The Pig as a Large Animal Model for Studying Anti-Tumor Immune Responses

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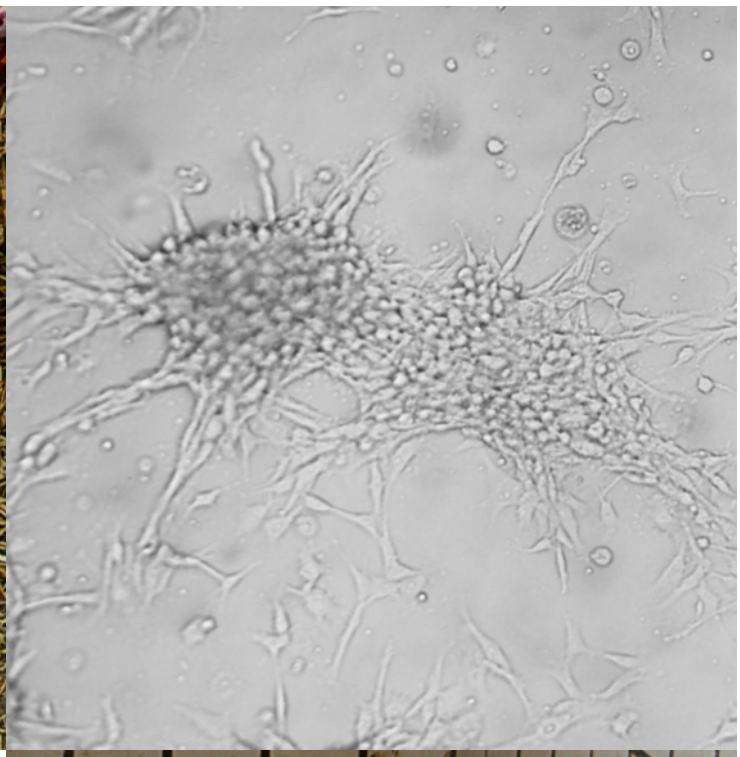

Ph.D. Thesis
Nana Haahr Overgaard
September 2017

DTU Vet
National Veterinary Institute

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Preface

This thesis is submitted to the Technical University of Denmark, National Veterinary Institute (DTU Vet), as part of the requirements to obtain the degree as doctor of philosophy (Ph.D).

The work was conducted partly at DTU Vet, Frederiksberg, Denmark in the Division of Immunology and Vaccinology and partly at the University of Illinois Urbana-Champaign, Illinois, United States in the Department of Animal Sciences. The work was conducted from October 2014 to September 2017.

In this thesis three papers are included in the result section; 1 published and 2 manuscripts in preparation. Prior to the papers themselves, a combined summary of the major findings is briefly presented. A few additional findings relevant for the interpretations are included as well.

Additionally, the thesis is comprised of an introduction, a discussion, a conclusion, and perspectives for the work. Together, these chapters introduce topics relevant for the data presented in the papers, discuss the data in relation to the literature, and describe the future directions for the work.

Acknowledgement

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I would also like to acknowledge DTU in general, the Danish Council for Independent Research, the Idella Foundation, Karolinska Institutet, The Danish Cancer Society, the Scandinavian Society for Immunology, the European Federation of Immunological Societies and the International Union of Immunological Societies Veterinary Immunology Committee for financial support. Thanks for giving me the opportunity to conduct this research and present my data at international conferences.

Lastly very special thanks to my entire family and all of my friends for unconditional love and understanding, when this work demanded all of my time. I am incredibly grateful for your never-ending encouragement and supportive natures. This would not have been possible without you, and I will be forever grateful.

All the best,

Nana Haahr Overgaard, Copenhagen, September 2017

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Summary

The immune system plays a crucial role in cancer development and progression. Cancer immunoediting encompasses three phases: elimination, equilibrium, and escape; together, describing the complex interplay between tumor and immune cells. Specifically, the immune system both protects against cancer but also generates a selective pressure, which may lead to selection of tumor cell variants with reduced immunogenicity; thereby, increasing the risk of tumor escape. Cancer immunotherapy includes treatment strategies aimed at activating anti-tumor immune responses or inhibiting suppressive and tumor-favorable immune mechanisms. One of the promising arms of cancer immunotherapy is peptide-based therapeutic vaccines; yet, no such vaccine has been approved for use in human oncology. For many years, mouse models have provided invaluable understanding of complex immunological pathways; however, the majority of preclinical results are lost in translation from mice to humans. In particular, the success rate when translating therapeutic cancer vaccines has been extremely low; thus leaving room for improvement.

The overall aim of this Ph.D. project was to investigate the potential for the pig as a large animal model for cancer immunology research and preclinical testing of cancer immunotherapies. We hypothesized that a physiologically relevant model with high degree of homology with humans can provide a crucial link between murine studies and human patients. This may increase the success rate when translating preclinical findings in the future.

As T cells are important mediators of anti-tumor immune responses, we first developed an immunization protocol allowing the induction of a cytotoxic T lymphocyte (CTL) response and evaluation of the effect of vaccine antigen dose. Göttingen minipigs received intraperitoneal (i.p.) injections with tetanus toxoid, an exogenous model antigen, formulated in CAF09 adjuvant. We demonstrate induction of a polyfunctional CTL response upon low antigen dose immunization, while a CAF09-formulated high antigen dose generates antigen-specific IgG antibodies.

Secondly, we investigated the effect of antigen dose, when immunizing Göttingen minipigs against Indoleamine 2,3-dioxygenase (IDO); an endogenous target relevant for cancer immunotherapeutic purposes. By repeated i.p. administration of CAF09-adjuvanted IDO-derived peptides, we show a vaccine-induced break in the peripheral tolerance towards IDO and the establishment of an antigen-specific cell-mediated immune (CMI) response. When comparing the different CAF09-formulated antigen doses, we demonstrate the induction of a CMI-dominant response upon exposure to a low endogenous peptide dose. In contrast, a mixed CMI and humoral immune response could be shown following repeated high peptide dose immunization. Together, our data underline the importance of correctly determining the first-in-human vaccine antigen dose, which may be more accurately predicted in a large animal like the pig.

Finally, we performed a T-cell focused immunological characterization of the novel transgenic Oncopig model. Following injection with an adenoviral vector Cre-recombinase (AdCre), these animals develop sarcomas at the injection site resulting from expression of two mutant transgenes: $KRAS^{G12D}$ and $TP53^{R167H}$. We demonstrate pronounced T-cell infiltration to the tumor site with a specific enrichment in both regulatory and cytotoxic subsets when compared to peripheral blood. Thus, Oncopig subcutaneous tumors can be classified as *hot* in accordance with the Immunoscore classification.

In an *in vitro* setup, we show immune-mediated specific lysis of autologous tumor cells, underlining the capacity of the Oncopig immune system to mount a cytotoxic anti-tumor response. Using the results from RNA-seq analysis, we propose a potential mechanism for *in vivo* inhibition of anti-tumor cytotoxicity based on elevated expression of the immunosuppressive genes *IDO1*, *CTLA4*, and *PDL1* within Oncopig leiomyosarcomas. As a high rate of spontaneous regression of subcutaneous tumors occurs over time, we speculate that the anti-tumor immune responses become dominant at the later stages post AdCre injection; eventually leading to tumor elimination. Combined, our data support that the Oncopig provides a crucial platform for studying anti-tumor immune responses in a large *in vivo* system, although the model currently only allows preclinical testing of therapeutics against the early stages of cancer.

Dansk sammendrag

Immunsystemet spiller en vigtig rolle i cancer udvikling og progression. Begrebet cancer immunoediting omfatter tre faser: eliminering, ligevægt og flugt. Tilsammen beskriver disse faser det komplekse samspil mellem immunceller og tumor: Immunsystemet kan nemlig både beskytte mod cancer, men også danne et selektivt pres, hvorved der sker en selektion af tumor varianter med reduceret immunogenicitet. Derved er risikoen for tumor flugt øget. Cancer immunterapi omfatter behandlingsformer rettet mod aktivering af anti-tumor immunresponser eller hæmning af suppressive og tumor-favorable immunmekanismer. Et af de lovende områder indenfor cancer immunterapi er peptid-baseret terapeutiske vacciner, dog er en sådan vaccine endnu ikke godkendt til behandling af patienter. I mange år har musemodeller medvirket til en uvurderlig forståelse af komplekse immun signaleringsveje, men størstedelen af de prækliniske resultater mistes i translationen fra mus til mennesker. Der er især en utrolig lav succesrate, når terapeutiske cancer vacciner oversættes til humant brug, hvilket understreger, at der er plads til forbedringer.

Det overordnede formål med dette Ph.D. projekt er at undersøge potentialet for grisen som en stor dyremodel for cancer immunologi forskning samt præklinisk testning af cancer immunterapier. Vores hypotese er, at en fysiologisk relevant dyremodel med stor homologi til mennesker kan fungere som et værdifuldt led mellem musestudier og humane patienter. Dette kan muligvis øge den fremtidige succesrate, når prækliniske resultater skal oversættes til klinikken. Siden T celler er vigtige spillere i eksekveringen af et anti-tumor immunrespons, starter vi med at etablere en immunisering protokol, der tillader induktion af et cytotoksisk T lymfocyt (CTL) respons samt undersøger effekten af vaccine antigen dosis. Göttingen minigrise modtog intraperitoneale (i.p.) injektioner med tetanus toxoid, et eksogent model antigen, formuleret i CAF09 adjuvant. Vi demonstrerer induktion af et polyfunktionelt CTL respons efter immunisering med en lav antigen dosis, hvorimod en CAF09-formuleret høj antigen dosis genererede antigen-specifikke IgG antistoffer.

Derefter undersøger vi effekten af antigen dosis, når Göttingen minigrise immuniseres mod Indoleamine 2,3-dioxygenase (IDO), et endogent protein, som er relevant for cancer immunterapeutiske formål. Efter gentagne i.p. immuniseringer med CAF09-formuleret IDO peptider påviser vi et vaccine-induceret brud i den perifere tolerance mod IDO samt demonstrerer etableringen af et antigen-specifikt cellemedieret immun (CMI) respons. Ved sammenligning af de forskellige CAF09-formulerede antigen doser kan vi vise induktion af et CMI-dominant respons ved immunisering med lav dosis endogene peptider, hvorimod et blandet CMI og humoralt immune respons kunne påvises efter gentagne immuniseringer med CAF09-formuleret høj antigen dosis. Vores data understreger vigtigheden af korrekt bestemmelse af den "først-i-menneske" vaccine antigen dosis, hvilket potentielt kan forudsiges mere præcist i en stor dyremodel som grisen. Til slut laver vi en T-celle fokuseret immunologisk karakterisering af den nye transgene Oncopig model. Efter injektion med en adenoviral vector Cre-recombinase (AdCre) danner disse grise sarkomer lokalt ved injektionsstedet som et resultat af ekspression af de to muterede transgener: $KRAS^{G12D}$ og $TP53^{R167H}$. Vi demonstrerer udtalt T celle filtration til tumoren med specifik øgning i mængden af regulatoriske og cytotoksiske populationer sammenlignet med perifert blod. Derved kan Oncopig subkutane tumorer i henhold til Immunoscore klassificeringen betegnes som *hot*. I en *in vitro* opsætning viser vi immunmedieret specifik lysis af autologe tumor celler, hvilket understreger kapaciteten af Oncopig modellens immunsystem til at generere et cytotoksisk anti-tumor respons. Ved RNA-seq analyse foreslår vi en mulig mekanisme for *in vivo* hæmning af den påviste anti-tumor cytotoksisitet baseret på øget ekspression af de immunsupprimerende gener *IDO1*, *CTLA4* samt *PDL1* i Oncopig leiomyosakomer. Grundet en høj rate af spontan regression af subkutane tumorer over tid spekulerer vi i, at anti-tumor immunresponser bliver dominante på de sene stadier efter AdCre injektion, hvilket kan resultere i eliminering af tumor. Vores data støtter, at Oncopig modellen er en værdifuld platform til undersøgelse af anti-tumor immunresponser i et stort *in vivo* system, selvom modellen på nuværende tidspunkt kun tillader præklinisk testning af terapier rettet mod de tidligere stadier af cancer.

List of Manuscripts Included

Paper I **Overgaard NH**, Frøsig TM, Jakobsen JT, Buus S, Andersen MH, Jungersen G. 2017. Low Antigen Dose Formulated in CAF09 adjuvant Favours a Cytotoxic T-cell Response Following Intraperitoneal Immunization in Göttingen Minipigs.

Vaccine 2017 Sep. doi.org/10.1016/j.vaccine.2017.08.057

Paper II **Overgaard NH**, Frøsig TM, Jakobsen JT, Buus S, Andersen MH, Jungersen G. 2017. Repeated Immunization with a CAF09-Formulated Low Peptide Dose Predominantly Induces a Cell-Mediated Immune Response Towards Indoleamine 2,3-Dioxygenase.

Manuscript in preparation

Paper III **Overgaard NH**, Principe DR, Jakobsen JT, Rund LA, Grippo PJ, Schook LB, Jungersen G. 2017. Genetically Induced Tumors Invoke a Robust Anti-Tumor Immune Response in the Oncopig Model.

Manuscript in preparation

List of Manuscripts Not Included

Overgaard NH, Frøsig TM, Welner S, Rasmussen M, Ilsøe M, Sørensen MR, Andersen MH, Buus S, Jungersen G. 2015. Establishing the pig as a large animal model for vaccine development against human cancer.

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Schachtschneider KM, Schwind RM, Newson J, Kinachtchouk N, Rizko M, Mendoza-Elias N, Grippo P, Principe D, Park A, **Overgaard NH**, Jungersen G, Garcia KD, Maker AV, Rund L, Ozer H, Gaba RC, Schook LB. 2017. The Oncopig Cancer Model: An Innovative Large Animal Translational Platform for Hematology and Solid Tumor Oncology.

Front Oncol 2017 7:190. doi 10.3389/FONC.2017.00190

Principe DR, **Overgaard NH**, Diaz AM, Torres C, McKinney R, Dawson DW, Rund LA, Grippo PJ, Schook LB. $KRAS^{G12D}$ and $TP53^{R167H}$ Cooperate to Induce Pancreatic Carcinoma in *Sus Scrofa* Pigs.

Nature Communications, in review (2017)

Ozer H, Jensen TW, Schachtschneider KM, Schwind RM, **Overgaard NH**, Darfour-Oduro KA, De AK, Rund LA, Gaba RC, Ray CE, Singh K, Schook LB. Characterization of the Porcine CD34 Gene and Development of a Monoclonal Antibody Identifying CD34p.

Submitted to Experimental Hematology

Abbreviations

1MT	1-methyl-tryptophan
AdCre	Adenoviral vector Cre-recombinase
CAF09	Cationic adjuvant formulation 09
CAR	Chimeric antigen receptor
CMI	Cell-mediated immune
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
FDA	Food and Drug Administration
GEM	Genetically engineered mouse
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IDO	Indoleamine 2,3-dioxygenase
I.p.	Intraperitoneal
IRES	Internal ribosome entry site
MDSC	Myeloid-derived suppressor cell
MeLiM	Melanoblastoma-bearing Libechov minipig
MHC	Major Histocompatibility Complex
NK cell	Natural killer cell
NKT cell	Natural killer T cell
NSCL	Non-small cell lung cancer
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PDX	Patient-derived xenograft
PFU	Plaque forming units
rAAV	Recombinant adeno-associated virus
SCID	Severe combined immunodeficiency
SLA	Swine leukocyte antigen
TAA	Tumor-associated antigen
TALEN	Transcription activator-like effector nucleases
TCR	T-cell receptor

TDO	Tryptophan-2,3-dioxygenase
TIL	Tumor infiltrating lymphocytes
TLR	Toll-like receptor
Tregs	Regulatory T cells
TT	Tetanus toxoid

CHAPTER I. Introduction

Cancer and the Immune System

Cancer Immunoediting

Cancer has recently surpassed cardiovascular disease as the leading cause of death worldwide[1]. The increasing necessity to address the unmet therapeutic needs of cancer has driven research into fields such as how the immune system influences cancer development and progression. The term immunosurveillance has traditionally been used to describe how the immune system can protect the host from tumor development[2]. However, as immunocompetent individuals still develop tumors, the hypothesis of immunosurveillance being a fully protective mechanism is challenged[3]. It has become well-recognized that the interplay between tumor cells and the immune system is extremely complex, and the ability of tumor cells to avoid immune destruction has been included as an official hallmark of cancer[4]. Cancer immunoediting describes the complex interplay, in which the immune system not only protects against cancer but also induces tumor-sculpting mechanisms leading to reduced immunogenicity of tumor cell variants[5,6]. The concept of cancer immunoediting is composed of three phases, namely *elimination*, *equilibrium* and *escape*[7,8] (Figure 1). The kinetics, by which each of the three cancer immunoediting steps occurs, is speculated to differ between tumors; with aggressive tumors accelerating faster through these phases[8,9].

The *elimination* phase encompasses the original concept of immunosurveillance, where the innate and the adaptive immune system collaborate to destroy the developing tumor[6,10] (Figure 1A). Although more work is needed to fully elucidate the mechanisms behind this anti-tumor immunity, it is known to be partly mediated by release of cytotoxic granules from $CD8^+$ T cells and Natural Killer (NK) cells, in addition to cytokine release from $CD4^+$ T cells and Natural Killer T (NKT) cells[11] (Figure 1A).

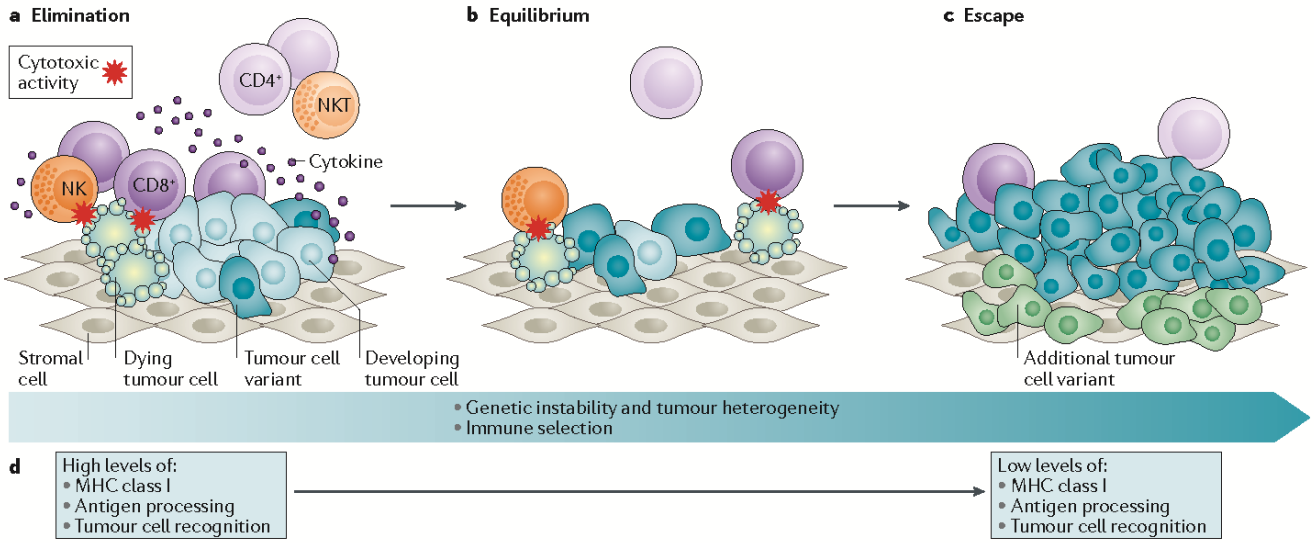

Figure 1. Cancer immunoediting: from immunosurveillance to tumor escape. (A) In the elimination phase, the immune system is in control and provides anti-tumor activity by direct delivery of cytotoxic granules from $CD8^+$ T cells and NK cells. Moreover, cytokines are released from $CD4^+$ T cells and NKT T cells. (B) During the equilibrium phase, tumor cell variants with reduced immunogenicity expand, while the immune system continues to attack and destroy other tumor cells. (C) The tumor variant with reduced immunogenicity continues to expand and gives rise to additional variants as well. At this stage, the immune system is no longer capable of recognizing the tumor cells; thus, resulting in tumor escape. (D) Several changes occur during the process of cancer immunoediting. Towards the escape phase, the expression of MHC class I molecules on the surface of tumor cells is reduced. Also, the processing of antigen might be defect and the tumor cell recognition will be reduced. Figure from[11]. Abbreviations: NK cell, natural killer cell; NKT cell, natural killer T cell; MHC, Major Histocompatibility Complex.

A more detailed mechanism behind the *elimination* phase has been proposed by Dunn et al (2002)[6]. In brief, the tumor becomes invasive when reaching a size which requires a distinct blood supply; controlled in part by the production of angiogenic proteins. Such invasive growth results in small disruptions in the adjacent tissue; thereby, inducing inflammation, which leads to intratumoral infiltration of innate immune cells like dendritic cells (DCs), NK cells, NKT cells, γδ T cells, and macrophages. Upon recognition of tumor cells, these innate immune subsets produce IFN-γ which can induce tumor cell death by anti-proliferative and apoptotic mechanisms. Moreover, these innate immune cells produce chemokines with the capacity to limit blood vessel formation. Tumor cell debris can then be taken up by DCs, which migrate to the draining lymph node and induce tumor-specific $CD4^+$ T helper cells and

tumor-specific $CD8^+$ T cells. Finally, these activated T cells home to the tumor, where the $CD8^+$ T cells in particular mediate anti-tumor activities[6]. If the immune system succeeds in completing this phase, the host is cleared of cancer with no clinical symptoms or progression to the additional editing stages[6,10].

However as well as protecting the host, anti-tumor immunity can also induce tumor-sculpting mechanisms resulting in tumor editing under Darwinian selective pressure[5,8,12,13]. Consequently, tumor cell variants with increased capacity to avoid immune recognition can develop; thereby, entering the *equilibrium* phase (Figure 1B). This is a dynamic equilibrium which might last for several years and is believed to be the longest of the three phases[6,8,14]. Several underlying molecular mechanisms which may contribute to reduced immunogenicity of cancer cells during the equilibrium phase have been suggested both at the genetic and the epigenetic level. In particular, increased genetic instability, reduced Major Histocompatibility Complex (MHC) class I expression, and defective antigen processing have been implicated in reducing tumor immunogenicity and facilitating tumor escape[8,10,15–22] (Figure 1D). Enhanced secretion of immunosuppressive cytokines by tumor cells, increased induction of regulatory T cells (Tregs), and tumor insensitivity towards IFN-γ have also been reported as important factors[23–26].

After a prolonged sub-optimal immune response, selected tumor cell variants with reduced immunogenicity can become insensitive to immune recognition; consequently, resulting in uncontrolled tumor growth. This is referred to as the *escape* phase[6–8,27] (Figure 1C). The tumor is now capable of growing in a fully immunocompetent environment, although the degree of immune cell infiltration still affects the patient's prognosis[28–30]. Specifically, the density, location, and the functional orientation of these intratumoral immune cells are crucial measurements in predicting prognosis and response to therapy[31–34]. Together, these factors are referred to as the immune contexture and form the basis of the Immunoscore; a novel approach for staging cancer patients[30,33]. Using this strategy, human tumors are classified as *hot* or *cold* depending on the degree and nature of intratumoral immune cell infiltrates[35,36]. Currently, the Immunoscore functions as a prognostic tool for colorectal cancer patients only; however, the broader applicability for this approach still remains to be

validated in many cancer types[36]. In general, more work is still needed to fully understand the complex interplay between cancer and the immune system.

T Cells in Cancer

T cells are key players in mediating anti-cancer immunity[37–39]. However, T cells are clonally selected to prevent autoimmunity by deletion of self-specific T cells; a process referred to as central tolerance[40,41]. Thus, a major challenge with establishing an anti-cancer immune response is the endogenous nature of the antigens, and the induction of an anti-tumor T-cell response is fully dependent on the T-cell repertoire remaining after the induction of the central tolerance[42].

The T-cell receptor (TCR) is essential for T-cell recognition of antigens, including tumor antigens. The TCR is a multi-subunit complex consisting of co-receptors (CD4, CD8, or both) in addition to the αß chains or the less conventional γδ chains[43,44]. Upon ligation of the TCR, signaling events are mediated through another important component of the TCR, namely the CD3 molecule[45]. $CD4^+$ T cells become activated by interaction with *exogenously*-derived peptides presented in the context of the MHC class II molecule expressed on antigen presenting cells[46]. The MHC class II molecule has an open-ended peptide binding groove, which allows binding of long peptides usually 12-25 amino acid residues or even whole proteins[47–49]. In contrast, both ends of the MHC class I binding groove are closed; thus, allowing only short peptides of approximately 8-12 amino acid residues to be presented[50–52]. The MHC class I molecule is expressed by all nucleated cells and presents *endogenously*-derived peptides to $CD8^+$ T cells[53–55]. Importantly, the mechanism referred to as cross-presentation allows certain DC subsets to present *exogenously*-derived peptides in complex with MHC class I[56,57]; thereby, enabling the induction of a cytotoxic T lymphocyte (CTL) response towards antigens not expressed by DCs, such as those on tumors.

In humans, T-cell reactivity towards a tumor-associated antigen (TAA) was first demonstrated towards the protein encoded by the melanoma antigen-encoding gene[58]. This

underlines that tumor cells can indeed be targets of CTL immunity. Despite several cancers displaying an enrichment of both $CD4^+$ and $CD8^+$ tumor infiltrating lymphocytes (TILs), very little is currently known about why only certain tumors become heavily infiltrated[9]. Amongst other factors, chromosomal instability, mutational load, TIL proliferation, and attraction of T cells to the tumor site itself are thought to influence the degree of intratumoral T cells[59–61]. An abundant T-cell infiltrate is associated with increased survival in melanoma patients[62], and the presence of $CD3^+$ TILs, $CD8^+$ TILs as well as a high CD8/FoxP3 T-cell ratio appear to have a positive impact on patient survival in several cancer types[63–65]. Notably, these TILs need to be proliferating in order to correlate with good prognosis[66]. Thus, the presence of TILs alone is not sufficient to provide anti-tumor immunity, as for instance $CD8^+$ TILs have been shown to express surface markers associated with T-cell exhaustion[67–69]. This indicates that the T cells within the tumor might not necessarily be functionally active.

In addition, the memory stage of the $CD8^+$ TILs is also important. Central memory $CD8^+$ T cells are reported to be superior in providing anti-tumor immunity when compared to $CD8^+$ T cells displaying an effector memory phenotype[70]. Moreover, the actual location of the T cells within the tumor, as suggested by the Immunoscore, is also an important prognostic factor. This is clearly shown in colorectal cancer patients, where the presence of $CD8^+$ T cells within the tumor nest correlates with better survival when compared to patients displaying $CD8^+$ T-cell infiltration to the stroma or the invasive margin of the tumor[71]. Although prognostic correlates for $CD4^+$ T cells are less clear, a high representation of Tregs as determined by CD4, CD25, and FoxP3 expression, has been shown to correlate with poor prognosis and response to therapy[72,73]. When compared to $CD8^+$ T cells, the $CD4^+$ T-cell compartment appears to be more plastic and play dual roles; directly shown by the ability of $CD4^+$ T cells to shift between pro-tumorigenic and anti-tumorigenic stages[74,75]. Although $CD8^+$ T cells are usually referred to as anti-tumorigenic, suppressive $CD8^+$ T cells can be readily detected in tumors[76,77]. This underlies the complex nature and plasticity of the T-cell pool in general.

Therapeutic Cancer Vaccines

Treatment strategies involving the induction of anti-cancer immune responses or inhibition of suppressive immune mechanisms are referred to as cancer immunotherapy. In 1992, bolus injection with interleukin 2 was approved by the U.S. Food and Drug Administration (FDA) as the first cancer immunotherapy for use in human oncology[78]. In 2013, cancer immunotherapy was awarded breakthrough of the year[79], and the field has received extensive attention ever since.

One arm of cancer immunotherapy is therapeutic vaccines. Especially based on results in murine models showing a crucial therapeutic role for cytotoxic $CD8^+$ T cells in cancer, the majority of the therapeutic vaccines are aimed at activating this immune cell population[80]. To date, the prostate cancer vaccine Provenge® (Sipuleucel-T)[81] is the only therapeutic cancer vaccine approved for human use. Therapeutic cancer vaccines encompassing selected peptides, often $CD8^+$ T-cell epitopes, have intriguing potential[82]. Many clinical trials involving peptide-based therapeutic vaccines have been performed[83], but none has currently been approved by the U.S. FDA or the European Medicines Agency[84–86]. Table 1 outlines some of the main advantages and disadvantages of using peptide-based therapeutic vaccines.

Advantages	**Disadvantages**
Readily synthesized, cost-effective	MHC class I restriction
Off-the-shelf reagent	Short peptides do not need processing; risk of tolerance induction
Stable under many storage conditions	Peptidases can rapidly degrade the peptides
Safe, very low toxicity	Peptides with low binding affinity to MHC might be poorly immunogenic
Effectively induce T-cell responses	Low magnitude of the immune response
Enable direct monitoring of the induced response	Risk of induced immune response being transient
Defined epitopes, reduced risk of autoimmunity	
Repeated boosting injections feasible	

Table 1. Advantages and disadvantages of peptide-based therapeutic vaccines. Table modified from[38].

The first benefit of peptide-based vaccines compared to many cancer treatments is that they do not rely on blood or biopsy sampling prior to treatment[87]. This is in contrast to labor-intensive therapies such as Provenge®, which involves leukapheresis of peripheral blood and subsequent intravenous re-infusion of *ex vivo* generated DCs[81]. Peptide-based therapeutic vaccines are cost-effective and easy to produce, as the peptides simply need to be synthesized and formulated in an adjuvant system. Moreover, peptides are fairly stable under many storage conditions. This, in addition to the before-mentioned advantages, makes several rounds of injection feasible (Table 1). Although targeting an endogenous protein poses the risk of autoimmune development[88,89], peptide-based therapeutic vaccines have generally shown low or no toxicity in human patients[90,91]. Therefore, the approach is acknowledged as relatively safe; in particular in situations where defined TAA-derived epitopes are used as targets (Table 1). Therapeutic cancer vaccines have efficiently generated antigen-specific T-cell responses towards TAAs[92–96], and due to the development of several MHC-based technologies detecting antigen-specific T cells[97], the vaccine-induced immune response of the patient can be monitored over time.

A crucial limitation to broadly distributing the use of peptide-based therapeutic vaccines is the MHC class I restriction[80,82] (Table 1). As the peptides are designed to specifically bind to certain MHC class I alleles, the group of patients eligible for receiving a given vaccine is fully dependent on their MHC class I profile. Moreover, endogenous peptides, in particular those with low binding affinity towards the MHC class I molecule, might be poorly immunogenic (Table 1). Consequently, the endogenously-derived TAA peptides need to be presented to the immune system under immunogenic rather than tolerogenic conditions[98]. To facilitate such immune activation, vaccines often consists of an adjuvant with different kinds of immune modulators in addition to their antigenic target[99,100]. Both short peptides, solely comprising one or several minimal epitopes[93,101–103], and long synthetic peptides, comprising a number of epitopes and potentially also some MHC class II-binding peptides[104–106], have been used in therapeutic vaccines. However, as short peptides do not need antigen processing prior to binding to MHC class I molecules; they might be presented by non-professional antigen presenting cells and trigger tolerance or T-cell anergy[38]. As a result, immunization with short

peptides might not result in immune activation[107,108] (Table 1). Another challenge for peptide-based therapeutic vaccines is the risk of the peptides being rapidly degraded by peptidases upon injection (Table 1). This further underlines the importance of both the peptide formulation and the vaccine delivery itself. Lastly, the magnitude of the immune response generated upon administration of peptide-based therapeutic vaccines is often fairly low, transient, and might not result in clinical benefit for the patient[38] (Table 1). Although more work is needed, the ability of therapeutic vaccines to induce anti-tumor immune responses underlines their potential as a future treatment strategy.

Indoleamine 2,3-dioxygenase as a Vaccine Target

A promising target within cancer immunotherapy is the intracellular enzyme Indoleamine 2,3-dioxygenase (IDO)[109]. In addition to the classical IDO1 enzyme, IDO2 has been discovered. This enzyme shares the critical catalytic residues and a 43% sequence similarity with IDO1[110–112]. As IDO2 is much less studied[113], The protein IDO1 will from this point onwards simply be referred to as IDO. Overall, the function of IDO is to induce tolerance and regulate immune responses. Specifically, IDO catalyzes the first and rate-limiting step in the breakdown of the essential amino acid tryptophan[114–116] (Figure 2).

