UNIVERSITY OF THESSALY
SCHOOL OF HEALTH SCIENCES
FACULTY OF MEDICINE
GREECE

And

NATIONAL INSTITUTES OF HEALTH
CENTER OF CANCER RESEARCH
NATIONAL CANCER INSTITUTE
USA

DOCTORAL DISSERTATION

The role of cellular immunity in the treatment of cancer

Vasiliki Stravokefalou

Submitted to the Faculty of Medicine, School of Health Sciences, University of Thessaly in fulfillment of the requirements for obtaining a PhD diploma.
USA, 2023

The role of cellular immunity in the treatment of cancer

3-members Advisory Committee

Konstantinos Dimas, Professor, Department of Pharmacology, School of Medicine, University of Thessaly, Larissa, Greece

George N. Pavlakis, Senior Investigator, Head on Human Retrovirus Section at Center for Cancer Research, NCI, Frederick, USA

Nikolaos Sakellaridis, Professor, Department of Pharmacology, School of Medicine, University of Thessaly, Larissa, Greece

7-members Defense Committee

Konstantinos Dimas, Professor, Department of Pharmacology, School of Medicine, University of Thessaly, Larissa, Greece

George N. Pavlakis, Senior Investigator, Head on Human Retrovirus Section at Center for Cancer Research, NCI, Frederick, USA

Nikolaos Sakellaridis, Professor, Department of Pharmacology, School of Medicine, University of Thessaly, Larissa, Greece

George Vassilopoulos, Professor, Department of Hematology – Internal Medicine, School of Medicine, University of Thessaly, Larissa, Greece

Dimitrios Bogdanos, Professor, Department of Internal Medicine and Autoimmune Diseases, School of Medicine, University of Thessaly, Larissa, Greece

Ourania Tsitsilonis, Professor of Immunology, Department of Biology, National and Kapodistrian University of Athens, Athens, Greece

Dimitra Mangoura, Senior Investigator, Vice Director of the Basic Research Center, BRFAA, Athens, Greece

Acknowledgements

This dissertation was completed at the National Cancer Institute (NCI) of National Institutes of Health (NIH) in the United States through a collaboration agreement with the Faculty of Medicine of University of Thessaly in Greece. During this journey, there were several people in both my professional and personal life that marked this unique experience with their insight, guidance, and emotional support.

To begin, I would like to thank my advisory committee, Dr. George N. Pavlakis (NCI) and Professors Konstantinos Dimas and Nikolaos Sakellaridis (University of Thessaly) for providing me with the resources and support necessary to pursue my academic goals. I am grateful to Dr. George N. Pavlakis for granting me the valuable opportunity to work in his lab, but also for providing me with their scientific expertise and guidance every step of the way. This opportunity contributed remarkably to my academic and personal growth. I would like to express my gratitude to Prof. Konstantinos Dimas, whose support and guidance helped me overcome challenges and successfully complete this dissertation. I would like to additionally acknowledge Prof. Nikolaos Sakellaridis (University of Thessaly) and to recognize the importance of the collaborative agreement that exists between the University of Thessaly and National Cancer Institute that enabled the completion of this dissertation. My deep appreciation goes to the rest of Ph.D. advisors and mentors, Drs. Dimitris Stellas (NCI), Sevasti Karaliota (NCI) and Antonio Valentin (NCI) for their time, effort, and understanding in helping me succeed in my studies. Dr. Dimitris Stellas has been a great research mentor for more than 10 years and this endeavor would not have been possible without him. Special thanks to a very important colleague who contributed tremendously to the experimentation of this dissertation, Dr. Sevasti Karaliota, especially in the studies of EO771 model and the discovery of the novel dendritic cell population. I would also like to thank her for the everyday personal support and discussions. Special thanks to Antonio Valentin for his support, scientific inspiration, and insightful input. I would also like to thank Dr. Barbara K. Felber and my colleagues and friends from Pavlakis/Felber's labs for everyday discussions, especially Hrishikeshi Pandit, Santhi Devasundaram, Cristina Bergamaschi and Jennifer Bear. Special acknowledgments go to our collaborators for their invaluable contributions. I had the pleasure of collaborating with Dr. Serguei Kozlov (NCI) and I would like to recognize his meaningful insight and support. I am also thankful to Theresa Guerin, Jerome Schlomer, Norene O'Sullivan and Laura Bassel for the productive collaboration and their technical assistance throughout my research. Also, to Matthew Angel from the CCR Collaborative Bioinformatics Resource for the bioinformatic analysis. Additionally, I would like to give special acknowledgments to the following individuals who assisted me in completing the project: Bethany A. Nagy and Breana

Myers from the Laboratory Animal Sciences Program; Batiar Karim, Tammy Beachley, Andrew Warner and Donna Butcher from the Molecular Histopathology Laboratory; K. Klarmann, from CCR-Frederick Flow Cytometry Core Laboratory; T. Bao, J. Sheety, and M. Mehta from Sequencing Facility; X. Wu and N. Bubunenko from Genomics Laboratory, Frederick National Laboratory for Cancer Research (FNLCR) for technical assistance; and T. Jones (NCI) for administrative support. Research was funded by the Intramural Research Program of NCI (Drs. Pavlakis and Felber) and was also supported by Admune/Novartis through a collaborative agreement with the NCI/NIH, USA (Dr. Pavlakis).

This achievement could not have been possible without the support of my family and friends in Greece and the US. I would like to express my deepest appreciation to my beloved friend Colin J. Reitman for his everyday support over all these demanding years; my mother Gianna Nikolakopoulou and my sisters Theoni and Iliana Stravokefalou for their love and support and my niece Aggeliki Vardaka for being a source of life. This dissertation is dedicated to my child self, who could have never dreamt of this career achievement but never stopped trying.

Vasiliki Stravokefalou
Washington DC
USA

Summary in English

This dissertation aimed to investigate the potential role of the cytokine hetIL-15 (heterodimeric interleukin-15) as an immunotherapeutic drug for the treatment of breast and pancreatic cancer using murine cancer models that recapitulate the human diseases. IL-15 is a cytokine that has been shown to enhance the anti-tumor responses through the stimulation of several leukocyte populations of the cellular immunity, including cytotoxic $CD8^+$ T lymphocytes and Natural Killer (NK) cells. Due to these properties, IL-15 has been suggested by the National Cancer Institute (NCI) as a promising immunotherapeutic drug for the treatment of cancer. Studies by Dr. Pavlakis' lab revealed that the native form of IL-15 is a heterodimeric protein composed by the IL-15 molecule and the IL-15 receptor a (IL-15Rα) which is the *in vivo* active form of the cytokine in mice and humans. This complex is termed heterodimeric IL-15 (hetIL-15) and has an extended half-life and better efficacy compared to the recombinant single chain IL-15 produced in *Escherichia coli*. Here, the studies of hetIL-15 administration, systemically and peritumorally, in different mouse cancer models of breast (4T1 and EO771) and pancreatic (orthotopic, IV and transgenic KPC) cancer provide evidence of the strong anti-tumor and anti-metastatic potency of hetIL-15. Utilizing the 4T1 model of TNBC, hetIL-15 systemic administration exhibited strong anti-metastatic activity reducing the metastatic foci while increasing the cytotoxic effector cells (NK and $CD8^+$ T cells) in the lung parenchyma of the tumor-bearing mice. hetIL-15 was found not only to reduce the metastasis in the lungs, but also to control the dissemination of circulating tumor cells (CTCs), resulting in improved therapeutic benefit in combination with chemotherapy and surgery. The data suggest that this anti-cancer response is achieved by the systemic expansion of $CD8^+$T and NK cells and reduction of the immunosuppressive PMN-MDSCs (polymorphonuclear – myeloid derived suppressor cells) mediated by hetIL-15 monotherapy and enhanced by combination with chemotherapy. Importantly, 4T1 tumor resection accompanied by the pre- and post-surgery administration of hetIL-15 alone or in combination with doxorubicin led to the cure of approximately half of the animals and the establishment of immunological memory against 4T1 cancer cells. EO771 mouse model of triple negative breast cancer (TNBC) showed the highest sensitivity in hetIL-15 monotherapy upon peritumoral administration. The treatment led to complete tumor regression in 40% of the mice, extension of animal survival, induction of immunological memory against EO771 cancer cells and control of metastatic disease in the lungs. Immune phenotyping of the hetIL-15-treated EO771 primary tumor revealed that the cytokine reshaped the tumor microenvironment accumulating cytotoxic lymphocytes (NK and $CD8^+$ T cells), conventional type 1 dendritic cells (cDC1s) and a novel dendritic cell population, defined as $CD103^{int}CD11b^+$DCs, which was found to correlate with EO771 tumor regression. Single-cell

transcriptomic analysis revealed that this distinct DC population shares phenotypic and gene expression characteristics with both cDC1s and cDC2s and has transcriptomic profiles akin to monocyte derived DCs (moDCs). Similarly, systemic hetIL-15 monotherapy decreased the metastatic foci in the lungs of the orthotopic, intravenous and transgenic KPC model increasing the NK and $CD8^+$ T cells in the organ without, however, extending animal survival due to insufficient control of the primary tumor. Although hetIL-15 monotherapy did not show a consistent control of the primary tumor growth, an extensive tumor necrosis was observed concurrently with a moderate increase in the frequency of cytotoxic cells (NK and $CD8^+$ T cells) indicating anti-tumor activity. The novel $CD103^{int}CD11b^+$DC population was also found elevated in the pancreatic tumor upon hetIL-15 treatment. However, attempts to optimize the treatment schemes combining hetIL-15 with chemotherapy or targeted agents against mesothelin, a tumor associated antigen found increased in pancreatic cancer, were not found to be beneficial.

The data presented in this study suggest that hetIL-15 is a promising cytokine for the treatment of TNBC and pancreatic cancer due to its anti-cancer and anti-metastatic properties. The reduction of the metastatic foci in the lungs and CTCs in the blood due to the systemic immune response against 4T1 tumor suggests that hetIL-15 may be a potent anti-metastatic therapy. The similar anti-metastatic effects of hetIL-15 treatment in the EO771 and KPC cancer models further support its anti-metastatic potential. Moreover, hetIL-15 treatment as a single agent or combined with chemotherapy in mice that underwent surgical excision of the 4T1 primary tumor, both pre- and post-surgery, resulted in favorable therapeutic outcomes with approximately half of the animals being cured. These findings suggest that hetIL-15 may be a promising treatment option for resectable breast cancer. Furthermore, hetIL-15 controls EO771 primary tumor growth by effectively coordinating a local immune response that leads to complete EO771 tumor regression. This potent anti-tumor activity is orchestrated by the activation of $CD8^+$T and NK cells, as well as by the increase of tumor infiltration of cDC1 and $CD103^{int}CD11b^+$ DC subpopulation, which is a unique dendritic cell population, most closely related to moDCs. Although hetIL-15 did not show a major effect in delaying the pancreatic tumor, findings of anti-tumor activity were observed by the presence of extensive tumor necrosis and the accumulation of cytotoxic cells and the novel DC subpopulation. Overall, hetIL-15 anti-cancer effects on the TNBC and KPC mouse models, with the consistent anti-metastatic effect, holds promise for the future clinical use of the cytokine as a therapeutic agent against cancer. If these properties are successfully reproduced in humans, hetIL-15 may provide additional therapeutic options for breast and pancreatic cancer patients.

Περίληψη στα ελληνικά

Η παρούσα διδακτορική διατριβή πραγματεύεται την αξιολόγηση της κυτταροκίνης hetIL-15 (heterodimeric interleukin-15 – ετεροδιμερής ιντερλευκίνη 15) ως ανοσοθεραπευτικού φαρμάκου για τη θεραπεία του καρκίνου του μαστού και του παγκρέατος χρησιμοποιώντας καρκινικά μοντέλα ποντικών που μιμούνται την ανθρώπινη νόσο. Η IL-15 είναι μια κυτταροκίνη που ενισχύει τις αντι-καρκινικές αποκρίσεις μέσω της ενεργοποίησης διαφόρων λευκοκυττάρων της κυτταρικής ανοσίας, συμπεριλαμβανομένων των κυτταροτοξικών $CD8^+$ Τ λεμφοκυττάρων και των κυττάρων NK (Natural Killer – φυσικών φονέων). Λόγω αυτών των ιδιοτήτων, η IL-15 έχει προταθεί από το Εθνικό Αντικαρκινικό Ινστιτούτο (National Cancer Institute, NCI) των ΗΠΑ ως ένα αρκετά υποσχόμενο ανοσοθεραπευτικό φάρμακο για τη θεραπεία του καρκίνου. Μελέτες της ομάδας του Δρ. Παυλάκη αποκάλυψαν ότι η ενδογενής μορφή της IL-15 είναι μια ετεροδιμερής πρωτεΐνη που αποτελείται από το μόριο της IL-15 και τον IL-15 υποδοχέα α (IL-15 receptor a, IL-15Rα), η οποία αποτελεί την *in vivo* ενεργοποιημένη μορφή της κυτταροκίνης στα ποντίκια και τον άνθρωπο. Το σύμπλοκο ονομάζεται ετεροδιμερής IL-15 (hetIL-15) και έχει αυξημένο χρόνο ημίσειας ζωής και βελτιωμένη αποτελεσματικότητα σε σύγκριση με την ανασυνδυασμένη μορφή του μορίου της IL-15 που παράγεται από την *Escherichia coli*. Στην παρούσα διατριβή παρέχονται δεδομένα που αποδεικνύουν την ισχυρή αντι-καρκινική και αντι-μεταστατική δράση της IL-15 όταν χορηγείται με συστημικό τρόπο ή κοντά στον πρωτογενή όγκο σε μοντέλα ποντικών με καρκίνο του μαστού (μοντέλα 4T1 και EO771) και του παγκρέατος (ορθοτοπικό, ενδοφλέβιο και διαγονιδιακό KPC μοντέλο). Αξιοποιώντας το 4T1 μοντέλο του τριπλά αρνητικού καρκίνου του μαστού (triple negative breast cancer, TNBC) δείχθηκε ότι η συστημική χορήγηση της hetIL-15 έδρασε ως αντι-μεταστατικό φάρμακο μειώνοντας τη μετάσταση και αυξάνοντας τα κυτταροτοξικά λεμφοκύτταρα (NK και CD8+ Τ κύτταρα) στο παρέγχυμα του πνεύμονα των ποντικών που έφεραν τον 4T1 όγκο στο μαστό. Στο 4T1 μοντέλο, η hetIL-15 όχι μόνο μείωσε τη μετάσταση στους πνεύμονες, αλλά περιόρισε και τη διασπορά των καρκινικών κυττάρων στην κυκλοφορία (circulating tumor cells, CTCs) με αποτέλεσμα να ενισχύεται το θεραπευτικό αποτέλεσμα κατά το συνδυασμό της με τη χημειοθεραπεία και τη χειρουργική αφαίρεση του όγκου. Φαίνεται ότι η αντι-καρκινική αυτή δράση επιτυγχάνεται με τη συστημική αύξηση των $CD8^+$Τ και NK κυττάρων και τη μείωση των ανοσοκατασταλτικών PMN-MDSCs (polymorphonuclear – myeloid derived suppressor cells, πολυμορφοπύρηνα ανοσοκατασταλτικά κύτταρα προερχόμενα από μυελώδη κύτταρα) που προκαλείται από τη μονοθεραπεία της hetIL-15 και ενισχύεται έπειτα από το συνδυασμό της με τη χημειοθεραπεία. Τέλος, η χειρουργική αφαίρεση του 4T1 πρωτογενούς όγκου συνοδευόμενη από τη χορήγηση της hetIL-15 ως μονοθεραπείας και συνδυαστικά με τη δοξορουβικίνη, προ-εγχειρητικά και μετεγχειρητικά,

οδήγησε στην ίαση περίπου του μισού αριθμού των ζώων και την διατήρηση της ανοσολογικής μνήμης ενάντια στα 4T1 καρκινικά κύτταρα. Το δεύτερο προκλινικό μοντέλο του τριπλά αρνητικού καρκίνου του μαστού που εξετάστηκε ήταν το EO771, το οποίο έδειξε τη μεγαλύτερη ευαισθησία στη hetIL-15 μετά τη χορήγηση της κοντά στον πρωτογενή όγκο. Τα αποτελέσματα έδειξαν την πλήρη εξάλειψη του EO771 όγκου στο 40% των ποντικών, την επιμήκυνση της επιβίωσης των ποντικών, την επαγωγή της ανοσολογικής μνήμης ενάντια στα EO771 καρκινικά κύτταρα και την καταστολή της μετάστασης στους πνεύμονες. Η ανάλυση του ανοσολογικού φαινοτύπου του EO771 όγκου φανέρωσε ότι η κυτταροκίνη αναδιαμορφώνει το μικροπεριβάλλον του όγκου συναθροίζοντας τα κυτταροτοξικά λεμφοκύτταρα (NK και CD8+ T κύτταρα), τα κλασικά δενδριτικά κύτταρα τύπου 1 (conventional type 1 dendritic cells, cDC1s) και ένα ξεχωριστό πληθυσμό δενδριτικών κυττάρων, που ορίστηκε ως $CD103^{int}CD11b^{+}$DCs και βρέθηκε να συσχετίζεται με την εξάλειψη του EO771 όγκου. Η single-cell μεταγραφική ανάλυση αποκάλυψε ότι αυτός ο καινούριος τύπος δενδριτικών κυττάρων μοιράζεται φαινοτυπικά και γονιδιακά χαρακτηριστικά με τα δενδριτικά κύτταρα τύπου 1 και 2 (cDC1s and cDC2s) και έχει μεταγραφικά προφίλ παρόμοια με τα δενδριτικά κύτταρα που προέρχονται από τα μονοκύτταρα (monocyte derived DCs, moDCs). Ομοίως, η συστημική χορήγηση της μονοθεραπείας της hetIL-15 μείωσε τη μετάσταση στους πνεύμονες των ποντικών με παγκρεατικό καρκίνο, στο ορθοτοπικό, ενδοφλέβιο και διαγονιδιακό KPC προκλινικό μοντέλο, αυξάνοντας τα κυτταροτοξικά κύτταρα (NK και $CD8^{+}$T) στο όργανο χωρίς ωστόσο να επιμηκύνει την επιβίωση των ζώων λόγω της μη επαρκούς καθυστέρησης της ανάπτυξης του πρωτογενούς όγκου. Αν και η hetIL-15 δεν έδειξε σταθερή δράση στην καθυστέρηση της ανάπτυξη του παγκρεατικού όγκου, παρατηρήθηκε εκτενής νέκρωση εντός του, με ταυτόχρονη μέτρια αύξηση των κυτταροτοξικών κυττάρων (NK και $CD8^{+}$T) υποδεικνύοντας δράση κατά του όγκου. Επίσης, ο καινούριος πληθυσμός δενδριτικών κυττάρων, $CD103^{int}CD11b^{+}$DC, βρέθηκε αυξημένος εντός των παγκρεατικών όγκων μετά τη χορήγηση της hetIL-15. Ωστόσο, προσπάθειες να βελτιωθούν τα θεραπευτικά σχήματα συνδυάζοντας την hetIL-15 με χημειοθεραπεία ή στοχευμένη θεραπεία ενάντια στο σχετιζόμενο με τον παγκρεατικό καρκίνο μόριο της μεσοθηλίνης, δεν ευόδωσαν.

