEI could be poised to attack tumors through another cellular therapy, sickle cells. These cells have the capacity to stick in the tumor vasculature, rupture, and release cytotoxic compounds directly at the tumor site [158, 159].

Long term, progression free survival was achieved in many animals throughout this study. HLA-DQ8 mice are able to respond favorably to 50 μg doses of SEG

and SEI and these responses can be directed towards an established B16-F10 tumor and can provide memory responses in rechallenged mice. However, these tumors responses are specific. The treatment of an established LLC tumor proved difficult in all strains of mice used. Interestingly, anti-PD1 and -PD-L1 therapy did prolong survival when tested in HLA-DQ8 mice. Studies were not done in C57BL/6 mice due to the lack of SE response in B16-F10 models. Experiments using different concentrations of LLCs at implant yielded varying results and where used to compare B16-F10 implant load. LLC models could use a lower number of cells at implant as the survival of 10,000 cells vs 250,000 cells is negligible; however, 10,000 B16-F10 cells can lead to variable tumor outgrowth. SEG/SEI activated a subset of T cells and skewed the immune contexture towards T cells expressing Tbet, IFNy, perforin, and granzymes b at day 13 post treatment. Rechallenge studies >200 days post implant yielded 100% survival >70 days post rechallenge. The data presented throughout this document provide evidence to support investigation into incorporating SEG and SEI into current clinical repertoire for treating melanoma.

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