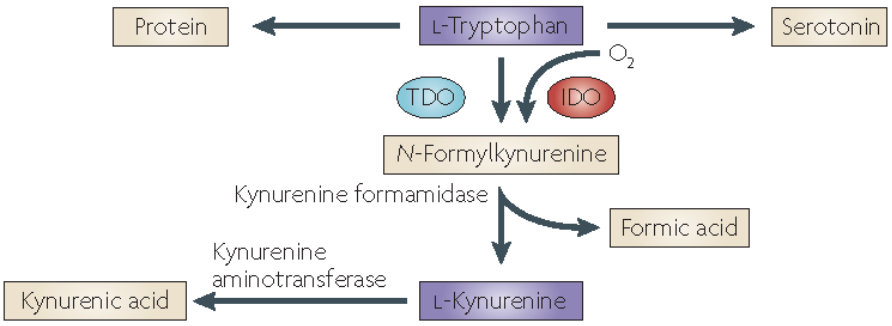

Figure 2. IDO catalyzes the conversion of tryptophan to kynurenine. The intracellular enzyme IDO catalyzes the breakdown of the amino acid tryptophan to kynurenine and other metabolites; thereby, depleting the level of tryptophan available in the tumor microenvironment. Figure modified from[114]. Abbreviations: IDO, Indoleamine 2,3-dioxygenase; TDO, Tryptophan-2,3-dioxygenase.

In several human cancers, an overexpression of *IDO1* or an accumulation of IDO$^+$ cells have been reported, which is usually associated with a worse prognostic outcome[117–120]. For instance, an increased level of IDO in colorectal cancer patients has been shown to correlate with liver metastasis and reduced intratumoral T-cell infiltration[118]. IDO can be produced by the tumor cells themselves[121] as well as innate cells like tumor-associated macrophages and myeloid-derived suppressor cells (MDSCs)[122,123]. It has recently been suggested that local IDO production in the tumor microenvironment contributes to recruitment of MDSCs and enhances their suppressor function[113]. Also, DCs can be induced to express IDO upon exposure to IFN-γ[124–126]. Moreover, CD4$^+$ T cells can trigger IDO activity in DCs by ligation of the CD80/CD86 molecules[127]. In the tumor microenvironment, IDO plays an immunosuppressive role and contributes to tumor escape by affecting T-cell function and survival[128–131]. In particular, IDO reduces CD8$^+$ effector T cell-mediated cytotoxicity[132,133]. The first proposed mechanism for this relies on effector T cells being very sensitive to tryptophan starvation. Therefore, the IDO-mediated intratumoral depletion of tryptophan results in inhibition of T-cell proliferation, induction of cell cycle arrest, and increased T-cell susceptibility to the apoptotic pathway[125,134–136]. The other proposed mechanism, by which IDO can suppress T-cell function and proliferation, is by an accumulation of toxic tryptophan-derived catabolites[137,138]. Further, IDO-producing DCs have been shown to induce conversion of CD4$^+$ T cells to Tregs rather than to the inflammatory Th17 cells[139–141]. In addition, IDO can affect NK cells by inducing downregulation of their activating receptors, which makes them more prone to apoptosis[114].

In terms of IDO as a target for immunotherapeutic purposes, several clinical trials have analyzed different IDO-inhibiting compounds[142]. The tryptophan analogue 1-methyl-tryptophan (1MT), which inhibits the enzymatic activity of IDO, has been heavily studied in mouse models[114]. Administration of 1MT has shown to potentiate the effect of chemotherapy; subsequently resulting in regression of established tumors in mouse models[128,143]. When it comes to T-cell reactivity, IDO-derived peptides have been demonstrated as epitopes for both CD4$^+$ and CD8$^+$ T cells[144–147]. Despite this, only four registered clinical trials involve a peptide-based therapeutic vaccine targeting IDO (Table 2).

Cancer	Status	Phase	Adjuvant	Combination	Trial ID
Metastatic melanoma	Not yet recruiting	I / II	Montanide ISA-51	Nivolumab, PD-L1 peptide	NCT03047928
MM	Terminated	II	Montanide ISA-51	GM-CSF, Temozolomide Imiquimod	NCT01543464
NSCL	Completed	I	Montanide ISA-51	Imiquimod	NCT01219348
MM with metastasis	Completed	I	Montanide ISA-51	Ipilimumab	NCT02077114

Table 2. Overview of clinical trials testing an IDO-targeting peptide-based therapeutic vaccine. Data obtained from[142]. Combination indicates administration of other treatments in combination with the vaccine. Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; MM, malignant melanoma; NSCL, non-small cell lung cancer; PD-L1, programmed death-ligand 1; TLR, toll-like receptor.

The first of these trials listed, NCT03047928, is a phase I/II trial yet to recruit patients. This trial involves a combination therapy with administration of Nivolumab, a monoclonal antibody against the programmed cell death protein 1 (PD-1), and a vaccine consisting of one long programmed death-ligand 1 (PD-L1)-derived peptide and one long IDO-derived peptide; formulated together in the Montanide ISA-51 adjuvant.

NCT01543464 is a phase II trial, which has been terminated due to diminished recruitment. However, the planned setup was a vaccine consisting of a short IDO-derived peptide together with a survivin-derived peptide formulated in Montanide ISA-51 and administered together with granulocyte-macrophage colony-stimulating factor (GM-CSF), the toll-like receptor (TLR)-7 agonist Imiquimod, and the chemotherapy drug Temozolomide.

The NCT01219348 phase I trial has been successfully completed. Here, non-small cell-lung cancer patients have been treated with a short IDO-derived peptide formulated in Montanide ISA-51 and delivered together with Imiquimod. The treatment has been demonstrated to be well-tolerated with low toxicity and successfully induced antigen-specific $CD8^+$ T-cell responses[148].

Lastly, NCT02077114 is a phase I trial, where malignant melanoma patients with metastatic lesions have been treated with a long IDO-derived peptide formulated in Montanide ISA-51 and administrated together with Ipilimumab, a monoclonal antibody against the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)[149]. Again, the IDO-derived peptide vaccine has shown minimal toxicity. No clinical benefit has been observed upon combination therapy with Ipilimumab, although IDO-reactive T cells have been induced following treatment[149].

Together, these trials support that IDO-specific T cells can be activated upon peptide-based therapeutic immunization; however, the clinical benefit to the patients generally remain limited. These studies show there is potential, although more research is needed. One of the important things to consider is the choice of animal model for preclinical testing.

Mouse Models of Cancer Immunology

For many years, mice have been the most commonly used animal model for immunological research and have provided understanding of complex immunological pathways[150–153]. This in part owes to mice displaying reduced genetic variation, short generation intervals, easy maintenance, and the large number of commercially available reagents[150,154]. In cancer immunology, the most widely used mouse models involve inoculation of histocompatible tumor cell lines into recipient mice; often of C57B/6 or BALB/c background[152,155,156]. These syngeneic tumor models offer several advantages including reproducible tumor growth and simplicity in measuring tumor development over time, especially if the tumor cells are inoculated subcutaneously[151,152,157]. However, the tumor cell lines tend to grow aggressively post injection, which causes studies to be terminated within relatively short time due to ethical considerations. Furthermore, the tumor cell lines differ in their intrinsic immunogenicity; therefore, the resulting tumor microenvironment often does not represent what is seen in human patients[158,159].

Syngeneic mouse models are immunocompetent, albeit they do not offer the opportunity for testing human targets. For this reason, syngeneic models are increasingly replaced by genetically engineered mouse (GEM) models, human xenograft, and patient-derived xenograft (PDX) models[157]. An almost unlimited number of GEM models exist, but the general idea for cancer research purposes is to delete, mutate, or overexpress genes known to be crucial for cellular transformation and malignancy[160]. The GEM models are very useful for studying the effect of a certain mutation and how it affects tumor progression in an immunocompetent host[160–163]. Despite this, GEM models often still fail in mimicking the complexity of human tumors[160].

Another alternative are xenograft models which involve the transplantation of human cancer cell lines, or patient-derived tumor cells in the case of PDX models, into immunodeficient mice[164–166]. Although these models offer a promising system for evaluating human personalized anti-cancer therapies, they are fairly expensive, labor-intensive, and time-consuming[167,168]. Also, the arising tumor is not exposed to any immune-mediated pressure due to the lack of an endogenous immune system. To try and accommodate the limitations in using an immunodeficient host, humanized mice have been developed. These mice are either genetically engineered to carry human genes[162] or were developed by engraftment of human immune cells into an immunodeficient host[169–172]. As humanized mice are often on the *Il2rg*$^{-/-}$ background, they lack both lymph nodes and Peyer's patches[173–175]. Furthermore, humanized mice are challenged in their capacity to restore MHC class I and II-selecting elements, which are crucial for shaping the T-cell repertoire[176]. It is becoming increasingly recognized that mice often poorly mimic human diseases, including cancer[177,178]. Table 3 outlines some of the limitations in using mouse models for cancer research.

Difference between mice and humans	Limitation to cancer research
Body size, life-span, and number of cell divisions	Humans are approximately 3,000 bigger, live 30-50 times longer, and human cells undergo ~10^5 more cell divisions
Tissue architecture	Surgical procedural training in mice is not possible
Basal metabolic rate	The murine basal metabolic rate is about seven times higher. Altered levels of by-products like endogenous oxidants and mutagens arise, which might affect cancer susceptibility
Risk of spontaneous cancer development	Murine cells have increased genetic instability and a lower threshold for development of genetic and epigenetic changes.
Telomerase expression	Human somatic cells suppress telomerase expression, which is then reactivated during cancer development

Table 3. Limitations to the use of mouse models for human cancer research. Some of the important differences between mice and humans are outlined together with the limitation associated with this. References[154,179–186].

It is well-recognized that animal models need to be fully immunocompetent in order to properly mimic human immune responses[157,187]. Despite some mouse models being immunocompetent, they often still display a very narrow MHC class I representation due to inbreeding. Consequently, this might result in unrepresentative results when compared to outbred animals and humans[150]. This in addition to the limitations outlined in table 3 have driven the field of cancer immunology towards alternative models. Our expertise lies within the field of porcine models; however, alternative large animal models will also be introduced in the next paragraphs.

Large Animal Models of Cancer Immunology

Canine Models

As cancer in dogs occurs spontaneously and displays similar characteristics to human disease, canine models are becoming more widely used in preclinical cancer research[188–190]. In reflection of this, the National Cancer Institute has recently launched a 'Comparative Oncology Program' designing, sponsoring, and executing trials in dogs in order to test novels anti-cancer drugs prior to human clinical trials[191]. There are several advantages unique to the canine models. Since dogs are companion animals, they often live together with humans; therefore, they are exposed to some of the same environmental risk factors and might to a certain extent have a diet similar to humans[192,193]. As with humans, a correlation between spontaneous tumor incidence and age is found in dogs[194]. From an evolutionally point of view, dogs are more closely related to humans than are mice[195,196]. The high degree of homology in the human and canine genome makes analysis of DNA damage as well as epigenetic changes during tumor development and progression possible in outbred dogs[195,197,198].

The canine immune system shows a close homology to the human counterpart[199–201]. Since canine tumors in dogs arise in an immunocompetent host, canine models enable the design of experiments which elucidate the complex interplay between cancer cells and the immune system. Using human antibodies towards T-cell markers it is now possible to distinguish canine activated T cells and central memory T cells by flow cytometry[201]; thus, providing an important tool for vaccine research purposes. Despite being limited in scope to date, some studies have evaluated tumor immune cell infiltrates in canine cancer models. Flow cytometric analysis has shown the presence of both $CD4^+$ and $CD8^+$ TILs within canine mammary tumors[202]. Another study using dogs with metastatic lesions showed an increased CD4/CD8 T-cell ratio, which also correlated with decreased survival rate[202]. In studies of canine B cell lymphoma, a worse prognosis was found in dogs with increased representation of tumor-associated macrophages, MDSCs, and Tregs[203–205], and CTL-mediated killing of autologous lymphoma cells has been demonstrated *in vitro*[204].

For immunotherapy purposes, canine tumor models offer a very powerful research tool. As monoclonal antibodies blocking CTLA-4, PD-1, and PD-L1 have shown impressive results in the clinic, it is desirable to have a preclinical animal model expressing these molecules. CTLA-4, PD-1, and PD-L1 expression have all been shown in canine tumors[206,207]. In fact, the PD-1/PD-L1 pathway in dogs is associated with T-cell exhaustion, as often reported for humans[207]. Due to limitations in commercially available canine reagents, detailed studies with checkpoint inhibitors in dogs are yet to be performed[194]. Although further investigation is needed, chimeric antigen receptor (CAR) T cells have shown promising results in dogs as a proof-of-concept[208,209]. Therefore, dogs might in the future serve as an important model in elucidating the adverse events often observed upon CAR T-cell therapy[210].

In terms of cancer vaccine trials in dogs, whole tumor cell lysate vaccines have been tested either as combination therapy or stand-alone treatment[211–213]. In 2007, a xenogeneic DNA vaccine (Oncept®) targeting the human tyrosinase protein was the first therapeutic vaccine to be approved for treatment of canine oral melanoma[214,215]. In addition, canine vaccine trials targeting the telomerase reverse transcriptase, heat-shock proteins, and the human vascular endothelial growth factor protein have been performed[196,214,216]. Notably, these trials all share the aim of treating cancer in dogs rather than using the canine tumor models as a link between rodent studies and human clinical trials. However, a DC-based vaccine in combination with IFN-γ administration has been demonstrated to improve the clinical outcome in tumor-bearing dogs; thereby, supporting the use of canine models for preclinical testing of human anti-cancer therapies[217].

Despite the many benefits of canine cancer models, their use for therapeutic cancer vaccine development has a number of important drawbacks. The low number of known canine tumor antigens[216], the increasing ethical regulation of experiments on companion animals[193], and the limited number of commercially available reagents undeniably make canine translational research more difficult[194]. Although dogs are more outbred than mice, modern dog breeds are the results of line inbreeding; thus, questioning whether canine models can properly mimic human heterogeneity[154]. Therefore, while canine models provide some important advantages over murine models, there is still a need for alternative large animal cancer models

Non-Human Primate Models

Amongst all animals, non-human primates are the ones most closely mirroring human genetic composition, immune system, and physiology[218–221]. Hence, these animals offer a unique opportunity to study complex immune mechanisms and enhance the knowledge of several human diseases. In particular, non-human primates have been invaluable as models for understanding infectious diseases like acquired immune deficiency syndrome, malaria, and hepatitis C infection[219,222,223]. This especially owes to the fact that only closely related species share similar pathogen susceptibilities[221]. However, while humans and non-human primates share many immunological similarities, crucial differences do exist between the two species[224]. Humans express six MHC class I genes, whereas up to 22 active MHC class I genes have been shown in rhesus macaques[225]; thus, challenging the relevance for testing T cell-based assays in non-human primates.

Regarding cancer, only one study has reported the development of a non-human primate model for the design of a cancer vaccine; against the virus causing Kaposi sarcomas in humans[226]. In general, the number of studies using non-human primates as a tumor model is very limited and includes mainly a few case studies[154]. One of the reasons for the dearth of non-human primate cancer models is that the incidence of tumor susceptibility between humans and non-human primates has been demonstrated to be rather different[218]. While the exact rate of spontaneous cancer in wild non-human primate populations remains unknown, experimental models display a very low cancer incidence; thus, questioning their relevance as a translational tumor model for human cancer research[194,227].

It can be speculated that differences in cancer incidence might be caused by the different exposure to environmental risk factors, variations in life-span, and of course genetic differences existing between humans and non-human primates[218]. However, a detailed analysis of genes involved in human cancer showed that the same genes are not only present, but also highly conserved in chimpanzees[218]; thus, suggesting that similar mechanisms of oncogenesis exist in the two species. On the other hand, differences in epigenetic profiles, for instance DNA methylation, patterns are reported for humans and non-human primates[228].

Additional limitations to non-human primate cancer models exist, including high cost, housing challenges, ethical regulation, breeding difficulties, as well as a limited number of commercially available reagents[194]. These provide significant challenges to the broader use of non-human primates as a model in cancer immunology research.

Porcine Models

Pigs are valuable models for studying immune responses towards infections[229–231]. Moreover, porcine models are becoming increasingly used for human biomedical research and as unique research tools for surgical procedural training[232–234]. The advancement in using porcine models is due to the high degree of homology in anatomy, physiology, size, cell biology, key metabolizing enzymes, genetics, and epigenetics between pigs and humans[235–245]. In addition, the life-span of the pig also offers an opportunity to monitor and characterize disease development and progression over a human-relevant amount of time[154,237,246]. Importantly for cancer research, porcine somatic cells, as with human cells, suppress telomerase activity in most tissues, which is then reactivated during tumorigenesis[186,247]. Although mice are closer to humans phylogenetically, pigs and humans share a higher similarity in protein structure[248]. A detailed comparison of immune related genes across several species revealed that pigs are more closely related to humans at the immunome level than are mice[229]. In addition, the number of species-unique immune related genes is considerably lower in pigs than in mice[229].

Overall, the porcine immune system comprises the same immune cell populations as demonstrated in humans[231,249]. However, some important differences do exist between the porcine and the human immune system. Porcine peripheral blood comprises a large number of γδ T cells; sometimes representing up to 50% of the total blood lymphocyte population in young animals[250]. In contrast, the representation of γδ T cells in human peripheral blood sampled across the world is less than 10%[251]. Although the functional properties of γδ T cells are not fully understood, it is suggested that these cells display both cytolytic activity and

capacity to perform antigen presentation[252]. In addition, the porcine T-cell pool comprises a large proportion of $CD4^+$ T cells co-expressing the CD8α homodimer in peripheral tissues[253,254]. In pigs, these $CD4^+CD8\alpha^+$ T cells are defined as an activated/memory $CD4^+$ T-cell population recognizing antigens in the context of MHC class II[252,255]. As this $CD4^+$ T-cell population expresses the $CD8\alpha^+$ homodimer, expression of the CD8ß molecule is commonly used to define porcine CTLs[249,252]. The porcine Treg population expresses markers similar to the human population; namely CD4, CD25, and FoxP3[252,256]. Although there is a high degree of homology and conserved structural motifs between humans and pigs, recent findings indicate that some inflammasome-related pathways do differ between the two species upon infection[248].

Although pigs have provided valuable findings in infectious diseases, porcine models have had limited use thus far in experimental oncology. The two most common cancer types found in pigs are lymphosarcoma and melanoma[257]. Porcine skin is very similar to human skin both in terms of morphology and functional characteristics[258]; thus, providing a unique model for studying skin cancers like melanoma. For many years, the Sinclair minipig and the melanoblastoma-bearing Libechov minipig (MeLiM) model have been the two most commonly used porcine spontaneous melanoma models, although the underlying genetic changes resulting in the melanoma development are not well-understood[257,259]. Despite this, a study in the MeLiM model has contributed to a better understanding of melanoma progression and identification of a potential marker of malignancy in human melanoma[260]. In recent years, porcine severe combined immunodeficiency (SCID) models have also been developed[261–266]. As in the rodent equivalents, porcine SCID animals lack T and B cells; hence allowing them to be used for xenotransplantation studies including engraftment of human tumor and immune cells.

To expand the use of pigs in experimental oncology, several genetically modified porcine models for human cancer have now been developed (Table 4). By overexpressing the human *GLI2* gene, it was possible to develop a model with basal cell carcinoma-like lesions[267]. In

addition, colorectal cancer[268,269] and breast cancer[270,271] models were developed; although these animals either lacked *in vivo* tumor development or showed issues with lethality (Table 4). Modification of either the tumor suppressor gene *TP53* or the oncogene *KRAS* has enabled the development of porcine models giving rise to various cancer types (Table 4). Mutational silencing of the *TP53* tumor suppressive pathway is observed in approximately 33% of human cancers[272]. Such mutations in the *TP53* gene are often associated with increased cell proliferation, survival, invasiveness, as well as metastasis[273]. The porcine models express the $TP53^{R167H}$ dominant negative mutation, which is equivalent to the frequently observed $TP53^{R175H}$ mutation in humans[272,274]. Upon expression of $TP53^{R167H}$, the pigs develop both lymphoma and osterogenic tumors[275] (Table 4).

Cancer type	Target genes	Genetic modifications and clinical pathology	References
Basal cell carcinoma	*GLI2*	Constitutive human transgene expression. Basal cell carcinoma-like lesions.	[267]
Colorectal cancer	*APC*	Truncating mutation resulting in premature stop codon. Dysplastic adenomas in the large intestine (precancerous lesions).	[268]
	APC	TALEN-mediated knockout. No *in vivo* tumor development tested.	[269]
Breast cancer	*V-H-Ras*	Transgenesis. V-H-Ras transgene. No tumor development.	[270]
	BRCA1	Loss of exon 11 by rAAV-mediated gene targeting. Lethal with animals dead at day 18.	[271]
Various cancers	*TP53*	$TP53^{R167H}$. Dominant negative allele by gene targeting vector DNA. Inducible transgene overexpression. Tumor histopathology to be determined	[276]
	TP53	$TP53^{R167H}$. Dominant negative allele by rAAV-mediated gene targeting. Lymphoma and osterogenic tumors.	[274]
	KRAS	Floxed $KRAS^{G12D}$. Oncogenic activation. Inducible transgene overexpression. Tumor histopathology to be determined.	[275]
	KRAS & *TP53*	Floxed, bicistronic $KRAS^{G12D}$ cDNA and $TP53^{R167H}$ cDNA. Oncogenic activation and dominant negative allele, respectively. Inducible transgene overexpression. Mesenchymal tumor formation	[272]

Table 4. Genetically modified porcine models for cancer research. Inspired from[237,257]. Abbreviations: rAAV, recombinant adeno-associated virus; TALEN, transcription activator-like effector nucleases.

Furthermore, the *RAS* gene is mutated in approximately 25% of all human cancers; with *KRAS* being the most commonly mutated isoform[272]. The RAS protein is a GTPase driving cellular proliferation and oncogenic RAS especially promotes pro-growth, pro-angiogenic, and anti-apoptotic signals[277]. Specifically for $KRAS^{G12D}$, this oncogenic activating mutation promotes metastasis in human pancreatic cancer in part by downregulation of E-cadherin[278]. Although histopathology is yet to be determined, a porcine model with inducible $KRAS^{G12D}$ has been developed[275] (Table 4). Upon xenotransplantation, *in vitro* transformed porcine mesenchymal stem cells expressing both the $TP53^{R167H}$ mutation and the $KRAS^{G12D}$ mutation have successfully established tumors in immunodeficient mice[279]. However, the only transgenic pig combining both the $TP53^{R167H}$ dominant negative mutation and the $KRAS^{G12D}$ oncogenic activating mutation is a model known as the Oncopig[272]. To generate this model, porcine oocytes received the adenoviral vector Cre-recombinase (AdCre)-inducible expression construct (displayed in Figure 3) by somatic nuclear transfer.

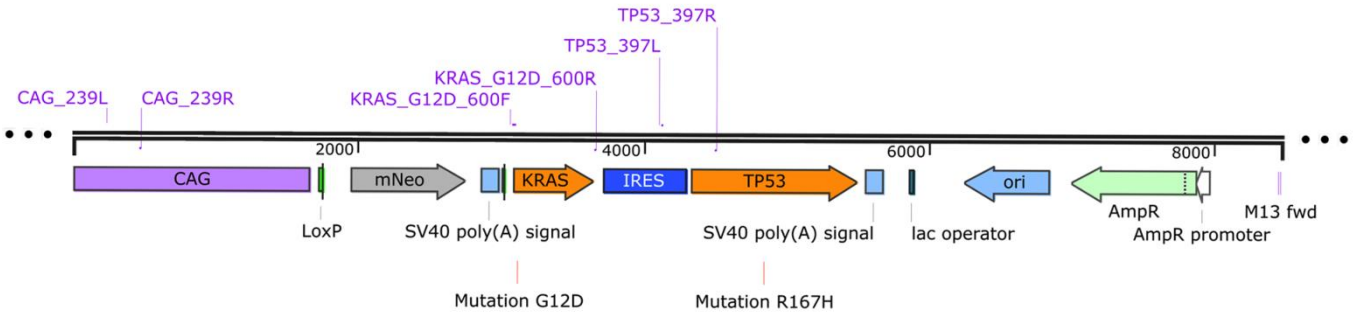

Figure 3. The AdCre-inducible vector encodes two mutated transgenes in the Oncopig model. Each cell in the transgenic Oncopig has the vector encoding $KRAS^{G12D}$ and $TP53^{R167H}$. Upon exposure to AdCre, these two transgenes will be expressed; subsequently resulting in tumor formation at the site of AdCre injection. Figure from[272]. Abbreviations: AdCre, adenoviral vector Cre-recombinase; IRES, internal ribosome entry site.

The expression of the two mutations is under control of the CAG promoter. Due to the internal ribosome entry site (IRES) element, bicistronic expression of the mutated transgenes, *KRAS*^{G12D} and *TP53*^{R167H}, is possible (Figure 3). Since every cell in the Oncopig has this expression construct, the model enables induction of a broad range of cancer types upon exposure to AdCre[272].

For immunological purposes, knowledge regarding the swine leukocyte antigen (SLA), the porcine MHC molecule, is crucial. The original Oncopig male used to breed these offspring was homozygous for SLA-2*03:01, a SLA class I allele, and the transgenes (*KRAS*^{G12D} and *TP53*^{R167H}) (Lawrence B. Schook, personal communication). For this reason, the F1 animals used for experiments are transgene heterozygous and express the SLA-2*03:01 allele, which can be used for vaccine design and T-cell assays. *In vivo* induction of sarcomas with regional leiomyosarcomas has been shown upon intramuscular, testicular, and subcutaneous injection of AdCre to Oncopigs[272].

Successful *in vitro* transformation of eleven different Oncopig cell lines have been established, as described in detail elsewhere[154]. In addition, *in vivo* Oncopig models for hepatocellular carcinoma[280] and pancreatic ductal adenocarcinoma (Principe et al, 2017, Nature Communication, in review) have recently been validated. Despite immunohistochemistry detection of infiltrating $CD3^+$ T cells in Oncopig hepatocellular carcinoma[280], no prior immunological research has been performed in the model. Knowledge regarding the immunological landscape of Oncopig tumors is crucial in order to determine, whether the model may serve as a relevant platform for studying anti-tumor immune responses and for preclinical testing of immunotherapies.

CHAPTER II. Purpose and Research Goals

The field of cancer immunotherapy has shown impressive results; however, a large fraction of the promising preclinical results obtained in rodent models are lost in the translation to human patients. From this, we hypothesized that the success rate when translating clinical trials can be increased by using an intermediate large animal model; thus, providing a link between murine studies and human patients. Therefore, the overall aim of this Ph.D. project was to investigate the potential for pigs as large animal models for studying anti-tumor immune responses and for preclinical testing of cancer immunotherapies.

Specifically, the research goals of this series of studies were:

1. To design an immunization strategy allowing the induction of an antigen-specific CTL response in pigs
2. To investigate if it is possible to break peripheral tolerance towards IDO, an important target in cancer immunotherapy, by immunizing pigs with cationic adjuvant formulation 09 (CAF09)-formulated porcine IDO-derived peptides.
3. To determine if the vaccine antigen dose influences the type immune response generated in pigs following immunization.
4. To establish protocols allowing characterization of the immunological landscape of Oncopig tumors with respect to T cells in particular.
5. To evaluate if endogenous anti-tumor immune responses are present in the Oncopig model.

CHAPTER III. The Major Findings

Summary of Results

Since the majority of findings obtained in animal models are lost in translation to clinical cancer trials[178], we investigated the potential for the pig as large preclinical animal model for studying anti-tumor immune responses. Using tetanus toxoid (TT) as a model antigen formulated in CAF09 adjuvant, we established an intraperitoneal (i.p.) immunization protocol allowing the induction of a CTL response in Göttingen minipigs (Paper I). Furthermore, we compared three different antigen doses (1μg, 10μg, and 100μg) and evaluated their potential influence on the vaccine-induced immune response. Generation of a CTL response was inversely correlated with the CAF09-formulated antigen dose following three immunizations. The induction of a polyfunctional T-cell response was found only upon low antigen dose immunization, while antigen-specific IgG antibodies developed in response to administration of a high dose TT protein.

Next, we investigated the effect of antigen dose for an endogenous protein. We showed that repeated i.p. delivery of CAF09-formulated long IDO-derived peptides to Göttingen minipigs successfully broke peripheral tolerance towards this endogenous target relevant for cancer immunotherapy (Paper II). An antigen-specific cell-mediated immune (CMI) response was established across all groups (1 μg, 10μg, and 100μg antigen dose) with no difference in the level of IFN-γ producing cells. IDO-specific IgG antibodies were produced predominantly in response to a CAF09-adjuvanted high peptide dose. Together, low antigen dose immunization against an endogenous target induced a CMI-dominant response, whereas a high antigen dose formulated in CAF09 adjuvant generated a mixed CMI and humoral immune response.

To investigate potential killing of IDO^+ cells following immunization, we performed a fluorescence-based *in vivo* cytotoxicity assay. Although some animals showed a tendency towards target-specific lysis following re-infusion of IDO-pulsed cells, no convincing *in vivo* reactivity was demonstrated. However, this assay is the first of its kind in a porcine model

and may serve as an important tool for monitoring and tracking immunological responses *in vivo*.

Finally, we investigated the potential for the transgenic Oncopig for studying anti-tumor immune responses (Paper III). We characterized the immunological landscape of Oncopig tumors (induced following AdCre injection) and demonstrated pronounced T-cell infiltration which was independent of tumor site. The existence of a tumor did not seem to alter the systemic immune landscape, as no difference in the composition of immune cells in peripheral blood was observed between tumor-bearing pigs and healthy controls. The intratumoral T-cell compartment showed enrichment of both FoxP3-expressing T cells and cytotoxic CD8ß$^{+}$ T cells when compared to peripheral blood. Pronounced perforin and granzyme B expression were demonstrated in the tumors; further underlining the presence of cytotoxic intratumoral immune cells. To determine if the Oncopig immune system poses the ability to target and lyse tumor cells, we adapted our fluorescence-based cytotoxicity assay for *in vitro* use. By co-culturing immune effector cells with labeled control cells and tumor target isolates, we showed tumor-specific killing in an effector:target cell ratio dependent manner. Finally, RNA-seq analysis revealed elevated expression of *IDO1*, *CTLA4*, and *PDL1* in Oncopig leiomyosarcoma tumors. This suggested a potential mechanism for *in vivo* inhibition of anti-tumor immunity at the early time points post AdCre injection.

Long term studies revealed spontaneous regression of most Oncopig tumors. From this, it can be speculated that there is equilibrium between immune activation (intratumoral cytotoxic cells) and suppression (FoxP3^{+} T cells and elevated expression of *IDO1*, *CTLA4*, and *PDL1*) at the early time points post AdCre injection, while anti-tumor immune responses become dominant over time. Combined, our data support that pigs, and in particular the Oncopig, provide an important platform for studying anti-tumor immune responses. With more in-depth understanding of how this anti-tumor immunity and spontaneous regression are mediated, the model may serve as a large and physiologically relevant animal model for evaluation of future preclinical cancer immunotherapies.

Paper I

Overgaard NH, Frøsig TM, Jakobsen JT, Buus S, Andersen MH, Jungersen G

Low Antigen Dose Formulated in CAF09 Adjuvant Favours a Cytotoxic T-cell Response Following Intraperitoneal Immunization in Göttingen Minipigs

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Low antigen dose formulated in CAF09 adjuvant Favours a cytotoxic T-cell response following intraperitoneal immunization in Göttingen minipigs

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ABSTRACT

The relationship between the antigen dose and the quality of an immune response generated upon immunization is poorly understood. However, findings show that the immune system is indeed influenced by the antigen dose; hence underlining the importance of correctly determining which dose to use in order to generate a certain type of immune response.