Αυτή η διατριβή παρουσιάζει δεδομένα που προτείνουν την hetIL-15 ως μια υποσχόμενη κυτταροκίνη για τη θεραπεία του τριπλά αρνητικού καρκίνου του μαστού και του παγκρεατικού καρκίνου λόγω των αντι-καρκινικών και αντι-μεταστατικών ιδιοτήτων που επιδεικνύει. Η μείωση της μετάστασης στους πνεύμονες και των CTCs στο αίμα λόγω της συστημικής ανοσο-απόκρισης ενάντια στον 4T1 όγκο προτείνει ότι η hetIL-15 ίσως αποτελεί μια ισχυρή αντι-μεταστατική θεραπεία. Τα παρόμοια αποτελέσματα της αντι-μεταστατικής δράσης της hetIL-15 στα EO771 και KPC μοντέλα ενισχύουν περαιτέρω την πιθανή δυναμική της κυτταροκίνης ως αντι-μεταστατική θεραπεία. Επιπλέον, η μονοθεραπεία με hetIL-15 ή ο συνδυασμό της με χημειοθεραπεία σε

ποντίκια που υποβλήθηκαν σε χειρουργική αφαίρεση του πρωτογενούς όγκου 4T1, τόσο πριν όσο και μετά τη χειρουργική επέμβαση, οδήγησε σε ευνοϊκά θεραπευτικά αποτελέσματα, με περίπου το ήμισυ των ζώων να θεραπεύονται. Αυτά τα ευρήματα υποδεικνύουν ότι η hetIL-15 μπορεί να αποτελέσει μια ελπιδοφόρα επιλογή θεραπείας για τον χειρουργήσιμο καρκίνο του μαστού. Επιπλέον, η hetIL-15 ελέγχει την ανάπτυξη του πρωτογενούς όγκου EO771, συντονίζοντας μια τοπική ανοσοαπόκριση που οδηγεί στην πλήρη εξάλειψη των EO771 όγκων. Αυτή η ισχυρή αντι-καρκινική δράση συντελείται μέσω της ενεργοποίησης των $CD8^{+}$T και NK κυττάρων, καθώς και την αύξηση της εισροής του υποπληθυσμού cDC1 και $CD103^{int}CD11b^{+}$ DC εντός του όγκου. Ο $CD103^{int}CD11b^{+}$ DC είναι ένας διακριτός υποπληθυσμός δενδριτικών κυττάρων που σχετίζεται στενά με τα moDCs. Αν και η hetIL-15 δεν έδειξε μεγάλη δραστικότητα στον έλεγχο της ανάπτυξης του παγκρεατικού όγκου, εντοπίστηκε εκτενής νέκρωση του όγκου και συσσώρευση κυτταροτοξικών κυττάρων και του καινούριου πληθυσμού δενδριτικών κυττάρων, $CD103^{int}CD11b^{+}$ DC, που φανερώνουν δράση κατά του όγκου. Συνολικά, η αντικαρκινική δράση που επιδεικνύει η hetIL-15 στα προκλινικά TNBC και KPC μοντέλα καρκίνου, υπόσχεται τη μελλοντική κλινική χρήση της κυτταροκίνης ως θεραπείας κατά του καρκίνου. Εάν αυτές οι ιδιότητες αναπαραχθούν επιτυχώς σε ανθρώπους, η hetIL-15 μπορεί να παρέχει επιπλέον θεραπευτικές επιλογές για ασθενείς με καρκίνο του μαστού και του παγκρέατος.

Dedicated to my child self who would be proud of this achievement

Table of contents

Outline

To provide a clear structure and guide for the reader, the following outline summarizes the main parts of the dissertation, including the theoretical framework (introduction), experimental procedure (material and methods), results and discussion of this research. Introduction has 3 chapters: cancer, immune system, and cancer immunotherapy. Chapter 1 describes cancer disease and provides more detailed information of the anatomy, pathophysiology and current treatment of the specific cancer types that this dissertation focuses on, i.e., breast and pancreatic cancer. The chapter ends with information on metastasis. Chapter 2 covers the immune system providing an overview of the immune cells and types of immunity. Lastly, chapter 3 refers to cancer immunotherapy describing the history, tumor immunology and the immunotherapeutic approaches (with a focus on cytokines). Materials and methods, results and discussion sections describe the research of this dissertation and are divided in two main projects of breast and pancreatic cancer. The cancer models that were utilized were the EO771 and 4T1 orthotopic breast cancer mouse models representative of the human triple negative breast cancer (TNBC) and the KPC transgenic or orthotopic mouse models that resembles the human pancreatic ductal adenocarcinoma (PDAC). Section 1 of the results is further divided into two subsections presenting the findings on the two different breast cancer models of EO771 and 4T1, and section 2 describes the findings on the KPC pancreatic cancer model. Prior to introduction, a list with the names of the 3- and 7- members committees, acknowledgements and summaries in English and Greek are provided. My resume together with the list of my publications are given after the discussion, at the end of the dissertation.

Objective

This dissertation aims to explore the anti-cancer immunotherapeutic properties of the hetIL-15 cytokine, a relatively newly discovered cytokine complex with promising properties against cancer. The study uses mouse models of TNBC (4T1 and EO771) and pancreatic (KPC) cancer to examine the efficacy of hetIL-15 as a single agent or in combination with chemotherapy, targeted therapy, or surgery, while also assessing changes in the immune population landscape, both locally and systemically. The findings from this study will shed light on how hetIL-15 exerts its activity and could provide valuable information for future research in this field.

Introduction

Chapter 1 - Cancer

1.1 Overview

Cancer is a genetic disease of multiple steps. It arises due to the accumulation of mutations in an evolutionary fashion. These mutations derived from errors of the replication machinery during cellular replication and could eventually lead to the loss of the normal regulatory processes that inhibit the uncontrolled divisions. Thankfully, there are numerous intrinsic and extrinsic mechanisms that suppress carcinogenesis, but under certain conditions they might all fail.

Early references to cancer are dated more than thousands of years ago in ancient Egypt, India, and Greece. The first descriptions come from Egyptian papyruses that refer to treatment methods around 1600 BC that were also mentioned in Indian manuscripts. The term "cancer" is attributed to Hippocrates, a Greek physician, that described the shape of the tumors as crabs (ancient Greek word for crab was "karkinos"). In ancient times the therapy that was suggested was surgical removal of tumors and treatment with ointments that contained arsenic. (Bozzone, 2009)

To date, more than 100 different cancers are known according to the US National Cancer Institute (SEER Training Modules). They are usually named after the type of organ or tissue they originate from, and they are grouped into six major categories: Carcinoma, Sarcoma, Lymphoma, Myeloma, Leukemia and Mixed types. Carcinoma is considered the malignancy that derived from epithelial tissues, and it accounts for 80-90 % of all cancer types. Laborious efforts started in the 20th century with the aim to unfold cancer development mechanisms and discover effective anti-cancer therapies. Modern anti-cancer therapeutic approaches involve surgery, chemotherapy, hormone therapy, radiation therapy, targeted therapy, stem cell transplant, and the most recent one, immunotherapy.

Cancer is one of the most deleterious public health problems and according to estimates from the World Health Organization (WHO) is included in the list with the leading causes of death (by Cause and Age, 2020). The global cancer statistics estimated that 19.3 million new cancer cases and almost 10.0 million cancer deaths occurred in 2020 (Sung et al., 2021). Breast cancer was the most commonly diagnosed cancer with an estimated 2.3 million new cases (11.7%), followed by lung (11.4%), colorectal (10.0 %), prostate (7.3%), and stomach (5.6%) cancers (**Fig. 1**). The cancer type with the most cancer deaths remained the lung cancer (18%), followed by colorectal (9.4%), liver (8.3%), stomach (7.7%), and female breast (6.9%) cancers (**Fig. 1**). Global cancer burden is estimated to be increased by 47% with 28.4 million new cancer cases until 2040.

The intrinsic and extrinsic mechanisms that are involved in the cancer development are linked with the hallmarks of cancer that have been suggested by Hanahan and Weinberg in 2000 (Hanahan and Weinberg, 2000). To deconvolute the complexity of the multistep process of carcinogenesis, the authors suggested six hallmarks of cancer: constant proliferative signaling, evasion of the growth suppressors, cell death resistance, replicative immortality, induction of angiogenesis and signaling of invasion and metastasis. Genome instability and chronic inflammation are two key factors that facilitate tumor progression and the acquisition of hallmark characteristics. In 2011, Weinberg and Hanahan updated the hallmarks of cancer to include two more: reprogramming of energy metabolism and immune escape (Hanahan and Weinberg, 2011), bringing the hallmarks to the total of eight (**Fig. 2**). In this publication they also highlighted the role of the tumor microenvironment (TME) formation with the recruitment or priming of numerous accessory cells that interact with the cancer cells and support their growth. It is now known that tumor cells closely synergize with TME to escape the host immunosurveillance mechanism and avoid destruction. More recently Hanahan published additional prospective hallmarks of cancer in an effort to encourage the discussion of the hallmark scheme: phenotypic plasticity, epigenetic reprogramming, polymorphic microbiomes and senescent cells (Hanahan, 2022).

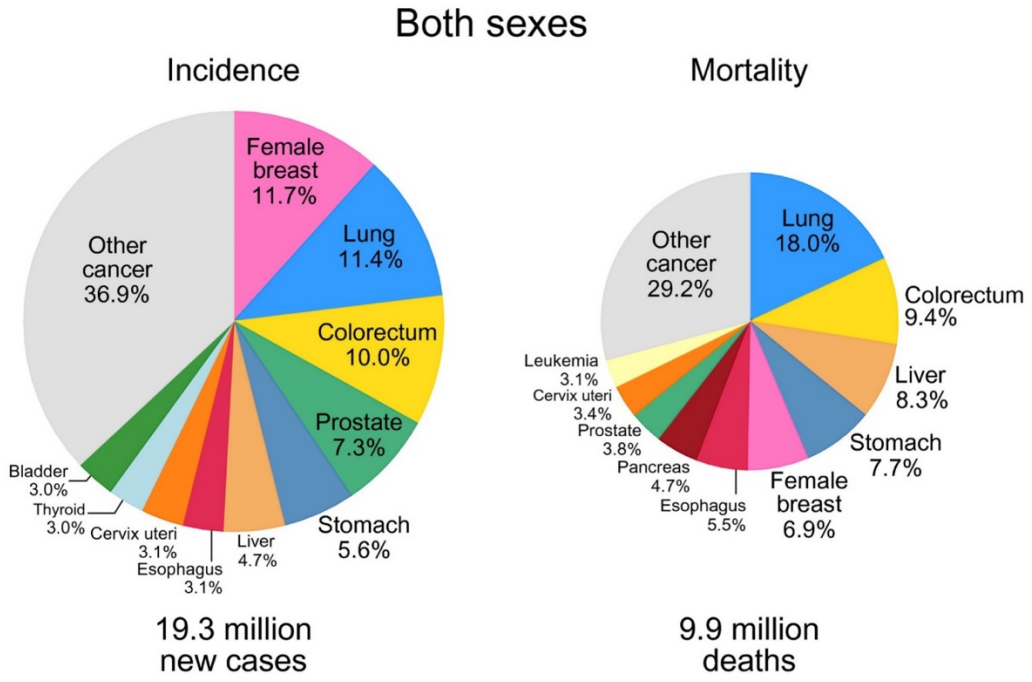


Figure 1: Distribution of cases and deaths for the top 10 most common cancers in 2020 for both sexes. (Sung et al., 2021)

One of the most contemporary approaches in cancer treatment is immunotherapy that has revolutionized anti-cancer therapy as it is demonstrated not only to effectively treat major types of cancer but to additionally promote durable responses in patients with advanced cancer (Mellman et al., 2011). Immunotherapy is the type of cancer therapy that this dissertation focuses on.

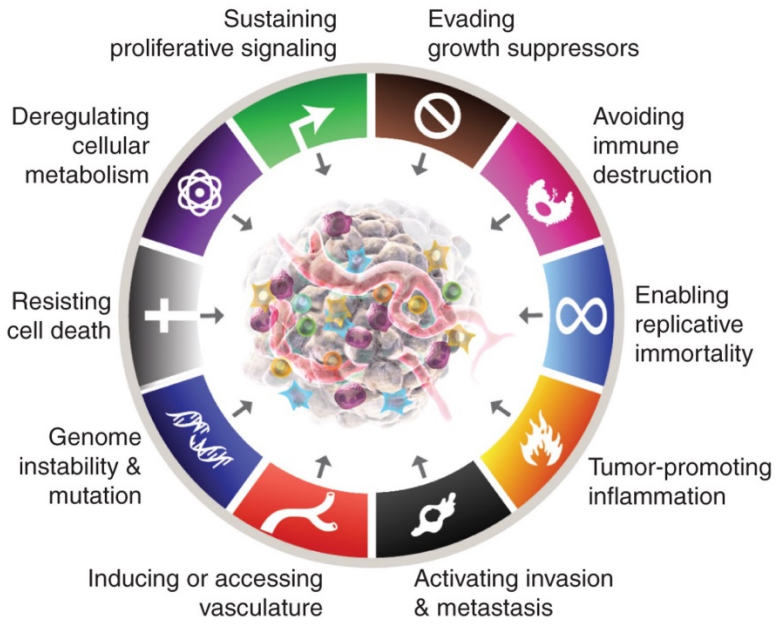


Figure 2: The hallmarks of cancer (including the two traits that enable the progression: genome instability and inflammation) (Hanahan, 2022).

1.2 Breast Cancer

1.2.1 Anatomy and physiology of breast

Human breasts are located on the anterior aspect of the thorax and serve as a milk-secreting organ for lactation. Breasts are paired mammary glands and one of the distinguishing features found in mammals. The organ consists of glands, adipose and connective tissue. Mammary glands are modified tubuloalveolar apocrine sweat glands that contain 15–20 lobes, which in turn are comprised of many lobules. Lobules are referred to as terminal ductules or acini, which are responsible for the milk production in response to prolactin. The lobules drain into a series of ducts and collectively into a single duct which opens onto the surface of the nipple (**Fig. 3**). Mammary glands consist of an epithelial bilayer. The inner part of the bilayer is formed by the luminal cuboidal cells and the outer part by the myoepithelial cells. Myoepithelial cells participate in the

process of milk ejection during lactation. The epithelial part of the mammary gland constitute 10 to 15% of its total volume. (Aranda-Gutierrez and Diaz-Perez, 2022, McGuire, 2019)

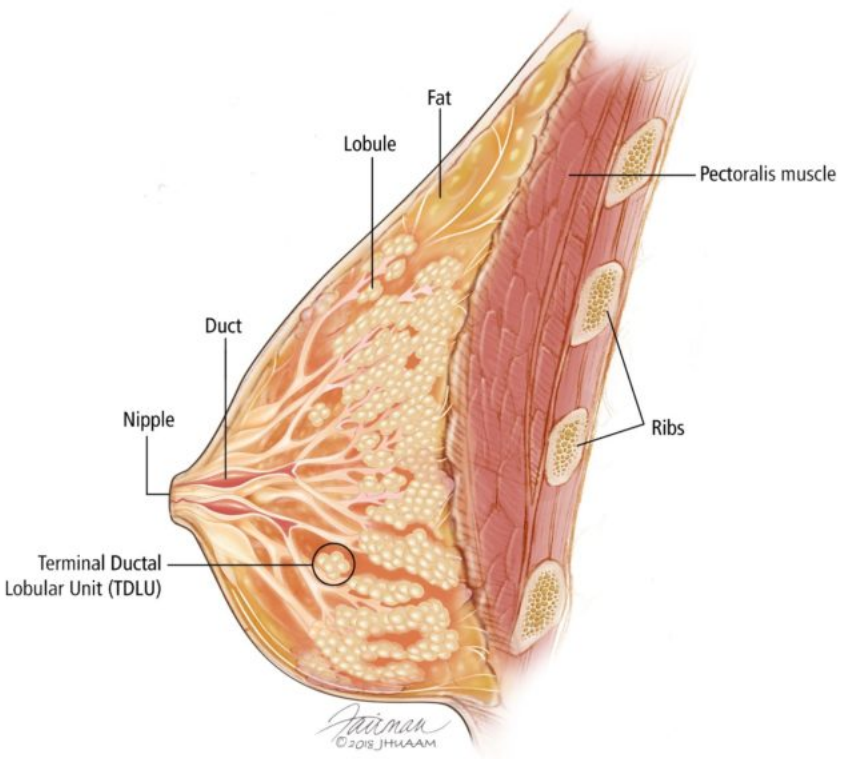


Figure 3: Breast Anatomy (https://pathology.jhu.edu/breast/overview)

1.2.2 Pathophysiology and molecular characteristics of breast cancer

Breast cancer is the leading cause of cancer-related deaths in women globally, according to CLOBOCAN 2020 estimates (Sung et al., 2021). The new cases are estimated at 2.3 million (11.7% of total) and the deaths at 685,000 (6.9% of total). Breast cancer is divided into two classes with respect to its histopathological confinement in the tissue or not: in situ and invasive. In situ or invasive breast cancer can be classified in one of the four subclasses: ductal, lobular, mucinous and papillary carcinoma. A different type of classification was proposed by Perou and Sorlie (Perou et al., 2000) based on the histological evaluation of specific molecular characteristics. These characteristics include the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). The positive or negative expression of these proteins classifies breast cancer in four subtypes: luminal A (ER^+, PR^+ and $HER2^-$), luminal B (HER^-): ER^+, PR^- and $HER2^-$ or luminal B ($HER2^+$): ER^+, $PR^{+/-}$ and $HER2^+$, HER2 positive tumors (ER^-, PR^-, $HER2^+$) and basal-like (triple negative phenotype; ER^-, PR^-, $HER2^-$) (**Table 1**) (Nascimento, 2020). ER+ tumors (luminal A and B) are the most common and account for up to 75% of all cases

(particularly in postmenopausal women >50 years). TNBC accounts for 10% to 20% of all breast carcinomas and is one of the most challenging breast cancer subtypes with a poor prognosis (Kumar & Aggarwal, 2016). Transcriptomic analyses have more precisely classified breast cancers adding the normal breast-like and claudin-low subtypes in the previous four subtypes. (Prat and Perou, 2011, Harbeck et al., 2019)

Table 1: Classification of molecular subtypes of breast cancer and therapies (Nascimento, 2020)

Molecular Subtypes	Luminal A	Luminal B		HER2+	TN
		(HER2-)	(HER2+)		
Biomarkers	ER+ PR+ HER2- Ki67low	ER+ PR- HER2- Ki67high	ER+ PR-/+ HER2+ Ki67low/high	ER- PR- HER2+ Ki67high	ER- PR- HER2- Ki67high
Frequency of Cases (%)	40–50	20–30		15–20	10–20
Histological Grade	Well Differentiated (Grade I)	Moderately Differentiated (Grade II)		Little Differentiated (Grade III)	Little Differentiated (Grade III)
Prognosis	Good	Intermediate		Poor	Poor
Response to Therapies	Endocrine	Endocrine Chemotherapy	Endocrine Chemotherapy Target Therapy	Target Therapy Chemotherapy	Chemotherapy PARP Inhibitors

ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2.