To investigate this area further, we used Göttingen minipigs as an animal model especially due to the similar body size and high degree of immunome similarity between humans and pigs. In this study, we show that both a humoral and a cell-mediated immune (CMI) response can be generated following intraperitoneal immunization with tetanus toxoid (TT) formulated in the CAF09 liposomal adjuvant. Importantly, a low antigen dose induced more TT-specific polyfunctional T cells, whereas antigen-specific IgG production was observed upon high-dose immunization. Independent of antigen dose, intraperitoneal administration of antigen increased the amount of TT-specific cytotoxic CD8β$^+$ T cells within the cytokine-producing T-cell pool when compared to the non-cytokine producing T-cell compartment.

Taken together, these results demonstrate that a full protein formulated in the CAF09 adjuvant and administered to pigs via the intraperitoneal route effectively generates a cytotoxic T-cell response. Moreover, we confirm the inverse relationship between the antigen dose and the induction of polyfunctional T cells in a large animal model. These finding can have implications for the design of upcoming vaccine trials aiming at establishing a cytotoxic T-cell response.

1. Introduction

Vaccines can contain different amounts of target antigen; however, it is not well known how the antigen dose influences the quality of a resulting immune response. Relatively few studies directly investigate this, although an inverse relationship between antigen dose and the duration of delayed type hypersensitivity has been proposed [1]. Also, it has been hypothesized that more T cells and antigen are required for Th2 than Th1 responses [2]. Recent findings further support an inverse relationship between the antigen dose and the induction of CD4$^+$ T-cell polyfunctionality and functional avidity in both mice and humans [3–5].

Given that the antigen dose can influence the immune response, correctly determining the first-in-human dose based on preclinical animal studies becomes even more crucial, and translating findings from preclinical vaccine research is dependent on animal models reliably mimicking human patients. Previously, the body weight of the animal alone has been used for extrapolation; but due to resulting unsuccessful clinical trials, using the body surface area (BSA) of the animal has been a suggested approach [6]. However, the BSA method still shows extreme inaccuracy [7]; suggesting the need for further improvement in strategies converting animal

Abbreviations: BSA, body surface area; Cat, catalogue number; CMI, cell-mediated immune; CTL, cytotoxic T lymphocyte; DC, dendritic cell; i.m., intramuscular; i.p., intraperitoneal; s.c., subcutaneous; SEB, *staphylococcal enterotoxin B*; SFC, spot forming cells; TT, tetanus toxoid.

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doses to human equivalents in order to reliably study the effect of antigen dose on the immune response.

In contrast to rodents; the porcine metabolic rate, important metabolic enzymes, and the immunome closely resemble the human counterparts [8–11]. Moreover, pigs are fully immune competent and display high MHC-allelic diversity with the number of known porcine MHC class I alleles continuously expanding due to an improved detection method [12]. For vaccine research to be reliably translated to humans, it is crucial to perform the preclinical tests in an animal model with a fully competent immune system [13,14]; further supporting the potential in using pigs as a large animal model in the interphase from early rodent work to clinical trials in humans.

In this study, we hypothesised that a cytotoxic immune response can be generated in pigs following intraperitoneal (i.p.) immunization. Moreover, we hypothesized that the quality of the resulting immune response is influenced by the antigen dose. Tetanus toxoid (TT) was used as a model antigen and formulated in CAF09; a dimethyldioctadecylammonium bromide liposomal adjuvant with synthetic monomycolyl glycerol and the TLR3 agonist poly I:C as immune modulators [15]. We i.p. administered 10-fold titrations of the full TT protein to Göttingen minipigs and investigated effects of antigen dose on the humoral and cell-mediated immune (CMI) response to further evaluate the potential of pigs for translational vaccine research.

2. Materials and methods

2.1. Pigs

Fifteen Göttingen minipigs aged ~2 to 4.5 months and derived from four different litters were purchased from Ellegaard A/S (Sorø, Denmark), housed at the National Veterinary Institute, Technical University of Denmark (Frederiksberg C, Denmark) and randomized into three groups based on sex, litter, and weight ($n = 5$). Animal procedures were carried out in accordance with both national and international guidelines, and all procedures comply with the ARRIVE guidelines. The institutional committee as well as the Danish Animal Experiments' Inspectorate (Ethical approval ID: 2012−15−2934−00557) approved all procedures.

2.2. Immunizations

Animals received either 1 μg, 10 μg, or 100 μg of purified TT (State Serum Institute, batch: T 262-01) formulated in the CAF09 adjuvant as previously described [15]. The CAF09 adjuvant was kindly provided by Dennis Christensen (Statens Serum Institut, Copenhagen, Denmark). Each immunization was comprised of 1 ml CAF09 and 1 ml TT diluted in 10 mM Tris buffer. Immunizations were delivered via the intraperitoneal (i.p.) route using an 18G × 2″ needle; no anaesthesia was used. Animals were primed and subsequently boosted twice with two week intervals (Supp. Table 1).

2.3. Cell isolation

Blood was collected into sodium heparinized vacutainer tubes (BD Diagnostics, catalogue number (cat.): 362753) and purified using SepMate tubes (StemCell Technologies, cat.: 85450) according to manufacturer's protocol. In brief, the blood was diluted in PBS/2%FBS (ThermoFischer Scientific, cat.: 10082147) and separated using Lymphoprep (StemCell Technologies, cat.: 07851). Following separation, the cells were counted using the Nucleocounter NC-200 (Chemometec, Allerød, Denmark).

2.4. IFN-γ ELISpot

MultiScreen$_{HTS}$ IP Filter Plates (Merck Millipore, cat.: MSIPS4510) were pre-wet in 35% ethanol (v/v in sterile milliQ water) and coated with 5 μg/ml mouse anti-swine IFN-γ antibody (ThermoFischer Scientific, cat.: MP700) overnight at 4 °C. The plates were blocked with AIM V™ media (ThermoFischer Scientific, cat.: 12055091), no serum, for at least one hour at 37 °C. To each well, 2×10^5 freshly isolated PBMCs were added and incubated for 20 h at 37 °C in the presence of 1.5 μg/ml TT, 1.5 μg/ml *staphylococcal enterotoxin B* (SEB) (Sigma Aldrich, cat.: S4881) as positive control, or media alone. Biotin Mouse Anti-Pig IFN-γ (BD Biosciences, cat.: 559958) was used at 1 μg/ml for detection with incubation for 1 h at room temperature (RT). Streptavidine-Alkaline Phophatase conjugate (Sigma Aldrich, cat.: 11 089 161 001) was diluted 1:2000 and added to the plates with incubation on a shaking table for 1 h at RT. Finally, 100 μl/well of BCIP®/NBT Liquid Substrate System (Sigma Aldrich, cat.: B1911) was added and spot development was terminated after five minutes. The plates were allowed to air-dry in the dark. The AID EliSpot Reader version 6.0 (Autoimmun Diagnostika GmbH, Strassberg, Germany) was used for analysis. Data is shown with subtraction of the background levels of spot forming cells (SFCs) from culturing with media alone.

2.5. IgG ELISA

The 96-well polysorp plate (ThermoFischer Scientific, cat.: 475094) was coated with 0.125 μg/ml TT and incubated overnight at 4 °C. Serum samples, diluted 1:10,000, were added to the plate with incubation on a shaking table for 1 h at RT. Biotinylated goat anti-pig IgG (Bio-Rad, cat.: AAI41), was diluted 1:20,000 and used as secondary antibody with incubation on a shaking table for 1 h at RT. HRP-conjugated streptavidin (ThermoFischer Scientific, cat.: N100) diluted 1:8000 was added; the plate was incubated on a shaking table for 1 h at RT. Finally, tetramethylbenzidine (Kem-En-Tec, cat.: 4380 L) was added and the reaction was terminated with 0.5 M sulfuric acid after five min at RT. A microplate reader (ThermoFischer Scientific) was used to determine the absorbance at 450 nm; corrections for unspecific background were done by subtraction of the signal at 650 nm.

2.6. Flow cytometry

Antibodies were used at pre-determined concentrations (details in Supp. Table 2). PBMCs were stimulated for 16 h with 2 μg/ml TT, media alone, or 1 μg/ml SEB as a positive control, followed by 6 h culturing in the presence of 10 μg/ml Brefeldin A (Sigma-Aldrich, cat.: B7651-5MG). Cells were surface stained for 30 min at 4 °C with antibodies against CD3 and CD8β in combination with a live/dead stain. Fixation/Permeabilization Solution Kit (BD Biosciences, cat.: 554714) was used according to manufacturer's protocol. Intracellular cytokine staining was conducted using antibodies against IFN-γ, TNF-α, and perforin for 30 min at 4 °C. Samples were acquired on an LSRFortessa (BD Bioscience) flow cytometer, and 200,000 viable $CD3^+$ cells were recorded for analysis. Data was analysed using FlowJo Data Analysis Software version 10.

2.7. Statistical analysis

Despite low numbers of animals, the data were analysed by parametric analyses as non-baseline data passed the Shapiro-Wilk normality test and presumably represent normally distributed populations. Results are thus shown as the mean or the mean ± SEM and statistical comparisons were performed using either paired or unpaired Student's *t*-test. GraphPad Prism version

7.00 for Windows (California, United States) was used for statistical analysis. $P < 0.05$ (*) was considered significant, and $P < 0.005$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****) are indicated.

3. Results

3.1. Immunization with a low antigen dose drives a CMI response

We firstly evaluated the amount of IFN-γ produced during the immunization trial. Prior to immunization, all animals were TT naïve as demonstrated by the lack of IFN-γ SFCs at day 0 (Fig. 1A). A quantification of the IFN-γ SFCs in response to CAF09-formulated TT revealed that a CMI response was generated in all the groups already at day 27 (Fig. 1B). The immune response in each group was enhanced by an additional immunization as indicated by the presence of more IFN-γ SFCs at day 41; most pronounced in the 1 μg and 10 μg group (Fig. 1B). Additionally, we investigated whether several rounds of i.p. immunization induced a humoral immune response. No TT-specific IgG antibodies were detected in serum samples prior to the first immunization in any of the groups (Fig. 1C). Two immunizations were sufficient to generate TT-specific IgG antibodies only in the 100 μg dose group; however, all groups displayed a humoral response to TT following three injections (Fig. 1C). A comparison of the three immunization groups revealed that animals receiving 1 μg TT produced a stronger TT-specific IFN-γ response when compared to animals receiving 100 μg TT (Fig. 1D). In contrast, immunization with a high antigen dose induced a stronger humoral immune response (Fig. 1E).

3.2. T-cell-derived IFN-γ is enhanced by immunization with a low antigen dose

Having established that the dose of immunizing antigen affected the subsequent IFN-γ responses detected by *ex vivo* IFN-γ ELISpot, we further investigated the effect of antigen dose directly on T cells. The capacity of T cells to produce IFN-γ against TT following *in vitro* re-stimulation at day 41 was determined by flow cytometry; a representative gating strategy is depicted in Supp. Fig. 1.

Although numbers of IFN-γ^+ TT-specific T cells were small, the flow cytometric plots clearly indicated that T cells derived from the 1 μg, and somewhat also the 10 μg group, were IFN-γ^+ while animals receiving 100 μg of CAF09-formulated TT did not seem to respond (Fig. 2A). This was substantiated by a statistically significant higher percentage of T cells producing IFN-γ against TT in animals receiving 1 μg of antigen compared to 100 μg immunized pigs (Fig. 2B). Interestingly, a titration effect could be observed across the groups (Fig. 2B); thus suggesting an inverse relationship between the percentage of IFN-γ^+ T cells and the CAF09-formulated antigen dose. Analysis of the $CD3^-$ population did not reveal IFN-γ producing cells in response to TT (data not shown).

3.3. TT-specific cytotoxic CD8β^+ T cells are increased within the IFN-γ^+ T-cell population

Given that the antigen dose when formulated in CAF09 is inversely correlated with the amount of IFN-γ responsive T cells, we further investigated whether the phenotype of the T cells was also affected by the antigen dose. The CD8β marker was used to distinguish between cytotoxic and helper T cells as previously described [16]. The ratio between CD8β^+ and CD8β^- T cells was evaluated in both the IFN-γ^- and the IFN-γ^+ T-cell population for all groups (Fig. 3A–C). When quantifying the ratios, a significant increase in CD8β^+ T cells was detected in the IFN-γ^+ T-cell population for both the 1 μg (Fig. 3D) and the 10 μg group (Fig. 3E). In the high dose group, four out of five animals also showed a tendency towards an increase in CD8β^+ T cells within the IFN-γ^+ T-cell population (Fig. 3F). Taken together, these results demonstrate that the TT-specific CTLs are increased within the IFN-γ^+ T-cell population independently of the antigen dose formulated in CAF09.

3.4. TNF-α^+ T cells are slightly increased when immunizing with a low antigen dose

In addition to IFN-γ, TNF-α is an important effector molecule produced by cytotoxic $CD8^+$ T cells [17]. For this reason, we investigated whether TNF-α was also affected by the antigen dose. The ability of T cells to produce TNF-α in response to TT was again evaluated using flow cytometry; a representative gating strategy is outlined in Supp. Fig. 1. Across all groups and in all individual animals, TNF-α-producing T cells were readily detectable (Fig. 4A). When comparing the percentage of TNF-α^+ T cells, no difference could be observed between the 1 μg and the 10 μg groups, while four out of five pigs in the 100 μg group were non-responders (Fig. 4B). Although non-significant, a trend towards an inverse relationship between CAF09-formulated antigen dose and the ability of T cells to produce TNF-α could thus be observed (Fig. 4B).

3.5. TT-specific cytotoxic CD8β^+ T cells are increased within the TNF-α^+ T-cell population

Since the cytokine-producing T-cell population was shifted towards a cytotoxic phenotype when measuring IFN-γ (Fig. 3), we speculated whether this would also be the case for TNF-α. The relationship between cytotoxic and helper T cells, as determined by the expression of the CD8β molecule, was determined within the TNF-α-producing and TNF-α^- T-cell population (Fig. 5A–C). An increase in the amount of CD8β^+ T cells in the TNF-α^+ population was observed for all groups, when comparing to the TNF-α^- population (Fig. 5A–C). This observation was clearly supported by a statistical analysis of the CD8β^+/CD8β^- ratio in the TNF-α-producing and non-producing T-cell population. Here, a significant increase in cytotoxic CD8β^+ T cells within the TNF-α^+ T-cell population was demonstrated for all the groups (Fig. 5D–F). Together, these results show a specific increase in CTLs within TNF-α^+ T-cell population independent of the CAF09-formulated antigen dose.

Moreover, perforin has been reported to be an important effector molecule for CTLs [18]. Therefore, we also investigated the effect of antigen dose on the ability of CTLs to produce perforin in response to TT. A substantial population of perforin$^+$CD8β^+ T cells was detected in all animals (Supp. Fig. 2A). Despite this, no difference was observed when comparing the percentage of perforin$^+$CD8β^+ T cells across the groups (Supp. Fig. 2B); hence showing that the production of perforin is independent of the antigen dose when administered in CAF09 adjuvant.

3.6. Low antigen dose induces more TT-specific polyfunctional T cells

The ability to induce polyfunctional $CD4^+$ T cells in humans has been shown to be inversely correlated with antigen dose following intramuscular (i.m.) immunization [4]. Therefore, we investigated whether an i.p. administration route had similar effect on the ability to induce polyfunctional T cells in response to CAF09-adjuvanted TT. Flow cytometric analysis of re-stimulated PBMCs harvested at day 41 was performed using a gating strategy as depicted in Supp. Fig. 1. T cells producing both TNF-α and IFN-γ were detected in both the 1 μg and the 10 μg group; however, this population of double-cytokine-positive T cells appeared to be mostly absent in the high dose group (Fig. 6A). When quantifying the percentage of TNF-α^+IFN-γ^+ T cells across the three groups, a

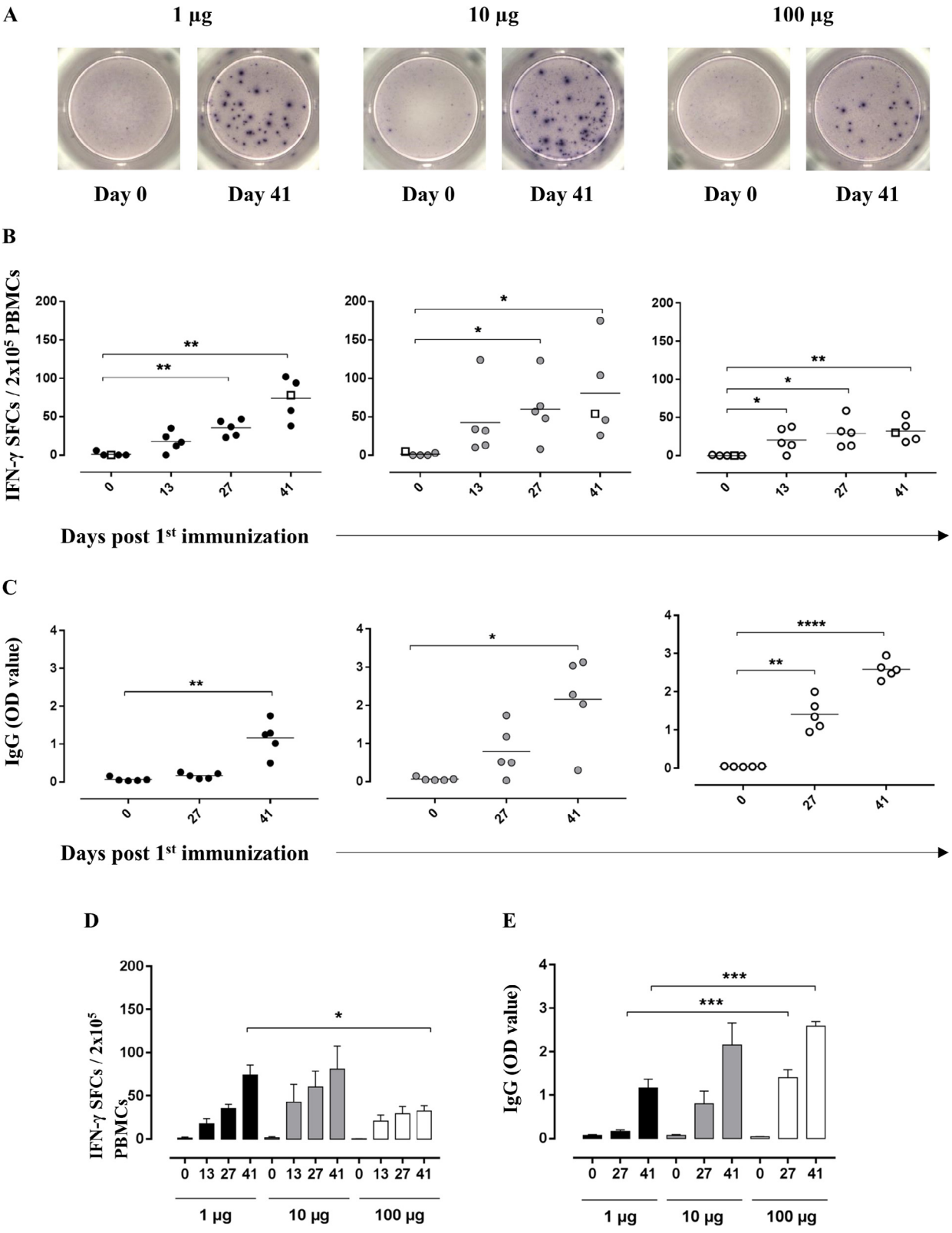

Fig. 1. Immunization with a low antigen dose preferentially drives a CMI response. Göttingen minipigs were intraperitoneally immunized with either 1 µg, 10 µg, or 100 µg of tetanus toxoid formulated in the CAF09 adjuvant. Immunizations were administered three times with two weeks in between. All animals were blood sampled prior to each immunization and two weeks post the last injection. (A) IFN-γ ELISpot images at day 0 and 41 from one representative animal in each group in response to tetanus toxoid. (B) Quantification of IFN-γ ELISpot responses against tetanus toxoid from animals receiving 1 µg (black circles), 10 µg (grey circles), or 100 µg (white circles). Open squares indicate the representative animal shown in (A). Data is presented as spot forming cells (SFCs) per 2×10^5 PBMCs with indication of the mean. (C) ELISA-based detection of anti-tetanus IgG in serum samples from animals immunized with 1 µg (black circles), 10 µg (grey circles), or 100 µg (white circles). Data is shown as OD values with indication of the mean. (D) Comparison of IFN-γ SFCs in response to tetanus toxoid across all groups and for each time point. Data is shown as mean ± SEM. (E) Comparison of the anti-tetanus IgG production across all groups and for each time point. Data is shown as mean ± SEM. Statistical evaluation by paired student's *t*-test (B and C) or unpaired student's *t*-test (D and E), ($n = 5$).

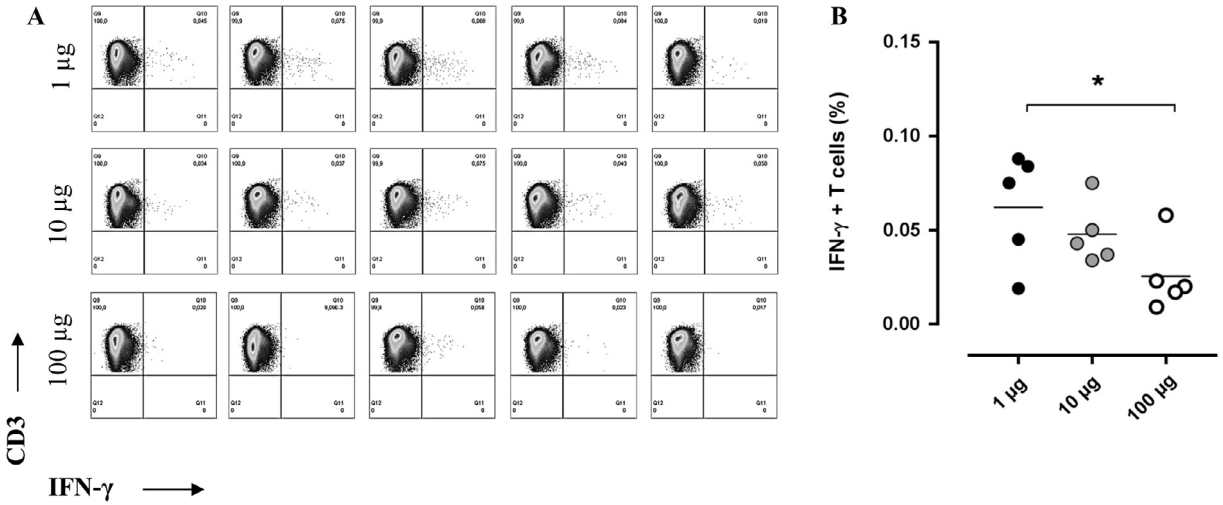

Fig. 2. Flow cytometry corroborates the inverse relationship between antigen dose and the percentage of IFN-γ^+ T cells. PBMCs purified at day 41 were stimulated *in vitro* with tetanus toxoid and IFN-γ production was determined by flow cytometry. Analysis included pre-gating on single, viable $CD3^+$ cells. (A) Flow cytometric plots showing IFN-γ^+CD3^+ cells in the 1 µg (upper panel), 10 µg (middle panel), and 100 µg group (lower panel). Individual animals in each group are shown and horizontally aligned. (B) Percentage of IFN-γ-producing T cells across all groups with indication of the mean. Numbers indicate the percentage of IFN-γ^+ T cells as a proportion of total T cells. The background level of IFN-γ-producing T cells in response to media alone were at least 2-fold lower when compared to stimulation with TT or ≤0.03%, while in average 0.36% of the T cells produced IFN-γ in response to the positive SEB stimulation. Statistical evaluation in (B) by unpaired student's *t*-test, ($n = 5$).

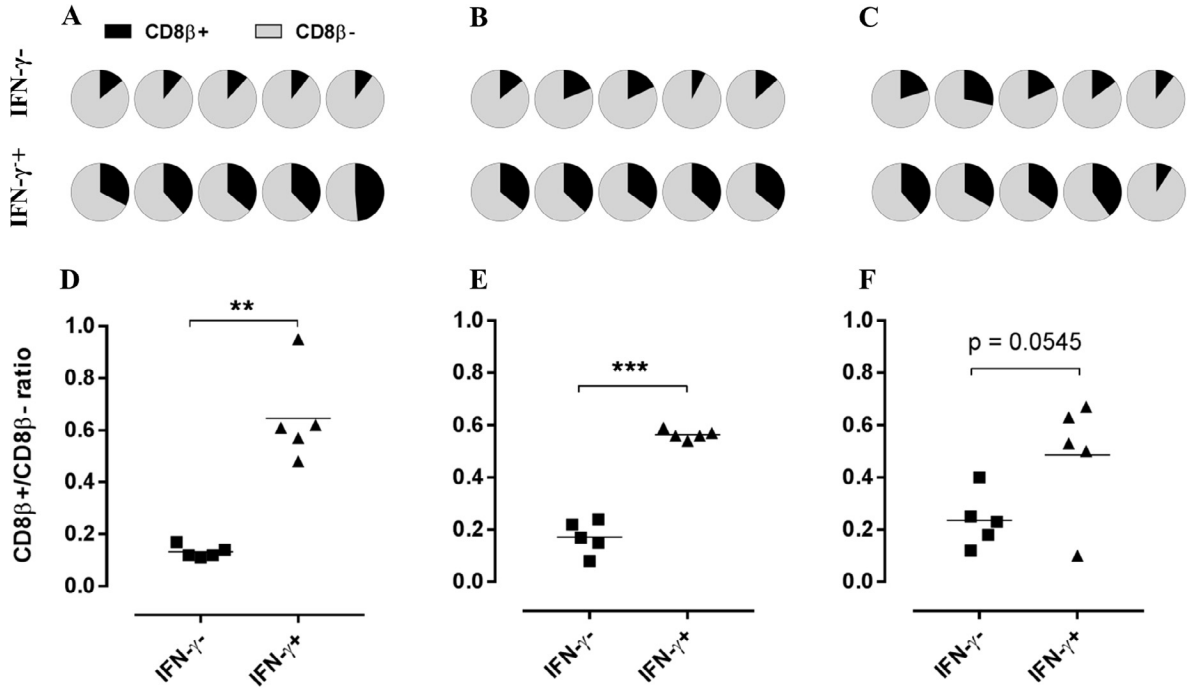

Fig. 3. Tetanus-specific cytotoxic $CD8\beta^+$ T cells are increased within the IFN-γ^+ T-cell population. PBMCs were harvested at day 41 stimulated *in vitro* with tetanus toxoid. By flow cytometry, CD8β expression was individually determined in both the IFN-γ^- and the IFN-γ^+ T-cell population. Pie charts from animals immunized with either 1 µg (A), 10 µg (B), or 100 µg (C) tetanus toxoid showing the distribution of $CD8\beta^-$ (grey) and $CD8\beta^+$ T cells (black) in both the IFN-γ^- (upper panel) and the IFN-γ^+ (lower panel) T-cell population. Individual animals in each group are shown. The $CD8\beta^+/CD8\beta^-$ ratio in both the IFN-γ^- (squares) and the IFN-γ^+ T-cell subsets (triangles) of animals immunized with 1 µg (D), 10 µg (E), or 100 µg (F) of tetanus toxoid are shown with indication of the mean. Statistical evaluation in D, E, and F by paired student's *t*-test ($n = 5$).

clear titration effect could be observed with a low dose specifically inducing more polyfunctional T cells (Fig. 6B). It should be noted that only the 1 µg group clearly demonstrated a population comprising IFN-γ^+ single-producing T cells (Fig. 6A).

4. Discussion and conclusions

During this study, we showed the induction of a CTL response when administrating CAF09-formulated TT via the i.p. route in Göttingen minipigs. A low antigen dose resulted in a predominant CMI response, whereas a high dose favoured TT-specific IgG production. Previously, TT has been used as a model antigen in pigs [19], and a study reported the animals to be antigen naïve prior to immunization [20]. Our data confirmed this; hence showing that the anti-TT response was indeed vaccine-induced.

Our observed cell- and antibody-mediated responses are not surprising, as the anti-TT response has been reported to be a mixture between Th1 and Th2 [21,22]. Humans i.m. immunized against alum-adjuvanted TT showed a strong $CD4^+$ T-cell response [23], whereas we demonstrated an increased amount of CTLs

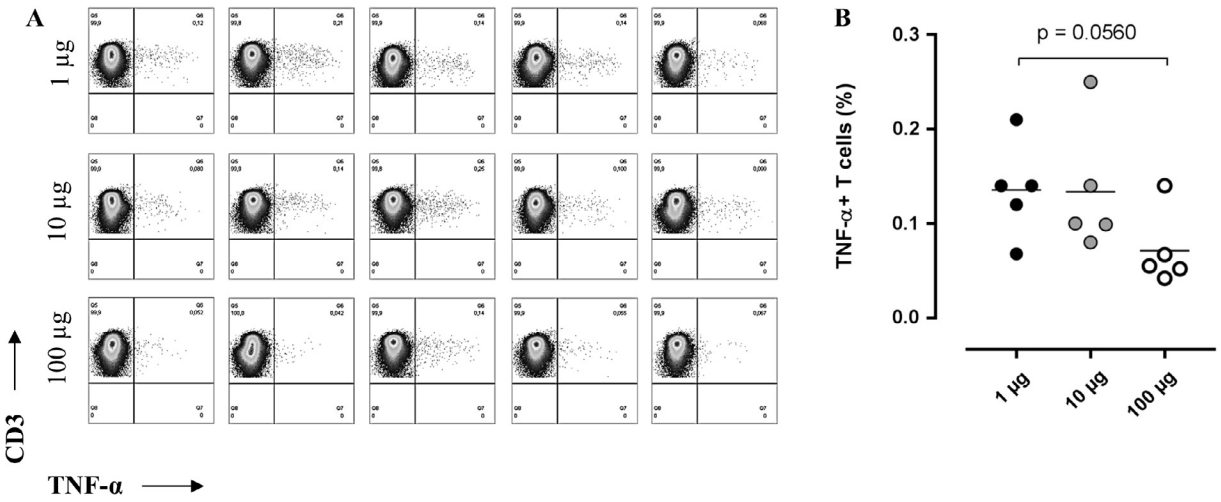

Fig. 4. Lowering the antigen dose tends to trigger a higher percentage of TNF-α^+ T cells. PBMCs were purified at day 41 and stimulated *in vitro* with tetanus toxoid. Production of TNF-α was determined by flow cytometry, and pre-gating included selection of single, viable $CD3^+$ cells. (A) Flow cytometric plots showing TNF-α^+CD3^+ cells in the 1 µg (upper panel), 10 µg (middle panel), and 100 µg group (lower panel). Individual animals in each group are shown and horizontally aligned. (B) Amount of TNF-α-producing T cells across all groups with indication of the mean. Numbers indicate percentage of TNF-α^+ T cells as a proportion of total T cells. The background level of TNF-α-producing T cells in response to media alone were at least 2-fold lower when compared to stimulation with TT or ≤0.06%, while in average 0.98% of the T cells produced TNF-α in response to the positive SEB stimulation. Statistical evaluation in (B) by unpaired student's *t*-test, ($n = 5$).

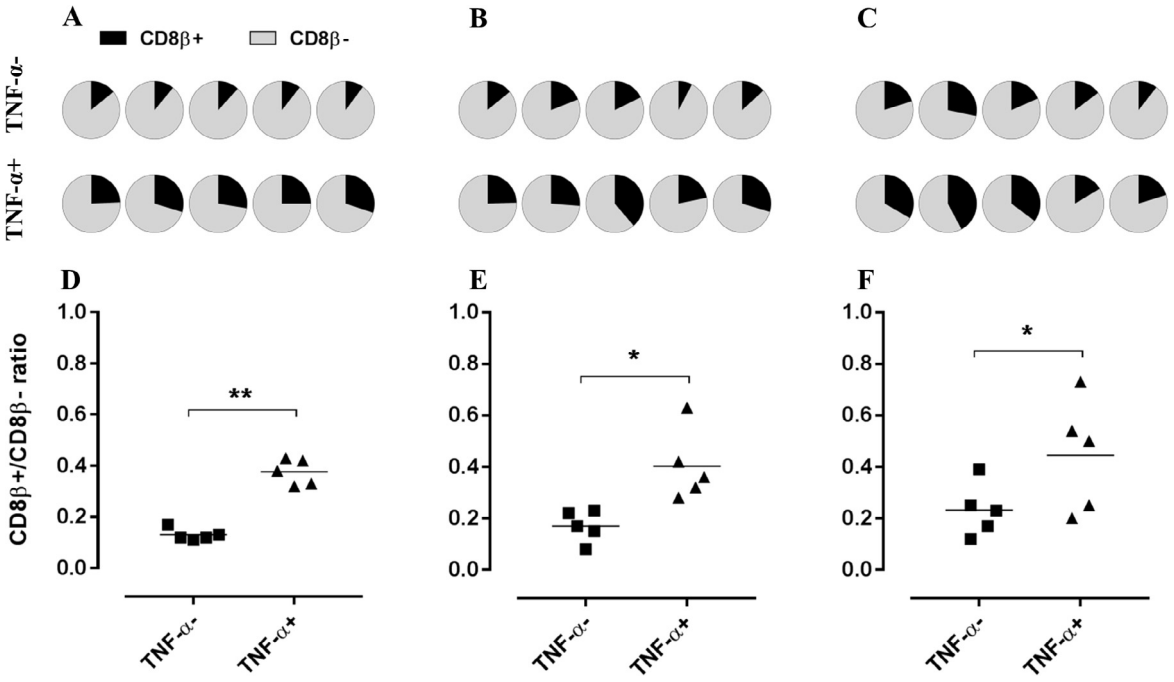

Fig. 5. The TNF-α^+ T-cell population comprises an increased representation of cytotoxic $CD8\beta^+$ T cells. PBMCs were purified at day 41 and stimulated *in vitro* with tetanus toxoid. Flow cytometry analysis included pre-gating on single, viable $CD3^+$ cells, and the CD8β expression was then individually determined in both the TNF-α^- and the TNF-α^+ T-cell subset. Pie charts from animals immunized with either 1 µg (A), 10 µg (B), or 100 µg (C) of tetanus toxoid showing the distribution of $CD8\beta^-$ (grey) and $CD8\beta^+$ T cells (black) in both the TNF-α^- (upper panel) and the TNF-α^+ (lower panel) T-cell population. Individual animals in each group are shown. The $CD8\beta^+/CD8\beta^-$ ratio in both the TNF-α^- and the TNF-α^+ T-cell subset from animals immunized with 1 µg (D), 10 µg (E), or 100 µg (F) of tetanus toxoid are shown with indication of the mean. Statistical evaluation in D, E, and F by paired student's *t*-test ($n = 5$).

within the pools of IFN-γ and TNF-α producing T-cells. This discrepancy likely reflects the differences in adjuvants and delivery route. It is well known that the immune response generated upon vaccination differs depending on which TLR is activated [24,25] and i.p. administration of cationic liposomes like CAF09 is superior in generating strong CTL responses when compared to subcutaneous (s.c.) and i.m. injection in mice [26]. Establishment of a CTL response against a full protein is dependent on cross-presentation by dendritic cells (DCs); the process by which extracellular antigen is taken up and presented in the context of MHC class I [27,28]. Specifically for i.p. immunizations in mice, vaccine self-drainage to lymphoid organs was shown to efficiently provide antigen to cross-presenting DCs [26]. Upon i.p. immunization in pigs, self-drainage might also play an important role; thus enabling DCs to effectively prime naïve $CD8^+$ T cells and induce a strong CTL response. Hence, the observed inverse relationship between antigen dose and the induction of a polyfunctional CMI response might be differently affected with the use of a different adjuvant system

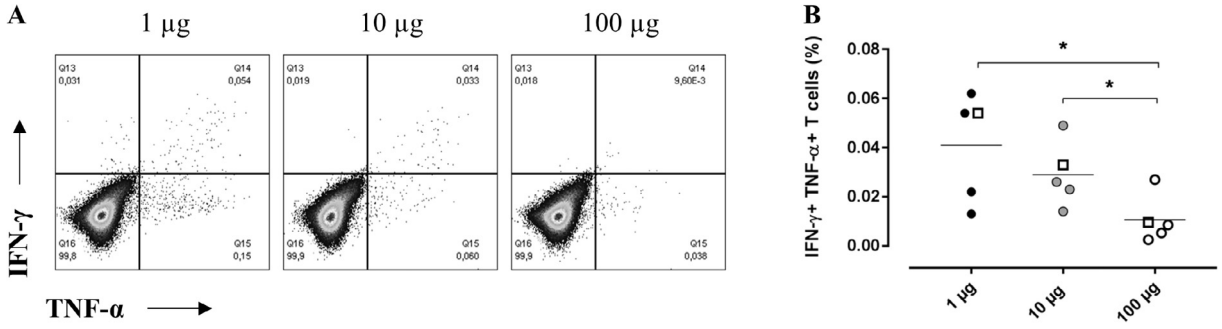

Fig. 6. A lower antigen dose increases the percentage of IFN-γ^+TNF-α^+ T cells. PBMCs were purified at day 41 and stimulated *in vitro* with tetanus toxoid. Cytokine production was determined by flow cytometry, and the analysis included pre-gating on single, viable $CD3^+$ cells. (A) Representative flow cytometric plots showing IFN-γ^+TNF-α^+ T cells in the 1 μg (left), 10 μg (middle), and the 100 μg (right) group. (B) Percentage of IFN-γ^+TNF-α^+ T cells as a proportion of total T cells across all groups. Open squares indicate the representative animal shown in (A). Statistical evaluation in (B) by unpaired student's *t*-test, ($n = 5$).

comprising other TLR agonists than poly I:C or the use of other delivery routes.

The antigen dose has previously been shown to influence the immune response following immunization [29,30]. In both mice and humans, immunization with a low dose protein induced high frequencies of $CD4^+$ T cells producing IL-2, IFN-γ, and TNF-α [3,4]. In contrast, our data showed a specific increase in CTLs within the cytokine-producing T-cell pool. Notably, the studies reporting a specific induction of polyfunctional $CD4^+$ T cells were in response to *Mycobacterium tuberculosis*-derived antigens [3,4], and protection against this bacteria is known to be dependent on a $CD4^+$ T-cell response [31–34]. Overall, these studies and our data all support an inverse relationship between CAF09-formulated antigen dose and the induction of polyfunctional T cells.

Moreover, the antigen dose has been reported to influence the avidity and quality of CTLs [35–37]. In addition, the expression level of inhibitory receptors like PD-1 and CTLA-4 on $CD4^+$ T cells was found to be decreased, when mice were immunized with a low antigen dose [5]; Future studies should evaluate the effect of antigen dose on both the quality and the activation/memory stage of the TT-reactive T cells in pigs in order to select the optimal strategy for establishment of a vaccine-induced cytotoxic immune response. In conclusion, our results showed that it is possible to induce a CTL response by i.p. delivering a CAF09-formulated protein in pigs. Moreover, we confirmed the inverse relationship between the antigen dose and the induction of polyfunctional T cells previously demonstrated in mice and humans. The T-cell subsets affected might differ depending on the antigen in question; however, the antigen dose clearly affects the immune response induced by immunization. Therefore, correctly determining the first-in-human dose becomes even more important. Due to its similarities in both metabolism and immunome with humans, we believe that pigs can serve as an important animal model for preclinical optimization of vaccine doses.

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Conflict of interest statement

The authors declare no conflicts of interest.

Authors and contributors

Experimental design: NHO, TMF, and GJ. Performed the experiments: NHO, JTJ, and TMF. Data analysis and interpretation: NHO, JTJ, and GJ. Drafted the manuscript and figures: NHO. Manuscript revision: NHO, TMF, JTJ, SB, MHA, and GJ. All the authors approved the final manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.08.057.

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Outline of the immunization trial.

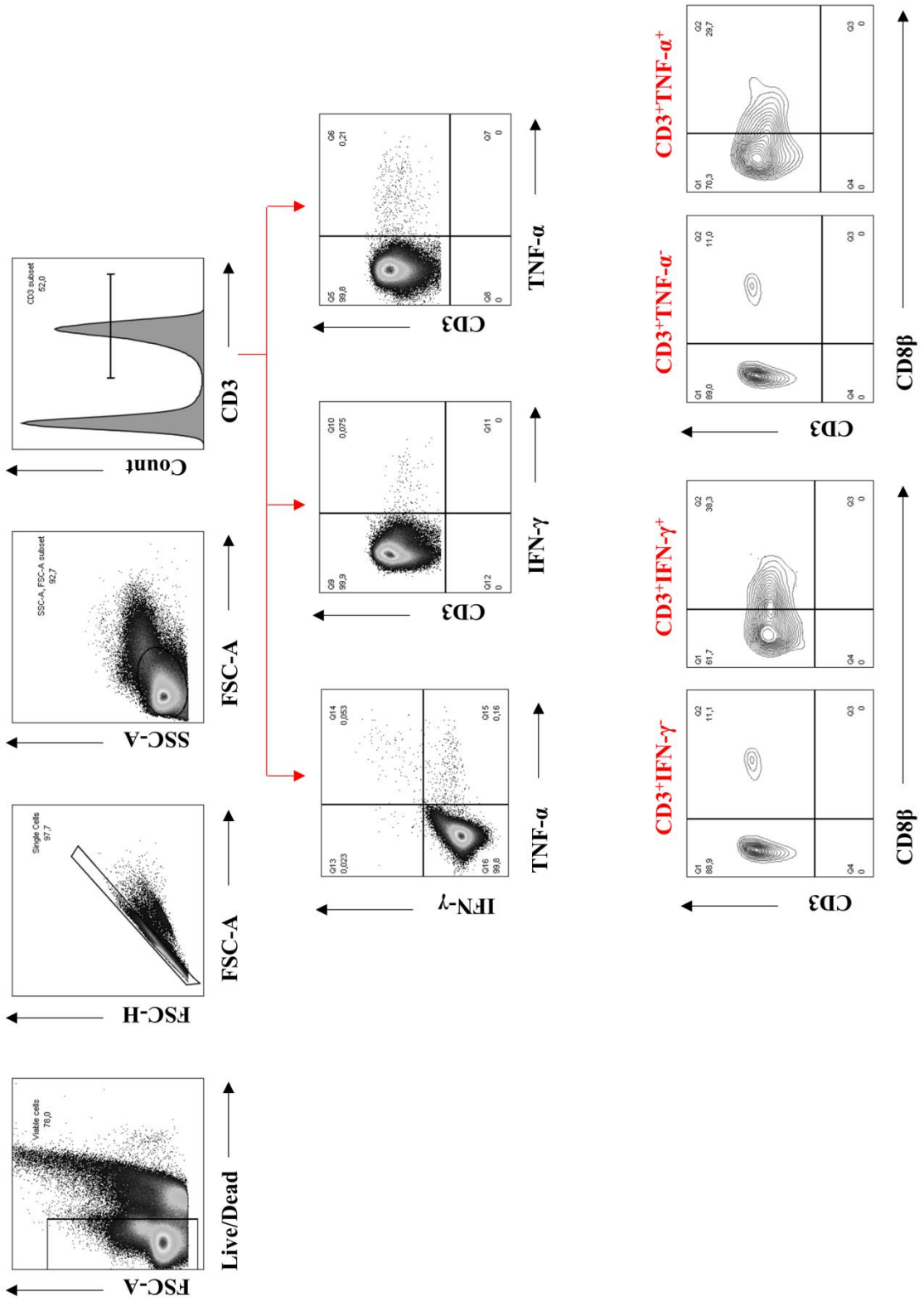
# Animals	Immunization	Day 0	Day 13	Day 27	Day 41
5	CAF09 + 1 µg	Immunization and blood	Immunization and blood	Immunization and blood	Blood only
5	CAF09 + 10 µg				
5	CAF09 + 100 µg				

Supplementary Table 1. Outline of the immunization trial. Göttingen minipigs were intraperitoneally immunized with tetanus toxoid formulated in the CAF09 adjuvant. A total of 15 animals were split into three groups receiving either 1 µg, 10 µg, or 100 µg of tetanus toxoid for immunization (*n=5*). Each animal was immunized three times with a two-week interval in between. Blood samples were drawn prior to each immunization as well as two weeks post the last immunization.

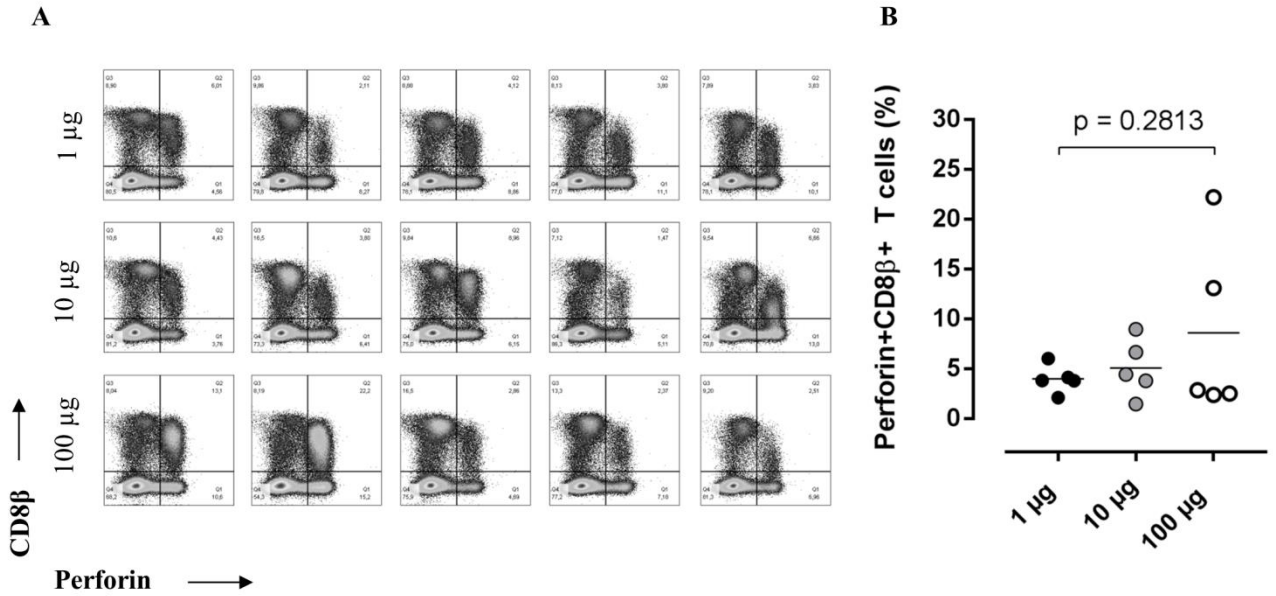
Antibodies for flow cytometry

Marker	**Conjugate**	**Isotype**	**Clone**	**Final concentration**	**Source**
CD3	Unconjugated	Mouse IgG1	PPT3	5 µg/ml	Southern Biotech
CD8 β	Unconjugated	Mouse IgG2a	PG164A	5 µg/ml	Washington State University
Live/Dead	Aqua	N/A	N/A	1:1000	ThermoFischer Scientific
IFN- γ	AF647	Mouse IgG1	CC302	50 µg/ml	Serotec
TNF- α	PerCP-Cy5.5	Mouse IgG1κ	MAb11	3 µg/ml	Biolegend
Perforin	PE	Mouse IgG2bκ	dG9	3 µg/ml	Biolegend
IgG2a goat anti-mouse	PE-Cy7	Goat IgG	N/A	1.25 µg/ml	Southern Biotech
IgG1 rat anti-mouse	BV421	Rat LOU	N/A	0.4 µg/ml	BD Biosciences

Supplementary Table 2. Antibodies used for flow cytometry. Primary and secondary antibodies were all titrated prior to use. The final concentrations used for flow cytometry staining are indicated together with conjugation, isotype, clone, and source details.


FSC-A
Live/Dead
Viable cells
76,0
FSC-H
FSC-A
Single Cells
97,7
SSC-A
FSC-A
SSC-A, FSC-A subset
92,7
Count
CD3
CD3 subset
52,0
IFN-γ
TNF-α
Q13
0,023
Q14
0,053
Q15
0,16
Q16
99,8
CD3
IFN-γ
Q9
99,9
Q10
0,075
Q11
0
Q12
0
CD3
TNF-α
Q5
99,8
Q6
0,21
Q7
0
Q8
0
CD3
CD8β
CD3+IFN-γ-
Q1
88,9
Q2
11,1
Q3
0
Q4
0
CD3+IFN-γ+
Q1
61,7
Q2
38,3
Q3
0
Q4
0
CD3+TNF-α-
Q1
89,0
Q2
11,0
Q3
0
Q4
0
CD3+TNF-α+
Q1
70,3
Q2
29,7
Q3
0
Q4
0

Supplementary Figure 1. Representative gating strategy used for flow cytometry. For flow cytometric analysis, cells were firstly gated on viable cells by selection of the aqua-negative population. Single cells were then selected based on the FSC-A/FSC-H relationship. Lymphocytes were subsequently selected; the fixation of the cells for intracellular staining results in the FSC-A/SSC-A plot appearing more squeezed when compared to non-fixed cells. T cells were gated based on $CD3^+$ staining, and the final analysis included detection of both IFN-γ^+, TNF-α^+, and IFN-γ^+TNF-α^+ T cells. CD8β staining is shown for $CD3^+$IFN-γ^-, $CD3^+$IFN-γ^+, $CD3^+$TNF-α^-, and $CD3^+$TNF-α^+ cells.



Supplementary Figure 2. The level of perforin produced by CD8β$^+$ T cells in response to tetanus toxoid is independent of antigen dose. Production of perforin following *in vitro* stimulation with tetanus toxoid was determined by flow cytometry in PBMCs harvested at day 41. The cells were pre-gated on single, viable CD3$^+$ cells. (**A**) Flow cytometric plots of perforin$^+$CD8β$^+$ cells from animals receiving either 1 µg (upper panel), 10 µg (middle panel), or 100 µg (lower panel) of tetanus toxoid. Individual animals in each group are shown and horizontally aligned. (**B**) Percentage of perforin-producing CD8β$^+$ T cells as a proportion of total T cells is shown for all groups with indication of the mean. Statistical evaluation in (B) by unpaired student's t-test (*n=5)*.

Paper II

Overgaard NH, Frøsig TM, Jakobsen JT, Strube ML, Sørensen MR, Buus S, Andersen MH, Jungersen G

Repeated Immunization with a CAF09-Formulated Low Peptide Dose Predominantly Induces a Cell-Mediated Immune Response Towards Indoleamine 2,3-Dioxygenase

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Manuscript in preparation

Repeated Immunization with a CAF09-Formulated Low Peptide Dose Predominantly Induces a Cell-Mediated Immune Response Towards Indoleamine 2,3-Dioxygenase

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Keywords: Cancer immunotherapy, immunization, Indoleamine 2,3-dioxygenase, antigen dose, Major Histocompatibility Complex, large animal model

Abbreviations:

CMI	Cell-mediated immune
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
IDO	Indoleamine 2,3-dioxygenase
i.p.	Intraperitoneal
i.v.	Intravenous
MHC	Major histocompatibility complex
PBMC	Peripheral blood mononuclear cell
SFC	Spot forming cells
SLA	Swine leukocyte antigen
TT	Tetanus toxoid

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Abstract

The relationship between antigen dose and the immune response remains poorly understood especially for endogenous proteins. Since the antigen dose of an exogenous protein has been demonstrated to affect the immune response, we set to determine whether repeated immunization with different peptide doses of an endogenous and cancer-relevant target influences the immune response. Due to the high degree of homology with humans, we used Göttingen minipigs as a large animal model and immunized against Indoleamine 2,3-dioxygenase (IDO); a promising cancer immunotherapeutic target. Three different doses of porcine IDO-derived 30-31mer peptides formulated in CAF09 liposomal adjuvant were administered via the intraperitoneal route. Following repeated immunization, IDO-specific IFN-γ producing cells were readily detectable across all groups; thus, demonstrating a break in peripheral tolerance towards IDO. Interestingly, a CAF09-formulated low antigen dose predominantly induced an antigen-specific cell-mediated immune (CMI) response, while a mixed CMI and humoral immune response was observed upon high peptide dose immunization. Using an *in vivo* cytoxicity assay, a trend towards target-specific lysis following re-infusion of IDO-pulsed cells was demonstrated in a few animals. However, no general tendency towards IDO-specific cytoxicity could be observed; thus, supporting that immunization as a stand-alone treatment may not be sufficient to induce lysis of an endogenous target *in vivo*. Together, our data show that repeated immunization with CAF09-formulated peptides can break peripheral tolerance towards IDO in a large and physiologically relevant animal model. In addition, our data underline the importance of the vaccine antigen dose and supports that the pig may serve as a large preclinical model for cancer vaccine research.

1. Introduction

The potential for immunological control of cancer is an intensely investigated topic. In 2013, cancer immunotherapy was awarded breakthrough of the year [1], and peptide-based therapeutic vaccines are one of the promising arms within the field. Several clinical trials have been performed [2]; however, no peptide-based therapeutic vaccine has yet received approval by the U.S. Food and Drug Administration or the European Medicines Agency [3–5]. A major challenge to cancer vaccine development is the immunological tolerance existing towards endogenous tumor-associated antigens. As the majority of self-reactive T cells undergoes clonal deletion in the thymus to avoid autoimmunity [6, 7], the induction of an anti-tumor cell-mediated immune (CMI) response relies on the T-cell repertoire remaining post the induction of central tolerance [8].

A promising target within cancer immunotherapy is Indoleamine 2,3-dioxygenase (IDO). This intracellular enzyme regulates immune responses and induces tolerance by catalyzing the first rate-limiting step in the breakdown of tryptophan [9–11]; an essential amino acid for effector T cells [12, 13]. The lack of tryptophan locally in the tumor microenvironment and the accumulation of downstream metabolites block T-cell proliferation, polarize $CD4^+$ T cells towards a regulatory phenotype, and render T cells susceptible to the apoptotic pathway [14–16]. In several human cancers, an overexpression of *IDO* or an accumulation of IDO^+ cells have been linked to poor patient prognosis [12, 17–19]. In terms of T-cell reactivity, both IDO-specific $CD4^+$ and $CD8^+$ T cells have been demonstrated [20–23].

The majority of preclinical vaccine research has been performed in rodent models; however, it is becoming increasingly recognized that mice often poorly mimic human diseases [24, 25]. In contrast, the porcine and the human immune systems are far more analogous [26]. The homology in size, anatomy, physiology, genetics, epigenetics, pathology, and metabolism with humans [27] underlines the potential for the pig as a large animal model for studying human diseases.

The porcine major histocompatibility molecule (MHC) is referred to as swine leukocyte antigen (SLA). Based on a next-generation sequencing (NGS) approach [28], Göttingen minipigs expressing the SLA-2*03:01 allele were selected for the vaccine trial. Synthetic 30-31mer IDO-derived peptides comprising *in silico* predicted SLA-2*03:01-binding 8-11mer peptides, potential $CD8^+$ T-cell epitopes, were

designed. Göttingen minipigs were immunized via the intraperitoneal (i.p.) route with the 30-31mer IDO-derived peptides formulated in CAF09; a dimethyldioctadecylammonium bromide liposomal adjuvant comprising synthetic monomycolyl glycerol and the TLR3 agonist poly I:C [29]. Using this immunization strategy, we show a break in peripheral tolerance and establishment of an IDO-specific immune response in this large animal model. While a CAF09-formulated high peptide dose generated a mixed CMI and humoral immune response towards IDO, immunization with a low peptide dose induced an antigen-specific CMI-dominant response. Combined, these data demonstrate the importance of peptide dose and suggest that the pig may serve as a physiologically relevant large animal model for preclinical cancer vaccine research.

2. Methods

2.1 Animals

Fifteen Göttingen minipigs were purchased from Ellegaard A/S (Denmark), maintained at the National Veterinary Institute, Technical University of Denmark, and randomized into groups based on SLA-class I allele profile, sex, litter, and weight ($n=5$). All animal procedures were approved by the institutional committee and the Danish Animal Experiments' Inspectorate (Ethical approval ID: 2012−15−2934−00557). All procedures comply with the ARRIVE guidelines.

2.2 NGS-based SLA-typing

RNA extraction and subsequent generation of cDNA were performed as previously described [30]. The SLA-profile of each animal was determined using a NGS-based approach described elsewhere [28]. Four of the fifteen animals included in the study did not conclusively express the SLA-2*03:01-allele and were distributed into each of the immunization groups (two in the high peptide dose group).

2.3 Peptide library design

The Uniprot database (http://www.uniprot.org/uniprot/F6K2E8) was used to obtain the porcine IDO protein sequence. Using the NetMHCcons1.1 server [31], 8-11mer potential SLA-2*03:01-binding peptides were identified within the IDO sequence; a total of ten peptides were synthesized and referred

to as peptide 1-10 (**Table 1**). Four long 30-31mer peptides; referred to as IDO1, IDO2, IDO3, and IDO4, were selected for immunization (**Table 1**); each comprising at least two SLA-2*03:01-predicted binders (peptide 1-10). The peptides were purchased (Pepscan, Presto BV) and contained a free acid at the C-terminal as well as a free amine at the N-terminal. All peptides were dissolved to a concentration of 5 mg/ml in sterile DMSO followed by five min sonication.

2.4 Peptide-MHC affinity ELISA

The ability of peptide 1-10 to form peptide-MHC complexes with SLA-2*03:01 was evaluated as previously described [32]. Briefly, seven-point 5-fold titration dilutions of each peptide starting from a final concentration of 16.7 µM were folded for 48 hours with SLA-2*03:01 heavy (final concentration 2 nM) and β2m light chains (final concentration 15 nM) generated in *E. coli* for determination of the K_D value. An 11-point 2-fold dilution standard curve using a pre-folded human HLA-A2 in complex with β2m and the peptide FLPSDYFPSV [33] was included to calculate the absolute sample complex concentrations.

2.5 Immunizations

Animals were immunized with either 1 µg, 10 µg, or 100 µg of each immunization peptide (IDO1, IDO2, IDO3, and IDO4) formulated in CAF09 adjuvant as previously described [29]; the adjuvant was kindly provided by Dennis Christensen from Statens Serum Institut, Denmark. For each injection, animals received 2 ml immunization comprised of 1 ml CAF09 and 1 ml of peptide pool diluted in 10 mM Tris buffer. A total of nine immunizations were performed, distributed at day 0, 14, 27, 41, 70, 83, 97, 173, and 186. All injections were delivered via the i.p. route; no anesthesia was used. At day 70, 83, and 97; tetanus toxoid (TT) was mixed into the vaccine formulation in similar concentration as the IDO peptides for each group. An experimental outline can be found in **Supplementary table 1**.

2.6 Peripheral blood mononuclear cell isolation

Animals were blood sampled using sodium heparinized vacutainer tubes (BD Diagnostics), and peripheral blood mononuclear cells (PBMCs) were purified using SepMate tubes (StemCell Technologies) according to manufacturer's protocol. Briefly, the blood was diluted 1:1 in PBS/2%FBS (Thermo Fischer Scientific) and separated using Lymphoprep (StemCell Technologies). If necessary,

red blood cells were lysed using an in-house made lysis buffer. The cells were counted using the Nucleocounter NC-200 (Chemometec).

2.7 IFN-γ ELISpot

IFN-γ ELISpot responses were evaluated from day 0 to 111 (**Supplementary table 1**). The general assays details have been described elsewhere [34]. In brief, the plates were coated with 5 µg/ml mouse anti-swine IFN-γ antibody (Thermo Fischer Scientific). AIM V^{TM} media (Thermo Fischer Scientific) was used for blocking, and $1x10^5$-$2x10^5$ PBMCs were added to each well with incubation in the presence of 1.5 µg/ml IDO1-IDO4, 1.5 µg/ml *staphylococcal enterotoxin B* (SEB) (Sigma Aldrich) as positive control, or media alone. Biotin mouse anti-pig IFN-γ antibody (BD Biosciences) was used at 1µg/ml. Streptavidine-alkaline phophatase conjugate (Sigma Aldrich) was diluted 1:2000. Each well received 100µl BCIP®/NBT liquid substrate system (Sigma Aldrich) and spot development was terminated after five min. The AID EliSpot Reader version 6.0 (Autoimmun Diagnostika GmbH) was used for analysis. Data is shown as spot forming cells (SFCs) per $2x10^5$ PBMCs with subtraction of the background IFN-γ spot numbers from PBMCs cultured with media alone.

2.8 IgG ELISA

The presence of antigen-specific IgG antibodies was evaluated in serum samples from day 0 to 111 (**Supplementary table 1**) using an indirect ELISA as described elsewhere [34]. Briefly, the plates were coated with 1 µg/ml of IDO1, IDO2, IDO3, and IDO4. Serum samples were diluted 1:40 and incubated with biotinylated goat anti-pig IgG (Bio-Rad); diluted 1:20,000. HRP-conjugated streptavidin (Thermo Fischer Scientific), diluted 1:8000, was added followed by addition of tetramethylbenzidine (Kem-En-Tec) for 5-10 min. The reaction was terminated with 0.5 M sulfuric acid. The absorbance at 450 nm was determined using a microplate reader (Thermo Fischer Scientific); corrections for non-specific background were done by subtraction of the 650 nm signal.

2.9 *In vivo* cytotoxicity

Animals were immunized nine times prior to performing an *in vivo* cytotoxicity assay. Freshly isolated PBMCs were washed twice in PBS to remove any serum and counted using the Nucleocounter NC-200. A total of $15x10^7$ cells per animal were isolated and split into two groups. Target cells were labeled with Cell Proliferation Dye eFluor450® (TermoFischer Scientific) and the control cells with Cell Proliferation Dye eFluor670® (Thermo Fischer Scientific) according to manufacturer's protocol. Dyes have previously been swapped to make sure no dye-specific effect occurs. The control and target cells were cultured overnight at 37°C, 5% CO_2. Target cells were pulsed with a pool of peptide 1-10 (10 µg/ml of each peptide) for 1 hour at 37°C, 5% CO_2. Control cells remained non-pulsed. Correct labelling was evaluated using flow cytometry prior to intravenous (i.v.) re-infusion. The animals were fasted from the day before and anaesthetized using an intramuscular injection with 1 ml/10-15kg of Zoletil mix (tiletamine 12.5 mg/ml, zolazepam 12.5 mg/ml, xylazin 12.5 mg/ml, ketamine 12.5 mg/ml, and butorphanol 2.5 mg/ml). For i.v. administration, a 22GA 0.9 x 25 mm venflon (BD Bioscience) was inserted in the ear vein and flushed with 2 ml sterile PBS. A 1:1 mixture of target and control cells, resuspended in approximately 1.8 ml PBS, was injected followed by flushing with 4 ml sterile PBS. Animals were blood sampled by venipuncture from the jugular vein 10 min post administration of the cells, and PBMCs were isolated as already described. Isolated PBMCs were acquired using an LSRFortessa (BD Bioscience), and the ratio between target and control cells was compared at 10 min (baseline samples) and 24 hours post injection. Data were analyzed using FlowJo Data Analysis Software version 10. Cells from one animal in the 1 µg group were not stained properly prior to injection and left out of analysis.

2.10 Statistical analysis

Despite low numbers of animals, the data were analysed by parametric analyses as 85-100% of datasets showing a significant difference to baseline data passed the Shapiro-Wilk normality test. Thus, results are shown as the mean ± SEM. Statistical comparisons were performed using either paired or unpaired Student's t-test, and GraphPad Prism version 7.00 for Windows (California, United States) was used for all statistical analysis. $P<0.05$ (*) was considered significant, and $P<0.005$ (**) is indicated.

3. Results

3.1 The immunization peptides encompass potential CD8$^+$ T-cell epitopes with the ability to form peptide-MHC complexes with SLA-2*03:01

Immunization with long synthetic peptides has been shown to generate more efficient and long-lasting cytotoxic T lymphocyte (CTL) responses when compared to immunization with a minimal CTL epitope alone [35–38]. For this reason, the selected immunization peptides were naturally occurring 30-31mers containing *in silico* predicted 8-11mer SLA-2*03:01-binding peptides. Ten peptides were predicted as either strong binders (%rank ≤ 0.50%) or weak binders (%rank ≤ 2.00%) (**Table 1)**. The capacity of the ten peptides to form peptide-MHC complexes with SLA-2*03:01 was investigated using a peptide-MHC affinity ELISA. K_D values, indicative of the peptide-MHC binding affinity, were ranging from 448 nM to 25,457 nM (**Table 1**). In detail, 40% of the predicted strong binders had a K_D value < 500 nM, while 20% of the predicted weak binders had a K_D value < 5,000 nM. As different MHC class I alleles bind peptides with different size, affinity, and immunogenicity [39], we did not attempt to conclude on the hierarchy of the peptides based on the K_D values. Nevertheless, seven of the ten predicted peptides showed complex formation with SLA-2*03:01 (**Table 1**); thereby, the peptides may be presented to CD8$^+$ T cells *in vivo*.