Major pathways that are disrupted in breast cancer are related to genes that are involved to ER (luminal A and B group fall into this pathway), amplification of a gene region that contains the ERBB2 gene (encodes HER2) and an expression signature of genes involved in the cell cycle and cellular proliferation (both HER2-positive tumors and TNBC fall into these pathways). The most frequently mutated and/or amplified genes in the breast tumor cells as reported in a series of early breast cancers are *TP53* (41%), *PIK3CA* (30%), *MYC* (20%), *PTEN* (16%), *CCND1* (16%), *ERBB2* (13%), *FGFR1* (11%) and *GATA3* (10%). Luminal A type has a high prevalence of *PIK3CA* mutations (49%), whereas basal-like tumors of *TP53* mutations (84%). Mutations in the tumor suppressor genes *BRCA1* (Breast cancer gene 1) and *BRCA2* (Breast cancer gene 2) are also involved in breast cancer development. Mutations in these genes are highly correlated with increased inherited risk of breast tumorigenesis by the age of 80. Other genes that have been revealed by next generation sequencing (NGS) to be involved are *ATM, CHEK2, PALB2* and *STK1*. (Harbeck et al., 2019)

1.2.3 Current Treatment

There are five major stages of breast cancer: stage 0, which is ductal carcinoma in situ (DCIS) with no metastatic potential, and stages I through IV, which are used for invasive breast cancer. Stage I comprises tumors that are smaller or with minimal invasion and negative nodes; Stage II and III include cases with increasing tumor or nodal extent, and Stage IV defines those who present with distant metastases. (AJCC, 2017) Breast cancer is curable in ~70–80% of patients with early-stage and non-metastatic disease. Advanced and metastatic breast cancer is currently incurable with metastases being the main cause of death and a median overall survival of 2–3 years. Advanced breast cancer includes inoperable tumor that has not spread yet (locally advanced), and metastatic (stage IV) breast cancer involves the metastatic spread to the common sites of the bone, lungs and liver. Surgery or radiation and systemic therapy are the major therapeutic strategies for the treatment of breast cancer. Systemic therapy includes endocrine therapy, targeted therapy (anti-HER2 therapy, PARP inhibitors for BRCA mutation carriers and others) and chemotherapy. Recently, immunotherapy was included in the therapeutic protocols for TNBC which is considered the most immunogenic from all the breast cancer types. FDA approved anti-PD1 therapy (pembrolizumab) for the treatment of early TNBC. (Harbeck et al., 2019, Schmid et al., 2020)

Adjuvant (after surgery) endocrine therapy and chemotherapy are very effective against early breast cancer and have significantly decreased mortality. In luminal early breast cancer adjuvant endocrine therapy is standard for at least 5 years after surgery. Patients with ER- positive and/or PR-positive breast cancers receive endocrine therapy to block ER activity independently of HER2 status. Estrogen promotes breast cancer development by engaging ER located in the nucleus. Endocrine therapy, such as tamoxifen, inhibits estrogen effects by binding ER. Other drugs in this class of therapy block the production of estrogen, such as aromatase inhibitors. In HER2-positive early breast cancer that are defined by protein overexpression or gene amplification, the standard of care includes neoadjuvant (before surgery) chemotherapy together with anti-HER2 therapy (monoclonal antibodies: trastuzumab and pertuzumab). Standard chemotherapy regimens include among other agents, anthracyclines and taxanes, which are the standard of care for TNBC. Similarly with HER2-positive early breast cancer, TNBC is treated with chemotherapy in the neoadjuvant setting. Another therapeutic approach is the postoperative radiation therapy, which is shown to improve disease-free and overall survival for patients with early breast cancer. The mechanism of action among others is also related to the induction of immunogenicity that causes a systemic immune response which can lead to the elimination of the residual tumor cells or to the abscopal effect. Radiation therapy also plays a crucial role in eliminating metastases from bone, brain and soft tissue, among others. (Harbeck et al., 2019)

1.2.4 Animal models of breast cancer

Mouse breast cancer models are a key tool for the study of breast cancer biology and therapy. Although there are differences between mice and human breast cancer development, there are certain models that are very useful for studying specific subtypes. These are either spontaneous, induced or transgenically driven. The induced models can be produced by viruses (e.g., mouse mammary tumor virus, MMTV), chemical carcinogens, hormonal agents, and ionizing radiation. Additionally, cancer cell lines can be directly implanted to compatible host animals. These are the most commonly used models for screening therapeutics. In this case both animal and human cell lines or tissues are grafted in syngeneic and non-syngeneic hosts in different sites (subcutaneous, orthotopic, and systemic).

A syngeneic approach uses cancer cells and a host from the same genetic background avoiding the need for immunocompromised animals. There are numerus murine breast cancer cell lines that have been isolated from spontaneous, chemically induced, or transgenic tumors. A very well characterized is the 4T1 cell line, which originated from one spontaneous tumor in a BALB/cfC3H mouse. From this spontaneous tumor a series of TNBC cell lines derived (67NR, 168FARN, 4TO7, 66cl4, and 4T1) exhibiting different tumorigenic and metastatic characteristics (Aslakson and Miller, 1992). The 4T1 cell line after orthotopic inoculation, grows rapidly at the primary site and forms metastases in lungs, liver, bone, and brain in 3–6 weeks depending on the initial inoculation density. The 4T1 model is ideal for the study of TNBC and the metastatic progression because it is aggressive and highly efficient, whereas the metastatic sites recapitulate very well the human metastatic disease. (Pulaski and Ostrand-Rosenberg, 2001). EO771 medullary breast adenocarcinoma is another cell line for modelling TNBC in mice. They originate from a spontaneous tumor in a C57BL/6 mouse and they are also metastatic. (Ewens et al., 2005). Both breast cancer cell lines were used for the development of the animal models of cancer used for the *in vivo* studies performed in this dissertation.

In contrast to syngeneic models, xenograft tumors derived from human cell lines require host animals with compromised immunity. It is now well known that the immune system plays a major role in cancer progression, which makes the dependence of immunocompromised hosts a great limitation of xenograft models (Sereti et al., 2018). However, there are various human cell lines that have been isolated from patients with various subtypes of breast tumors and have driven the research for many decades. Most of the well-established human breast cancer cell lines were derived from metastases rather than from primary tumors. MCF-7 cell line was established in 1973 and is one of the most widely used xenograft model of breast cancer. It is noninvasive and represents a model for early-stage disease (Soule et al., 1973). Another human cell line is the MDA-MB-231, which is commonly used for modelling triple negative late-stage breast cancer and

when inoculated orthotopically produces xenografts that metastasize to lymph nodes (Cailleau et al., 1974). (Welsh, 2013)

Genetically engineered mouse models (GEMMs) are another major category for modeling breast cancer. Most of the transgenic models were designed to express specific promoters, which are highly active in the luminal epithelial cells of the mouse mammary gland. The most commonly used are the MMTV (Dudley et al., 2016), the whey acidic protein (WAP) (Ranganathan et al., 1999), and the b-lactoglobin (BLG) (Palmer, 1934). These promoters are hormone dependent but generally they drive genes expression at puberty (MMTV) or pregnancy (WAP and BLG). The first transgenic model expressed the c-Myc oncogene specifically in mammary epithelial tissues by the MMTV promoter. To date, thousands of murine models of breast cancer exist that not only express activated oncogenes, but also lack tumor-suppressor genes or are generated by more advanced techniques including inducible promoters such as the tetracycline regulators and conditional techniques such as the Cre/lox system. Some of the most commonly used are MMTV–Ras, MMTV–Neu models (Neu gene is the murine homolog of the human ErbB2 that codes for HER2), MMTV–PyVMT model (expresses the polyoma virus middle T antigen) and models with loss of function of the tumor-suppressors p53, BRCA1 and BRCA2. (Welsh, 2013)

1.3 Pancreatic Cancer

1.3.1 Anatomy and physiology of pancreas

Pancreas is an organ of the gastrointestinal system that is located in the upper abdomen behind the stomach and connected with other abdominal organs including the spleen, stomach, duodenum and colon (**Fig. 4**). The organ has a dual function: exocrine and endocrine. The exocrine gland participates in the digestion process by making and secreting digestive enzymes, ions and water into the duodenum. The endocrine gland synthesizes peptide hormones that are secreted in the blood and are responsible for the maintenance of glucose homeostasis. The exocrine activity is performed by acinar and ductal cells that produce digestive enzymes and sodium bicarbonate to facilitate food processing and nutrients absorption. Most of the pancreas mass consists of the cells with exocrine activity (90-95%). There are different groups of proteolytic enzymes depending on the molecules they digest: proteolytic enzymes (e.g., trypsin, chymotrypsin), lipolytic enzymes (e.g. lipase, phospholipase, and esterase), glycolytic enzymes (e.g., lactase and amylase) and nucleolytic enzymes (e.g, ribonuclease and deoxyribonuclease). The secretion of digestive enzymes is regulated by food consumption, neurotransmitters and hormones. The role of pancreas in digestion process is vital and could lead to severe malnutrition if compromised.

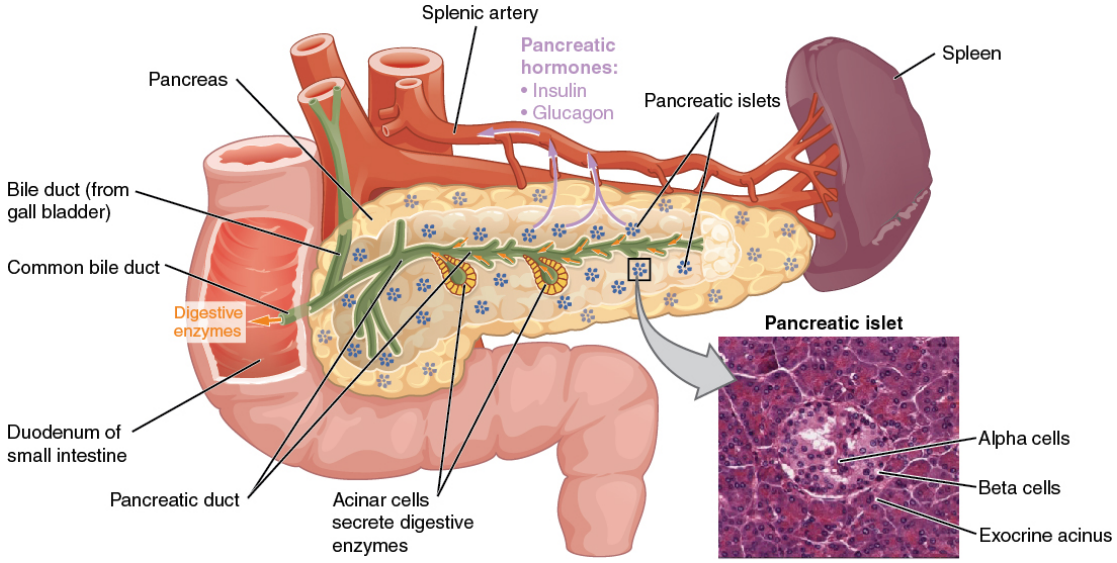


Figure 4: Pancreas anatomy (https://www.olivelab.org/the-pancreas-overview.html)

Langerhans islets are responsible for the endocrine function that produce hormones such as glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide (PP). The islets consist of different types of cells, namely the alpha cells (α-cells), beta cells (β-cells), delta cells (δ-cells), PP cells (also known as F-cells) and the epsilon cells (ε-cells), which have distinct roles in hormone type that produce and secrete. Examples are α-cells that produce glucagon and β-cells that produce

insulin and represent the dominant population (around 80% of the islet cells). The endocrine portion of the organ represents only 1–2% of the total mass of the organ. (Pandol, 2010, Leung, 2010, Beger, 2018)

1.3.2 Pathophysiology and molecular characteristics of pancreatic cancer

Pancreatic cancer is among the most aggressive and lethal malignant neoplasms. It is the seventh leading cause of cancer-related death worldwide with similar incidence (n=496.000) and mortality (n=466.000) rates according to GLOBOCAN 2020 estimates. The overall 5-year survival rate of pancreatic cancer is 9%, which is the lowest among all cancer types. (Sung et al., 2021)

Pancreatic cancer includes all the malignant neoplasms that evolve from the pancreas. However more than 90% are pancreatic ductal adenocarcinomas (PDAs), followed by the rare types of neuroendocrine tumors, acinar carcinomas, colloid carcinomas, pancreatoblastomas and solid-pseudopapillary neoplasms. (Kleeff et al., 2016) Pancreatic carcinogenesis starts with the formation of precursor lesions, most typically pancreatic intraepithelial neoplasias (PanINs). It can also evolve from intraductal papillary mucinous neoplasms or mucinous cystic neoplasms. Evidence shows that they arise from a progenitor cell population within the pancreas that has stem-cell-like properties. This population derives either from centroacinar cells located at the junction of acini and ductal epithelium or from mature acinar cells that transdifferentiate into ductal cells, in a process called acinar-to-ductal metaplasia (ADM). It has also been proposed that a duct epithelial stem cell could also be the progenitor. PanINs are represented by three stages that are characterized by gradual atypia of the duct epithelium, called PanIN-1, PanIN-2 and PanIN-3 (**Fig. 5**). PanIN-3 corresponds to carcinoma in situ. The most mutated genes in pancreatic cancer are: *KRAS2*, *CDKN2A*, *TP53* and *SMAD4*. *KRAS2* oncogene upregulation is the most common genetic mutation in pancreatic cancer present in almost all the cases. *KRAS2* encodes a member of the RAS family of the GTP-binding proteins that is responsible for a wide range of cellular functions, including proliferation and cell survival. *CDKN2A* (also known as *p16* or *INK4A*) is the most common inactivated tumor suppressor gene in pancreatic cancer, followed by the *TP53* gene. *SMAD4* is inactivated in approximately 55% of pancreatic cancers and its loss results in aberrant signaling by the transforming growth factor β (TGF-β). (Iacobuzio-Donahue, 2012, Makohon-Moore and Iacobuzio-Donahue, 2016)

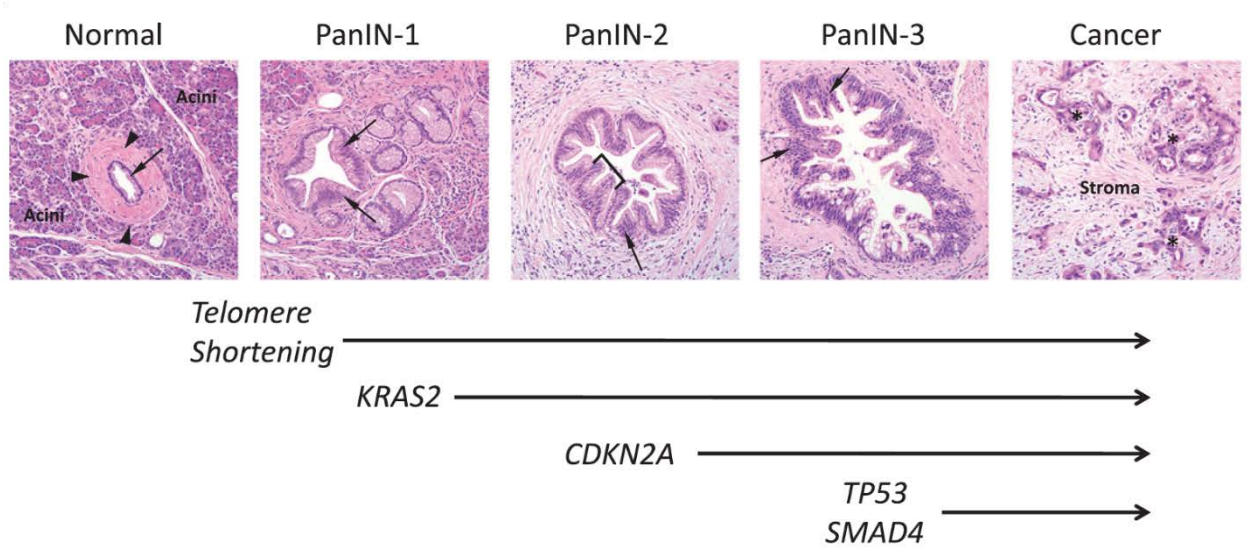


Figure 5: Progression model from intraepithelial neoplasia to pancreatic cancer (Iacobuzio-Donahue, 2012)

One of the unique characteristics of PDA is the dense desmoplastic stroma that surrounds the pancreatic tumor cells supporting their growth. The stroma constitutes up to 90% of the tumor volume and includes extra cellular matrix components, including laminins, fibronectins, collagens and hyaluronan. It is considered to originate from cancer-associated fibroblasts (CAFs) that derived from healthy pancreatic stellate cells after stimulation due to injury or chronic inflammation. There are distinct types of CAFs named myofibroblastic or inflammatory. Another characteristic of PDA biology is the presence of macrophages type-2 and myeloid-derived suppressor cells (MDSCs) that inhibit T lymphocytes infiltration and thus the anti-tumor response contributing to immune escape. These myeloid populations are often elevated in PDA patients and are found to be correlated with negative clinical prognosis. (Orth et al., 2019)

Attempts in identifying PDA subtypes by sequencing technology have been useful, but not straightforward, as different groups suggest different categorization (Collisson et al., 2019). Nevertheless, some subtypes are shared between the studies: classical/canonical subtypes are characterized by epithelial-like gene expression and quasi-mesenchymal/basal-like subtype by a more mesenchymal gene expression signature and poorer prognosis.

1.3.3 Current treatment

The available treatments for pancreatic cancer are very limited and with very low efficacy. Pancreatic cancer shows unique resistance to traditional chemotherapy and radiation therapy and even early-stage patients with the option of surgery have a 5-year survival rate <31%. Unfortunately, most patients are often in advanced stages at the time of the diagnosis, which is another disadvantage of the disease as these patients have no effective therapeutic options. It is

also typical that pancreatic cancer patients rarely develop symptoms before the advanced stages. (Wang et al., 2021)

Gemcitabine has been the first-line therapy since 1997 and together with other chemotherapeutic regimens are currently the best therapeutic options. These regimens include FOLFIRINOX (5-fluorouracil (5-FU), leucovorin, oxaliplatin, and irinotecan) and nab-paclitaxel plus gemcitabine, which however have shown a moderate increase in overall survival (11.1 months and 8.5 months, respectively, compared to 6.7 months of gemcitabine monotherapy) with considerable toxicity (Von Hoff et al., 2013, Conroy et al., 2011). Additional options in the era of targeted therapy (cetuximab (anti-epidermal growth factor receptor, EGFR), bevacizumab (anti-vascular endothelial growth factor, VEGF), axitinib (VEGFR1-3 inhibitor), and aflibercept (decoy receptor for VEGF-A, -B and placental growth factor (PIGF)) have failed to improve overall survival, while erlotinib (EGFR inhibitor) combined with gemcitabine showed a marginal clinical benefit (Moore et al., 2007). Newer classification as described above have revealed useful information of the PDA heterogeneity that could be utilized for the design of new therapeutic strategies. These strategies may include innovative immunotherapies and combination therapies with increased anti-tumor activity. (Orth et al., 2019)

1.3.4 Animal models of pancreatic cancer

There are both murine-based and patient-derived models for the study of human pancreatic cancer in the preclinical setting. The first category includes syngeneic, human cell line-based xenografts and genetically engineered mouse models (GEMMs), and the second, models that originate from established cell lines and patients' tumors, named patient-derived xenografts (PDXs). Syngeneic mouse models of PDA are developed by the subcutaneous or orthotopic inoculation of compatible murine pancreatic cell lines [such as Panc02 cell line that was derived from a C57BL/6 mouse through induced carcinogenesis, primary cell lines derived from GEMMs etc.]. The orthotopic models are considered more biologically relevant as the subcutaneous inoculation is not performed at the derived organ, but at an ectopic location. Immunocompromised mice like nude or SCID (severe combined immunodeficiency) must be used for the PDXs so the foreign cells will not be rejected, which is a limitation for the use of these models in immunotherapy studies (Sereti et al., 2018, Magouliotis et al., 2022). However, they have been used successfully in personalized therapies as they can be tested directly in the tumor of each patient. (Pham et al., 2021, Hwang et al., 2016). *Ex vivo* 3D models such as spheroids and organoids, have also been developed and successfully used for the evaluation of pancreatic tumor resistance to therapeutics. While the field of organoid technology holds promise, it faces several limitations, including challenges in developing and maintaining organoids, as well as a lack of

inter-organ communication and numerous elements of the tumor microenvironment (TME), such as immune cells. (Sereti et al., 2023)

One of the most clinically relevant available animal models of pancreatic cancer is the KPC GEMM: Pdx1-Cre;KrasG12D;Trp53R172H (Hingorani et al., 2005). PDX1 (pancreatic and duodenal homeobox 1) is a transcription factor that is expressed in pancreatic tissue and participates in its development. KPC mouse expresses the PDX1 promoter (embryonic day 8.5) that enables the expression of Cre recombinase in the acinar, ductal and islet cells of the pancreatic tissue. The model has conditional point mutations in the oncogene *KRAS* (G12D) and the tumor suppressor gene of *TRP53* (R172H) that mimic the clinically occurring mutations. Without Cre recombinase, the two mutated genes are not expressed due to the presence of a termination sequence (lox-stop-lox) upstream of the genes. Cre-mediated recombination excises the termination sequence and enables the expression of the mutated genes leading to pancreatic tumor formation in 4 to 6 months after birth.

1.4 Metastasis

Metastasis is the process that transforms localized cancer into a systemic disease and is responsible for most of the cancer-related deaths (~90%) (Chaffer and Weinberg, 2011). The metastatic process involves a sequence of events that starts with the invasion of primary tumor cells to the surrounding tissue, the intravasation and survival in the circulation as circulating tumor cells (CTCs) and the extravasation and eventual colonization to the metastatic site (Chambers et al., 2002). Despite the advances in cancer research, the treatment options for cancer patients with advanced metastatic disease are not effective.