3.2 Repeated i.p. immunization with CAF09-formulated long IDO-derived peptides induces an antigen-specific CMI response

We firstly evaluated if repeated i.p. immunization with CAF09-formulated peptides was sufficient to break peripheral tolerance and induce an antigen-specific CMI response. Following seven immunizations, animals immunized with 1 µg CAF09-adjuvanted peptides displayed significant IFN-γ production in response to all four peptides (IDO1-4) when compared to baseline samples (**Fig. 1a-d**, left panel). An intermediate peptide dose showed some sporadic, yet not significant, responses when compared to baseline samples (**Fig. 1a-d**, middle panel). As for the low dose group, animals immunized with a CAF09-formulated high peptide dose displayed IDO-specific IFN-γ$^+$ cells in response to re-stimulation with all four peptides (**Fig. 1a-d**, right panel).

3.3 The magnitude of the CMI response is independent of CAF09-formulated peptide dose

As both low and high antigen dose significantly induced IFN-γ^+ cells in response to IDO-derived peptides when compared to baseline samples (**Fig. 1**), we evaluated whether the level of IFN-γ SFCs differed between the groups. No statistical significant difference could be observed between the levels of IFN-γ-responsive cells towards any of the four peptides (**Fig. 2**), and the kinetics, by which the responses developed, was also rather similar between the groups (**Fig. 2**). Together, the magnitude of the anti-IDO CMI response generated upon repeated i.p. immunization was independent of the CAF09-formulated peptide dose. The addition of TT in the immunization protocol did not affect the CMI response generated towards IDO, as the magnitude of the IFN-γ response was already increasing at day 70 (prior to the first TT injection).

3.4 A CAF09-formulated high peptide dose induces antigen-specific IgG antibodies

We have recently shown that a high exogenous antigen dose formulated in CAF09 adjuvant induces antigen-specific IgG antibodies in Göttingen minipigs [34]. Using an indirect ELISA, we evaluated if the amount of IDO-specific IgG antibodies generated upon immunization was also affected by the antigen dose. When compared to the seronegative baseline samples, immunization with a CAF09-formulated low peptide dose did not induce any sustained humoral immune response (**Fig. 3a-d**, left panel). Significant IgG-production was observed in the intermediate dose group only in response to IDO3 and IDO4 (**Fig. 3a-d**, middle panel). Upon repeated immunization with a CAF09-formulated high peptide dose, a humoral immune response was demonstrated for all the peptides; however, only anti-IDO2 and anti-IDO4 IgG production were statistically significant when compared to baseline samples (**Fig. 3a-d**, right panel).

3.5 The magnitude of the IDO-specific humoral immune response correlates with peptide dose

As expected, no difference in the baseline levels of IgG antibodies was observed across the groups (**Fig. 4**). Repeated immunization with a CAF09-adjuvanted high peptide dose significantly induced more IDO-specific IgG antibodies towards all four peptides when compared to the 1 µg group (**Fig. 4**). Animals in the intermediate peptide dose group were superior in generating antigen-specific IgG antibodies, when compared to the low peptide dose group, for IDO3 and IDO4 only (**Fig. 4**). Combined, our data demonstrate that the vaccine-induced humoral immune response correlates with

the dose of an endogenous peptide formulated in CAF09 adjuvant. Again, no adjuvant effect of TT was observed.

3.6 Re-infusion of fluorescently labeled IDO-pulsed cells does not reveal target-specific lysis

In order to evaluate the quality of the CMI response, we developed a porcine *in vivo* cytotoxicity assay directly measuring the capacity of immune-mediated target cell lysis. The assay was based on re-infusion of fluorescently labeled autologous control and target cells. For all groups, control and target cell populations were detectable in the baseline blood samples withdrawn 10 min post re-infusion (**Fig.5a**, upper panel). However, the control and target cell populations were more pronounced 24 hours post injection (**Fig. 5a**, lower panel); suggesting that 10 min might not be the optimal time point for baseline sampling. The ratio between control and target cells was used to assess potential killing of IDO-pulsed cells. A few animals displayed an increase in control:target cell ratio 24 hours post i.v. injection, although the overall trend did not reveal *in vivo* specific lysis of IDO-pulsed cells (**Fig.5b-d**).

4. Discussion

In this study, we showed that it is possible to break peripheral tolerance towards an endogenous antigen in Göttingen minipigs by repeated i.p. immunizations with CAF09-formulated peptides. All animals were antigen-naïve prior to the first injection, as no pronounced antigen-specific CMI or humoral immune response was detectable in baseline samples. Hence, the observed anti-IDO immune response was vaccine-induced.

In outbred pigs, we have previously shown induction of a weak, yet detectable, CMI response towards CAF09-formulated IDO-derived peptides following two subcutaneous immunizations [30]. However, the responses appeared rather transient; thus, we set to optimize our immunization strategy. Since murine studies have shown that i.p. delivery of a CAF09-formulated antigen is superior in generating a CTL response when compared to subcutaneous injection [40], we repeatedly immunized Göttingen minipigs via the i.p. route. While the peptide pool in the previous study contained 20mer overlapping IDO-derived peptides [30], our four immunization peptides (**Table 1**) were specifically designed to

contain potential $CD8^+$ T cell epitopes, as this T-cell subset is a key mediator of anti-tumor immune responses [41].

In this current study, we showed peptide-MHC class I complex formation for 70% of the predicted SLA-2*03:01-binding peptides. Despite this, the *in vivo* processing of the 30-31mer immunization peptides remains unknown. Therefore, the immunization peptides might encompass $CD4^+$ T-cell epitopes, and the IFN-γ produced in the PBMC cultures could originate from $CD8^+$ T cells, $CD4^+$ T cells, and/or $CD4^+CD8\alpha^+$ T cells. Since activation of natural killer cells or γδ T cells is independent of peptide presentation by MHC molecules [42, 43], the IFN-γ response to our immunization strategy with long synthetic peptides is unlikely to depend on these cells. Importantly, we have recently shown, in the same animals, that repeated immunization with CAF09-adjuvanted full-length exogenous protein via the i.p. route generated a CTL response rather than a T helper cell response [34]. This, in conjunction with the demonstrated peptide-SLA-2*03:01 complex formation, suggests that IDO-specific cytotoxic $CD8^+$ T cells are activated using this immunization strategy. However, numbers of IDO-specific $CD8^+$ T cells were too few to analyze by phenotypic characterization or SLA-peptide tetramers in flow cytometry.

In humans, peptide-based therapeutic immunization has shown successful induction of anti-tumor immune responses, but the magnitude of the response is often low, transient, and might not correlate with clinical benefit [44]. We performed an *in vivo* porcine cytotoxicity assay to evaluate the quality of the induced anti-IDO CMI response. Comparison of the relationship between control and target cells at baseline (10 min) and 24 hours post re-infusion did not show convincing *in vivo* cytotoxicity towards IDO-pulsed target cells, although a few animals displayed potential target-specific lysis. The baseline blood sample for *in vivo* cytotoxicity assays is commonly withdrawn 10 min post i.v. injection in smaller animals [45]. To our knowledge, this assay has never been performed in a large animal like the pig. Thus, we speculate a potential delay in the lungs, which is not an uncommon phenomenon upon i.v. administration of cells [46, 47]. Consequently, 10 min might be too early for withdrawal of the baseline sample. Further studies should evaluate different time points for the baseline, before any conclusions can be made regarding the impact of antigen dose on the *in vivo* quality of the CMI response.

Surprisingly few studies evaluate the influence of antigen dose on the immune response, but the majority have suggested that low antigen dose favors a Th1 response, whereas a Th2 response is induced upon exposure to a high antigen dose [48–50]. Specifically, the number of responsive $CD4^+$ T cells in conjunction with the antigen dose was suggested to determine the Th1/Th2 nature of the immune response [51, 52]. Moreover, an inverse relationship between antigen dose and the induction of a polyfunctional $CD4^+$ T-cell response has been demonstrated in mice and humans [53–55]. We recently evaluated the TT-specific immune response in the same animals and demonstrated induction of a humoral immune response upon a CAF09-formulated high antigen dose, while a low antigen dose induced a polyfunctional CTL response [34]. To our knowledge, our IDO-immunization trial is the first study evaluating the dose effect of an endogenous vaccine antigen in a large animal model. Interestingly, our findings support that repeated immunization with low dose endogenous peptides specifically induces a CMI-dominant response. Combined, our data show the importance of vaccine antigen dose and suggest that the pig may serve as a valuable large animal model for future preclinical testing of cancer immunotherapies.

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Conflict of interest

The authors have no conflicts of interest to declare.

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Experimental design: NHO, TMF, and GJ. Experimental work: NHO, TMF, and JTJ. Data analysis and interpretation: NHO, MLS, MR, and GJ. Manuscript and figure preparation: NHO. Manuscript revision: NHO, TMF, JTJ, MLS, MR, SB, MHA and GJ. All the authors approved the final manuscript.

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Peptide	Sequence	Length	%Rank	Predicted binder	K_D (nM)
IDO1	MALDWWSPMDNSWKIFEEYHIDEDLGFALP	30 aa	-	-	-
Peptide 1	ALDWWSPM	8 aa	0.50	SB	N/D
Peptide 2	HIDEDLGFAL	10 aa	0.17	SB	909
IDO2	NSWKIFEEYHIDEDLGFALPNPLEELPHPY	30 aa	-	-	-
Peptide 2	HIDEDLGFAL	10 aa	0.17	SB	909
Peptide 3	ALPNPLEEL	9 aa	1.50	WB	25457
IDO3	LLDITSSLHKALEVFHQIHEYVDPKLFFNVL	31 aa	-	-	-
Peptide 4	LLDITSSL	8 aa	0.50	SB	N/D
Peptide 5	YVDPKLFF	8 aa	0.80	WB	17746
Peptide 6	YVDPKLFFN	9 aa	2.00	WB	4729
Peptide 7	YVDPKLFFNV	10 aa	0.12	SB	448
Peptide 8	YVDPKLFFNVL	11 aa	0.07	SB	468
IDO4	GSAAGFLQEMRTYMPPAHRNFLHSLESGPS	30 aa	-	-	-
Peptide 9	FLQEMRTYM	9 aa	2.00	WB	N/D
Peptide 10	YMPPAHRNFL	10 aa	0.80	WB	6918

Table 1 The immunization library consists of four long IDO-derived peptides comprising potential $CD8^+$ T-cell epitopes. Göttingen minipigs were immunized with four IDO-derived 30-31mer peptides (referred to as IDO1, IDO2, IDO3, and IDO4). Each immunization peptide was designed to contain either potential strong binders (SB) and/or potential weak binders (WB) based on NetMHCcons1.1 prediction towards the SLA-2*03:01 allele with indication of the %rank score. The location of each 8-11mer peptide within the given immunization peptide is indicated. Peptide 2 is part of both IDO1 and IDO2; hence listed twice. The K_D values were obtained using a peptide-MHC affinity ELISA with recombinant SLA-2*03:01. Abbreviation: ND = not determined.

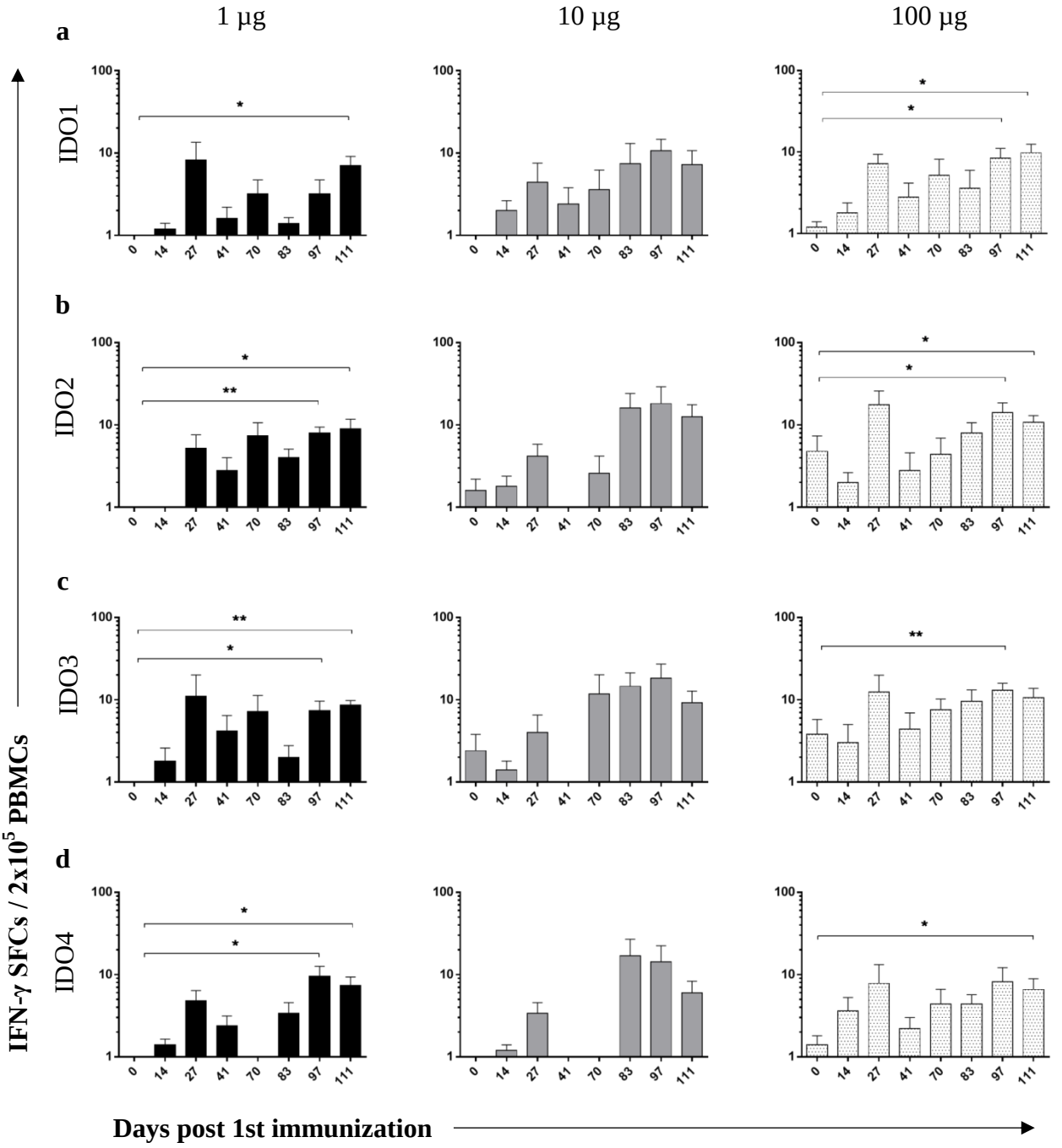

Fig. 1 Intraperitoneal administration of both a low and high dose IDO-derived peptides induces a cell-mediated immune response. Göttingen minipigs were i.p. immunized with IDO-derived peptides formulated in CAF09 adjuvant. IFN-γ ELISpot responses at the indicated time points from animals receiving either 1 µg (black bars), 10 µg (grey bars), or 100 µg (white spotted bars) of IDO1 (a), IDO2 (b), IDO3 (c), or IDO4 (d) are shown. Background values are subtracted, and the data is shown as number of IFN-γ spot forming cells (SFCs) per $2x10^5$ PBMCs; bars represent mean values ±SEM, (*n=5*). Statistical analysis on non-transformed data by paired Student's t-test.

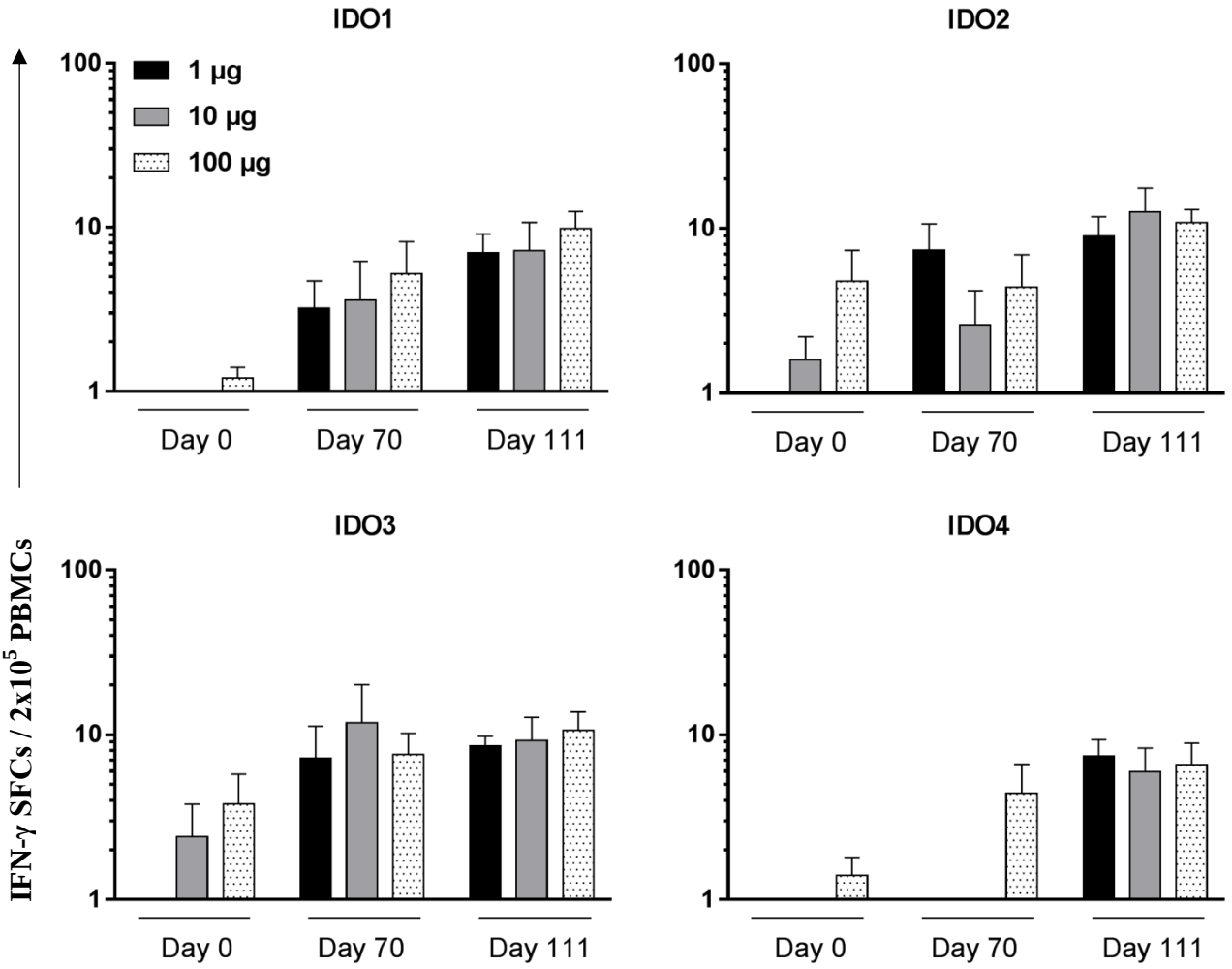

Fig. 2 The level of IDO-specific IFN-γ SFCs is independent of the antigen dose. Göttingen minipigs were immunized i.p. with IDO-derived peptides formulated in CAF09 adjuvant. The level of IFN-γ SFCs in response to IDO1, IDO2, IDO3, and IDO4 were evaluated across the treatment groups. Animals receiving 1 µg (black bars), 10 µg (grey bars), or 100 µg antigen (white spotted bars) were compared. Data is shown as IFN-γ SFCs per $2x10^5$ PBMCs. Background values were subtracted. Bars represent mean values ±SEM, (*n=5*). Statistical analysis on non-transformed data by unpaired Student's t-test.

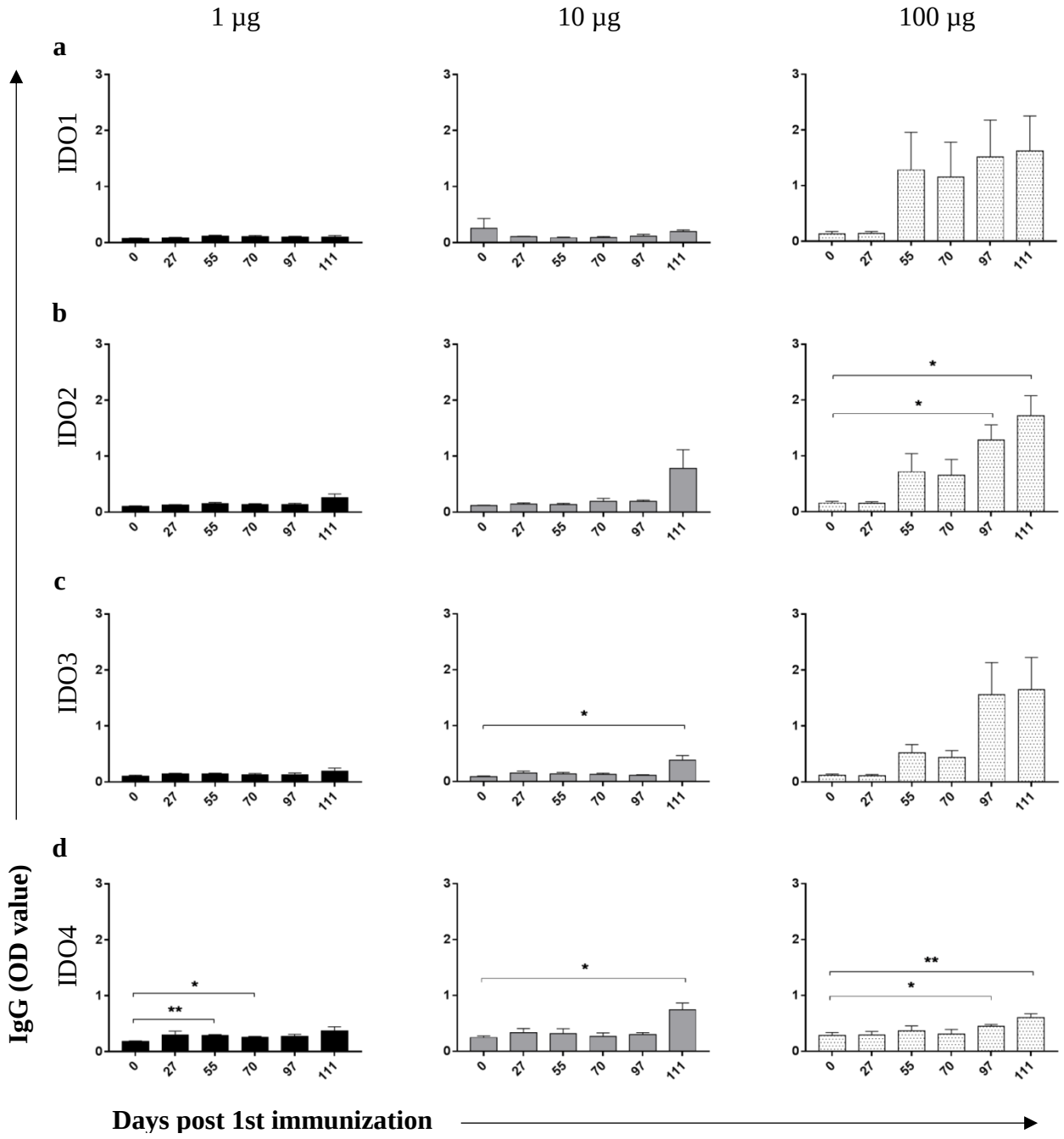

Fig. 3 Immunization with a high peptide dose generates IDO-specific IgG antibodies Göttingen minipigs were i.p. immunized with IDO-derived peptides formulated in CAF09 adjuvant. Antigen-specific IgG antibodies were evaluated in serum samples using an indirect ELISA. Anti-IDO IgG responses towards IDO1 (a), IDO2 (b), IDO3 (c), and IDO4 (d) are shown from animals receiving 1 µg (black bars), 10 µg (grey bars), or 100 µg antigen (white spotted bars). Data is shown as optical density (OD) values; bars represent mean values ±SEM, (*n=5*). Statistical analysis by paired Student's t-test.

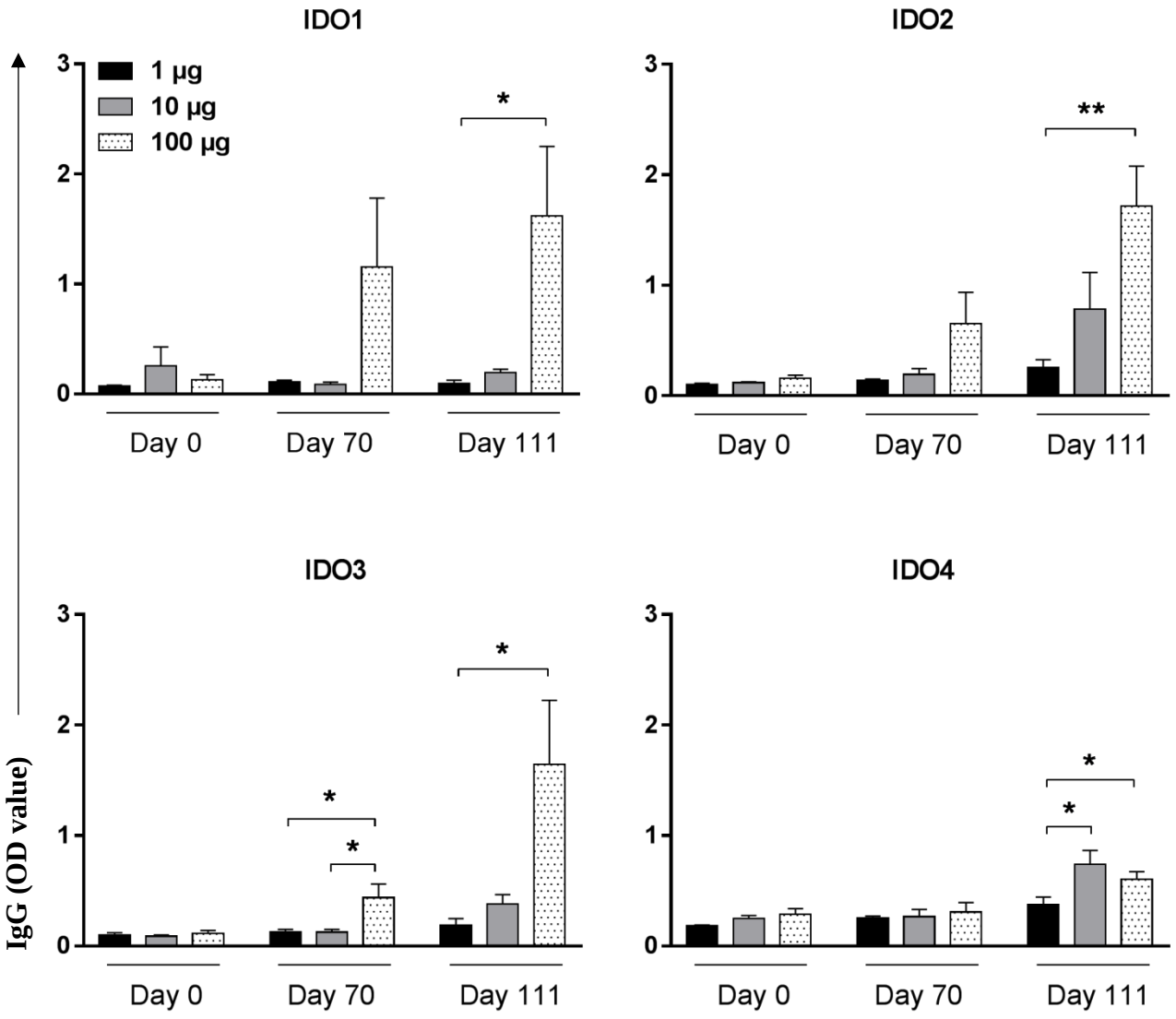

Fig. 4 The level of vaccine-induced antigen-specific humoral immune response correlates with the CAF09-formulated peptide dose. Göttingen minipigs were i.p. immunized with IDO-derived peptides formulated in CAF09 adjuvant. The level of IgG antibodies towards IDO1, IDO2, IDO3, and IDO4 in serum samples was evaluated across groups. Animals immunized with 1 µg (black bars), 10 µg (grey bars), or 100 µg (white spotted bars) were compared. Data is shown as optical density values; bars represent mean values ±SEM, (*n=5*). Statistical analysis by unpaired Student's t-test.

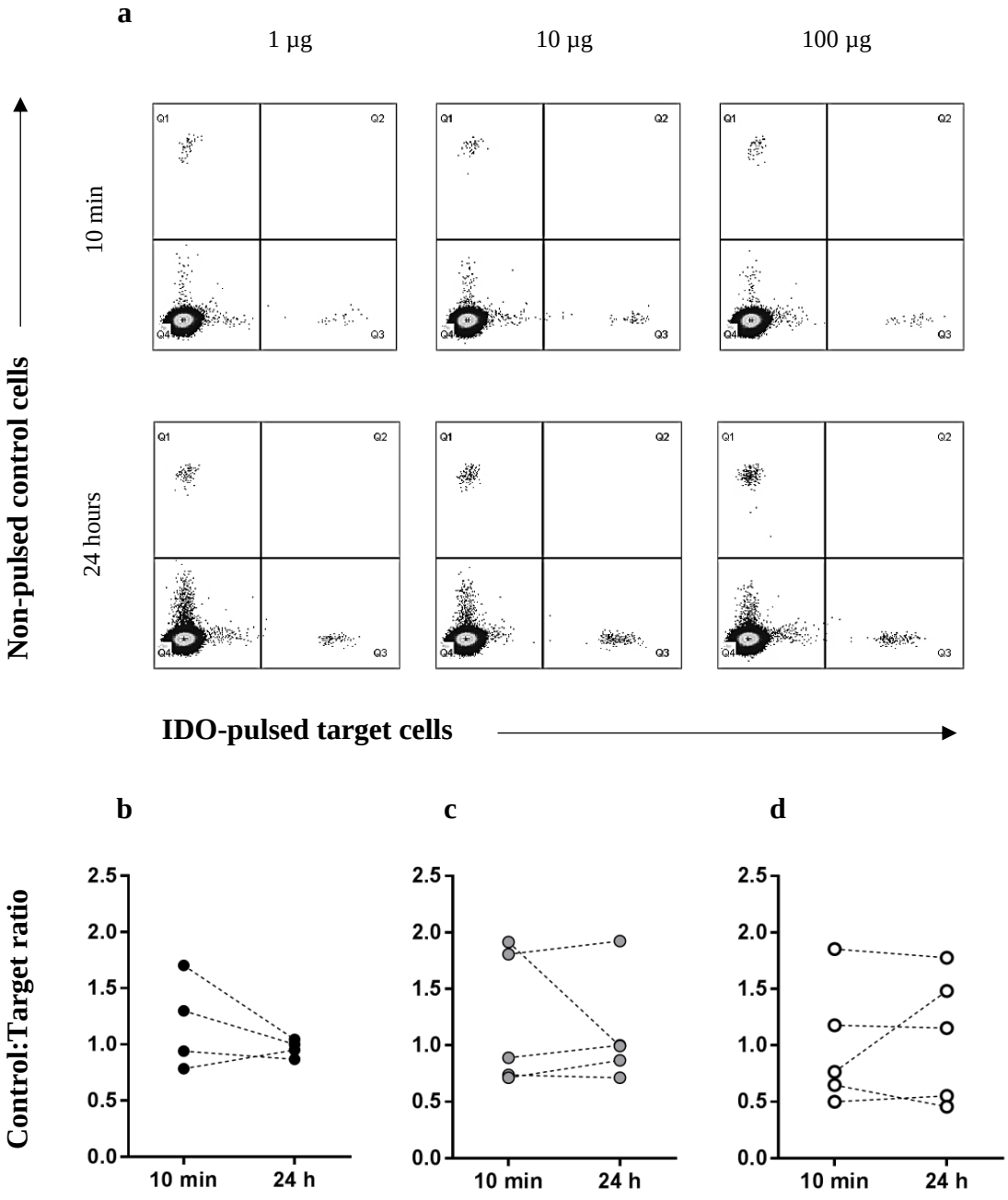

Fig. 5 Fluorescently labeled IDO-pulsed target cells are detectable but not specifically lysed following intravenous re-infusion to immunized donor animals. PBMCs were purified from all animals following nine rounds of immunization. Control cells remained non-pulsed (eFluor670-labeled) and target cells were pulsed with a pool of peptide 1-10 (eFluor450-labeled). A 1:1 mixture of control:target cells were intravenously re-infused into each donor animal for evaluation of *in vivo* cytotoxicity towards IDO-presenting cells. (a) The relationship between control and target cells was determined using flow cytometry on samples obtained 10 min post injection (baseline) and 24 hours post injection. Representative animals are shown. The control:target cell ratio was evaluated in animals immunized with 1 µg (b), 10 µg (c), and 100 µg (d) antigen.

Outline of immunization trial

Day	Treatment	ELISpot	IgG ELISA
0	IDO immunization	X	X
14	IDO immunization	X	-
27	IDO immunization	X	X
41	IDO immunization	X	-
55	-	-	X
70	IDO + TT immunization	X	X
83	IDO + TT immunization	X	-
97	IDO + TT immunization	X	X
111	-	X	X
173	IDO immunization	-	-
186	IDO immunization	-	-
195	-	-	-
200-203	*In vivo* cytotoxicity	-	-

Supplementary table 1 Outline of the immunization trial Göttingen minipigs were randomized into three groups and immunized seven times with either 1 µg, 10 µg, or 100 µg of IDO1-4, (*n=5*). The peptides were formulated in CAF09 adjuvant and delivered via the intraperitoneal route. The immunizations were performed with two week intervals; however, a resting period was included both after the 4^{th}. Tetanus toxoid was included in the immunizations at day 70, 83, and 97. ELISpot (purified PBMCs) and IgG ELISA (serum samples) were performed at the indicated time points. Finally, two additional immunizations were performed prior to an *in vivo* cytotoxicity assay. Abbreviations: TT, tetanus toxoid.

Paper III

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Genetically Induced Tumors Invoke a Robust Anti-Tumor Immune Response in the Oncopig Model

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Genetically Induced Tumors Invoke a Robust Anti-Tumor Immune Response in the Oncopig Model

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Abbreviations:

AdCre	Adenoviral vector Cre-recombinase
Cat	Catalogue number
CFSE	Carboxyfluorescein succinimidyl ester
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
HCC	Hepatocellular carcinoma
IDO1	Indoleamine 2,3-dioxygenase 1
IHC	Immunohistochemistry
I.m.	Intramuscular
PDL1	Programmed death-ligand 1
S.c.	Subcutaneous
Treg	Regulatory T cells

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Abstract

In recent years, immunotherapy has shown considerable promise in the management of several malignancies. However, the majority of preclinical studies have been conducted in rodents, the results of which often translate poorly to patients given the substantial differences between murine and human immunology. As the porcine immune system is far more analogous to that of humans, we set to determine whether pigs may serve as a supplementary preclinical model for testing such therapies. We have generated a large animal model, the Oncopig, with inducible tumor formation resulting from concomitant $KRAS^{G12D}$ and $TP53^{R167H}$ mutations under control of an adenoviral vector Cre-recombinase (AdCre). Following injection of AdCre, the transgenic Oncopig cells express the mutated transgenes, which results in tumor formation at the site of AdCre exposure. The objective of this study was to characterize the tumor microenvironment in this novel animal model with respect to T-cell responses in particular and to elucidate the potential use of Oncopigs for the preclinical testing of cancer immunotherapies. We observed pronounced T-cell infiltration to the tumors with a strong $CD8\beta^{+}$ predominance. Additionally, these intratumoral T cells were found to have increased expression of the cytotoxic marker perforin when compared to the circulating T-cell pool. Similarly, there was robust granzyme B staining localizing to the tumors; affirming the presence of cytotoxic immune cells within the tumor. In addition, the tumor displayed enrichment in regulatory cells as demonstrated by increased levels of FoxP3-expressing T cells when compared to peripheral blood. To investigate the immunogenicity of the tumor cells themselves, we developed a fluorescence-based *in vitro* porcine cytotoxicity assay and demonstrated pronounced killing of autologous tumor cells in an effector:target cell dependent manner. By RNA-seq analysis, we showed increased gene expression of Indoleamine 2,3-dioxygenase 1 (*IDO1),* Cytotoxic T-lymphocyte-associated protein 4 *(CTLA4),* and Programmed death-ligand 1 (*PDL1)* in Oncopig tumors, suggesting an *in vivo* suppression of T-cell effector functions. Combined, these results demonstrate the propensity of the porcine immune system to recognize and mount a cytotoxic response against tumor cells *in vitro*, and suggest that the Oncopig may serve as a valuable model for future preclinical testing of immunotherapies aimed at reactivating this tumor-directed cytotoxicity *in vivo*.

1. Introduction

For decades, preclinical studies pertaining to novel cancer therapies have relied on animal models of disease. Traditionally, rodents have been the gold standard for cancer research providing invaluable insights into the interplay between the immune system and tumor cells. However, despite these numerous advances, mice often fail to fully recapitulate human cancers, and many promising preclinical therapies have failed to have similar success in the clinic (1,2). Beyond the differences in disease pathogenesis and progression between rodents and humans (3–5), due to size constraints rodents often do not allow for the investigation of new surgical interventions (4,6). In light of the numerous obstacles presented by rodent models of disease, alternative model systems have been proposed, including zebrafish (7,8), cats (9), dogs (9–14), and pigs (15–22). Due to homology in physiology, anatomy, size, genetics, metabolism, life span, and immunome between humans and pigs (15,23–25), a porcine model may be extremely relevant for preclinical testing of cancer treatments. Further, in contrast to murine cells, both porcine and human somatic cells demonstrate suppressed telomerase expression in most tissues that is reactivated during cancer development (26,27). For this reason, induction of oncogenesis in humans and pigs generally requires a greater number of genetic defects than in mice (3,6). To determine the relevance of the pig as a platform for immunotherapy, we employed the Oncopig model with inducible oncogenic RAS and dominant-negative P53 (28). Upon exposure to an adenoviral vector Cre-recombinase (AdCre), the infected cells of the transgenic Oncopig acquire two driver mutations: $KRAS^{G12D}$ and $TP53^{R167H}$; two of the most common genetic abnormalities in human cancer (28,29).

The ability of tumor cells to avoid immune destruction has been included as a hallmark of tumorigenesis (30). To this end, immune checkpoint inhibitors have shown tremendous promise in the clinic (31–33). However, when predicting patient responsiveness to such immunotherapies, the number and types of intratumoral immune cells are a key factors (34–37). The Immunoscore suggests a new classification of cancer, where the tumor microenvironment plays an important role, and the relationship between intratumoral immune cells and patient prognosis is taken into account (38–40). This new approach currently serves as a prognostic tool for colorectal cancer; however, the universal applicability of the Immunoscore as a prognostic strategy in various cancer types remains to be fully validated (41). Given the importance of the intratumoral immune

cells in both prognosis and response to therapy, we performed a characterization of the immunological landscape in Oncopig tumors in order to evaluate the applicability of the model for studying anti-tumor immune responses and for future testing of immunotherapies in a large and relevant *in vivo* system.

2. Materials and Methods

2.1 Pigs

The *KRAS*^{G12D} and *TP53*^{R167H} floxed Oncopigs (28) were neither sex- nor age-matched, and all animals were housed at the University of Illinois, Urbana-Champaign, United States. F1 animals homozygous for the transgenes were used for experiments. All animal experiments were carried out in accordance with both national and international guidelines. The University of Illinois Institutional Animal Care and Use Committee (IACUC; Protocol number 14126) approved all procedures.

2.2 AdCre injections for tumor induction

All animals were anesthetized using an intramuscular (i.m.) injection of Telazol®-Ketamine-Xylazine, 1 ml/50 lbs. The AdCre (Ad5CMVCre-eGFP, Gene Transfer Vector Core, University of Iowa, batch: Ad3500 or Ad3743, catalogue number (cat.): VVC-U of Iowa-1174) was used for triggering tumors *in vivo*, and the preparation was previously described elsewhere (28,42). Briefly, AdCre was diluted with minimal essential medium (Corning, cat.: 50-011) containing 2 M calcium chloride resulting in a final concentration of calcium chloride of 0.01 M. Following dilution, the final concentration of AdCre ranged from $1x10^9$ to $2x10^9$ PFU/ml. The mixture was allowed to incubate at room temperature (RT) for 15 min prior to injection. For all subcutaneous (s.c.) injections, a total volume of 1 ml AdCre was injected. For i.m. injections, animals received 0.5 ml or 1 ml. All AdCre injections were carried out using a 21 gauge needle and completed within 45 min from the time of incubation. Animals were monitored every second day, and tumor measurements was carried out using a caliper. All animals were euthanized 7-21 days post AdCre injection.

2.3 Immunohistochemistry (IHC)

Tissues were fixed in 10% formalin and paraffin-embedded. Slides were sectioned at 4 µm interval and all subsequent steps were carried out at RT. Heat-induced epitope retrieval was carried out using a Menarini Access Retrieval Unit with a sodium citrate buffer (pH 6) for 1 min 40 sec at 125°C, full pressure. The slides were then loaded onto a Dako Autostainer and rinsed with a Tris/Tween buffer (pH 7.5) prior to treatment with Dako Real TM Peroxidase blocking solution (Agilent Technologies, cat.: S202386-2) for 5 min followed by buffer rinse (Tris/Tween, pH 7.5) for an additional 5 min. Slides were then treated with the primary antibody: Polyclonal Rabbit Anti-Human CD3 (Agilent Technologies, cat.: A045201-2) diluted in Dako universal diluent (Agilent Technologies, cat.: S080981-2) and stained for 30 min. Two rounds of 5 min buffer rinse (Tris/Tween, pH 7.5) were carried out prior to secondary staining with Dako EnVision+ System-HRP Labelled Polymer Anti rabbit (Agilent Technoligies, cat.: K400211-2) for 30 min. The slides were then rinsed twice (Tris/Tween, pH 7.5) and treated with 3,3'-diaminobenzidine (DAB)+ substrate-chromogen system (Agilent Technologies, cat.: K346889-2) for 10 min. Finally, the slides were washed thrice in H_2O and counterstained with Gills Haematoxylin (Sigma-Aldrich, cat.: GHS1128) for 27 sec followed by additional wash in H_2O.

2.4 Immunofluorescence

Tissues were fixed in 10% formalin, embedded in paraffin, and sectioned at 4 µm intervals. For immunofluorescence, slides were heated in a pressure cooker using DAKO Target Retrieval Solution (Agilent Technologies, cat.: S170084-2), blocked for 1 hour at RT with Innovex Background Buster (Innovex, cat.: NB306) with 5% Fc Receptor Block (Innovex, cat.: NB309), and incubated with primary antibodies against CD3 (Santa Cruz Biotech, cat.: sc-20047), CD8α (Santa Cruz Biotech, cat.: sc-7188), or Granzyme B (abcam, cat.: ab134933) at 1:100-200 overnight at 4°C. Slides were mounted in a DAPI containing medium (Santa Cruz) and visualized using either Alexa Fluor 488 (abcam, cat.: ab150113) or Alexa Fluor 594 (abcam, cat.: ab150080) conjugated secondary antibodies.

2.5 Cell isolation

Animals were blood sampled into BD sodium heparinized vacutainer tubes (BD Diagnostics, cat.: 362753) and purified using SepMate tubes (StemCell Technologies, cat.: 85450) according to manufacturer's protocol. Briefly, sodium heparinized blood was diluted 1:1 in PBS/2%FBS (ThermoFischer Scientific, cat.: 10082147) prior to separation using Lymphoprep (StemCell Technologies, cat.: 07851) with centrifugation settings at 1200 G for 20 min at 4°C. Cells were subsequently washed twice and counted using a hemocytometer. Viable cells were distinguished from dead cells using Trypan blue (Sigma-Aldrich, cat.: T0887). For isolation of cancer cells from *in vivo*-induced tumors; a 1 cm^3 tumor biopsy was harvested and cut into small pieces before incubation in pre-heated RPMI-1640 containing 2% FBS, 3 mg/ml Collagenase D (Sigma-Aldrich, cat.: COLLD-RO), 5 µg/ml DNase I (Sigma-Aldrich, cat.: 11284932001), and 1 µg/ml Dispase II (Sigma-Aldrich, cat.: 04942078001) for 90 min at 37°C. Samples were vortexed every 30 minutes to facilitate digestion. Cells were then passed twice through a 70 µm cell strainer to obtain a single cell suspension. Processing was completed within 6 hours for all cells. Cells were counted using the Nucleocounter NC-200 (Chemometec, Allerød, Denmark) and 10^7 cells per vial of PBMCs or tumor cells were cryopreserved for subsequent analysis. FBS/10%DMSO was used as freezing medium, and every vial was placed in a Mr. Frosty freezing container at -80°C within three minutes of exposure to DMSO. The vials were transferred to liquid nitrogen 24 h later for long term storage.

2.6 Flow cytometry

Antibodies were used at pre-determined optimal concentrations (**Supplementary Table 1**). Cryopreserved PBMCs and tumor cell suspensions were thawed in RPMI-1640/20%FBS and subsequently washed twice in PBS/0.5%FBS. The median viability post thawing was 91.7% as determined by the Nucleocounter NC-200, and ~$4x10^6$ cells per sample were stained for flow cytometry. The samples were then surface stained for 30 min at 4°C with a combination of anti-CD3, anti-CD4, anti-CD8α, anti-CD8β antibodies, and a live/dead stain allowing viable cells to be distinguished from dead cells. For detection of FoxP3, cells were fixed post surface staining using the Anti-Mouse/Rat Foxp3 Staining Set (ThermoFischer Scientific, cat.: 72-5775-40) according to manufacturer's protocol. Cells were then incubated with anti-FoxP3 antibody for 30

min at 4°C. For intracellular cytokine staining, samples were first cultured for 16 hours at 37°C, 5% CO_2 in RPMI-1640/10%FBS medium; serum was pretested in cell stimulation assays prior to use. As a positive control, 1 µg/ml PHA (Sigma-Aldrich, cat.: L4144) was used for stimulation. To block cytokine secretion, cells were then cultured for additional 6 hours in the presence of 10 µg/ml Brefeldin A (Sigma-Aldrich, cat.: B7651-5MG). Following surface stain with antibodies listed in Supplementary Table 1, cells were then fixed using the Fixation/Permeabilization Solution Kit (BD Biosciences, cat.: 554714) according to manufacturer's protocol and stained with a mixture of anti-IFN-γ, anti-TNF-α, and anti-perforin antibodies for 30 min at 4°C. To detect $KRAS^{G12D}$ by flow cytometry, the Fixation/Permeabilization Solution Kit was used directly with no pre-culturing in the presence of Brefeldin A. For all staining procedures, fluorescence minus one controls were included. Samples were acquired using an LSR II (BD Biosciences, Albertslund, Denmark) or an LSRFortessa (BD Bioscience, Albertslund, Denmark) flow cytometer, and the PMT voltages were adjusted based on a mixture of unstained cells resulting in a mean auto fluorescence intensity of ~10^2 for all fluorochromes. The data were analyzed using either FCS Express version 6 (De Novo Software) or FlowJo Data Analysis Software version 10. The analysis was performed on viable, single cells (lymphocytes or tumor cells) with the gating strategy being indicated in each figure legend. Examples of the gating strategies used for analysis are shown (**Fig. S1 & Fig. S2A-B**). For all samples, a minimum of 200,000 T cells were recorded for analysis.

2.7 *In vitro* cytotoxicity

Freshly isolated PBMCs and tumor cells were washed twice with PBS to remove any serum and counted using the hemocytometer and Trypan Blue. Effector cells (PBMCs) remained unlabeled. Control cells (PBMCs) and target cells (isolated tumor cells) were labeled with 10 µM eFluor450® and 5 µM eFluor670® Cell Proliferation Dye (eBioscience, cat.: 65-0842-85 and 65-0840-85), respectively, according to manufacturer's protocol. Briefly, cells were labeled for 10 min at 37°C in the dark and labeling was stopped by adding four-five volumes of cold RPMI-1640/10%FBS. The cells were then incubated on ice for 5 min covered in the dark followed by three washing steps with RPMI-1640/10%FBS. For culturing, a titration of effector:target cell ratio was carried out as follows: 0:1, 0.5:1, 1:1, and 2:1; culturing conditions were 37°C, 5% CO_2.

Samples were harvested at 10 min and 24 hours post co-culturing, fixed immediately with a 4%PFA solution (Fischer Scientific, cat.: 199431LT) to eliminate additional killing or cell turnover. Samples were washed twice in PBS/0.5%FBS and acquired using an LSR II (BD Biosciences) flow cytometer and data were analyzed using FCS Express version 6 (De Novo Software). PMT voltages were once again adjusted according to an unstained sample; the mean auto fluorescence value for each fluorochrome was adjusted to approximately 10^2. For each sample, ~$1.5x10^6$ cells were acquired for analysis. Percentage of specific killing was determined by comparing the percentage change in ratio between control and target cell populations at baseline and 24 hours post co-culture. For each individual animal, data were normalized to background levels of killing/cell turnover from wells with no effector cells added.

2.8 RNA-seq analysis

Previously produced RNA-seq datasets for Oncopig primary hepatocyte cell lines (*n=3*), transformed hepatocyte (hepatocellular carcinoma (HCC)) cell lines (*n=3*), primary fibroblast cell lines (*n=8*), and transformed fibroblast (soft-tissue sarcoma) cell lines (*n=4*) were downloaded from the ENA database (www.ebi.ac.uk/ena) under accession number PRJEB8646 (43,44). In addition, previously produced Oncopig skeletal muscle (*n=3*) and leiomyosarcoma tumor (*n=4*) RNA-seq datasets were downloaded from the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-3382 (28). Raw reads were trimmed, aligned to the swine reference genome (45), and assessed for differential gene expression as previously described (28,43,44)

2.9. Statistical analysis

Despite low numbers of animals, the data were analysed by parametric analyses as 80% of datasets showing a significant difference to baseline data passed the Shapiro-Wilk normality test. Results are shown as the mean ± SEM. Statistical comparisons of mean values were conducted using either paired or unpaired Student's t-test depending on the experimental setup. All statistical analysis was carried out using GraphPad Prism version 7.00 for Windows (California, United States). $P<0.05$ (*) was considered significant. $P<0.005$ (**) and $P<0.001$ (***) are

indicated. In order to take the false discovery rate into account, q-values rather than p-values were used for RNA-seq analysis (44,46). A q-value < 0.05 was considered significant.

3. Results

3.1 AdCre injection results in *KRAS^{G12D}* expression and formation of tumors, which are heavily infiltrated by T cells

To confirm tumorigenesis in this porcine model, Oncopigs were s.c. injected with AdCre, whereupon a tumor could be excised 7-21 days post injection (**Fig. 1A-B**). The tumor was localized to the s.c. tissue and did not invade the adjacent areas (**Fig. 1B**). Since the CAG promoter controls the expression of the two mutated transgenes, *KRAS^{G12D}* and *TP53^{R167H}*, showing the gene product of one or the other transgene is sufficient to confirm successful transformation. Therefore, the presence of *KRAS^{G12D}* was shown at the protein level using intracellular flow cytometry staining of single-cell suspensions obtained from tumor biopsies (**Fig. 1C**). Having confirmed the ability to induce tumors in the Oncopig, we then examined for the presence of intratumoral T cells. Tumor sections obtained from Oncopigs injected with AdCre at two different sites, s.c. and i.m., were stained for the common T-cell marker, CD3, and analyzed using IHC. Independent of the site of AdCre administration, $CD3^+$ cells were found to heavily infiltrate the tumors (**Fig. 1D-G**). Lymph node sections were used as positive controls to validate the $CD3^+$ staining (**Fig. S3A-B**). Since the site of AdCre administration did not affect the T-cell infiltration, s.c. tumors were used for the remaining parts of the study.

3.2 Comparison of circulating and intratumoral T cells reveals a preferential infiltration of $CD8\beta^+$ T cells to the tumor site

Given that T cells do infiltrate the tumors as shown by IHC, the next step was to address which T-cell subsets were present and whether the intratumoral T-cell pool differed from the circulating counterpart. Using flow cytometry, T-cell infiltration was confirmed in the tumor and in peripheral blood (**Fig. 2A**) with subsets of $CD4^+$ T cells (**Fig. 2B**), $CD8\beta^+$ T cells (**Fig. 2C**), and $CD4^+CD8\alpha^+$ T cells (**Fig. 2D**) being readily detectable. Quantification of the percentage of total

T cells revealed no difference between peripheral blood and tumor cell isolates (**Fig. 2E**), indicating that the PBMCs and tumor cell suspensions encompass similar T cells levels. A quantification of the different subsets revealed that the amount of $CD4^+$ T cells, as a percentage of total $CD3^+$ cells, was similar in the tumor and in peripheral blood (**Fig. 2F**). An increased percentage of $CD8\beta^+$ T cells was found at the tumor site (mean values: 39.7% in contrast to 13.3% for the PBMC samples) (**Fig. 2G**), indicating a specific infiltration of cytotoxic T cells to the tumor. In contrast to other species, pigs comprise a substantial $CD4^+CD8^+$ T-cell population (47); and the vast majority of this subset expresses the CD8α homodimer; a characteristic now associated with activation of porcine $CD4^+$ T cells (48). On the other hand, the expression of the CD8α/CD8β heterodimer is linked to conventional cytolytic $CD8^+$ T cells (49). As expected, we observed a pronounced proportion of the circulating $CD4^+$ T cells that expressed the $CD8\alpha^+$ molecule (**Fig. 2H**). This T-cell subset was also present in the tumor microenvironment; although there was an almost three-fold decrease when compared to peripheral blood (mean values: 9.4% versus 26.2%) (**Fig. 2H**).

3.3 The tumor microenvironment of Oncopigs contains cytotoxic immune cells.

To further investigate the nature of the intratumoral T-cell subsets in more detail, PBMCs and tumor samples were investigated for the presence of T cells positive for perforin, TNF-α, and IFN-γ. Using flow cytometry, perforin-producing T cells were observed both in peripheral blood and within the tumor itself (**Fig. 3A**), while T cells producing TNF-α or IFN-γ were not detectable without further stimulation. $CD4^+$ T cells, as expected, barely produced any perforin (**Fig. 3B**); however, a prominent $CD8\beta^+perforin^+$ T-cell population was detected in both peripheral blood and in the tumor (**Fig. 3C)**. When comparing the percentages between the two sites, a greater than four-fold increase in total perforin-producing T cells was observed in the tumor samples over peripheral blood samples (mean values: 26.9% versus 5.8%) (**Fig. 3D**). The very limited, yet still detectable, amount of perforin produced by the $CD4^+$ T cells (**Fig. 3B**) most likely originated from the $CD4^+CD8\alpha^+$ subset, which, using this gating strategy, was not excluded from the analysis (**Fig. S1 versus Fig. S2)**. No difference however, was observed in $perforin^+CD4^+$ T cells between the PBMC and the tumor samples (**Fig. 3E**). Interestingly, an almost three-fold increase in the percentage of $CD8\beta^+perforin^+$ T cells was found in the tumor

when compared to the PBMC samples (**Fig. 3F**); indicating a substantial cytotoxic infiltration to the tumor. To further investigate this observation, immunofluorescence on formalin-fixed tumor sections was performed. First, the pronounced infiltration of $CD3^+$ cells previously observed (**Fig. 1F**) was confirmed (**Fig. 3G**). Secondly, co-localization of the CD3 and the CD8α marker within the tumor was demonstrated, and the number of infiltrates was found to be substantial (**Fig. 3H**). Importantly, and to confirm the presence of cytotoxic immune cells, we examined the tumor for expression of granzyme B by immunofluorescence. DAPI was used as a counterstain, and a considerable amount of intratumoral granzyme B^+ cells were visualized (**Fig. 3I**); thereby, confirming the presence of cytotoxic cells within the tumor. Importantly, the percentage of $CD4^+$, $CD8\beta^+$, and $CD8\beta^+perforin^+$ T cells in PBMCs obtained from tumor bearing and non-tumor bearing pigs did not reveal any difference (**Fig. S4A-C**). An estimate of NK cell representation ($CD3^-CD4^-CD8\alpha^+$) revealed no significant differences between the NK cell percentage in PBMCs and intratumoral cell isolates (mean values: 8.7 versus 7.0, **Fig. S5**).

3.4 Oncopig tumors display increased levels of $FoxP3^+$ T cells

Tumor microenvironments often contain a mixture of immune cells. In addition to the cytotoxic subsets, which were already shown to be present, we looked for various regulatory T cells (Tregs) by flow cytometric detection of the FoxP3 marker. A pronounced population of T cells expressing FoxP3 was readily detected in both peripheral blood and within the tumor (**Fig. 4A**). When comparing the two sites, an elevated representation of $FoxP3^+$ T cells was found within the tumor (**Fig. 4B**), suggesting an intratumoral regulatory compartment. Similar percentages of $CD4^+CD8\alpha^-FoxP3^+$ T cells were found when comparing the PBMC and the tumor samples (mean values: 10.1% and 12.9%) (**Fig.4C**). Although not significant due to a high animal to animal variation, a strong tendency towards an increased amount of $CD4^+CD8\alpha^+FoxP3^+$ T cells in the tumor was observed when compared to peripheral blood (mean values: 16.0% and 2.1%) (**Fig. 4D**). In contrast, the circulating T-cell pool was comprised of a slightly higher amount of potential regulatory $CD4^-CD8\alpha^+FoxP3^+$ T cells; although the percentages were low in general (**Fig. 4E**).

3.5 Autologous tumor cells are specifically killed by immune cells

In addition to the regulatory cells, the tumor microenvironment of Oncopigs indeed comprised cytotoxic immune cells as determined by both flow cytometry and immunofluorescence. However, these data do not directly demonstrate an endogenous anti-cancer immune response. To investigate the capacity of the Oncopig immune system to lyse autologous tumor cells, we developed an *in vitro* fluorescence-based cytotoxicity assay. Isolated effector cells (non-labeled PBMCs) were co-cultured with either autologous targets (eFluor-450-labeled tumor cells) or autologous control cells (eFluor-670-labeled PBMCs); dyes were previously swapped to rule out any dye-specific bias (data not shown). PBMCs were used as control cells, since both healthy, adjacent skin and muscle cells isolated from the same site as the tumor did not allow a clear fluorescence separation.

Prior to assay initiation, correct labeling was verified for both control and target cells (**Fig 5A**). A 2-fold titration of the effector:target cell ratio was performed ranging from 0:1 – 2:1. Samples harvested 10 min post co-culture showed the baseline distribution of control and target cells (**Fig. 5B**, left plot). Notably, culture wells containing effector:control cells and effector:target cells were mixed only at the time of harvesting; samples were then fixed to stop potential additional killing or cell turn over and acquired straight away on the flow cytometer. To determine potential lysis of the tumor cells, samples were harvested 24 hours post co-culture and compared to the 10 min baseline samples (**Fig. 5B**, right plot). The percentage of specific tumor cell killing was quantified and each sample was normalized to its 0:1 effector:target control sample. Interestingly, a significant percentage of specific tumor cell killing was observed in an effector:target cell ratio dependent manner (**Fig. 5C**), thereby, for the first time directly showing an endogenous porcine anti-cancer immune response in the Oncopig model.

3.6. Oncopig tumors display elevated *IDO1, CTLA4,* and *PDL1* expression levels

Indoleamine 2,3-dioxygenase 1 (*IDO1),* Cytotoxic T-lymphocyte-associated protein 4 *(CTLA4),* and Programmed death-ligand 1 (*PDL1)* encode for proteins that are activated during tumor development in humans and play a role in suppressing immune responses, ultimately helping malignant cells escape T-cell mediated killing. In order to determine if these genes are

upregulated in Oncopig tumors, expression levels were investigated using previously produced Oncopig RNA-seq datasets (28,43,44). As expected, increased expression of *IDO1, CTLA4,* and *PDL1* was observed in Oncopig leiomyosarcoma tumors relative to control muscle samples (**Table 1**). No increased expression was observed in Oncopig transformed compared to primary cell lines, indicating the increased expression observed in Oncopig tumors is not simply a result of cellular transformation (**Supplementary Table 2**).

4. Discussion

Though valuable, mice have several inherent limitations in cancer research. In addition to size and anatomical constraints, inbred rodents also do not fully mimic the diversity seen in human patients. Therefore, to establish a more relevant disease model, we performed our studies in the Oncopig; increasing diversity by using non-sex- and non-age-matched animals and restricting the use of littermates. Given the substantial homology between the porcine and human immune system (24), the fully immunocompetent Oncopig model may be an excellent platform studying anti-tumor immune responses and for preclinical investigation of cancer immunotherapies.

To begin to assess the validity of the Oncopig model, we induced mutant transgene expression and tumor formation by s.c. delivery of AdCre. The resulting tumor microenvironment was heavily infiltrated by T cells displaying either a cytotoxic or regulatory phenotype. Theoretically, the increase in percentages of a certain cell subset within the tumor could result from either a consistent infiltration of these cells over time, intranodal proliferation, or efflux of other T-cell subsets from the tumor. For this reason, we do not conclude on exact numbers but report important differences in the representation of various T-cell subsets between the tumor and peripheral blood.

Although anti-tumor immune responses are often evaluated using IFN-γ as readout, granzyme B and perforin release are two highly specific measures of anti-tumor cytotoxicity (50–54). We observed pronounced intratumoral granzyme B production and increased levels of perforin-producing T cells. Combined, the data support a broad cytotoxic response to induced tumors. Nevertheless, the presence of the tumor indicates an intratumoral regulation of these cytotoxic cells.

We observed a robust subpopulation of T cells expressing FoxP3, both systemically as well as in the induced tumors. Recent findings suggest that human T helper cells can transiently upregulate FoxP3 upon activation, though only the T cells stably expressing FoxP3 were found to exhibit a suppressive nature (55). Therefore, the detection of FoxP3 in various intratumoral T-cell subsets in the Oncopig might indicate the presence of newly activated T cells. However, it is well established that FoxP3 is required for the development and maintenance of suppressive regulatory T cells (56,57). Moreover, FoxP3 has been suggested as an exclusive marker for the $CD4^+CD25^+$ Treg lineage in mice (58), and a suppressive $CD8\alpha^+CD25^+FoxP3^+$ T-cell subset has recently been observed in both mice and humans (59). Together, the significant infiltration of FoxP3-expresssing T cells to the tumor site in conjunction with the evident tumor mass suggest a regulatory role for this these immune cells in Oncopig tumors.

Although we show pronounced T-cell infiltration to the tumors, the anti-tumor immune responses demonstrated in our *in vitro* cytotoxicity could be mediated by other immune cell subsets present in the PBMC culture. Potential other subsets, which might mediate the anti-tumor response, include NK cells, γδ T cells, and NKT cells. In fact, porcine NK cells have been shown to display anti-tumor activities against a human cancer cell line (60); however, we did not observe *in vivo* specific NK cell infiltration to the tumor site. As T cells are key players in mediating anti-tumor immune responses (61–63), the significant T-cell infiltration to Oncopig tumors suggests a role for this immune cell subset in facilitating tumor-specific lysis.

In addition to the observed immune cell infiltration and anti-tumor immunity, increased expression of three genes involved in immune suppression (*IDO1, CTLA4,* and *PDL1*) was observed in Oncopig tumors but not in cell lines transformed *in vitro*. The lack of elevated expression *in vitro* indicates these genes are not simply upregulated as a result of cellular transformation, but rather in response to signals from the *in vivo* tumor microenvironment. The increased expression of *IDO1, CTLA4,* and *PDL1* in Oncopig tumors indicates suppression of T cells *in vivo*. Although we showed the capacity of the Oncopig immune system to mediate tumor-specific lysis *in vitro*, elevated expression of the immunosuppressive genes in conjunction with infiltration of regulatory T cells may explain the lack of evident *in vivo* anti-tumor cytotoxicity.

In conclusion, we performed an immunological characterization of Oncopig tumors, which revealed an intratumoral enrichment of cytotoxic and regulatory T cells. Moreover, we for the

first time showed *in vitro* anti-tumor immune responses in this large animal model, and propose a potential mechanism for *in vivo* suppression of anti-tumor immune responses based on elevated expression levels of *IDO1*, *CTLA4*, and *PDL1*. We believe that the Oncopig with its fully competent immune system and high degree of homology with humans provides a crucial platform for studying anti-tumor immune responses and potentially for future preclinical testing of immunotherapies.