1.4.1 Circulating Tumor Cells (CTCs)

During tumor progression, some cancer cells can acquire invasiveness and motility traits which will facilitate their invasion to the adjacent tissues. These cells are known as circulating tumor cells (CTCs). Millions of them are shed from the primary tumors, enter the circulation, and disseminate throughout the body ultimately forming metastatic lesions. This process, however, causes enormous stress to the cancer cells that leads to death of most of them. CTCs detection in patients' blood has become an emerging field, with several groups working on detection assays while clinical trials incorporating CTC counts in their protocols.

CTCs were first observed in the blood of a patient with metastatic cancer by Ashworth in 1869. He reported them as cells akin to primary tumor cells (Ashworth, 1869). However, subsequent assiduous studies revealed that there is distinction between CTC and primary tumor

cells which is based in genes related to the epithelial to mesenchymal transition (EMT) or stemness. This distinction is the principle for the molecular-based detection assays using epithelial and mesenchymal cancer markers. The ability to invade proximal tissues is highly related to EMT. The reverse process is called mesenchymal to epithelial transition (MET) and is necessary for the colonization of the metastatic tissues. Those CTCs that succeeded to establish colonies have accomplished the successful transition between these two phenotypes. The most common epithelial marker used for the detection of CTCs is EpCAM which is expressed in most cancer types and especially in breast and prostate cancer making the assay widely applied in these types. Other detection methods include the combination of epithelial and mesenchymal markers, such as EpCAM and N-cadherin, markers of EMT transition (E-cadherin, vimentin, and twist) or marker-independent methods (single-cell assays). Furthermore, hallmark markers of some cancer types are also used for the detection. Some of these markers are HER2, ER, prostate-specific membrane antigen (PSMA), folate receptor, survivin and MAGE A3. One limitation of the available detection technologies is that CTCs present heterogeneity in the expression of these markers among the different cancer types and patients. Thus, it is difficult to define the entire CTC population only with the currently available markers. (Lin et al., 2021)

The mechanisms that participate in cancer cells dissemination are not completely understood. There are reports that relate them to stroma-tumor cell interactions, hypoxia response, induction of metalloproteinases, angiogenesis and epithelial-mesenchymal transition (EMT). There are also reports of CTCs' interactions with cell populations which facilitate their escape from the immune system. Platelets, neutrophils, macrophages, myeloid-derived suppressor cells (MDSCs) and cancer-associated fibroblasts (CAFs) have been reported to interact with CTCs protecting them and promoting their survival. (Garrido-Navas et al., 2019, Rejniak, 2016)

Chapter 2 - Immune System

2.1 Overview

The immune system has been developed to maintain body homeostasis by providing protection against both exogenous and endogenous threats. These include foreign infectious agents and self-malignant cells that can be dangerous for the normal function of the organs and tissues. The immune system also maintains the balance between host and commensal organisms, promotes clearance of dead cells and tissues and induces wound healing. It can also mount responses against unharmful agents, such as allergens and self-antigens, creating allergies and autoimmune diseases, respectively. Moreover, the difficulty of the successful transplantations relies on the immune response against foreign antigens that the system recognizes on the allografts. (Rao, 2018)

The immune responses are orchestrated by a dynamic network of lymphoid organs and tissues, immune cells and signaling molecules that coordinate to react in response to a threat. The immune organs and tissues are divided into primary (or generative) and secondary (or peripheral) organs and tissues. The primary lymphoid organs are the bone marrow and thymus where the immune cells are produced (except of some tissue-resident macrophages, like microglial cells that originates from embryonic tissues (Ginhoux et al., 2010)) and mature for the most part. Further maturation takes place in the periphery, at the secondary lymphoid organs. These include the spleen, lymph nodes, and epithelial- and mucosa-associated lymphoid tissues that provide the environment for close contact and interaction. The lymphoid organs communicate with the rest of the body tissues with lymphatic and blood vessels. Recently, another type of lymphoid structure has been reported, the tertiary lymphoid tissues that are aggregates developed in autoimmune diseases, chronic infections and allograft rejections (Drayton et al., 2006). (Murphy and Weaver, 2016)

Hematopoietic stem cells (HSCs) are the progenitors of all blood cell types, including the white blood cells or leukocytes. Leukocytes play different roles in sensing and recognizing the threats, reacting and regulating the response and, finally, developing memory. They are divided into two main categories of myeloid and lymphoid lineages that have different morphological and functional characteristics (**Fig. 6**). The myeloid lineage derives from the common myeloid progenitor (CMP) that gives rise to granulocytes (neutrophils, eosinophils, basophils, and mast cells), monocytes/macrophages, dendritic cells, erythrocytes, and megakaryocytes. The lymphoid lineage derives from the common lymphoid progenitor (CLP) that gives rise to Natural Killer cells (NKs) and T and B lymphocytes. Mature immune cells reside in the peripheral tissues and circulate in the bloodstream and the lymphatic system. (Parkin and Cohen, 2001, Murphy and Weaver, 2016)

Immune cells constantly move responding to specific chemical stimuli, the cytokines. These are transiently secreted proteins with autocrine and/or paracrine actions targeting primarily the immune system. These inflammatory mediators exert growth, differentiation, and activation functions acting on specific cell-surface receptors of the producing or target cell. Cytokines direct immune cell trafficking and determine the nature of immune responses, which can be cytotoxic, humoral or allergic. (Lin and Leonard, 2019, Borish and Steinke, 2003)

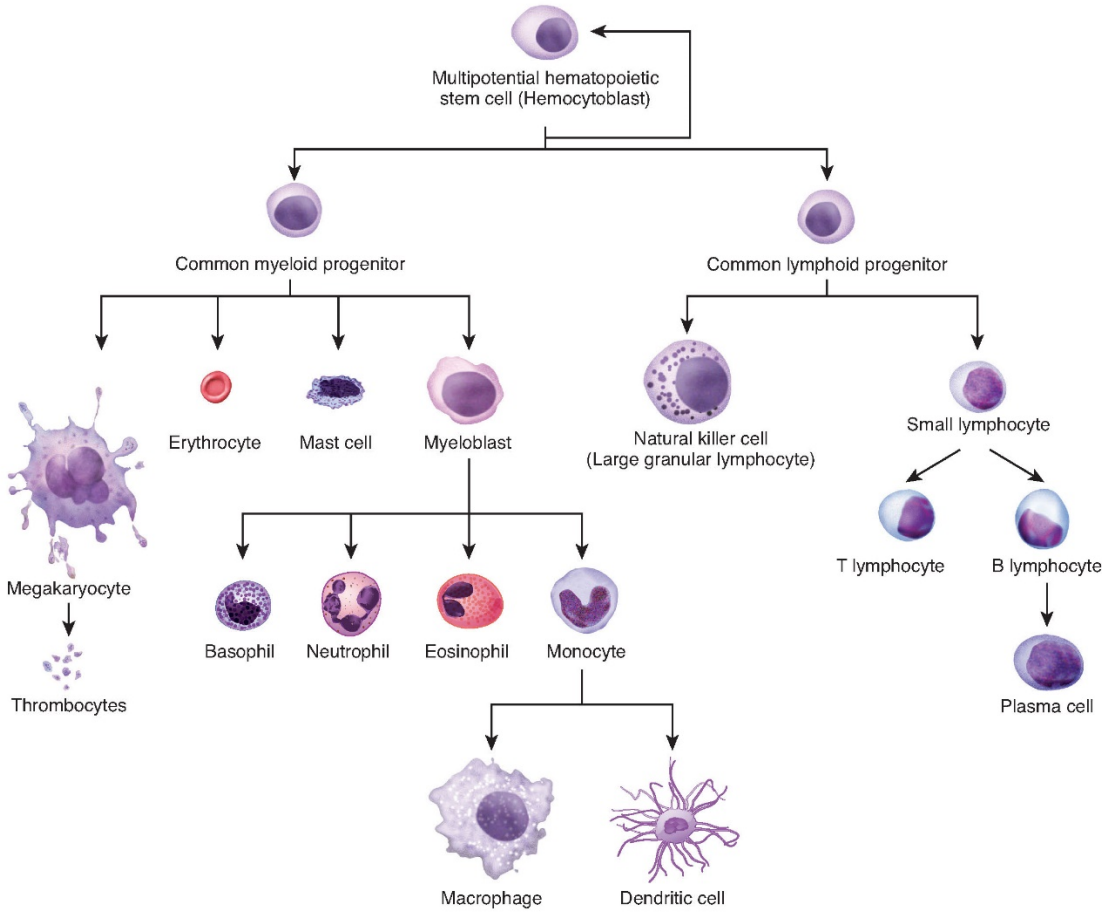


Figure 6: Hematopoiesis (image by A. Rad)

The immune system has evolved, acquiring some unique features that makes it very effective in protecting the body. One of them is the ability to distinct self from non-self, known as self-tolerance. The host defense mechanism must detect structural features of the invading pathogens that are distinct from the host cells. This discrimination is vital to prevent the immune system from damaging host tissues, which otherwise leads to autoimmune diseases. (Chaplin, 2010) Another crucial feature is the ability to create immunological memory after each exposure which provides immunity. The second exposure to an antigen induces a faster and stronger immune response. Immune memory was first documented in ancient Greece by the historian Thucydides

who noted that "the same man was never attacked twice" when he described the plague of Athens in 430 BC (Rutter, 2000). The conception of immune memory is widely regarded as one of the most significant contributions to public health in history. It is often attributed to Edward Jenner for his work in 1976, which has saved countless human lives. Jenner vaccinated a boy with cowpox which provided immunity to smallpox conceptualizing the importance of vaccination. (Murphy and Weaver, 2016)

2.2 Types of immunity

There is a main distinction between the types of leukocytes that relies on how specific they are in antigen recognition. This distinction classifies immunity into two types: the innate and the adaptive immunity. Innate immunity, appeared first during evolution, is inherited and includes leukocytes that target pathogens based on common structural patterns. Immune cells of the innate immunity include cell types of myeloid lineage, such as granulocytes, monocytes/macrophages, and dendritic cells, and some of the lymphoid lineage that are called innate lymphoid cells (ILCs), such as the Natural Killer cells (NKs). Development in immunology research throughout the years has revealed several additional cell subsets that appear to have innate–like properties, like NKT and γδ T cells (Godfrey et al., 2015). Adaptive immunity is developed in response to exposure to foreign antigens and has great sensitivity. Cells of adaptive immunity include B and T lymphocytes that carry receptors derived upon gene rearrangement able to recognize almost every antigen with great specificity. Although cells of innate immunity respond fast, they do not live long. In contrast, cells of adaptive immunity need time to develop but are long-lived cells that provide long-term protection through the immunological memory. (Rao, 2018, Murphy and Weaver, 2016)

2.2.1 Innate immunity

Innate immunity constitutes the first line of defense preventing pathogens' entrance and providing fast responding immune mechanisms. This immediate defense consists of anatomic and chemical barriers, circulating plasma proteins called complement and cells that recognize structural patterns on pathogens or signals derived from tissue damage. Skin and mucosal tissues are the first anatomic barriers that block microbes, through tight cell-cell contacts and mucus secretion, respectively. If host's first barriers are breached, the system will defend with innate chemical barriers and innate immune cells. The innate chemical barriers are antimicrobial enzymes (e.g., lysozyme that digests bacterial cell walls), peptides (e.g., defensins that directly lyse bacterial cell walls) and plasma proteins of complement that lyse pathogens directly or mediate their phagocytosis. The innate immune cells will be "notified" through inflammatory signals induced

by pathogens or damaged tissues. These inducers are pathogen associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) that sensor cells (e.g. macrophages and dendritic cells) recognize through their pattern recognition receptors (PRRs) (Takeuchi and Akira, 2010). Sensor cells respond either by killing the pathogens directly or by amplifying the response though the production of inflammatory signals, such as cytokines and chemokines. These signaling molecules inform effector cells (e.g., NKs) that there is a harmful invasion and activate them to eliminate it. (Chaplin, 2010, Murphy and Weaver, 2016)

Sensor cells appear as tissue resident cells with the ability to phagocyte pathogens. Phagocytosis is the process of engulfing microbes for elimination. Cells with phagocytic activity are monocytes/macrophages, granulocytes (neutrophils, eosinophils, basophils, and mast cells) and dendritic cells. Monocytes are the immature form of macrophages that circulate in the periphery. Signals from an infected area provided by epithelial cells will activate monocytes to migrate into the tissues. Monocytes that arrive at the infection site differentiate into mature macrophages. In addition to newly arrived macrophages, there are also resident macrophages that arise during embryonic development in all the tissues. Macrophages are also involved in wound healing and immune regulation. (Mosser and Edwards, 2008) The second major group of phagocytes are the granulocytes: neutrophils, basophils, and eosinophils. These cells contain granules filled with proteins that have microbicidal activity. Neutrophils are derived from bone marrow, and they are the most abundant population in the blood. They are the first that arrive at the inflammation site responding to signals coming from endothelial cells that have been stimulated by tissue resident macrophages. Neutrophils kill through the protein content of their granules or through respiratory burst by rapid increase of reactive oxygen species (ROS) production. They also form extracellular nets (containing degraded nuclei acids and histones) that trap the microorganisms. Neutrophils are implicated in tissue repair and remodeling, and recruitment and activation of the adaptive immune system. (Borregaard, 2010) Eosinophils and basophils are recruited to sites of allergic inflammation, and they defend the host against parasites. Another immune cell population that is implicated in allergic responses is mast cells that complete their maturation in tissues. The third group of phagocytes are the dendritic cells that play a central role in connecting the innate with the adaptive immunity. They reside in peripheral tissues and lymphoid organs. Immature dendritic cells travel via blood to enter peripheral tissues, where they uptake large amounts of extracellular matrix or phagocyte microorganisms. When they encounter a pathogen, they mature and migrate to lymphoid tissues. Dendritic cells do not use phagocytosis for killing microbes, but rather for breaking down microbe-derived proteins to generate peptide antigens. These peptides are bound to MHC class type I and II of dendritic cells and presented to T lymphocytes in lymphoid organs. Dendritic cells are considered professional antigen-presenting

cells (APCs), as they are the most effective immune cells in antigen-presentation. Antigen-presenting cells are also macrophages and B cells. NKs are innate immunity cells with cytotoxic capability. They are morphologically defined as large granular lymphocytes, and they recognize their target using a complex repertoire of activating and inhibitory cell surface receptors. NKs will be discussed in more depth in section 3.3.2 in the context of cancer. (Chaplin, 2010, Murphy and Weaver, 2016)

2.2.2 Adaptive immunity

Adaptive immunity comprises of two major types of cells, B and T lymphocytes. The two cell types are distinguished by their sites of differentiation. Both originate from the bone marrow, but while B cells complete their development in the organ, immature precursors of T cells migrate to thymus to finish maturation. B cells have receptor forms of immunoglobulin on their surface that mediate the antigen recognition, known as the B-cell receptors (BCRs). The secreted form of immunoglobulin is the antibody, which is produced by the terminally differentiated B cells, called plasma cells. Autoreactive B cells that bind self-antigens are negatively selected within the organ as a result of central tolerance. Their maturation continues after they migrate and home into the secondary lymphoid organs. T cells maturation occurs in thymus, which is the organ responsible for positive and negative selection of T lymphocytes. Lymphocytes' "education" ensures that naive T lymphocytes are not self-reactive and that they are MHC restricted, meaning that they recognize processed peptides that are presented in host MHC molecules. Educated T cells exit the thymus and enter the lymphoid organs and peripheral blood. Both B and T lymphocytes that emerge from the bone marrow and the thymus are naïve as they have not yet encountered their specific antigen. The priming of B and T lymphocytes takes place in the specialized environment of the lymph nodes. The effector T lymphocytes migrate to circulation in search of the corresponding antigen and the secreted antibodies are released into blood and tissue fluids where they will finally encounter their target. A subset of the effector B and T cells differentiates into memory cells that will rapidly respond in case of a second exposure. (Chaplin, 2010)

Adaptive immunity bifurcates in humoral and cellular immunity. The immune components of the adaptive immunity that are T cell mediated are considered parts of the cellular immunity. Cellular immunity also includes cells that participate in T cell activation and various cytokines that are released for their communication. Macrophages, NKs, DCs and T lymphocytes are all cells of the cellular immunity. Some of the key characteristics of these cells have already been discussed, but more in-depth review regarding their involvement in anti-cancer immunity follows in section 3.3.1. Humoral immunity is mediated by B lymphocytes and antibodies. As discussed, they differentiate into plasma cells upon antigen recognition, which are terminally differentiated cells

that produce and secrete antibodies. These antibodies are of five isotypes: lmmunoglobulin (Ig) M, lgD, lgG, lgA and lgE. Surface markers that are used to identify mouse B cells are: CD220 (CD45R), CD19, CD20 and MHC II. (Chaplin, 2010)

T lymphocytes carry receptors that are called T cell receptors (TCRs) and differ from the receptors on B cells. TCRs recognize antigens that are presented on the major histocompatibility complexes (MHC) class I and II by antigen presenting cells (**Fig. 7**). Together with the antigen presentation, APCs also provide co-stimulatory signals, such as B7 molecules (CD80 and CD86) that engage CD28 on T cells and prime them to effector T cells. T cells expressing TCRs that bind MHC Class I molecule mature into $CD8^+$ T cells, while those that express TCRs that bind MHC Class II molecule mature into $CD4^+$ T cells. $CD8^+$ T cells that are activated by antigen presentation on the MHC class I molecule are directly cytotoxic. $CD4^+$ T cells differentiate into different subtypes depending on the cytokine milieu in the microenvironment. The subtypes include T-helpers 1 (Th1), T-helpers 2 (Th2), T-helpers 17 (Th17), T regulatory cells (Tregs), T follicular helper cells (Tfh) and T-helpers 9 (Th9). $CD4^+$ T helper cells secrete cytokines that regulate the immune response. Th1 cells favor cellular immunity that targets intracellular pathogens by the activation of $CD8^+$ T cells, while Th2 cells favor humoral immunity to activate a response against extracellular threats by the activation of B cells. (Naing and Hajjar, 2017) BCR engagement with the specific antigen leads to B cell activation. Activated B cells secrete cytokines and present their recognized antigen on MHC class II molecules to $CD4^+$ T helper cells which are required for their full activation. Full activation induces their differentiation to antibody secreting plasma cells (**Fig. 7**). Specific clones of effector plasma cells arise in a process of clonal selection. (Murphy and Weaver, 2016)

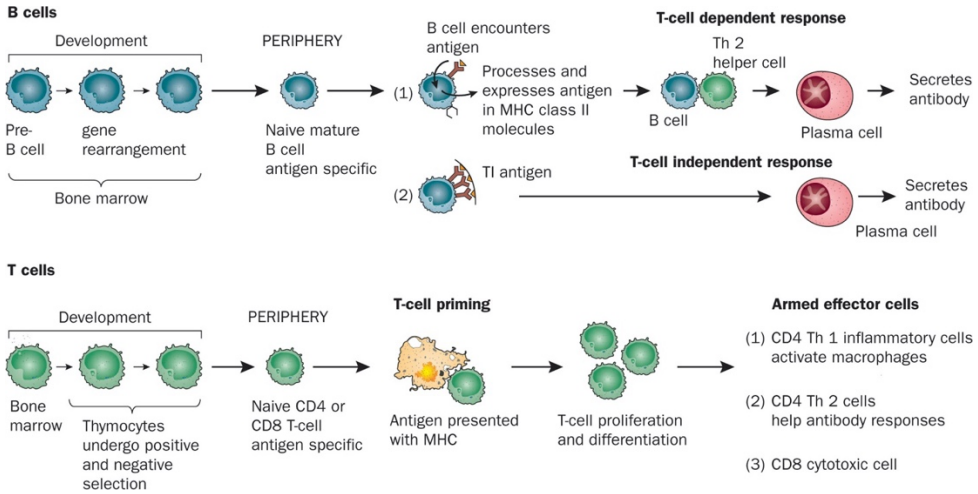


Figure 7: B And T cell priming and differentiation (Parkin and Cohen, 2001)

Chapter 3 - Cancer Immunotherapy

3.1. History

The idea that the immune system protects the host from tumor cell growth was conceived in the middle of the 20th century with some initial insights from the 19th century. As early as 1863, Virchow made the observation that tumors are infiltrated by leukocytes (Virchow, 1863). Some years later (1891), William B. Coley, the father of immunotherapy, was the first to practice the use of immune system against cancer using bacterial products named Colin's toxins (Starnes, 1992). He used a combination of two heat-inactivated bacteria of *S.pyogenes* and *S. marcecsens* based on some previous remissions observed in sarcoma patients infected with erysipelas. This regimen led to favorable long-term responses, but it was not widely accepted by medicine at that time as there was no scientific explanation for these results. After many years of experimental research, we can now comprehend the rationale behind Coley's successful results. One major premise is the cancer immunosurveillance, which is the process of patrolling and eliminating tumor cells by the immune cells (Kim et al., 2007). This process was discovered by Burnet in 1957 (Burnet, 1957) while it was suggested years earlier by Paul Ehrlich (1909). Several subsequent studies verified the important role of the immune system in inhibiting carcinogenesis (Yuzhalin and Kutikhin, 2014). However, clinical, and preclinical studies conducted in 1980s and 1990s really gained the interest around cancer immunotherapy. Talpaz and colleagues at the MD Anderson Cancer Center started treating CML patients with recombinant interferon alpha (Talpaz et al., 1983). In 1986, (IFN)-α was approved by the FDA for the treatment of hairy cell leukemia making cytokines the first immunotherapeutic approach to be approved as cancer treatment. For many years Rosenberg from National Cancer Institute was working on IL-2 treatment as monotherapy and the adaptive cell transfer of autologous T infiltrating cells that were expanded ex vivo using IL-2. In 1987, he published important data on clinical responses in patients with metastatic renal cell cancer (23%) and melanoma (29%) after high-dose IL-2 therapy (Rosenberg et al., 1989). Several clinical trials were followed showing objective responses mainly in patients with these cancer types (Rosenberg, 2014). This led the FDA to approve IL-2 as an anti-cancer drug for metastatic renal cell cancer and advanced melanoma in 1992 and 1998, respectively. Some other studies revealed the importance of IFN-γ signaling and the role of T cells in cancer immunosurveillance. When mice were treated with antibodies against interferon-γ (IFN-γ), their tumors were growing more aggressively (Dighe et al., 1994) and mice with damaged IFN-γ receptor or T cells were more susceptible to induced sarcoma (Kaplan et al., 1998, Engel et al., 1996, Engel et al., 1997). Some years later Shankaran et al. (Shankaran et al., 2001) reported that IFN-γ and lymphocytes prevented

tumorigenesis and shaped tumor immunogenicity, introducing the concept of cancer immunoediting.