Conflict of interest statement

The authors have no potential conflicts of interest to disclose.

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Authorship Contributions

Conceived and designed the experiments: N.H.O., L.A.R., L.B.S., and G.J. Performed the experiments: N.H.O., D.R.P., J.T.J., and L.A.R. Data analysis and interpretation: N.H.O, D.R.P., K.M.S., J.T.J., L.A.R., L.B.S., and G.J. Manuscript preparation: N.H.O., D.R.P., K.M.S., J.T.J., L.A.R., P.J.G., L.B.S., and G.J.

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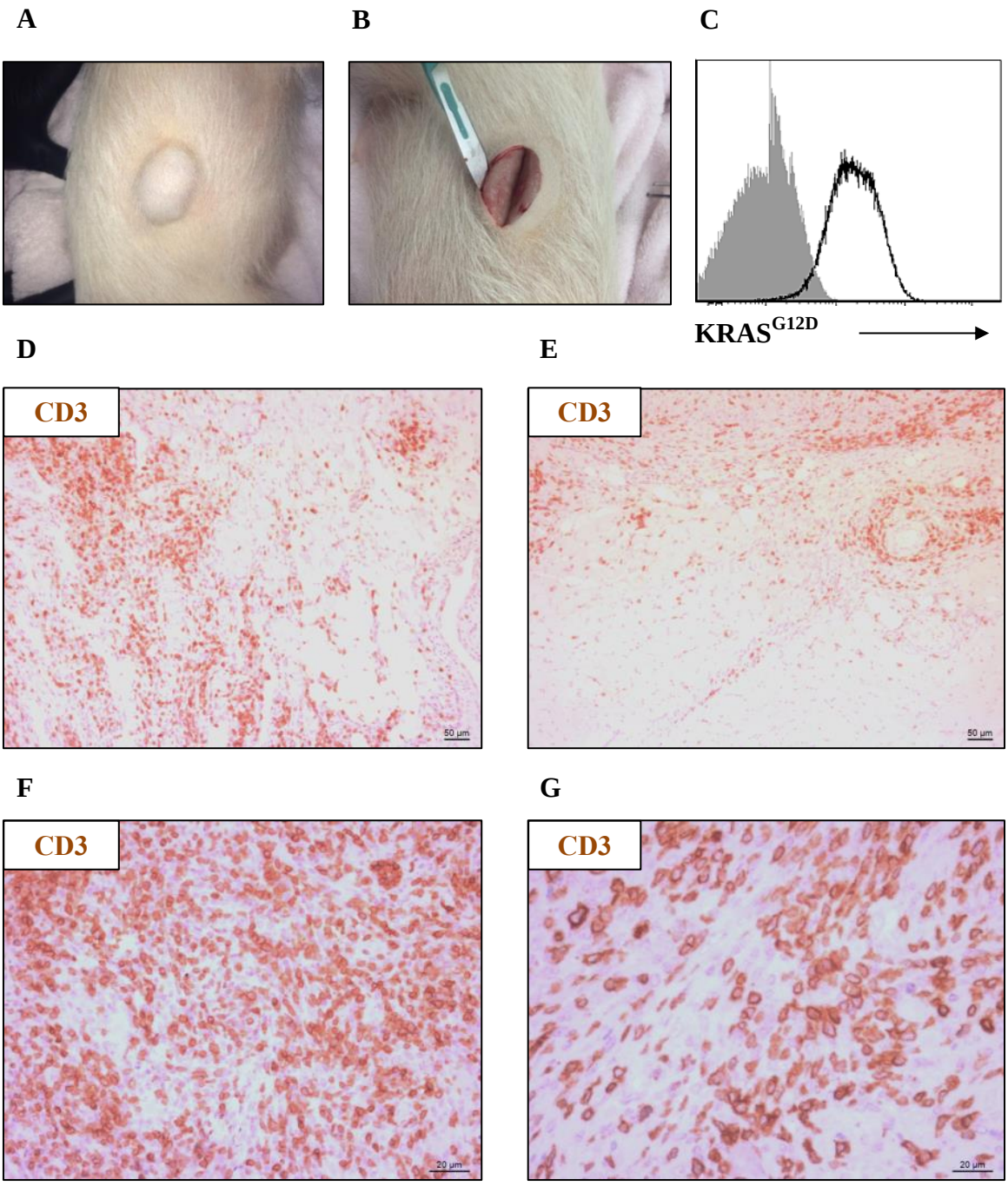


Figure 1. Oncopig tumors are heavily infiltrated by T cells. The *KRAS*^{G12D} and *TP53*^{R167H} floxed Oncopigs were subcutaneously injected with AdCre to induce tumorigenesis. (**A**) Representative image of subcutaneous tumor formation in Oncopigs 7-21 days post subcutaneous injection of AdCre *(n=6.)* (**B**) Cross-section of the subcutaneously formed tumor. Representative image is shown *(n=6)*. (**C**) Representative intracellular flow cytometric plot of *KRAS*^{G12D} expression in isolated tumor cells (white) with FMO control indicated (grey). Oncopigs were subcutaneously (**D, F**) or intramuscularly (**E, G**) injected with AdCre and tumor sections were harvested 20 days post injection. Representative immunohistochemistry images with detection of CD3^{+} cells at x10- (**D, E**) and x40- (**F, G**) magnification are shown *(n=3).*

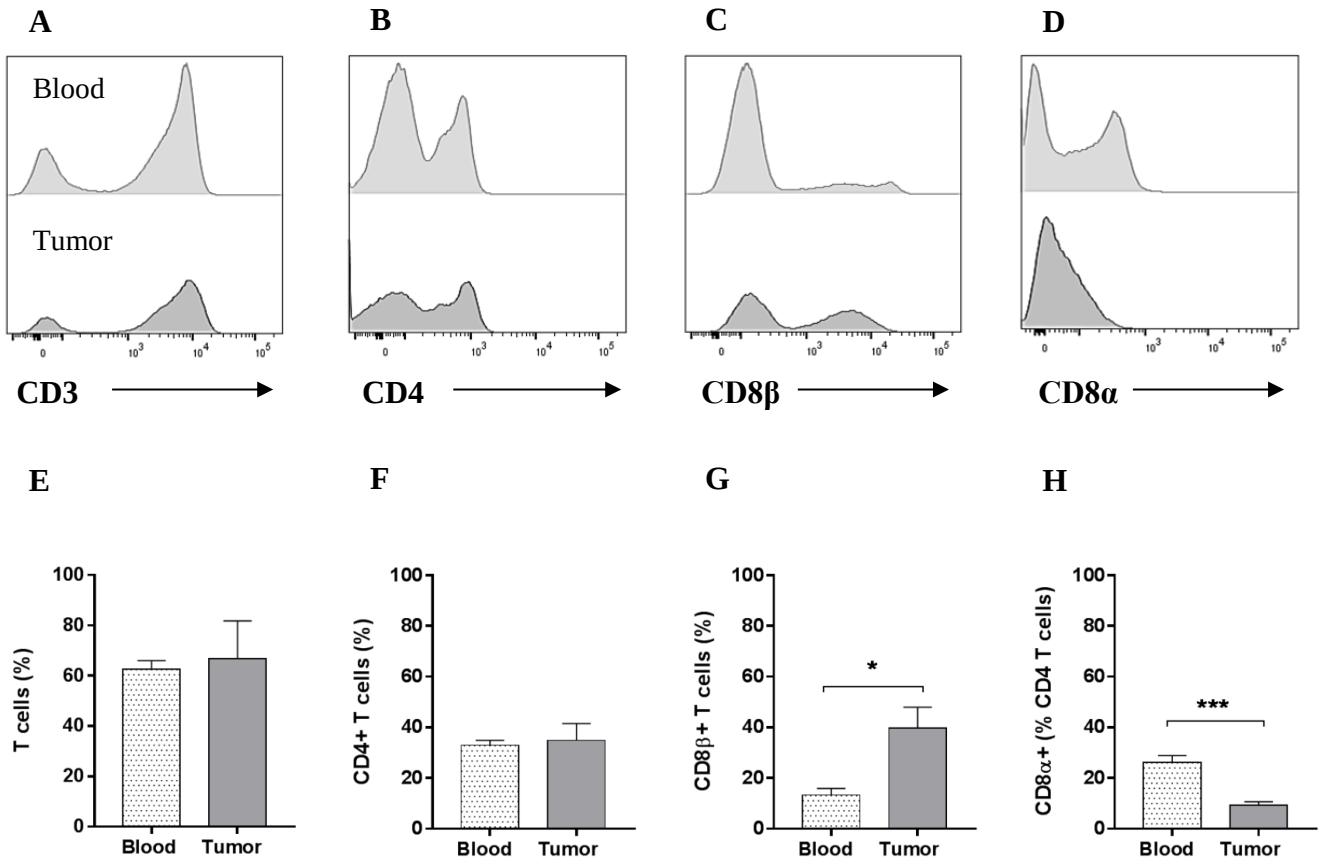


Figure 2. CD8β$^+$ T cells specifically infiltrate the established tumors. Oncopigs were subcutaneously injected with AdCre. PBMCs and tumor tissue were harvested 7-21 days post injection. Representative flow cytometric overlay plots from peripheral blood (upper) and tumor (lower) samples detecting total T cells (**A**), CD4$^+$ T cells (**B**), CD8β$^+$ T cells (**C**), and CD8α expression in CD4$^+$ T cells (**D**). (**E**) Numbers represent CD3$^+$ cells as a percentage of live cells. (**F**) Percentage of CD4$^+$ cells in live, CD3$^+$-gated cells. (**G**) Percentage of CD8β$^+$ cells in live, CD3$^+$-gated cells. (**H**) Percentage of CD8α$^+$ cells in live, CD3$^+$CD4$^+$-gated cells. Bars represent mean values ± SEM and data are from two independent experiments *(n=4-5)*. Statistical evaluation in (E), (F), (G), and (H) by unpaired Student's t-test.

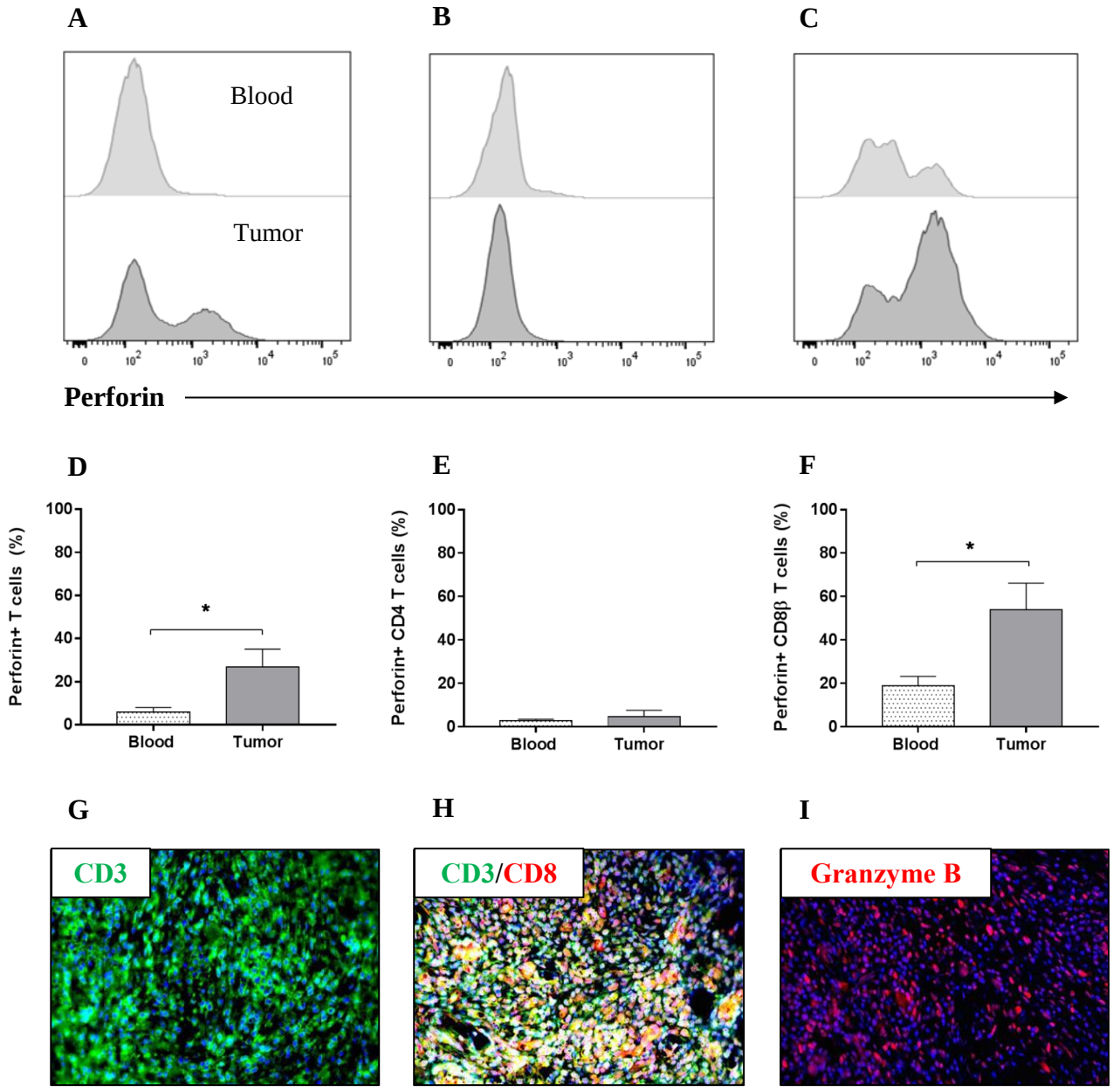


Figure 3. The tumor microenvironment is infiltrated by perforin$^+$ and granzyme B$^+$ immune cells. Oncopigs were subcutaneously injected with AdCre to induce tumor formation. PBMCs and tumor samples were harvested 7-21 days post injection. (**A**) Representative flow cytometric overlay plots from peripheral blood (upper) and tumor (lower) samples detecting perforin expression in total T cells (**A**), in CD4$^+$ T cells (**B**), and in CD8β$^+$ T cells (**C**). (**D**) Numbers represent perforin$^+$ cells as a percentage of live CD3$^+$-gated cells. (**E**) Percentage of perforin$^+$ cells in live, CD3$^+$CD4$^+$-gated cells. (**F**) Perforin$^+$ cells as a percentage of live, CD3$^+$CD8β$^+$-gated cells. Bars represent mean values ± SEM and data are from two independent experiments *(n=4-5)*. Statistical evaluation in (D), (E), and (F) by unpaired Student's t-test. (**G**) Detection of CD3$^+$ cells (green) in a tumor cross-section by immunofluorescence. (**H**) Immunofluorescence image detecting co-localization of CD3$^+$ (green) and CD8α$^+$ (red) cells in the tumor. (**I**) Detection of granzyme B$^+$ cells (red) in a tumor cross-section. DAPI (blue) used as nuclear counterstain for all immunofluorescence images.

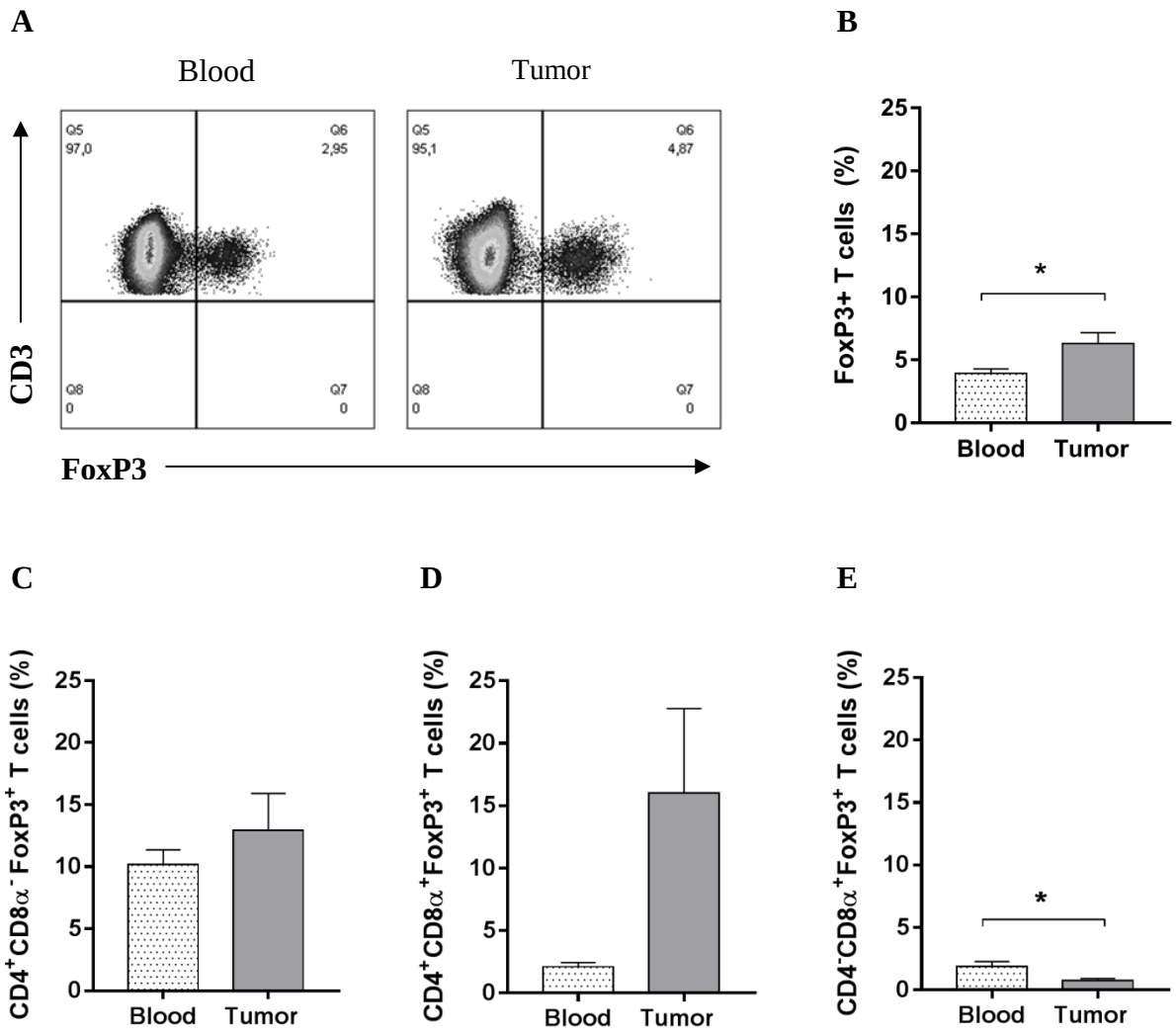


Figure 4. Oncopig tumors display elevated levels of FoxP3$^+$ T cells. Oncopigs were subcutaneously injected with AdCre. Peripheral blood and tumor samples were harvested 7-21 days post injection and analyzed for expression of FoxP3 by flow cytometry. (**A**) Representative flow cytometric plots from peripheral blood (left) and tumor (right) detecting total FoxP3$^+$ T cells. (**B**) Percentage of FoxP3$^+$ cells in live, CD3$^+$-gated cells. (**C**) Percentage of FoxP3$^+$ cells in live, CD4$^+$CD8α$^-$-gated T cells. (**D**) Percentage of FoxP3$^+$ cells in live, CD4$^+$CD8α$^+$-gated T cells. (**E**) Percentage of FoxP3$^+$ cells in live, CD4$^-$CD8α$^+$-gated T cells. All bars represent mean values ± SEM and data are from one experiment *(n=5).* Statistical evaluation in (B), (C), (D), and (E) by paired Student's t-test.

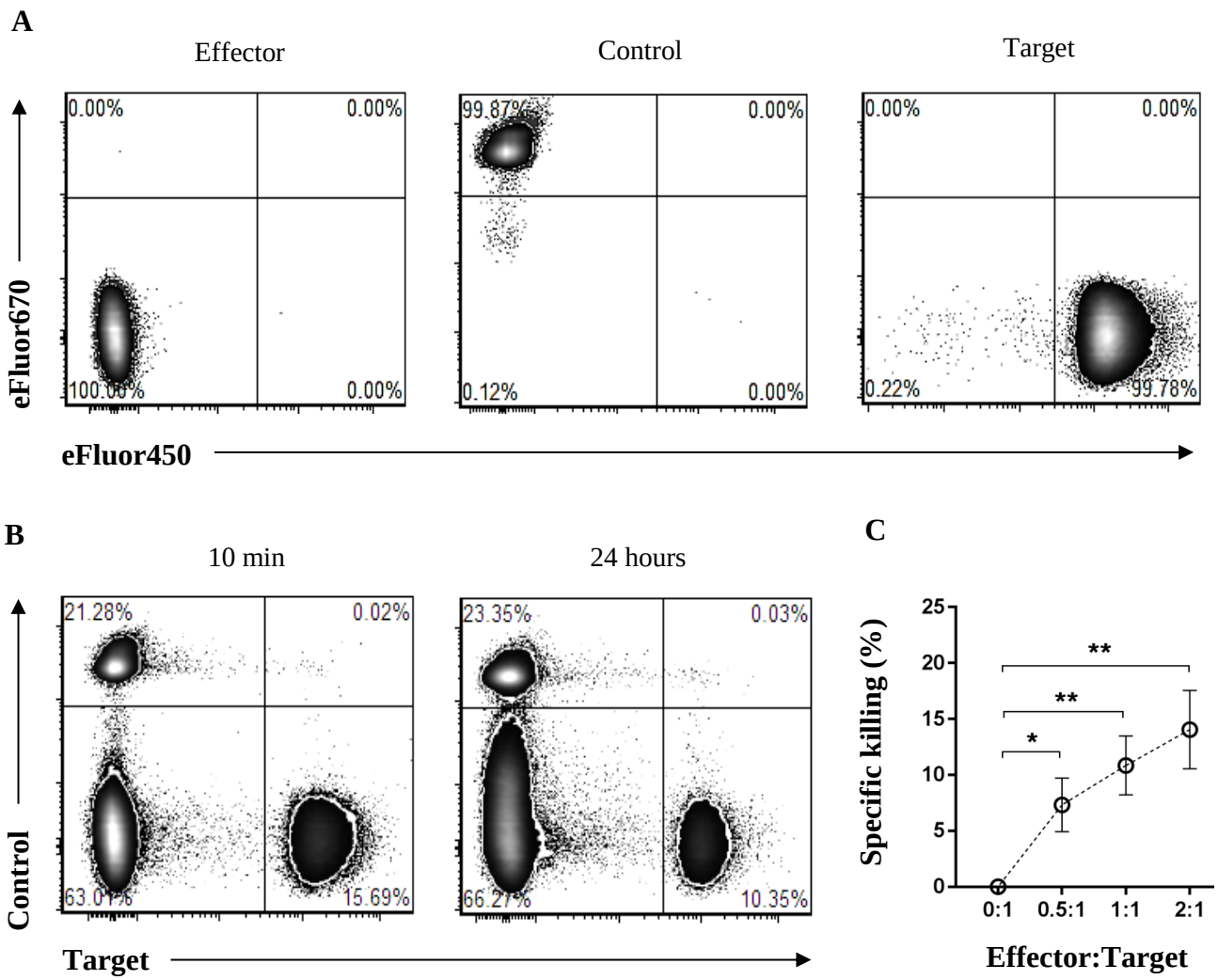


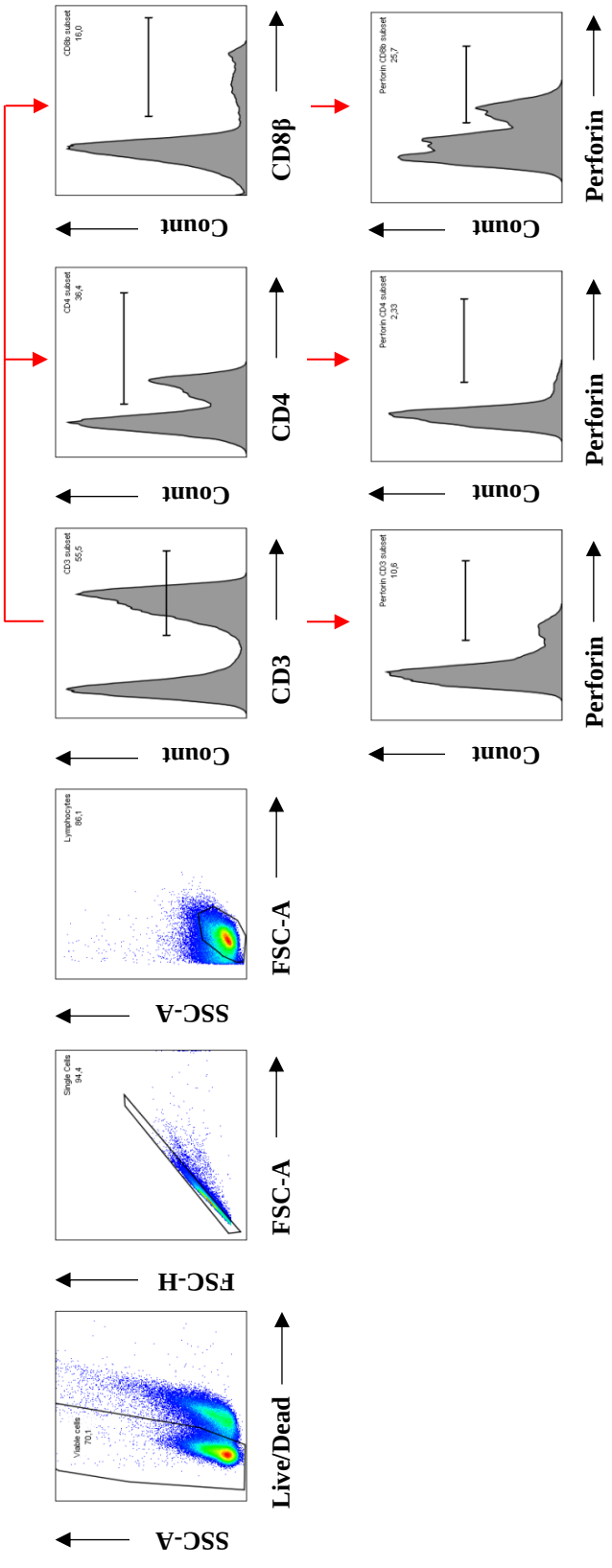
Figure 5. The Oncopig immune system specifically lyses autologous tumor cells *in vitro*. Oncopigs were subcutaneously injected with AdCre to induce tumor formation. Following tumor development (7-21 days post injection), tumor cells and PBMCs were harvested. (**A**) Isolated effector cells remained unlabeled with control cells and tumor cells being labeled with eFluor670 or eFluor450, respectively. (**B**) Representative flow cytometric plots of control and tumor cells at 10 min (baseline, left) and 24 hours (right) post co-culture. (**C**) Numbers show percentage specific killing of tumor cells; data was normalized to adjust for cell turnover in no-effector cells control cultures. A titration of the effector (E) to target (T) cell ratio is shown. Data are from four independent experiments and the data are pooled (*n=8*). Bars represent mean values ±SEM. Statistical evaluation in (C) by paired Student's t-test.

Gene	Skeletal Muscle (FPKM)	Leiomyosarcoma (FPKM)	Log2 fold change	p-value	q-value	Significant
IDO1	0.488057	3.80091	2.96122	5.00E-05	0.000233877	yes
CTLA4	0.133311	1.01914	2.93448	5.00E-05	0.000233877	yes
PDL1	0.343398	1.08631	1.66148	0.00075	0.00276049	yes

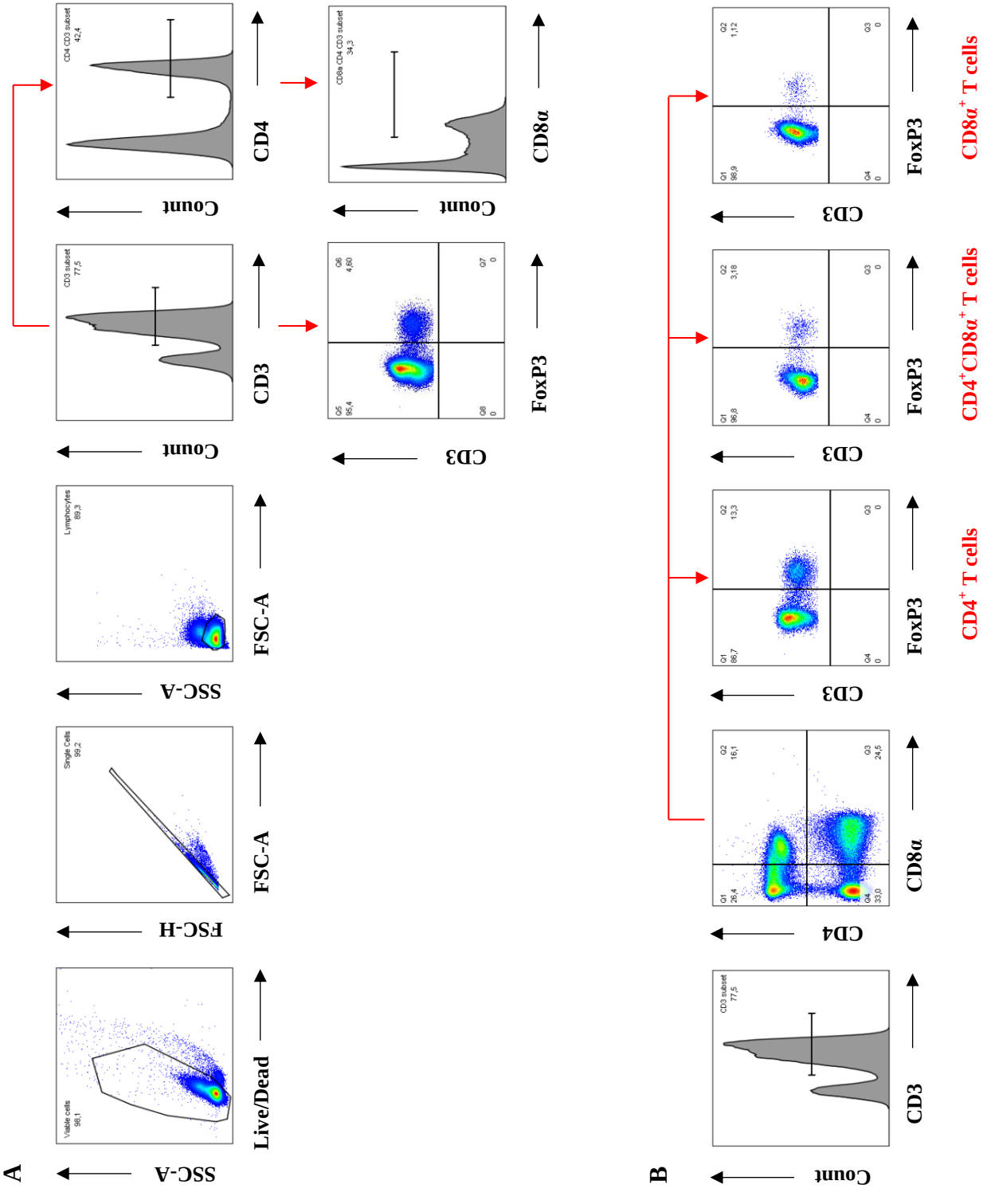
Table 1. Elevated IDO1, CTLA4, and PDL1 expression in Oncopig tumors. Expression values are given as fragments per kilobase of transcript per million mapped reads (FPKM). q-value < 0.05 is considered significant. Abbreviations: CTLA4, Cytotoxic T-lymphocyte-associated protein 4; IDO1, Indoleamine 2,3-dioxygenase 1; PDL1, Programmed death-ligand 1.