The journal *Science* selected cancer immunotherapy as the breakthrough of the year in 2013 (Couzin-Frankel, 2013). This followed the events of 2011, where the FDA approved two immunotherapeutic drugs. One was the dendritic cell vaccine sipuleucel-T (Provenge) for the treatment of metastatic castrate-resistant prostate cancer (Kantoff et al., 2010) and the other the revolutionary antibody ipilimumab that targets the immune checkpoint inhibitor (ICI) CTLA-4 (cytotoxic T-lymphocyte antigen 4) for the treatment of metastatic melanoma (Hodi et al., 2010). CTLA-4 was discovered by Brunet and colleagues several years earlier in 1987 (Brunet et al., 1987), but the researchers could not foresee its potential as anti-cancer agent at that time. In 1995, James Allison's group reported that blocking CTLA-4 led to completely regressed melanoma tumors in mice (Krummel and Allison, 1995). Allison discovered that CTLA-4 acts as a brake on T cells preventing them from attacking tumor cells. In 1999, the anti-CTLA-4 antibody was commercialized by Medarex (Princeton, New Jersey) under the name ipilimumab, but it was not before 11 years later those crucial results came from the first clinical trials. Bristol-Myers Squibb reported that melanoma patients treated with the antibody had an average survival of 10 months compared to 6 months of the control group, showing the first ever promising results in the battle against metastatic melanoma, which later led to the FDA approval. Another brake on T cells was identified in the early 1990s. This time was from Japan, where Hodjo and colleagues published that they identified a molecule expressed in dying T cells (Ishida et al., 1992). They named the molecule PD-1 (programmed death 1) and blocking it was proved to be very effective in patients with non–small-cell lung cancer (17%), melanoma (31%), or renal-cell cancer (29%) and with fewer side effects (Topalian et al., 2012). The combination of the two antibodies was proven even more effective with tumors vanished months after the start of the treatment and patients kept responding even after the treatment was discontinued (Wolchok et al., 2013). In a different approach, Rosenberg published encouraging results on engineered T cells with chimeric antigen receptor the so-called CAR T cells in 2010 (Kochenderfer et al., 2010), that was first described experimentally in 1993 (Eshhar et al., 1993). Later, Carl June from the University of Pennsylvania together with colleagues from Memorial Sloan-Kettering Cancer Center, reported complete remission of leukemia patients using CAR-T therapy after replacing the CD28 signaling domain with 4-1BB (Porter et al., 2011). In the following years, CAR-T therapy was applied in more cancer types and improved for better outcomes in response and safety. By the end of 2016, four different immune checkpoint inhibitors drugs (ipilimumab, nivolumab, pembrolizumab and atezolizumab) were approved by the FDA for the treatment of melanoma, renal cell carcinoma, lung cancer,

lymphoma and bladder cancer. Now, the list of the FDA-approved immune checkpoint inhibitors is longer as well as the indicated cancer types.

3.2 Cancer Immunoediting

Cancer immunoediting is the process of shaping tumor immunogenicity (**Fig. 8**). This process takes place through the tumor-host interaction and is composed of three phases (3Es): elimination, equilibrium, and escape (Vesely and Schreiber, 2013, O'Donnell et al., 2019). Cancer immunosurveillance acts as a defense mechanism against malignant cells that leads to cancer elimination. The immunosurveillance hypothesis was first proposed in 1957 by Thomas and Burnet (Thomas, 1982, Burnet, 1970) and during this phase, the immune effector cells recognize and destroy the malignant cells. This is the endpoint of cancer immunoediting in most cases. However, sometimes cancer clones are not eliminated, and they survive in a dormant mode entering the equilibrium phase. In this case the immune system keeps tumor under control constraining the growth. It is possible that tumor cells stay in this phase for the rest of the host's life. Sometimes though the immune system-tumor interaction leads some cancer clones to escape immune recognition. Tumor cells then grow in an immunologically unrestricted manner that leads to cancer. (Vesely et al., 2011, O'Donnell et al., 2018)

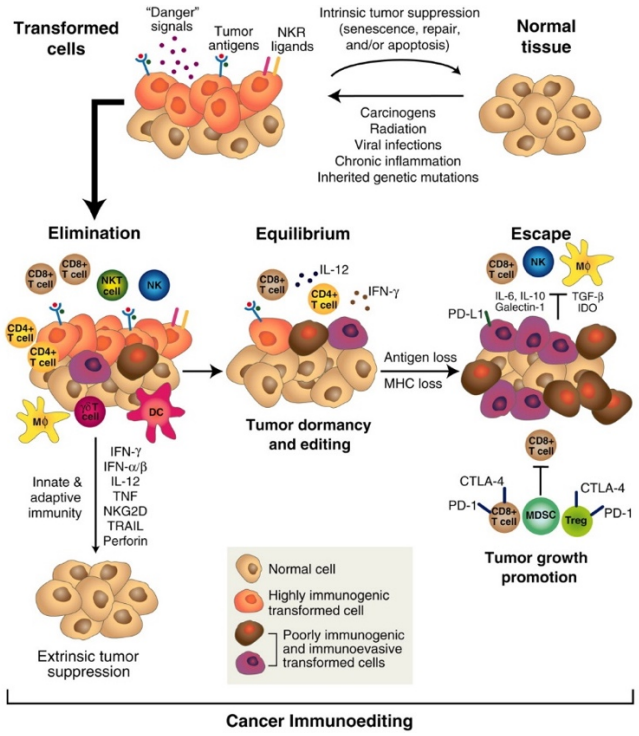


Figure 8: Cancer immunoediting process that includes the three phases of elimination, equilibrium, and escape. (Schreiber et al., 2011)

3.3 Cancer Immunology

Extensive research in basic biology during the past century set the stage for modern immunology and provided insights into the nature of interactions between tumor and immune cells. These insights shaped our understanding of how anti-tumor responses are mounted and why anti-tumor immunity sometimes fails. In an important publication of Mellman et al. the anti-tumor response is described as a 3-step process (Mellman et al., 2011) that was later referred as Cancer-Immunity Cycle (Chen and Mellman, 2013). First, DCs capture released tumor antigens from the tumor microenvironment (first step), and they subsequently migrate to the lymph nodes where they present them on MHC class I and II molecules to T lymphocytes (second step). Antigen-educated T cells will exit the lymph node, infiltrate the tumor site, and find their target (third step). However, these steps could be interrupted leading to the inhibition of an effective anti-cancer response.

Cancer-Immunity cycle steps are not optimally performed in cancer patients through various of interrupted mechanisms. Tumors often are not immunogenic enough or there are factors that prevent DCs maturation which impede them from detecting antigens. Additionally, inappropriate immune responses could lead to tolerance and T cell deletion, anergy or T regulatory cells (Tregs) activation that suppresses the anti-tumor responses. Tregs are specialized T cells that suppress the function of other T cells to maintain homeostasis. They are characterized by the expression of the transcription factor FOXP3 (forkhead box P3) and are cells with high affinity for self-antigens. They also express immunosuppressive cytokines such as IL10 and TGF-β. Targeting Tregs is a common strategy of cancer immunotherapy. (Naing and Hajjar, 2017) Other inhibitory mechanisms include the prevention of T cells from infiltrating the tumor bed. However, even if entrance is allowed, the tumor microenvironment is a hostile place that can utilize a variety or immunosuppressive mechanisms to prevent the recognition. The upregulation of immune checkpoint inhibitors (ICIs) (e.g., PD-L1 and PD-L2) is one of these mechanisms. Tumor cells overexpress ICIs which engage receptors on T cell surface (e.g., PD-1) causing T cell anergy or exhaustion. They also interfere with antigen presentation by downregulating MHC class I molecules, the expression of target tumor antigens and other components of the antigen processing machinery. Additionally, they release immunosuppressive molecules, such as indoleamine 2,3-dioxygenase (IDO), adenosine, prostaglandin E2, TGF-β and VEGF-A that could limit T-cell function directly or indirectly. In addition to Tregs, there are other types of suppressive cells that are recruited in the tumor bed, such as tumor associated macrophages (TAMs) that secrete pro-angiogenic cytokines and growth factors, such as ornithine, TGF-β and VEGF and myeloid derived suppressor cells (MDSCs) that release additional immunosuppressive molecules, such as arginase and nitrous oxide synthase. Cells of the tumor stroma, such as cancer associated fibroblasts and

cells of the tumor vasculature have been shown to contribute to T cell inhibition of proliferation, function, and adhesion. Cancer associated fibroblasts promote the immunosuppressive cells through the secretion of CCL2 and CXCL12 and secrete TGF-β that suppress effector T cells (Mellman et al., 2011). The goal of cancer immunotherapy is to overcome these inhibitory mechanisms and maintain a potent cancer immunity where the cycle will be uninterrupted.

3.3.1 T Cells

T lymphocytes are key components of anti-tumor responses. Cytotoxic $CD8^+$ T lymphocytes (CTLs) are the effectors that mediate direct killing of cancer cells and $CD4^+$ T cells are the regulators that either promote or inhibit the anti-tumor immune response. Peripheral and LN-resident T cells are divided according to their state of differentiation into naive, effector and memory T cells (**Fig. 9**). An additional T cell category includes the exhausted cells that are unable to function properly. This category has recently extensively studied because of the research in cancer immunotherapy. Conventional $CD4^+$ T cells and specifically Th1 helper cells (that regulate mainly cellular immunity) promote tumor control primarily through stimulation of $CD8^+$ T, NK cells and antibody responses, as well as through the secretion of pro-inflammatory cytokines, such as interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα), and in some specific cases though direct cytotoxicity against tumor cells. (Chen and Mellman, 2017, Tay et al., 2021)

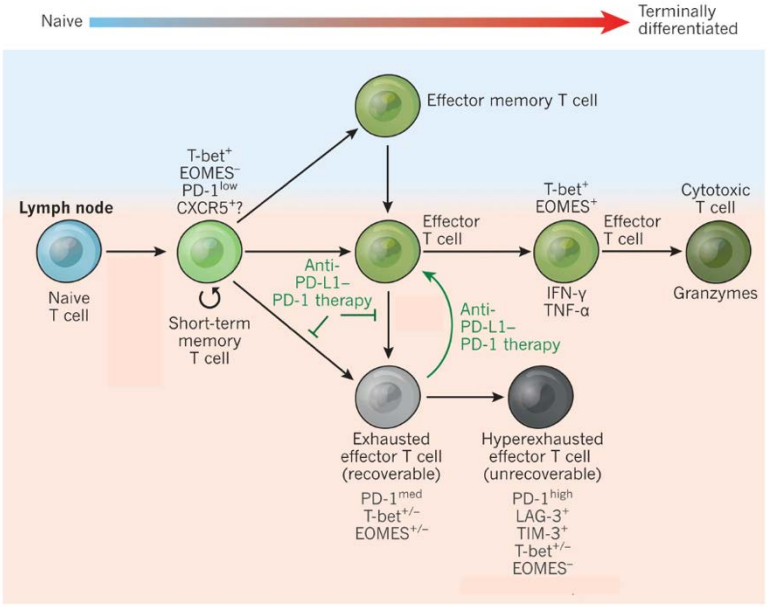


Figure 9: Differentiation of T cells (modified) (Chen and Mellman, 2017)

Regulation of T cells is orchestrated by co-stimulatory and inhibitory signals (**Fig. 10**). The co-stimulatory molecules include CD28, ICOS, 41BB, and OX40 that synergize with TCR

signaling to induce cytokine production and T-cell survival. T cells constitutively express CD28 that binds to B7 proteins (B7-1/CD80 or B7-2/CD86) on APCs providing the necessary co-stimulatory signals for their activation. PD-1, CTLA-4, T cell immunoglobulin mucin receptor 3 (Tim-3) and lymphocyte activation gene 3 protein (LAG3) are the co-inhibitory molecules that suppress T cell function serving as immune checkpoints. PD-1 and CTLA-4 are expressed in activated T cells. CTLA-4 is a CD28 homologue that has higher affinity for B7 molecules and upon engagement blocks CD28 co-stimulation. It is expressed by both $CD4^+$ and $CD8^+$ T cells and constitutively in Tregs. PD-1 is a member of the CD28 family that expressed on T cells. PD-1 ligands are PD-L1 and PD-L2 and expressed by APCs and tumor cells mostly in response to cytokines, such as IFNγ. PD-1 receptor does not interfere with co-stimulation, but it leads to B and T cell suppression through inhibiting signaling pathways downstream of TCRs and BCRs. Suppression also reduces $CD8^+$ T cell effector secretion of cytokines, such as tumor necrosis factor (TNF), interleukin-2 (IL-2) and interferon-γ (IFN-γ). These mechanisms have evolved to protect and maintain immune homeostasis, but tumors hijack them to escape immunosurveillance. (Naing and Hajjar, 2017, van der Leun et al., 2020)

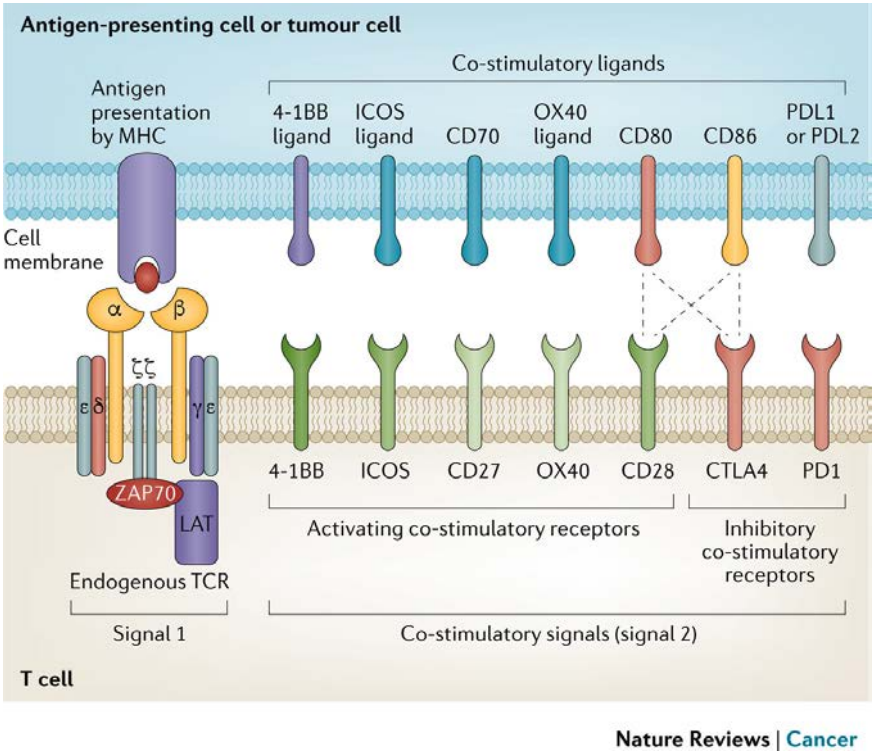


Figure 10: TCR signaling and co-stimulatory or co-inhibitory molecules (Fesnak et al., 2016)

Cytotoxic $CD8^+$ T cells kill the infected or malignant cells through cell-mediated cytotoxicity that induces the target to undergo apoptosis. The killing process takes place through

direct cell-to-cell contact after TCR-mediated target recognition and the formation of an immunological synapse. The mechanisms that involved in T cell-mediated cytotoxicity consist of death-inducing effector molecules, such as perforin (a pore-forming protein), granzymes (proteases that mediate cleavage of caspases and induce apoptosis), Fas-ligand (FasL) and TRAIL (TNF-related apoptosis-inducing ligand) that binds with their corresponding death receptors on the tumor cells triggering apoptosis (the so called extrinsic apoptotic pathway). Perforin and granzymes are released from granules that are located in CTLs and transported to the target cells to induce their lysis. Additionally, CTLs secrete cytokines, like IFN-γ, TNF-a and TNF-b, that induce the activation of macrophages and mediate the activation of anti-tumor pathways. (Halle et al., 2017)

Accumulated evidence correlates intra-tumoral T cell infiltration with improved survival in a wide range of solid tumors, such as melanoma, breast, lung, and other solid tumors. Immune checkpoint blockade therapies rely on the replenished activity of pre-existing or newly formed T cells that reside in the tumor bed and can recognize and eliminate tumor cells. Additional immunotherapeutic approaches have been designed focusing on boosting T cells anti-tumor function. More about these strategies are described in section 3.4.