Antibodies used for flow cytometry

Marker	Conjugate	Isotype	Clone	Supplier
CD3	Unconjugated	Mouse IgG1	PPT3	Southern Biotech (cat.: 4510-01)
CD3	FITC	Mouse IgG1	PPT3	Southern Biotech (cat.: 4510-02)
CD4	FITC	Mouse IgG2b	74-12-4	BD Biosciences (cat.: 559585)
CD4	PE-Cy7	Mouse IgG2b	74-12-4	BD Biosciences (cat.: 561473)
CD4	PerCP-Cy5.5	Mouse IgG2b	74-12-4	BD Biosciences (cat.: 561474)
CD8 α	AF647	Mouse IgG2aκ	76-2-11	BD Biosciences (cat.: 561475)
CD8 α	PE	Mouse IgG2aκ	76-2-11	BD Biosciences (cat.: 559584)
CD8 β	Unconjugated	Mouse IgG2a	PG164A	Washington State University (cat.: PG2020)
Live/Dead	Aqua	N/A	N/A	Thermo Fischer Scientific (cat.: L34957)
IFN- γ	AF647	Mouse IgG1	CC302	Serotec (cat.: MCA1783A647)
TNF- α	PerCP-Cy5.5	Mouse IgG1κ	MAb11	Biolegend (cat.: 502926)
Perforin	PE	Mouse IgG2bκ	dG9	Biolegend (cat.: 308106)
FoxP3	PE	Rat IgG2aκ	FJK-16s	eBioscience (cat.: 12-5773-82)
IgG2a goat anti-mouse	PE-Cy7	Goat IgG	N/A	Southern Biotech (cat.: 1080-17)
IgG1 rat anti-mouse	BV421	Rat LOU	N/A	BD Biosciences (cat.: 562580)

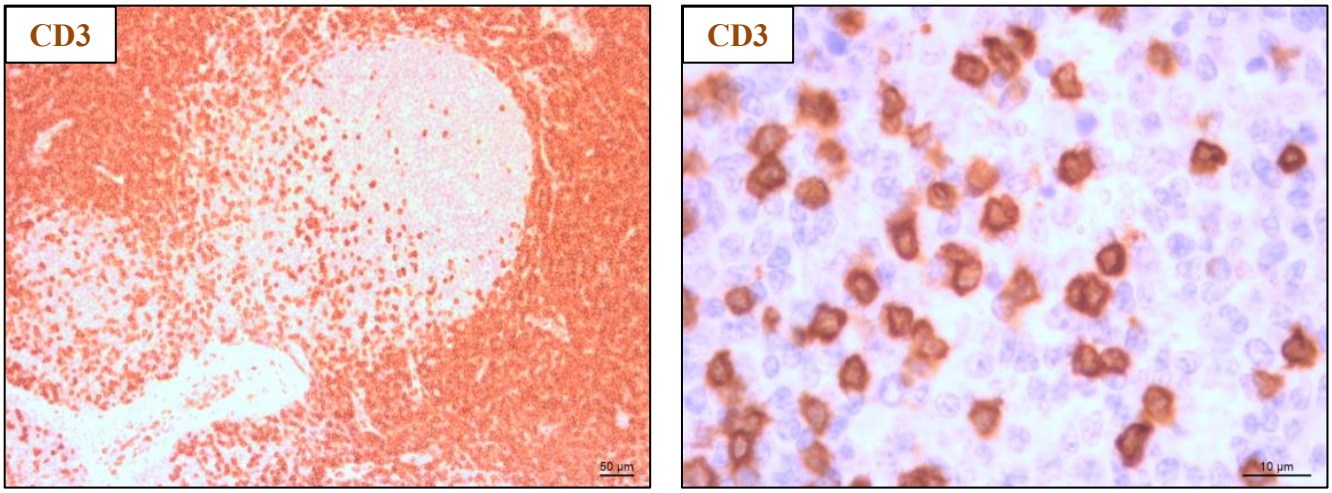


Supplementary Figure 1. Gating strategy used for the flow cytometric analysis of CD4$^+$, CD8β$^+$, and perforin$^+$ cells. Representative gating strategy used for flow cytometry. Firstly, cells were gated on viable cells, singlets, lymphocytes, and CD3$^+$ cells. Perforin production was determined directly in the overall T-cell population. CD3$^+$ cells were then further gated into CD4$^+$ and CD8β$^+$ cells with perforin expression being determined within each of these two T-cell subsets. Data is shown using a representative PBMC sample. The same approach was used for all tumor samples.

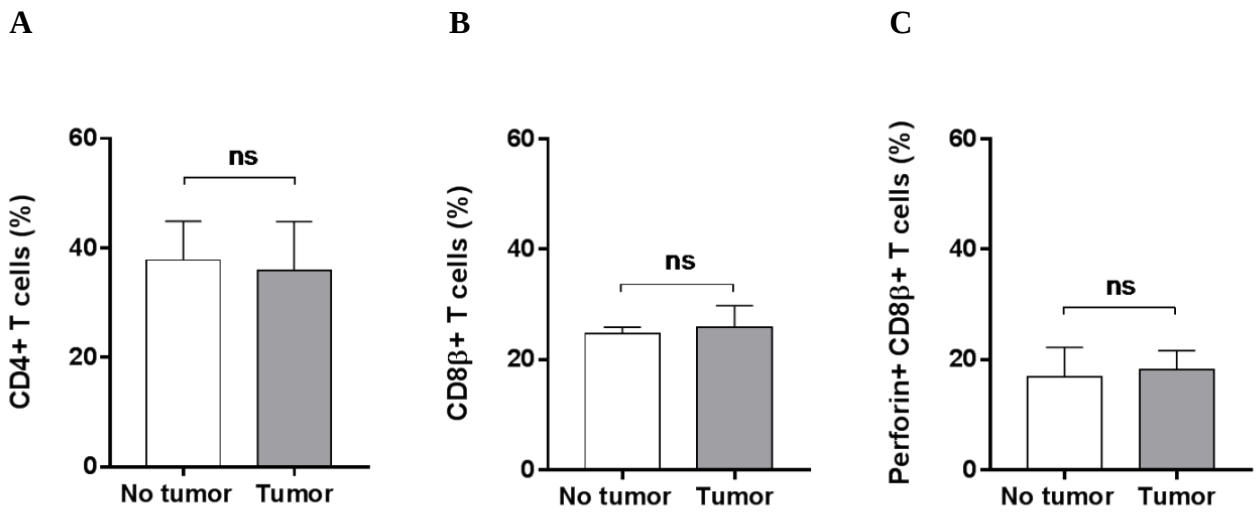

A
SSC-A
Live/Dead
FSC-H
FSC-A
SSC-A
FSC-A
Count
CD3
FoxP3
Count
CD4
Count
CD3
FoxP3
Count
CD8α
B
Count
CD3
CD4
CD8α
CD3
FoxP3
CD3
FoxP3
CD3
FoxP3
CD4+ T cells
CD4+CD8α+ T cells
CD8α+ T cells

Supplementary Figure 2. Gating strategy used for the flow cytometric analysis of $CD4^+CD8\alpha^+$ and $FoxP3^+$ cells. Representative gating strategy used for flow cytometry. (A) Detection of $CD4^+CD8\alpha^+$ T cells and FoxP3 expression in T cells overall. Firstly, cells were gated on viable cells, singlets, lymphocytes, and $CD3^+$ cells. FoxP3 expression was then determined directly in this population. Further gating on $CD3^+$ cells included the selection of $CD4^+$ T cells and lastly detection of CD8α within this population as a measure for $CD4^+CD8\alpha^+$ cells. (B) Detection of FoxP3 within the different T-cell subsets. $CD3^+$ cells were split into $CD4^+$, $CD4^+CD8\alpha^+$, and $CD8\alpha^+$ cells and the presence of $FoxP3^+$ cells within each of these three T-cell subsets was determined. Data is shown using a representative PBMC sample. The same approach was used for all tumor samples.

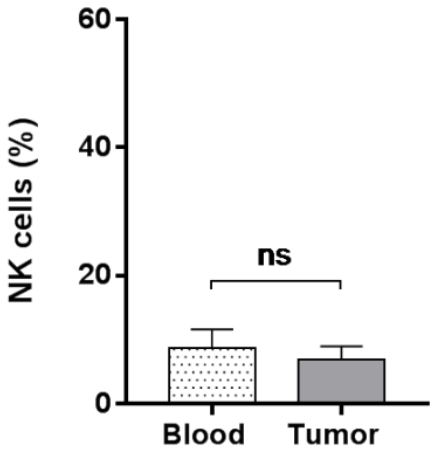
A **B**



Supplementary Figure 3. CD3$^+$ cells in Oncopig lymph nodes. Submandibular lymph nodes were harvested from tumor-bearing Oncopigs and analyzed for the presence of T cells by immunohistochemistry. Representative immunohistochemistry images with detection of CD3$^+$ cells at x10- (**A**) and x63- (**B**) magnification are shown *(n=5).*



Supplementary Figure 4. The presence of a tumor does not alter the systemic T-cell compartment. Peripheral blood samples from tumor-bearing and healthy controls (non-tumor-bearing) were harvested for comparison of their T-cell compartments. (**A**) $CD4^+$ T cells as a percentage of total live, $CD3^+$ cells. (**B**) Percentage of $CD8\beta^+$ T cells as a proportion of total live, $CD3^+$ cells. (**C**) Percentage of $perforin^+$ cells as a proportion of live, $CD3^+CD8\beta^+$ cells. Bars represent mean ± SEM and data are from one experiment (*n=3*). Statistical evaluation by unpaired Student's t-test.



Supplementary Figure 5. Natural killer cells are present but do not specifically infiltrate Oncopig tumors. Peripheral blood samples and tumor cell isolates were harvested for flow cytometric detection of Natural Killer (NK) cells. Numbers represent $CD3^{-}CD4^{-}CD8\alpha^{+}$ cells as a proportion of live cells. Bars represent mean ± SEM and data are from one experiment (*n=3*). Statistical evaluation by paired Student's t-test.

Gene	Primary Hepatocytes (FPKM)	HCC Cell Lines (FPKM)	Log2 fold change	P-value	Q-value	Significant
IDO1	1.15437	0.0406885	-4.82634	0.1494	0.23325	no
CTLA4	0	0	0	1	1	no
PDL1	1.15276	1.53313	0.411391	0.2771	0.370545	no

Gene	Primary Fibroblasts (FPKM)	Transformed Fibroblasts (FPKM)	Log2 fold change	P-value	Q-value	Significant
IDO1	0.0167633	0.676542	5.3348	0.3248	0.527807	no
CTLA4	0	0	0	1	1	no
PDL1	0.457239	0.308961	-0.565522	0.3923	0.595802	no

Supplementary Table 2. IDO1, CTLA4, and PDL1 expression in Oncopig cell lines. Expression values are given as fragments per kilobase of transcript per million mapped reads (FPKM). q-value < 0.05 is considered significant. Abbreviations: CTLA4, Cytotoxic T-lymphocyte-associated protein 4; HCC, Hepatocellular carcinoma; IDO1, Indoleamine 2,3-dioxygenase 1; PDL1, Programmed death-ligand 1.

Additional Findings

For vaccine studies where multiple immunizations are to be administered, the size of the tumor is important. The induced tumor needs to be sufficiently established to observe an effect on tumor growth, but if the growth rate is too aggressive it is not possible to test any therapies. For this reason, we set to determine the optimal concentration of AdCre for tumor induction. As dictated by ethical regulations, each animal received six injections with AdCre; three subcutaneous and three intramuscular injections using a two-fold titration of AdCre dose ranging from $2.5x10^8$ – $1.0x10^9$ plaque forming units (PFU). Tumor sizes were determined using ultrasound measurements (Figure 4).

Figure 4. Ultrasound measurements of intramuscular tumor sizes. Oncopigs were injected with three different doses plaque forming units (PFU) of AdCre. Ultrasound images of intramuscular tumors from one animal at day 16 post AdCre injection is shown.

When comparing the different doses, no difference in tumor growth was observed between low, intermediate, and high AdCre dose groups either in the subcutaneous or in the intramuscular tissue (Figure 5A-B). The subcutaneous tumors masses appeared to have a slightly less aggressive growth rate (Figure 5A) when compared to intramuscular tumors (Figure 5B). Strikingly, spontaneous clearance of both subcutaneous and intramuscular tumors was observed over time for all the animals included here (Figure 5).

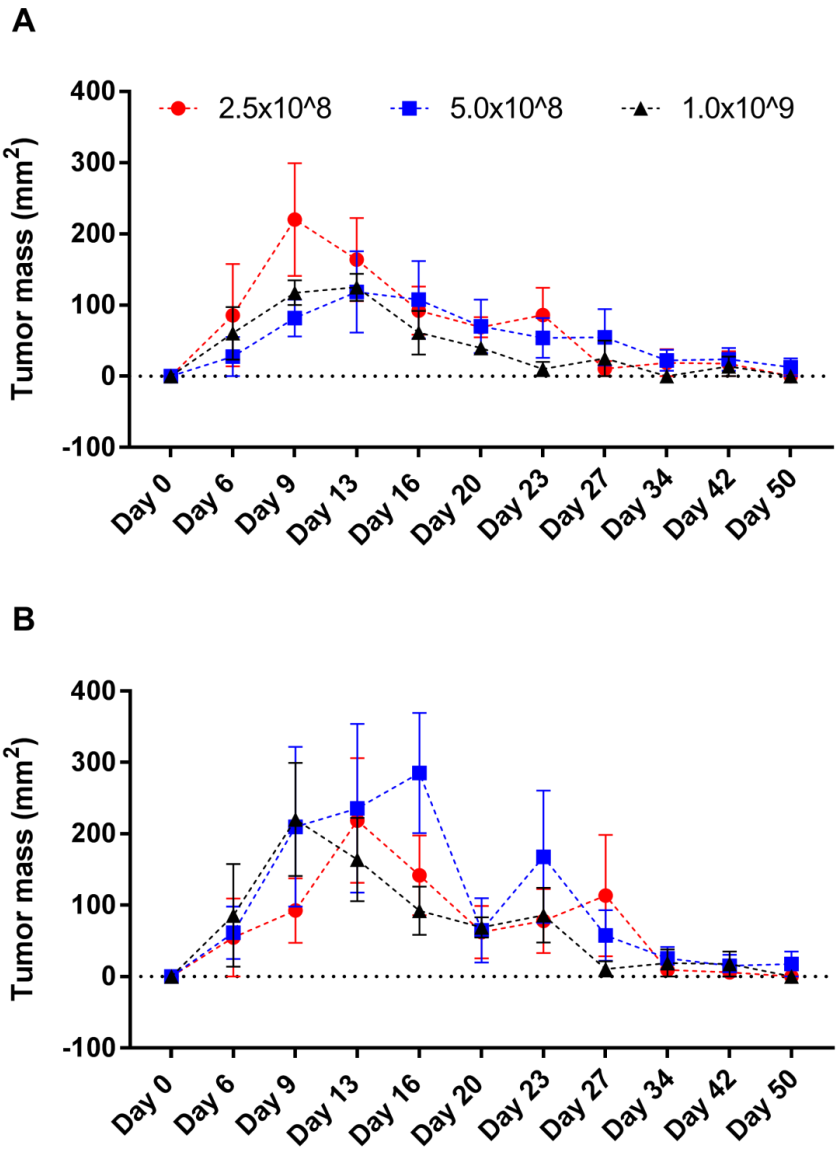


Figure 5. Tumor growth in subcutaneous and intramuscular tumors induced by different doses of AdCre. Animals were injected at six different sites with three different doses of AdCre ranging from $2.5x10^8$ – $1.0x10^9$ PFUs. Three sites received a subcutaneous injection and three sites received an intramuscular injection. Data show ultrasound measurements of subcutaneous (A) and intramuscular (B) tumor sizes. One animal has been left out of the high dose subcutaneous group due to no initial tumor formation at this particular site only (*n=3-4*).

While all other conditions show data from four animals, the high dose subcutaneous group only shows data from three. The fourth animal in this group was left out of analysis. Since the same animal developed tumors at the five other injection sites, we have no reason to believe that this lack of tumor formation was dose-related. In contrast, we believe that a technical error occurred during the injection of AdCre; thereby, justifying that this animal was removed from analysis of the high dose subcutaneous group.

In order to investigate whether the spontaneous regression over time resulted from lack of vascularization and subsequent necrosis, fine-needle aspiration of subcutaneous tumor samples were obtained 17 days post AdCre injection (*n=8*). Samples were sent for Haemotoxylin and Eosin staining followed by blind pathological assessment at the Veterinary Diagnostic Laboratory, University of Illinois, United States. The interpretations are shown in Table 5.

Tumor interpretation	**Necrosis**	**Suspected lymphocytic inflammation**
Sarcoma	Not detected	No
Sarcoma	Not detected	Yes
Sarcoma	Not detected	No
Sarcoma	Not detected	Yes
Sarcoma	Not detected	No
Sarcoma	Not detected	No
Suspected sarcoma	Yes	Yes. Potential suppurative inflammation
Suspected sarcoma	Yes	Yes

Table 5. Clinical pathology results from fine-needle aspirations of subcutaneous tumors obtained 17 days post AdCre injection (*n=8*).

Based on the pathological analysis, the tumors induced upon subcutaneous administration of AdCre were sarcomas (Table 5). Of the tumor biopsies tested, only two out of eight displayed evidence of necrosis, whereas half of them were suspected to have lymphocytic inflammation (Table 5); indicating that the tumor regression was probably not due to lack of vascularization. The lymphocytic inflammation is only referred to as *suspected*, since the observed increase in lymphocytes theoretically could result from the involvement of a peripheral lymph node or blood contamination during the process of fine-needle aspiration.

CHAPTER IV. General Discussion

In our series of studies we evaluate the potential for pigs as a large animal model for studying anti-tumor immune responses and for preclinical testing of immunotherapies against human cancer. The topics already discussed in Paper I-III will not be repeated here. Instead, a more general evaluation of pigs as cancer models, and Oncopigs in particular, follows.

As outlined in the introduction of this thesis, large animal models other than pigs exist. To date, canine models in particular have shown promise as immunotherapeutic models[206,207]. Despite this, the porcine immune system remains better characterized[281], as exemplified by comparison of NK cells between the two models. Porcine NK cells are well-described and express CD8α and NKp46[282,283]; the latter being a typical human NK cell marker[284,285]. In contrast, characterization of canine NK cells is more complicated[194]. Expression of NKp46 has been shown upon activation in a canine immune cell subset with phenotypic and functional characteristic of NK cells[286,287]. However, it remains to be fully evaluated whether these cells correspond to the human NK cell population. Overall, the porcine immunome shares substantial homology with the human counterpart[229]; thus, providing an important platform for translational immunology research.

With the exception of our previous proof-of-concept vaccine trial[288], there is to our knowledge no previous *in vivo* study using pigs as a model for cancer immunotherapy. The vaccine approach in our first trial was very different, as we immunized outbred pigs only twice and with 20mer overlapping peptides covering the entire IDO sequence. In this first study, the peptides were formulated in different adjuvant systems including, amongst others, CAF09. As determined by IFN-γ release, we showed induction of a weak immune response towards IDO following subcutaneous delivery of CAF09-formulated peptides in outbred pigs, although the response appeared to be rather transient[288]. Recent murine studies have shown that immunization of CAF09-formulated antigen via the i.p. route is superior in generating an antigen-specific CTL response when compared to subcutaneous administration[289].

Consequently, we altered our approach and established an i.p. immunization protocol with repeated administration of IDO-derived peptides.

Our results demonstrated that it was possible to break peripheral tolerance against an endogenous antigen relevant to human cancer (Paper II). Furthermore, we showed how the CAF09-formulated antigen dose affected the type of immune response generated upon repeated immunization (Paper I and Paper II). It is well-established that the tumor microenvironment possesses the ability to shape and limit the function of TILs[9]. Specifically, intratumoral T cells can be affected by secretion of inhibitory cytokines, limitation in nutrient availability as a result of metabolic competition, reduction of oxygen levels, as well as increase in lactate production[290–293]. Consequently, testing our vaccine strategy in a tumor model rather than healthy animals as we have done so far is an obvious next step. Since we showed increased expression of *IDO1* in Oncopig leiomyosarcoma tumors (Paper III), this model may provide a relevant platform for evaluating clinical benefit of IDO-targeted therapies including therapeutic immunization.

The various different large animal models presented in the introduction of this thesis each have advantages and disadvantages. Since cancer is not one disease and different tumor types require specific treatment strategies[294], a 'one size fits all' universal animal model for preclinical testing does not seem realistic. In our studies, pathological analysis of fine-needle aspiration samples obtained from subcutaneous Oncopig tumors were all blindly interpreted as sarcomas (Table 5); thereby, confirming previous results following subcutaneous injection of AdCre[272]. Recent RNA-seq analysis revealed that transcriptional characteristics of human sarcomas are recapitulated in Oncopig sarcomas[295], which supports the relevance of using Oncopigs for human sarcoma research.

Investigation of the immunological landscape of Oncopig tumors revealed pronounced T-cell infiltration with a mixed phenotype. Interestingly, we demonstrated immune-mediated tumor-specific killing *in vitro* in an effector:target cell ratio dependent manner. In paper III, all the studies investigating the anti-tumor immune responses were performed with tumor material obtained at day 7-21 post AdCre injection. As shown in Figure 5, this range covers

the peak in tumor mass; however, long-term studies revealed spontaneous regression of subcutaneous and intramuscular tumors (Figure 5). This currently limits the model to investigating mechanism of tumor killing or preclinical testing of therapeutics against the early stages of cancer.

Theoretically, the spontaneous Oncopig tumor regression could be non-immune mediated. Necrosis can be observed in aggressive tumors due to the absence of vascular support[296,297]; thus, we needed to rule out that the tumor clearance was simply the result of a necrotic tumor. Pathological analysis of fine-needle aspiration samples obtained from subcutaneous tumors 17 days post AdCre injection revealed that only 25% of the tumors demonstrated evidence of necrosis (Table 5). Thus while the spontaneous regression might partly be the result of necrosis, it is unlikely to fully explain the high rate of tumor clearance over time.

Our demonstrated tumor cell-directed *in vitro* cytoxicity supports the hypothesis that the tumor regression is likely to be immune mediated (Paper III). We attempted to evaluate the effect of tumor development in pigs receiving immunosuppressive treatment. Rather than administering chemotherapeutic drugs, we orally administered prednisone to pigs at different time points before, during, and after AdCre injection. However, the immunosuppressant treatment did not alter the rate of tumor regression or the systemic immune response (unpublished data). The lack of response to this mild immunosuppressive treatment is likely due to pigs being largely corticosteroid resistant[298]. Although future studies should fully determine which immune cell subsets are involved, the significant T-cell infiltration suggests a role for T cells in Oncopig tumor clearance *in vivo*.

Despite the abundant T-cell infiltration, Oncopig anti-tumor immune responses seem to be inhibited by an immunosuppressive tumor microenvironment at the early time points post AdCre exposure; as indicated by the observation that the tumor mass peaks between days 6-20 (Figure 5). Important mediators of immunosuppression include the proteins encoded by *IDO1*[128–133], *CTLA4*[299–301], and *PDL1*[302–304]. Elevated expression of these genes, which all impair T-cell effector functions, was demonstrated in Oncopig leiomyosarcoma tumor materials obtained at day 20 post AdCre injection (Paper III). Based on these data in

conjunction with Figure 5, it can be speculated that Oncopig subcutaneous tumors do not reach the escape phase potentially due to downregulation of *IDO1*, *CTLA4*, and *PDL1* gene expression over time. If so, this might allow reactivation of T-cell cytotoxicity *in vivo*; eventually leading to tumor clearance.

Many organs and tissues are not just passive recipients of infiltrating immune cells[305]; thus, some of the T cells within Oncopig tumors might derive from a resident T-cell compartment rather than from infiltrating T cells. The *in vitro* killing assay showed a certain percentage of tumor lysis in the absence of added PBMC (no effector cell control wells). This killing could result from either resident or infiltrating T cells being able to exhibit their effector functions *in vitro*. Interestingly, the tumor material used for the *in vitro* cytoxicity assay was obtained at time points at which RNA-seq data demonstrated elevated expression levels of the immunosuppressive genes *IDO1*, *CTLA4*, and *PD-L1* (Paper III). Therefore, the T cells present in the tumor cell isolates are not likely to exhibit effector functions *in vivo* at this time post AdCre injection due to the expression of these immunosuppressive genes. Nevertheless, they may be able to exhibit effector functions *in vitro* following the tumor digest, which would explain the rate of background killing. However, increase in tumor-specific lysis observed *in vitro* with a high ratio of added PBMC effectors (Paper III) clearly suggests that the added peripheral immune cells also play a role in mediating the tumor killing.

Spontaneous regression of human tumors is most commonly seen in neuroblastoma, renal cell carcinoma, lymphomas, and melanoma[306]. However, complete histological regression of human melanoma lesions is a rare occurrence limited to relatively few case studies[307]. In contrast, lesions of porcine melanoma models display a high tendency of spontaneous regression with the MeLiM model showing complete clearance in up to 96% of the cases[308,309]. The onset of spontaneous regression also appears earlier in pigs than in humans[310]. The first genome-wide time-dependent analysis elucidating some of the molecular mechanisms underlying spontaneous tumor regression in the MeLiM model demonstrated upregulation of several immune-related genes[310]. The initial process of spontaneous regression of melanoma

lesions included pronounced lymphocyte infiltration[310], which is in line with our results demonstrating a significant T-cell enrichment in Oncopig tumors (Paper III).

Having shown that T cells may play a role in spontaneous regression of Oncopig tumors, a critical next step is to elucidate potential T-cell targets within the tumors. Based on genomic data, the cancer antigenome has been defined and encompasses two main classes of tumor-specific antigens: self-antigens and neoantigens[311]. The *IDO1* gene encodes a non-mutated self-antigen, whereas the driver mutations $KRAS^{G12D}$ and $TP53^{R167H}$ in Oncopigs give rise to neoantigens. As only self-reactive T cells are deleted in the thymus, T cells reactive towards neoantigens are not subject to peripheral tolerance[312]. As we have shown pronounced $KRAS^{G12D}$ expression in tumors (Paper III), it can be speculated that this neoantigen is a T-cell target in Oncopigs. In a human colorectal cancer patient, $CD8^+$ T-cell reactivity towards $KRAS^{G12D}$ has been demonstrated[313]. However, targeting several passenger mutations, rather than a single driver mutation, is increasingly considered a more effective therapeutic approach[311]. One of the suggested reasons for this includes the much lower frequency of driver mutations, when compared to passenger mutations, presented on the surface of tumor cells[314]. As observed in the colorectal cancer patient displaying $KRAS^{G12D}$ T-cell reactivity, loss of the MHC class I allele presenting this neoantigen provides the tumor with an efficient escape mechanism[313]. In addition to a putative reduction in *IDO1*, *CTLA4*, and *PDL1* expression over time, it can be speculated that the MHC class I allele(s) presenting mutated neoantigens remains highly expressed on the surface of Oncopigs tumor cells. Recent findings clearly show that clonal neoantigens, when compared to sub-clonal ones, are superior targets for inducing anti-tumor immunity[315]. Consequently, evaluating the heterogeneity of the neoantigen repertoire in Oncopig tumors might improve our understanding of potential T-cell targets.

Although interesting from an immunological point of view, the spontaneous tumor regressions demonstrated in Figure 5 raise concerns with regards to long-term treatment studies in Oncopigs. However, in a separate experiment we restricted administration of AdCre to the main pancreatic duct, which sufficiently induced a tumor with morphological features consistent with human pancreatic ductal adenoma carcinoma (Principe et al, 2017,

Nature Communications, in review). This tumor showed no signs of regression, but was present even one year post AdCre injection. Furthermore, subcutaneous injection with an established hepatocellular carcinoma cell line showed no signs of regression 46 days post injection[280]. Together, these data underline that long-term tumorigenesis is indeed possible in the Oncopig model.

In general, tissue- and cell-specific differences between tumors do exist[316]. In reflection of this, the ability to induce tumors at basically any site in the Oncopig upon exposure to AdCre or by injection of an autologous tumor cell line is a clear advantage of the model. Since establishment of persistent tumors is possible in the model as mentioned above, a strict breeding scheme selecting animals with reduced anti-tumor immune responses might be a way to overcome the high rate of spontaneous tumor regression, especially if anti-tumor immunity is linked to expression of particular MHC class I alleles.

Combined, we provide evidence of anti-tumor immunity in the physiologically relevant Oncopig model; suggesting that it may serve as an invaluable platform for studying immune response to cancer. The elevated expression of three relevant immunotherapeutic targets (*IDO1*, *CTLA4*, and *PDL1*) further supports the potential for the Oncopig as a preclinical model, especially if a strict selective breeding scheme is established.

CHAPTER V. Conclusion

In our series of studies, we established an immunization protocol, where repeated i.p. injections with CAF09-formulated antigens induced both a CMI and humoral immune response in Göttingen minipigs. Using a low dose exogenous antigen, we showed induction of a cytotoxic and polyfunctional T-cell response, while a high antigen dose induced antigen-specific IgG antibodies. Although *in vivo* cytotoxicity towards IDO-pulsed target cells could not be demonstrated, our immunization protocol was sufficient to break the peripheral tolerance towards porcine IDO. For this endogenous target, we showed an inverse relationship between peptide dose and the induction of a CMI-dominant response. In contrast, a CAF09-formulated high peptide dose generated a mixed IDO-specific CMI and humoral immune response. Combined, these data underline the importance of antigen dose when designing vaccines strategies.

In the Oncopig model, we show pronounced intratumoral T-cell infiltration with enrichment of both Tregs and CTLs when compared to peripheral blood. Thus, Oncopig tumors can be classified as *hot* tumors in accordance with the Immunoscore classification. Moreover, we demonstrated elevated expression of the immunosuppressive genes *IDO1*, *CTLA4*, and *PDL1*. By adapting our cytotoxicity assay for *in vitro* use, we proved that the Oncopig immune system is capable of specifically lysing tumor cell isolates. However, long-term studies revealed a high rate of spontaneous regression of most Oncopig tumors. From this, it can be speculated that there is immune equilibrium, as indicated by the mixed regulatory and cytotoxic response, at the early time points post AdCre injection, while anti-tumor immune responses become dominant over time; eventually leading to tumor clearance. Together, our data support that the Oncopig provides an invaluable platform for investigating anti-tumor immune responses in a large and physiologically relevant model. Given that the rate of spontaneous regression can be reduced, for instance by selective breeding, the Oncopig is a promising model for preclinical testing of cancer immunotherapies.

CHAPTER VI. Perspectives

The use of pigs as a large animal model for studying anti-tumor immune responses and for preclinical testing of immunotherapies has intriguing potential. However, several aspects need to be elucidated further. Some of the specific questions are evaluated below.

How do pigs respond to checkpoint inhibition?

Although therapeutic cancer vaccines are promising, the response rate in patients receiving these types of vaccines is often low[317]. We showed a break in the peripheral tolerance towards IDO following repeated immunization, but the lack of *in vivo* cytotoxicity towards IDO-pulsed target cells supports that combination therapies, rather than immunization as a stand-alone treatment, is needed. The monoclonal antibodies targeting either CTLA-4 or PD-1 have shown impressive results in the clinic[318–322], and it will be interesting to test checkpoint inhibitors either alone or in combination with a therapeutic vaccine in the Oncopig model.

Which immune cells mediate the anti-tumor cytoxicity in Oncopigs?

Although we have strong indications of T-cell involvement in Oncopig anti-tumor immunity, there is a need for a thorough investigation determining exactly, which immune cells subsets are involved. While αß T cells have received a lot of attention, γδ T cells have been much less studied, although they have been demonstrated to have implications in cancer[323]. As γδ T cells represent a major T-cell population in pigs, it will be important to determine whether this immune cell subset plays a role in the elimination of Oncopig tumors. The memory stage of the various T-cell subsets within Oncopig tumors might also play a role as suggested for human cancer patients[29,324]. Hence, evaluation of T-cell memory is also needed.

What is the neoepitope landscape of Oncopig tumors and does it encompass T-cell targets?
Somatic mutations often result in tumor cells becoming less similar to self. For this reason, a high mutational load increases the likelihood of the tumor being recognized by the immune system[325–327]. The recognition of these foreign epitopes, referred to as neoepitopes, is a critical factor for tumor control[311,327–331]. In a recent study, melanoma patients were treated with a personal neoantigen vaccine, which was shown to be safe, effective, and induce polyfunctional T cells[332]. Thus, targeting neoantigens is an intriguing approach. Exploration of the Oncopig neoepitope landscape will determine, if the model can be used for preclinical testing of this kind of vaccines. Also, it might increase our understanding of the effective anti-tumor immunity in the Oncopig.

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