3.3.2 NK Cells

NK cells are effector cytotoxic cells that are present in the circulation and tissues with the ability to also produce cytokines. NKs represent a minor lymphocyte population in most tissues (from 2% in spleen to 10% in lungs in mice and from 2-18% in human peripheral blood). They were first identified as cells able to kill their targets without previous activation and were characterized as “large granular lymphocytes”. NKs develop in the bone marrow from NK progenitors and the cytokine IL-15 is required for their development and final maturation. The most common human NK receptor is CD56, which, however, is not expressed by murine NKs (although CD56 is expressed in murine brain tissue). NKs in mice are identified by anti-NK-1.1 (C-type lectin-like receptor, a PRR type) or anti-CD49b (DX5, integrin VLA-2α) antibodies. (Wu and Lanier, 2003, Vivier et al., 2008)

NKs express a wide range of activating and inhibitory cell-surface receptors that regulate their activity. Their primary function is to eliminate infected or malignant cells that lack self-MHC class I molecule through the expression of several inhibitory receptors such as KIR-L (killer cell inhibitory receptor-L). Healthy cells are protected through the inhibitory receptors on NKs that sense normal expression of the MHC I molecule on their surface. NKG2D, KIR-S (killer cell Ig-like receptors), TLR, and NLR are all activating receptors expressed on cell-surface of NKs that

activate them to kill their target. PAMPs binding to cell surface-activating receptors is an activating mechanism for NKs. (Wu and Lanier, 2003)

NK mediated cytotoxicity is performed by the same cytotoxic mechanisms that are used by CTLs, i.e., release of perforin and granzymes, Fas ligand expression or TRAIL. However, NKs also kill through ADCC (antibody-dependent cell cytotoxicity) by their Fc-γIII receptor (CD16) binding to Fc region of antibodies that opsonize the target for elimination. NKs are activated by cellular communication and cytokine signaling. DCs are key partners to NKs, essential for their activation and the ones that secrete vital cytokines for promoting NKs function, such as IL-2, IFN-α, IL-12, IL-15, and IL-18. Subsequently, NKs induce cytotoxicity and promote cytokine production, with IFN-γ being the most important because it is produced early in the immune reaction and coordinates the anti-tumor response by activating other cellular components (**Fig. 11**). (Wu and Lanier, 2003, Vivier et al., 2008)

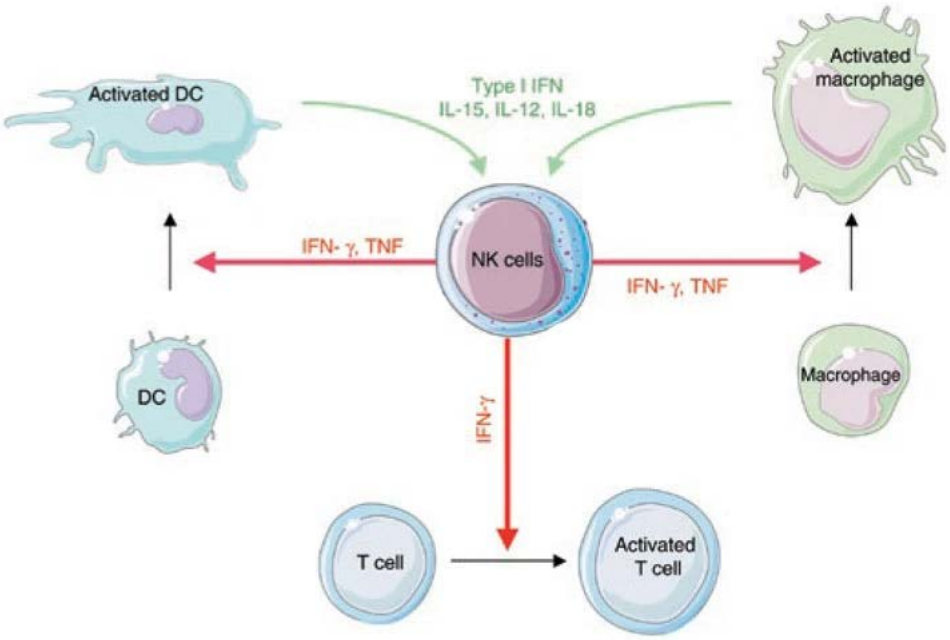


Figure 11: NKs support other cellular components. Following the stimulation by IL-15, type I IFN, IL-12 or IL-18, NKs can further boost the activation and maturation of DCs, macrophages and T cells through the secretion of IFN-γ and other cytokines. (modified) (Vivier et al., 2008)

NKs are considered important for the control of tumor growth and metastasis. Experimental tools that have been used to reveal these functions include mouse models and depletion studies. Some of the most widely used mouse models are the SCID or RAG1-/- or RAG2-/- immunodeficient mice that lack T and B development. Unfortunately, there are no mouse models that completely lack NKs, but only models that are NK defective, like NOD and beige mice. For

the depletion studies, anti-NK-1.1 and anti-glycolipid asialo-GM1 antibodies are used for depleting NK cell population. Although anti-NK-1.1 mAb is very effective, its use is restricted to C57B/L6 mouse strain. Cytokines are also used for studying NKs *in vivo* function, such as IL-2, IL-12, IL-15 and IFN-α/β that can booster NKs function. (Wu and Lanier, 2003)

Tumor cells evolve to escape from being targets of NKs. One of these mechanisms includes the proteolytic shedding of NKG2D ligands or NKG2D endocytosis. NKG2D is an activating receptor that upon stimulation induces cytotoxicity and increased cytokine expression by NKs. NKG2D ligands are expressed by transformed, infected or stressed cells and are different between mice and humans. Experiments have shown that chronic exposure of NKs to tumors bearing or shedding NKG2D ligands may negatively impact their function. Another factor that contributes to tumor escape is the lack of anti-tumor cytokines that leads to decreased stimulation and cytotoxicity, reduced secretion of subsequent cytokines to amplify the response and inadequate NK and DC interactions. Lastly, tumors are protected by immune recognition through the loss of the ligands that activate NKs, such as the downregulation of TRAIL ligand. Immunotherapeutic strategies that might hold therapeutic promise include the manipulation of the balance between inhibitory and activating NKs receptors, the sensitivity of the targets to NKs cytotoxicity and the NKs crosstalk with DCs. (Wu and Lanier, 2003, Terme et al., 2008)

3.3.3 Dendritic Cells

Dendritic cells (DCs) are the most efficient antigen presenting cells that acquire and process antigens for presentation to T cells in the lymph nodes (LNs). They are produced in bone marrow and can be found in all the peripheral tissues in small numbers, where they patrol for antigens. Recognition triggers internalization of the antigen and maturation of DCs. Maturation involves the upregulation of co-stimulatory and major histocompatibility complex I and II (MHC I and II) molecules to facilitate T cells priming. Mature DCs migrate to LNs and present the antigen to naïve T cells. DCs also regulate the immune responses primarily by instructing T cell differentiation and polarization. (Randolph et al., 2005)

DCs function by sampling and processing antigens for presentation to T cells while at the same time providing the necessary co-stimulatory molecules and cytokines for their complete activation. There are different pathways of antigen-processing and presentation that involve either MHC I or II molecules. The exogenous pathway of presenting internalized antigens on MHC I molecules is called cross-presentation and is performed by DCs against tumors and viruses that do not infect them directly. Priming of naïve $CD8^+$ T cells by cross-presenting DCs is called cross-priming. Activated effector $CD8^+$ T cells will then be in search of the specific antigen that is presented on the MHC class I of the target cell through direct presentation (endogenous pathway).

The exogenous pathway that involves peptides loaded on MHC II molecules is mediated by the antigen presenting cells: DCs, macrophages and B cells. Pathogens are captured by phagocytosis, pinocytosis and receptor-induced endocytosis. This pathway activates naive $CD4^+$ T cells to become effector cells, which in turn will produce cytokines that can activate the macrophages to destroy the pathogen (one of the $CD4^+$ Th1 subset roles) or they will serve as helpers to B cells to facilitate antibody production. Another pathway that mediates presentation on MHC II is the autophagy in which cytoplasmic proteins are degraded and presented by self-cells to induce tolerance. (Murphy and Weaver, 2016)

There are two major subsets of DCs in mice and humans: the conventional (cDCs) (or classical or myeloid DCs) and the plasmacytoid DCs (pDCs). The cDCs comprise two main populations, the $CD8^+$ and/or $CD103^+$ cDC1 subset (CD8 mostly in lymphoid tissues and CD103 mostly in non-lymphoid tissues) (BDCA-3 (CD141) expression in humans) and the $CD11b^+$ cDC2 subset, which are derived from common DC precursors in the bone marrow. cDC1s are characterized by the expression of the chemokine receptor XCR1 and their development is dependent on the expression of the transcription factors IRF8 and Batf3 (basic leucine zipper transcription factor ATF- like 3), whereas the development of cDC2s is mainly relies on IRF4. cDC1s are considered very effective cells in cross-presentation that participate in cellular immunity against intracellular pathogens and cancer activating $CD8^+$ T cells and type I Th cell responses. cDC2s have been suggested as inducers of $CD4^+$ T cell responses with a crucial role in cancer. $B220^+$ plasmacytoid DC (pDCs) subsets are derived from common DC and lymphoid progenitors and they do not exhibit potent priming of T cells. Finally, monocyte-derived DCs (moDCs) are a distinct DC subset that arise upon inflammation by monocytes and is CCR2 (CC-chemokine receptor 2)-dependent. Interestingly, some human cDC2s express markers of moDCs and vice versa, while “MoDC- like” cells are often classified as monocytes with high plasticity rather than DCs. Many therapies have been designed to target DCs in cancer. They focus on the activation of endogenous DCs with antigens and immunomodulators or they are DC-based vaccines. (BATF3)-dependent cDC1s are strong activators of anti-tumor immunity, as it has been reported that are necessary for the rejection of highly immunogenic tumors and cDC1-based vaccines have been shown anti-tumor activity reducing the tumor growth. Reports show that cDC2s and MoDCs may also cross-present antigens. Both cell populations participated in tumor-associated antigens (TAAs) presentation following treatment with chemotherapies, like anthracyclines in mice. (Wculek et al., 2019)

3.3.4 Myeloid Derived Suppressor Cells (MDSCs)

Myeloid derived suppressor cells (MDSCs) represent a heterogeneous population of pathologically activated neutrophils and monocytes characterized by the ability to suppress T and NK cell functions, and to support the metastatic process. Accumulation of MDSCs in secondary sites is found only in murine cancer models with metastatic capacity. The presence of MDSCs correlated with clinical cancer stage and the highest frequency of circulating MDSCs was found among stage IV patients with solid tumors and extensive metastatic burden. Two major subpopulations of MDSCs have been identified in mice and humans: monocytic-MDSCs (M-MDSCs) and polymorphonuclear-MDSCs (PMN-MDSCs). In humans, M-MDSCs and PMN-MDSCs are characterized as $CD11b^{+}CD14^{+}HLA\text{-}DR^{-/lo}CD15^{-}$ and as $CD11b^{+}CD14^{-}CD15^{+}/CD66b^{+}$ respectively, while in mice M-MDSCs are defined as $CD11b^{+}Ly6G^{-}Ly6C^{high}$ and PMN-MDSCs as $CD11b^{+}Ly6G^{+}Ly6C^{low}$. Chemotherapy has been reported to decrease the frequency of MDSCs in the periphery of both murine preclinical models and cancer patients (Condamine et al., 2015, Kowanetz et al., 2010, Bronte et al., 2003, Bronte et al., 2016).

MDSCs are generated from bone-marrow hemopoietic precursors in response to several cytokines including GM-CSF, G-CSF, M-CSF, VEGF, SCF, IL-6, and IL-13. MDSCs suppress T cell proliferation by the release of cytokines, such as IL-10 and TGF-β and through mechanisms that involve ROS (reactive oxygen species), iNOS (induced nitric oxide synthase) and Arg1 (arginase 1). NOS2 catalyzes the production of nitric oxide (NO) from L-arginine, while ARG1 converts arginine into ornithine and urea. Arginine is an amino acid that is required for T cell proliferation, activation, and function. Cells with upregulated these two enzymes can deprive the arginine from the milieu and suppress T cells. (Lim et al., 2020, Bruger et al., 2019)

3.4 Cancer Immunotherapy Strategies

Basic discoveries in T cell biology paved the way for designing cancer immunotherapy strategies that include both endogenous and synthetic immunotherapy approaches. T cells are capable of recognizing tumor-specific antigens that can be derived from oncovirus-related antigens, neoantigens (mutated proteins), temporally or anatomically restricted proteins (such as germ cell antigens or melanocyte differentiation antigens) or amplification of tumor-associated antigens (such as HER2). The neoantigens must be effectively cross-presented by antigen-presenting cells (APCs) and tumor cells in order to be immunogenic, which is a requirement for efficient immunosurveillance. (O'Donnell et al., 2018) Cancer immunotherapy strategies include cytokines, immune checkpoint inhibitors (ICIs), adoptive cell transfer and cancer vaccines.

3.4.1 Immune Checkpoint Inhibitors (ICIs)

Immune checkpoint inhibitors (ICIs) have revolutionized cancer treatment introducing the great potential of cancer immunotherapy. They have been proven to induce durable responses across multiple types of cancer. During carcinogenesis, tumors hijack the checkpoint inhibitory molecules to hide from the immune system. James P. Allison and Tasuku Honjo won the 2018 Nobel Prize in Physiology or Medicine for the discovery of immune checkpoints. Nowadays, first- or second-line clinical treatment schemes include ICIs, as single agents or in combination with chemotherapy, for about 50 cancer types. More than 3000 active clinical trials are testing ICIs, which represent the 65% of the total trials in oncology. (Robert, Robert, 2020)

The most well-known inhibitory molecules CTLA-4 and PD1 regulate T cell function by preventing their activation, which under physiological conditions, protect the host from autoimmunity. CTLA-4 is expressed by both conventional and regulatory T cells. As it was previously mentioned, CTLA4 receptor competes CD28 for binding to the B7 co-stimulatory molecules. CTLA4 has higher affinity for the ligands and is constitutively expressed on Tregs that suppress the immune system. In 2011, the first immunotherapeutic monoclonal anti-CTL4 antibody (mAb), ipilimumab, was approved by FDA for metastatic melanoma. No treatment before ipilimumab had shown any survival extension of the patients with stage IV melanoma. The development of mAbs targeting PD1 (pembrolizumab and nivolumab) and PDL1 (atezolizumab and durvalumab) followed rapidly. PD-1 does not interfere with co-stimulation but suppresses T cells by inhibiting signaling pathways downstream of TCRs upon binding to its ligands, PD-L1 and PD-L2. PD-1 is expressed on T cells and its blockade together with PD-L1 are the most promising therapeutics in oncology. The anti-PD-1 mAbs pembrolizumab and nivolumab were approved due to the objective responses in 40% of the patients with advanced melanoma. The combination with ipilimumab showed even better outcomes compared to the single agents leading to the approval of the combinatorial regimen in 2015. It is anticipated that the greatest benefits will be achieved by combination treatments of immunomodulators or other therapies that operate through immune modulation. Other co-inhibitory molecules that suppress T cell function serving as immune checkpoints are T cell immunoglobulin mucin receptor 3 (Tim-3), lymphocyte activation gene 3 protein (LAG3) and T-cell immunoreceptor with Ig and ITIM domains (TIGIT). TIM-3 also serves as a constraining factor to T cell effector function that is associated with exhaustion. TIM-3 has four ligands: galectin-9, HMGB, phosphatidyl serine and CEACAM-1 and its blockade has shown anti-tumor activity in several preclinical models of colon adenocarcinoma, melanoma, and sarcoma, while the combination with PD-1 is beneficial. LAG-3 is expressed on activated conventional T and Treg cells, B cells and plasmacytoid dendritic cells. The inhibitory signal of LAG-3 is transmitted upon binding to MHC class II molecules. LAG-3 co-expression

with PD-1 indicates exhaustion of T cells. Another inhibitory and exhaustion marker is TIGIT, which is expressed on Tregs and other immune cell populations. TIGIT blockade shows anti-tumor activity when combined with anti-TIM-3 or anti-PD-L1 inhibitors. Even though immune checkpoint blockade shows favorable responses to patients, there are adverse effects that are immune-related (irAEs) and require attention. Fortunately, most of them can be controlled with already available immune suppressants without affecting the anti-tumor activity of the immunomodulators. Overall, ICIs are powerful immunotherapeutic drugs that has changed cancer treatment in the recent years. However, there are some limitations that must be overcome to get the best out of the inhibitors blockade. These include the lack of understanding of ICIs mechanism of action, as well as the limited number of patients and indications that can benefit, highlighting the need for more effective and novel approaches. (Waldman et al., 2020, Khalil et al., 2016)

3.4.2 Adoptive Cell Transfer

Adoptive cell transfer (ACT) is personalized therapy of administering *ex vivo* cultured naturally occurring or engineered autologous T lymphocytes with anti-cancer activity. T cells are either selected and expanded from the tumor microenvironment (tumor-infiltrating lymphocytes, TILS) or engineered to express a synthetic TCR (sTCR) or a chimeric antigen receptor (CAR). (Rosenberg and Restifo, 2015, Khalil et al., 2016) Adoptive cell transfer gives the advantage of bypassing the *de novo* activation of an anti-tumor specific T cell population, which is a limitation of cancer vaccine strategies, particularly because the patients often are immune-compromised or highly tolerant to cancer-antigens. (Kalos and June, 2013)

3.4.2.1 Natural T Cells for ACT

The administration of natural autologous T cells has been successfully tested in melanoma patients and thus, expanded to other cancer types, such as cervical cancer, lymphoma, leukemia, bile duct cancer and neuroblastoma. However, consistent results of isolating anti-tumor reactive T infiltrating lymphocytes (TILS) has been reported mainly in melanoma.

The first who successfully performed ACT to treat melanoma patients was Steven A. Rosenberg at the National Cancer Institute of NIH in 1988 (Rosenberg et al., 1988). A discovery that made ACT possible was the description of IL-2, which is a T-cell growth factor that enabled the *ex vivo* culture of T lymphocytes. The approach includes the resection of the specimen and the dissection into fragments that are cultured with 6000 IU/ml of IL-2. After 2-3 weeks, the cultures will have only T lymphocytes that have previously grown and killed the tumor cells. The lymphocytes are tested for reactivity against the tumor and are expanded in the presence of an antibody that targets the epsilon subunit (in the CD3 complex of the TCR), which acts as feeder

for the lymphocytes together with IL-2. After another 2-3 weeks, up to 10^{11} lymphocytes can be obtained. Then, the patient undergoes lymphodepletion with preparative chemotherapy and subsequently the lymphocytes are reinfused back to the patient with IL-2 (720,000 IU/kg) (**Fig. 12**). Lymphodepletion is very important for successful ACT application that proved to increase the effectiveness of the ACT. It includes the use of cyclophosphamide and fludarabine that are believed to deplete the circulating MDSCs and Tregs. In addition, $CD8^+$ T cells persist in the peripheral blood of the patients even after a month of the treatment. Furthermore, it has been reported that lymphodepletion leads to IL-15 elevation in the circulation, which serves as a growth factor of the newly transferred lymphocytes. Adoptive cell therapy using autologous TILs is considered the most effective approach against metastatic melanoma for the induction of complete long-term regressions.

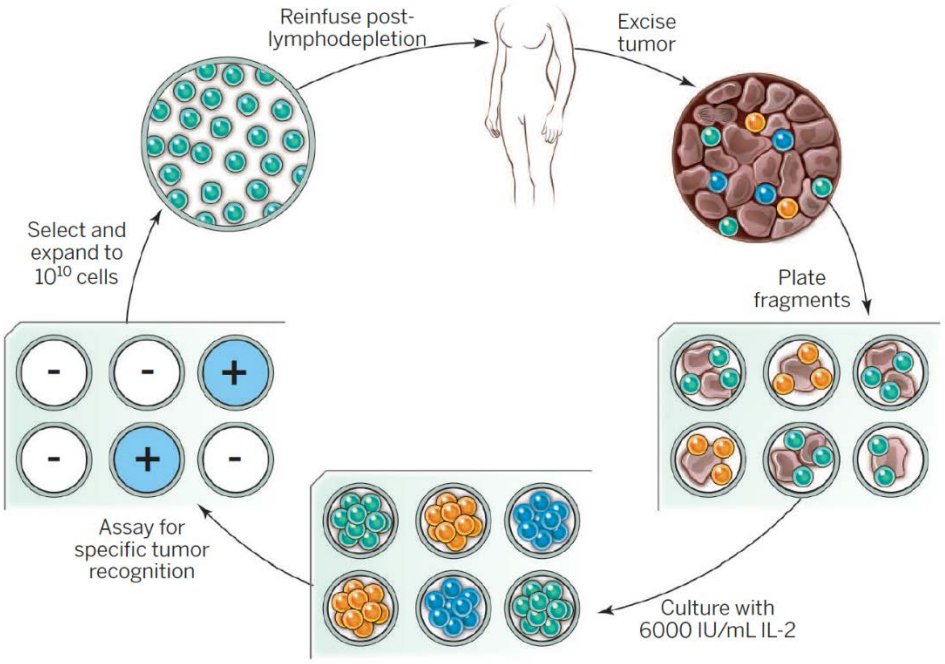


Figure 12: Protocol of the adoptive cell transfer (Rosenberg and Restifo, 2015)

However, ACT with autologous TILs has some limitations. It has been shown that T cells have low affinity TCRs for self-antigens, which reflects the impact of central tolerance. This observation together with the fact that tumor tumor microenvironment is immunosuppressive supports the notion that the use of autologous TILs may be suboptimal for killing tumor cells in patients with advanced cancers. (Kalos and June, 2013) Additionally, not all the patients that are diagnosed with metastatic melanoma can undergo the exhausting protocol and thus, they are excluded from entering the clinical trials. This is mostly related to the life-threatening effects of

lymphodepletion, and the demanding IL-2 based treatments that limit the therapy to patients with good performance status. Furthermore, the reasons why only some melanoma patients respond to the therapy remain unknown, as well as why successful ACT using naturally occurring T cells is restricted to melanoma. (Restifo et al., 2012)

3.4.2.2 Engineered T Cells for ACT

Recent advances have made possible to genetically engineer T cells in order to target antigens of interest. These gene-transfer-based strategies enabled the transduction of T cells using retroviruses or lentiviruses that encode a specific receptor for the target antigen. With these methods, TCRs are modified to exhibit high affinity and specificity against tumor antigens increasing the effector function of T cells. The modified receptor is either a transgenic TCR with α and β chains (αβTCR) or a chimeric antigen receptor (CAR) that is composed of the specific single-chain immunoglobulin variable region (scFv) linked to CD3-ζ and/or other costimulatory signaling domains, like CD28. Both endogenous and transgenic TCRs require MHC-mediated antigen presentation and co-stimulation signals for complete T activation. By contrast, CARs mediated recognition of antigens is independent of the MHC molecules. However, this restricts the recognition to only surface antigens. (Fesnak et al., 2016)

Engineered T cells have shown impressive results in the clinic, especially in patients with hematological malignancies. Adoptive transfer of CAR T cells that target the CD19 antigen (CART19) have induced complete remissions in patients with B cell malignancies. Also, TCR-engineered T cells specific for NY-ESO-1 have generated responses in multiple myeloma and synovial cell sarcoma patients. Among the benefits of the adoptive cell therapy with engineered T cells are the requirement for only one treatment for durable responses and the high response rate (>90% of patients with ALL). The limitations include the on target off-tumor toxicities as the targets are self-antigens which could be overcome with increase of specificity. The field constantly improves engineering strategies for safer and more effective CAR Ts. One of the hallmarks of this dynamic process is the replacement of the CD28 signaling domain with 4-1BB by Carl June and colleagues which resulted in a complete and durable remission of a pediatric patient. (Kalos and June, 2013)

3.4.3 Cancer Vaccines

Cancer therapeutic vaccines are designed to target tumor antigens that are unique to malignancy and not shared with the normal cells. Although all tumor associated antigens (TAAs) can be targeted, viral antigens and neoantigens are considered better targets as they can be easier recognized as non-self by the immune system. Cancer vaccines are attractive choices for cancer

treatment as they show low toxicities and are easy to use. However, the success rate is very low (4%) and the sipuleucel-T, which is the only currently approved cancer vaccine for the treatment of castration-resistant prostate cancer, prolongs the patients' lives just by some months. The promise for the future, though, seems to be the personalized recombinant cancer vaccines that target neoantigens. The design is assisted by next generation sequencing of genomic DNA from the patients' tumors. It remains to be seen if the results from the advanced clinical trials that are testing these personalized recombinant cancer vaccines will show high efficacy. (Waldman et al., 2020, Restifo et al., 2012)

3.4.4 Cytokines

Cytokines are small polypeptides or glycoproteins with a molecular weight typically less than 30 kDa that provide the necessary signals for orchestrating immune responses. Stanley Cohen and colleagues first used the term cytokine in 1974 to refer to a molecule that acts between cells, typically in the immune system. Cytokines include interleukins, interferons (IFNs), members of the tumor necrosis factor (TNF) superfamily, growth factors and chemokines. Cytokines are expressed in response to a stimulus and have short half-lives that limit them to exert their functions locally, in an autocrine or paracrine fashion (with some exceptions (Lin and Leonard, 2019)2019). They are secreted by immune and non-immune cells, such as mesenchymal and epithelial cells, and are tightly regulated. The target-cells of cytokines express high-affinity extracellular receptors that transmit the signal in the cell and often are shared between different cytokines. These receptors trigger intracellular signaling through JAK/STAT or NF-κB pathways that leads to transcription of specific genes. Concentration and exposure duration to cytokines are crucial since these factors shape the complex antagonistic or synergistic relationships among the different cytokines. (Propper and Balkwill, 2022)

In the last decades, preclinical studies revealed that some cytokines exhibit anti-cancer activity and have a crucial role in tumor progression and metastasis. Cytokines with anti-tumor activity either inhibit tumor growth directly or target the tumor indirectly by guiding cytotoxic effector cells against cancer cells. These cytokines include interleukin (IL)-2, IL-12, IL-15, IL-21, granulocyte macrophage colony-stimulating factor (GM-CSF) and interferon (IFN)-α. In contrast, some cytokines exert pro-tumor activity by promoting all the stages of the tumor development. Some examples of pro-tumor cytokines are TNF-a, TGF-β, CSF-1 or other cytokines that promote myeloid cells (e.g., CXCL18, CCL2, CCL3 and CCL5). (Berraondo et al., 2019, Waldmann, 2018)

Cytokines were the first immunotherapeutic approach to be approved by the FDA for the use against cancer. The first to approve was recombinant interferon (IFN)-α for the treatment of hairy cell leukemia in 1986, followed by recombinant IL-2 for metastatic renal cancer and

advanced melanoma in 1992 and 1998, respectively. IL-12, IL-15, IL-21, and GM-CSF have all been evaluated in clinical trials. Extensive review on IL-15, which is the cytokine that was evaluated in this study, follows at the next section 3.4.4.1. Cytokines as monotherapies have shown moderate anti-tumor efficacy, even with some of them approved for use in clinics. The moderate efficacy relies on some limitations in using cytokines as therapeutics. These include the short half-life, narrow therapeutic windows, high toxicities (in higher doses) or the activation of immunosuppressive mechanisms. Better clinical results of ICIs initially outplaced cytokines. However, the development of engineered cytokines with improved half-life, reduced toxicities and increased efficacy paved the way for their evaluation in combinations with other drugs. Towards this cause, clinical trials are expanding testing cytokines in combination schemes with immunomodulatory and chemotherapeutic drugs or vaccines. Some of these agents include ICIs, monoclonal antibodies (for ADCC induction) and signaling molecules (anti-CD40). Additionally, there are other approaches to increase cytokine activities that include engineering, fusion with antibodies or ICIs and alternative delivery routes (peritumoral). In a different strategy, the inhibition of cytokines that suppress the anti-tumor immune response have been also tested for anti-tumor activity. Ongoing clinical trials are testing this inhibition through antibodies, small molecules, cytokine traps and siRNAs. (Berraondo et al., 2019, Waldmann, 2018)

3.4.4.1 IL-15

Il-15 is a pleiotropic cytokine that plays a critical role primarily in function and homeostasis of NK, NKT and memory $CD8^+$ T cells. IL-15 was discovered by two independent groups in 1994 as a 14–15 kDa cytokine that acts as a T cell growth factor similar to IL-2 in the simian kidney epithelial cell line, and in the human T cell leukemia virus-1 cell line. It is a four-helix bundle protein, member of the common γ chain family of cytokines (other members include IL-2, IL-4, IL-7, IL-9, and IL-21). human IL-15 has 96% homology in sequence with simian IL-15 and 72% with mouse and rat IL-15, showing that is highly conserved among species. IL-15 acts in complex with IL-15 receptor a (IL-15Rα or CD215) that is trans-presented by the producer cell to the target cell. This IL-15/IL-15Ra complex binds to another receptor complex present on the target cell that is composed of the IL-2/IL-15 receptor β (IL-2/IL-15Rβ or CD122) and common γ chain inducing its stimulation. The β/γc complex is common between IL-15 and IL-2 and upon stimulation leads to JAK1 activation that phosphorylates STAT3 (via the β chain) and JAK3 that phosphorylates STAT5 (STAT5A, STAT5B) (via the γ chain). (Guo et al., 2017, Waldmann et al., 2020). Other signaling pathways that are stimulated upon IL-15/IL-15Ra binding include the production of effector and cytotoxic molecules, such as IFN-g, TNF-a, XCL1, Granzyme and Perforin, phosphorylation of the tyrosine kinases LCK and SYK, induction of BCL-2, stimulation

of the (PI3K)–AKT pathway and the RAS–RAF–MAP-kinase pathway, leading to activation of FOS- and JUN-containing transcription-factor complexes. (Waldmann, 2006) Other populations supported by IL-15 include γδ T cells, intestinal intraepithelial lymphocytes, and type-1 innate lymphoid cells (ILC1). IL-15 and IL-15R mRNAs have been found in several cell types, revealing the source of the cytokine complex in vivo. These include monocytes, macrophages, dendritic cells, stroma cells from bone marrow and lymph nodes, blood endothelial and intestinal epithelial cells. (Bergamaschi et al., 2021)

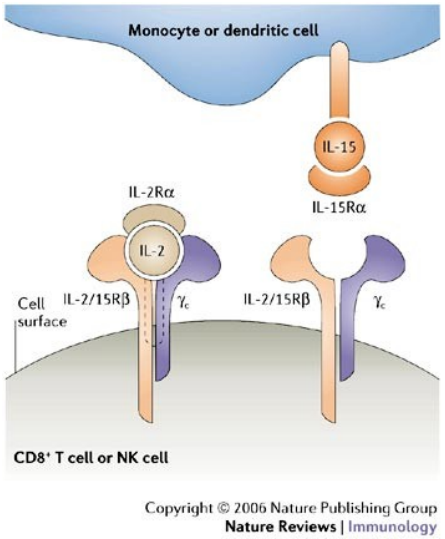


Figure 13: The different nature of IL-2 and IL-15 binding for signal transmission (Waldmann, 2006)

IL-15 was discovered as a cytokine that shares the same β/γc receptor with IL-2 (**Fig. 13**). IL-2, as mentioned before, was one of the first cytokines that was approved as an immunotherapeutic drug for the treatment of cancer. The fact that the two cytokines share the β/γc receptor was the basis to hypothesize that they might also share same functions, which started the process of evaluating IL-15 as a cancer immunotherapeutic drug. Indeed, they both stimulate T cell proliferation and induce NK cell generation and maintenance. However, they also have distinct functions that make them nonredundant cytokines for body homeostasis. IL-2 has a unique role in self-tolerance by eliminating self-reactive T cells through the activation-induced cell death (AICD) and the maintenance of peripheral Tregs. On the contrary, IL-15 is crucial for the maintenance of immunological memory by supporting the survival of $CD8^+$ memory T cells. IL-15 has minor effects on Tregs maintenance, which makes the molecule more suitable for use in cancer immunotherapy. (Waldmann, 2006, Waldmann et al., 2020) Furthermore, IL-2 and IL-15 different affinities to their unique receptors subunits IL-2a (CD25) and IL-15a, respectively, suggests that IL-15Ra is a component of the bioactive cytokine rather than a receptor subunit. IL-2Ra stabilizes

the heterotrimeric receptor complex increasing IL-2 affinity, whereas IL-15Ra already has a high affinity to IL-15 and is co-expressed by the same cells producing IL-15. Indeed, studies revealed that IL-15 *in vivo* is a heterodimeric complex formed by the IL-15 chain and the IL-15Ra, which are co-expressed by the same cell. The two chains of IL-15 and IL-15Rα associate in the endoplasmic reticulum due to their high binding affinity (kd ~10-11) and the complex is transported to the cell surface. It is released as bioactive soluble heterodimeric molecule upon proteolytic cleavage of IL-15Rα. hetIL-15 produced from human HEK293 cells has been shown to be well tolerated and bioactive in both mice and rhesus macaques. Pavlakis lab contributed to the discovery of the molecular mechanism of the complex and produced the native molecule that is named hetIL-15 (**Fig. 14**). They showed that hetIL-15 molecule is the *in vivo* active form of the cytokine in mice and humans (Bergamaschi et al., 2012; Bergamaschi et al., 2009; Bergamaschi et al., 2008b; Chertova et al., 2013).

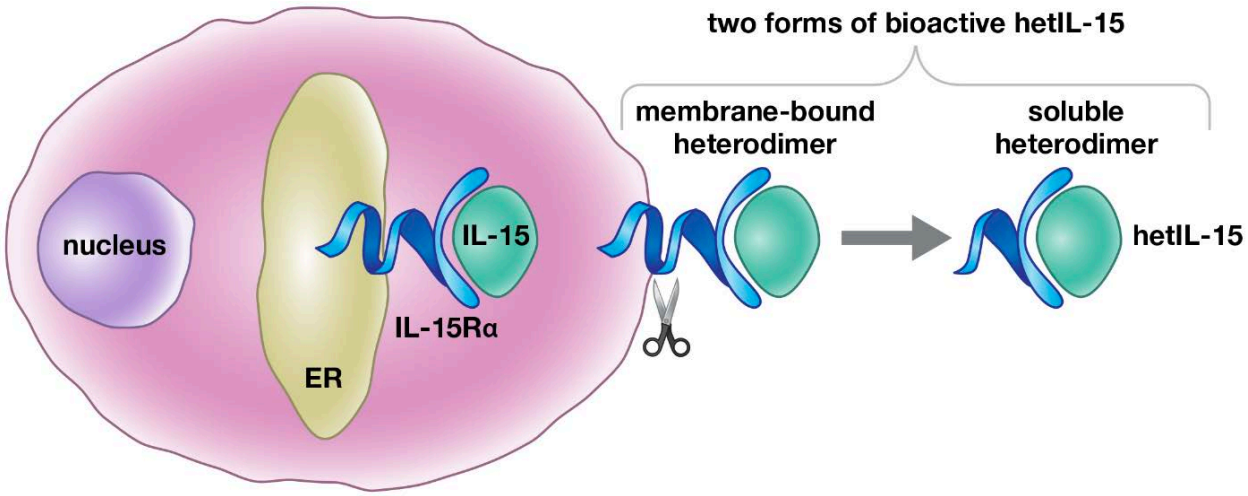


Figure 14: Schematic representation of hetIL-15 production. (Bergamaschi et al., 2021)

Early studies showed that recombinant single chain IL-15 (rhIL-15) produced in *Escherichia coli* has substantial anti-tumor activity (Munger et al., 1995). However, the short plasma half-life and immunogenicity in humans limited the therapeutic potential of the molecule. For that purpose additional efforts to improve IL-15 *in vivo* properties led to the generation of several more stable variants that combine IL-15 with IL-15Rα, such as hetIL-15FC (the C-terminus of soluble IL-15Ra is fused to the Fc region of human IgG1), N803 (IL-15 mutant (N72D) bound to the extracellular region of IL-15R fused to IgG1 Fc) and RLI (Receptor-Linker-IL-15 is a sushi domain fused to IL-15, via a 20-amino acid flexible linker). The promising results of these variants in several preclinical cancer models have advanced them to clinical trials for safety and efficacy evaluation as monotherapy or in combination with immune checkpoint inhibitors and/or chemotherapeutic

agents (Bergamaschi et al., 2021, Robinson and Schluns, 2017). All the variants and hetIL-15 have shown extended half-life and better efficacy compared to rhIL-15. Subsequent studies from Pavlakis lab have shown that hetIL-15 exhibits significant anti-cancer activity as monotherapy in several preclinical cancer models including B16 melanoma, MC38 colon carcinoma, EO771 breast adenocarcinoma and TC-1 carcinoma (Ng et al., 2017, Stellas et al., 2019, Bergamaschi et al., 2020). Additionally, hetIL-15 enhances adoptive cell transfer (ACT) in immunocompetent hosts promoting T lymphocyte infiltration into the tumor (Ng et al., 2017). hetIL-15 (NIZ985) has been tested as a single agent in a first-in-human study and is currently being evaluated in combination with anti-PD-1 antibodies (Spartalizumab and Tislelizumab) for the treatment of metastatic or unresectable tumors (NCT02452268) and solid tumors or lymphoma (NCT04261439). Results from the phase I NCT02452268 clinical trial showed that hetIL-15 as a single agent is well tolerated and induces IFN-γ production and expansion of cytotoxic lymphocytes in patients with several advanced cancers (Conlon et al., 2021a).

Materials And Methods

Cells and mice

EO771 (CH3 BioSystems), 4T1 (ATCC) and KPC (KPC223481+hMSLN-H06) cell lines were tested for mycoplasma (by PCR using standard mycoplasma testing protocol) and cultured in RPMI-1640 [+] L-glutamine medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum.

C57BL/6, BALB/c and *Rag-1* ko female mice were purchased from Envigo International Holdings, Inc., Charles River Laboratories (Wilmington, MA, USA) or Jackson Laboratory, respectively. The KPC transgenic model and the TPO (Thyroid Peroxidase) model were bred in Dr. Kozlov's laboratory. TPO mouse model has intrinsic tolerance for human mesothelin because it is genetically engineered to express the non-self-antigen in the thyroid gland under the thyroid-specific peroxidase (TPO) promoter (Hagerty et al., 2021). All animals were kept under pathogen-free conditions at the National Cancer Institute Animal Facility in Frederick. All animal studies were approved by the National Cancer Institute-Frederick Animal Care and Use Committee. NCI-Frederick is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals (National Research Council; 1996; National Academy Press; Washington, D.C.).

Animal studies and treatment

EO771 ($3x10^5$) and 4T1 tumor cells ($0.3\text{-}1x10^6$) were orthotopically inoculated in the fourth mammary fat pad of C57BL/6 and Balb/c mice, respectively. 4T1 tumor cells were also injected (10^4) through the lateral tail-vein (intravenously, IV) of 6-8 weeks old Balb/c mice. KPC cell line (10^4) was orthotopically inoculated in the pancreas of 8-10 weeks old TPO C57BL/6 mice.

For the orthotopic inoculation, the cells were resuspended in Dulbecco's PBS, and Matrigel was added at 1:3 dilution. Matrigel is a soluble and sterile extract of basement membrane proteins that forms a 3D gel at 37 °C (Kleinman and Martin, 2005) preventing cell leakage to adjacent tissues upon inoculation. Tumor size was measured by a digital caliper (breast tumors), or ultrasound imaging (pancreatic tumors) and tumor volume was calculated by the equation $L*W*H*\pi/6$. Treatment started when the tumor sizes reached a size from 20-80mm^3. The hetIL-15 molecule that was used in the studies was purified from HEK293 cells (Admune Therapeutic LLC/Novartis). hetIL-15 was administered peritumorally in PBS and Matrigel (EO771) or

intraperitoneally in PBS (4T1 and pancreatic studies). In some experiments, we used the hetIL-15Fc molecule, which is a fusion of hetIL-15 to the Fc fragment of human immunoglobulin G1 (IgG1). hetIL-15Fc was diluted in PBS, and in Matrigel at 1:4 dilution.

EO771 tumor-bearing C57BL/6 mice were treated peritumorally with vehicle (PBS) or hetIL-15Fc every 4 days at 5 ug/mouse. hetIL-15 was administered in Matrigel (Corning Inc.) at 1:4 dilution. For NK cell depletion, mice received 100μg of anti-NK1.1 mAb (clone PK13) or isotype IgG2a (BioXCell) which were administered intraperitoneally for 4 consecutive days before the inoculation and every 4 days after. Depletion of NK cells were confirmed through flow cytometry analysis of spleen and was consistently > 95%. In both orthotopic and IV models of 4T1 breast cancer model, the mice were randomized into four therapeutic groups: untreated (PBS) (eight IP injections), doxorubicin [(Doxorubicin Hydrochloride injection solution (Dox), Pfizer (three IV injections - 5mg/kg), hetIL-15 (eight IP injections - 3ug/mouse) and doxorubicin+hetIL-15 (combined schedule of monotherapies). The study endpoints were: (i) day 22 and (ii) day 18, post tumor cell inoculation, in the orthotopic and IV model, respectively.

We used two different agents in the TPO model that target mesothelin: the immunotoxin LMB100 and the anti-mesothelin antibody MB. LMB100 toxin is a de-immunized cytotoxic protein of Pseudomonas exotoxin A (PE24) (B-cell epitope silenced) fused to a humanized Fab fragment of SS1 antibody against the tumor-associated antigen mesothelin (MSLN) (Bauss et al., 2016). When the tumor reached a size of 20-80 mm^3 the mice were randomized in four groups: (i) control (PBS), (ii) LMB100 or MB (6 IP injections), (i) hetIL-15 (9 IP injections) and (iv) LMB100 or MB plus hetIL-15. Tumor growth was measured overtime until day 19. GEMM (genetically engineered mouse model) KPC mice develop pancreatic tumors at the age of around 15-weeks-old. When the tumors reached a size of 40mm^3, the mice were randomized in four groups: (i) control (PBS), (ii) gemcitabine (IP injections every 3 days - 100mg/kg), (iii) hetIL-15 (10 IP injections - 3ug) and (iv) gemcitabine plus hetIL-15 (3 injections and 7 injections, respectively).

In the survival studies, mice were sacrificed when the primary tumor reached a 2cm diameter or any other humane endpoints listed in the ACUC-approved animal protocol, such as 20% weight loss or acute morbidity.

Adoptive cell transfer

Recipient naïve C57BL/6 mice were challenged with 3×10^5 EO771 cells on day 0 and 7 days later the mice were irradiated with 600cGy. 8 days after tumor challenge, $CD8^+$T cells from spleen of naïve or hetIL-15 treated mice (rechallenged with EO771 tumor cells) were injected into

the EO771 tumor-bearing mice. Recipient mice were boosted with hetIL-15 IP injections (5μg/dose/mouse) every 3 days until the end point.

Rechallenge

Long-term surviving tumor-free mice previously cured upon hetIL-15 treatment, were rechallenged with 5×10^4 EO771 cells on day 68 (1^{st} challenge with 3×10^5 EO771 cells – day 0). The mice never grow EO771 tumors and were rechallenged for a second time on day 158 with 5×10^4 EO771 cells (4th right mammary pad) and with 5×10^4 KPC cells (3rd left mammary pad) as a negative control. Growth of individual EO771 and KPC tumors were monitored from the day of the 2nd rechallenge until the end point.

Metastasis evaluation

Metastasis evaluation in the blood and lungs of 4T1 model was performed by clonogenic assays according to Pulaski and Ostrand-Rosenberg protocol (Pulaski and Ostrand-Rosenberg, 2001). Briefly, RBC-lysed blood and lung cell suspensions were placed in a petri dish (100 mm) and cultured in a selection medium: complete RPMI-1640 [+] L-glutamine supplemented with 60μg/ml of 6-thioguanine (6-TG, Sigma) for 14 days. Lung cell suspensions were obtained by enzymatic digestion (Lung Dissociation Kit, mouse, Miltenyi Biotec) and mechanical dissociation (GentleMACS dissociator, Miltenyi Biotec) according to the manufacturer's protocol. At the end of the 14 days, colonies were fixed with 50% ice-cold trichloroacetic acid (TCA, Sigma) and stained with sulforhodamine B (0.04% SRB, Sigma) according to Orellana and Kasinski protocol (Orellana and Kasinski, 2016). The evaluation was performed macroscopically under the stereoscope counting the individual colonies and assigning the animals in one of the following ranges: 0, 1-50, 50-500 and >500 number of colonies. For the India Ink staining, India Ink (15%, Speedball) was injected into the trachea of the animal and the lungs were washed in PBS. Lungs were then stored overnight in Fekete's solution containing 300ml of 70% ethanol (Pharmco), 30ml of 37% formaldehyde (Sigma) and 5ml of glacial acetic acid (Sigma). The next day, the white pulmonary tumor nodules were counted macroscopically.

Histological analysis

Lungs were fixed in 10% neutral buffered formalin (NBF, Sigma) and paraffin embedded. Sections were stained with hematoxylin/eosin (H&E) or processed for immunohistochemistry (IHC). IHC automated staining was performed on Leica Biosystems' Bond RX with the following

conditions: Epitope Retrieval 1 (Citrate) 20’ for CD8a (eBioscience, 1:50), NK1.1 (clone 30-F11, BD Biosciences) and Ly6G/GR1 Granulocyte Marker (Origene, 1:100). The Bond Polymer Refine Detection Kit (Leica Biosystems) with the omission of the Post Primary Reagent was used, and an anti-rat secondary antibody (Vector Labs) was included. Isotype rat IgG2a antibody (BD Bioscience) was used in place of the primary antibodies for the negative controls. H&E and IHC slides were scanned using an Aperio AT2 scanner (Leica Biosystems) into whole slide digital images (one section was used for the analysis). Image analysis of positive-stained cells in lung tissue was performed using HALO image analysis software (v3.3.2541.300; Indica Labs). Positive-stained cells located in vessels or areas of artifact such as folds and tears were excluded from the analysis.

Splenic $CD8a^+$T cells isolation

Single-cell suspension of splenocytes were collected through a 100-mm cell strainer. The $CD8a^+$T cells isolation Kit (Miltenyi Biotec Inc.) was used for the isolation, according to the manufacturer protocol. Cells were isolated through negative selection using AutoMACS® Pro Separator (Miltenyi Biotec Inc.).

$CD11c^+$ cell isolation from tumors

EO771 tumors from control and hetIL-15 treated animals were enzymatically digested using the tumor dissociation kit (Miltenyi Biotec Inc.) and mechanically dissociated using the GentleMACS™ Dissociator (Miltenyi Biotec Inc.). Tissues were passed through 100-mm cell strainers (Falcon) and washed with PBS before proceeding to the isolation step. The $CD11c^+$ cells isolation Kit (Miltenyi Biotec Inc.) was used according to the manufacturer protocol. Cells were isolated through positive selection using AutoMACS® Pro Separator (Miltenyi Biotec Inc.).

Flow cytometry

Tumors and lungs were processed by enzymatic digestion and mechanical dissociation as mentioned above, and spleens and lymph nodes (LNs) were mechanically homogenized. Single-cell suspensions were obtained by filtering the homogenates using 100 μm cell strainers (Corning). Red blood cells were lysed using ACK lysis buffer (Lonza). Single cells were washed with PBS and stained (except blood) with a fixable aqua dead cell stain kit (ThermoFisher Scientific) for 30 min at 4°C. Next, the samples were surface stained with the following antibodies: CD45 (clone 30-F11, Biolegend), CD3 (clone 145-2C11, Biolegend), CD8a (clone 53-6.7, BD Biosciences),

NK1.1 (clone PK136), B220 (clone RA3-6B2, BD Biosciences), XCR1 (clone ZET), MHCII (clone M5/114.15.2), CD11c (clone N418), CD64 (clone X55-5/7.1), F4/80 (clone BM8), CD103 (clone M290), CD172a (clone P84), Ly6C (clone HK1.4), TREM-1 (clone TR3MBL1), CD101 (clone Moushi101), CX3CR1 (clone SA011F11) and GP2 (clone 2F11-C3), Ly6G (clone 1A8, BD Biosciences), CD49b (clone DX5, BD Biosciences), CD19 (clone 6D5, Biolegend) and CD11b (clone M1/70, ThermoFisher Scientific). For intracellular staining, cells were fixed and permeabilized using the Foxp3 staining buffer (ThermoFisher Scientific), following the manufacturer's instructions. After permeabilization, the cells were stained with Ki67 (clone B56, BD Biosciences), Granzyme B (clone GB12), IRF8 (clone V3GYWCH) and INF-γ (clone XMG1.2). The samples were acquired in a Fortessa flow cytometer (BD Biosciences), and the data were analyzed using FlowJo software (Version 10.8.0, Becton Dickinson and Company, Ashland, OR). PMN-MDSCs were characterized as $CD45^{+}lin^{neg}$ (CD3/CD49b/CD19/B220) $/CD11b^{+}/Ly6G^{+}/Ly6C^{low}$ and M-MDSCs as $CD45^{+}lin^{neg}$ (CD3/CD49b/CD19/B220) $/CD11b^{+}/Ly6G^{-}/Ly6C^{high}$. Some of the 4T1 and KPC tumors were excluded from the analysis because of an unusual percentage of B cells (> 5% of $CD45^{+}$) that was attributed to contamination with cells from the draining lymph node.

Gene expression analysis by nCounter PanCancer immune profiling panel

Tumors were mechanically disrupted in RLT buffer (QIAGEN), and RNA extraction was performed with RNeasy (QIAGEN) including on-column DNase I digestion according to the manufacturer's instructions. nCounter PanCancer Immune Profiling Panel (NanoString Technologies) was used to monitor the expression of a panel of 770 genes related to immuno-oncology. The mRNA molecules were counted with the NanoString nCounter at the Laboratory of Molecular Technology Advanced Technology Program, Frederick National Laboratory. Analysis was performed with a workflow written in R and through a user interface developed on the Foundry Platform (Palantir Technologies). Pathway enrichment analysis was performed using the GO database and the top 150 positively and negatively differentially expressed genes as defined by t-statistic.

Bulk RNA sequencing

Tumor-infiltrating DC subpopulations ($CD103^{+}$cDC1s, $CD11b^{+}$cDC2s and $CD103^{int}CD11b^{+}$DCs) and macrophages were sorted on a BD FACSAria II. For each cell subset, (4,000-20,000) viable cells were sorted directly into RLT buffer, flash frozen and stored at -80° C until RNA extraction. RNA was isolated using RNeasy Mini Kit (Qiagen) and removal of genomic

DNA (gDNA) was performed with the DNase I enzyme (Qiagen), according to manufacturer's recommendations. Library preparation was performed using NEBNext® Ultra™ II Directional RNA Library Prep Kit. At least 100 million reads per sample were used following the standard operating procedure at the Sequencing Facility - Illumina (CCR). Preprocessing, alignment, and gene-wise quantification steps were performed using the CCBR Pipeliner as implemented by NIH HPC Biowulf cluster. Downstream analysis and visualization were performed in R on the NIH Integrated Data Analysis Platform.

Single-cell RNA sequencing

Isolated tumor-infiltrating $CD11c^+$ populations from control and hetIL15-treated EO771-tumor bearing mice were processed into single-cell suspension. Approximately 10,000 cells from every sample were then loaded on one channel of the 10X chip and GEMs (Gel Beads-in-emulsion) were generated using the 10X Genomics Chromium Controller. 3' mRNA-seq gene expression libraries were then prepared using the Chromium Next GEM Single Cell 3′ Reagent Kits v3.1. These libraries were pooled and first run on NextSeq500 as asymmetric paired-end run with a read length of 28bp for Read 1,55bp for Read 2, and 8bp for the sample index read. The data from this run was used to calculate the re-pooling ratios for better balancing of the libraries, and the new pool of the six gene expression libraries was sequenced on a NovaSeq SP (100 cycle) run as asymmetric paired-end run with a read length of 28bp for Read 1, 75bp for Read 2, and 8bp for the sample index read. The data from the two sequencing runs for gene expression libraries was pooled for the final analysis. Cellranger v4.0.0 count matrices were analyzed on the NIH Integrated Data Analysis Platform. Quality control, merging, and clustering was performed using Seurat v3.1.5. Cells were serially annotated with scRNA-seq reference datasets from Brown et al. (Brown et al., 2019) and our own bulk RNA-seq dataset from FACS-purified populations with SingleR v1.0.0. Single-sample GSEA analysis was performed on cluster average gene expression using the GSVA v1.30.0 R package against dendritic cell pathways extracted from all collections in MSigDB (v6.2). Normalized enrichment scores were row scaled and plotted with heatmap v1.0.12. MSigDB dendritic cell pathways and Seurat clusters were clustered within the heatmap using Euclidean distances.

Multiplex RNA in situ hybridization staining

CD24a, *Mgl2*, and *Ccl17* expression was detected by staining 5μm FFPE tissue sections with RNAscope® 2.5 LS Probe –Mm-CD24a-C1 (ACD), RNAscope® 2.5 LS Probe –Mm-Mgl2-O1 (ACD), RNAscope® 2.5 LS Probe –Mm-Ccl17-C3 (ACD), and the RNAscope LS Multiplex

Fluorescent Assay (ACD) using the Bond RX auto-stainer (Leica Biosystems) with a tissue pretreatment of 15 minutes at 95°C with Bond Epitope Retrieval Solution 2 (Leica Biosystems), 15 minutes of Protease III (ACD) at 40°C, and 1:750 dilution of TSA-Cyanine 5 Plus, TSA-Fluorescein Plus and TSA-Cyanine 3 Plus (AKOYA), respectively. The RNAscope® 3-plex LS Multiplex Negative Control Probe (Bacillus subtilis dihydrodipicolinate reductase (*dapB*) gene in channels C1, C2, and C3) was used as a negative control. The RNAscope® LS 2.5 3-plex Positive Control Probe-Hs was used as a technical control to ensure the RNA quality of tissue sections was suitable for staining. Slides were digitally imaged using an Aperio ScanScope FL Scanner (Leica Biosystems).

Direct co-culture of DC with $CD8^+$ T cells

Sorted tumor-infiltrating $CD103^{int}CD11b^+$DCs were co-cultured with isolated splenic $CD8^+$T cells from naïve mice (ratio DC: $CD8^+$T cells, 1:10) in RPMI-1640 supplemented with 10% FBS, 1% penicillin – streptomycin, GM-CSF (100 U/mL) and IL-2 (30IU/mL). After 24hrs incubation, the cells were harvested, washed, and analyzed by flow cytometry to determine IFN-γ expression.

Tumor surgeries and pre- and post-treatment

Tumor resections were performed one week post 4T1 cells (0.35×10^6) inoculation (tumor volume $130mm^3$). The treatments started before and continued after surgery following a neoadjuvant and adjuvant setting. On day 4 (tumor size $60mm^3$), the mice were randomized into five groups: (1) untreated (PBS) without tumor resection, (2) untreated (PBS) with resection only, (3) doxorubicin-treated with resection, (4) hetIL-15-treated with resection (5) combination-treated with resection. The dosing schedule was the same as mentioned above except for the additional hetIL-15 administration on day 4 (total of nine injections). The study endpoint was day 22, and mice showing tumor regrowth after surgery were excluded from the analysis.

Rechallenge experiment

4T1 tumor cells ($5x10^4$) were subcutaneously injected in the flanks of 18-20 weeks old naïve Balb/c mice (not previously challenged with 4T1 cells, control group) or survivors after tumor resection and hetIL-15 or combination therapy (considered as one group). Cells were resuspended in PBS and Matrigel was added at 1:3 dilution. The rechallenge was performed 90 days after the

first challenge. The animals were monitored, and the tumors were measured for 19 days post-inoculation.

Statistical analysis

Statistical analyses were performed using Prism 9.2.0 (GraphPad) Software (San Diego, CA, USA). Differences were evaluated by 1-way ANOVA or unpaired parametric Student's t test when the groups were 2. Ordinary one-way ANOVA (analysis of variance) and Tukey's multiple comparisons tests analysis was used to compare the different groups (when >2) or Dunette's multiple comparisons test analysis to compare each experimental group to tumor-free group only. Two-way ANOVA or mixed-effects analysis model and Tukey's multiple comparisons test were used to compare the tumor growths among the groups overtime. Survival analysis was done using Log-rank (Mantel-Cox) test. Pearson correlation was used to test the relationship between cell count and tumor volume. Significant p values were annotated as follows *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Results

Section 1

1. Breast cancer immunotherapy

1.1 Triple negative breast cancer (TNBC)

1.1.1 4T1 murine breast cancer model

1.1.1.1 hetIL-15 in combination with chemotherapy delays 4T1 tumor growth and extends animal survival

The anti-cancer activity of hetIL-15 was also evaluated in the 4T1 murine model of TNBC as a single agent or in combination with doxorubicin. 4T1 tumor cells were orthotopically inoculated in Balb/c mice and, when the tumors became palpable, the mice were randomized into four therapeutic groups: untreated (vehicle), doxorubicin, hetIL-15 and combination group. The treatment was performed according to the schedule shown in **Fig. 15**.

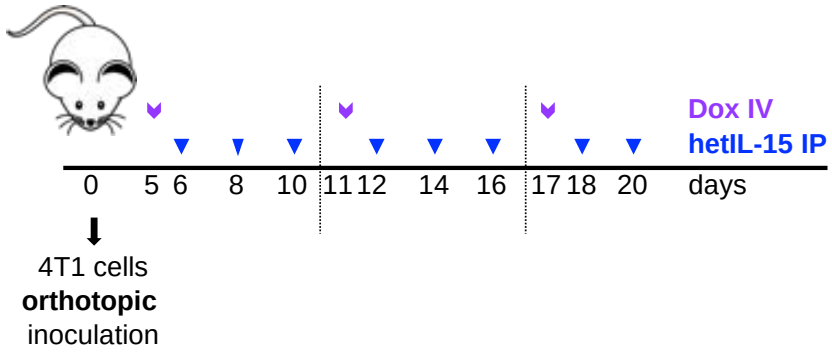


Figure 15: Therapeutic scheme of Balb/c mice orthotopically injected with 4T1 cells and treated with doxorubicin and hetIL-15.

The efficacy of the different treatments was initially evaluated by monitoring the growth of the primary tumor and by animal survival. Mice in the three groups that received therapy showed a significant delay in tumor growth compared to the untreated animals (**Fig. 16, left panel**). The most prominent delay was observed among mice treated with the combination therapy. However, compared to doxorubicin monotherapy, the two agents together result in a marginal improvement in the control of the primary tumor. When the different treatments were evaluated for animal survival, doxorubicin-treated mice showed a moderate benefit, whereas mice in the combination group had significantly extended survival compared to all the other groups (**Fig. 16, right panel**).

hetIL-15 did not show benefit in delaying animal survival. These results indicate that the combination therapy has additive anti-tumor effects, especially in extending animal survival.

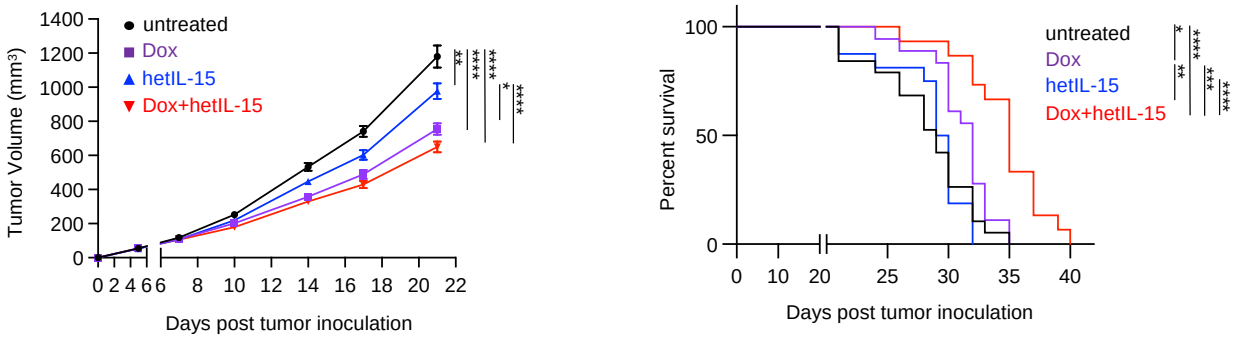


Figure 16: Mean of tumor growth curve overtime up to day 21 (**left panel**) and Kaplan-Meier survival curve (**right panel**) of 4T1 tumor-bearing Balb/c mice in the four groups.

1.1.1.2 hetIL-15 alone or in combination with doxorubicin exerts potent anti-metastatic effect in both lungs and blood

Although hetIL-15 monotherapy did not show any benefit in survival, when the mice were evaluated for the presence of pulmonary metastatic foci (measured as tumor areas at their individual endpoint), it was found that hetIL-15 exerted a strong anti-metastatic effect (**Fig. 17, left panel**). hetIL-15 co-administration with doxorubicin resulted in an even better control of metastasis. The tumor areas covering the lung tissue in untreated and doxorubicin-treated groups were found to be approximately 16%, whereas these areas were reduced to approximately 5.5% and 2.5% in the hetIL-15- and combination-treated mice, respectively (**Fig. 17, right panel**).

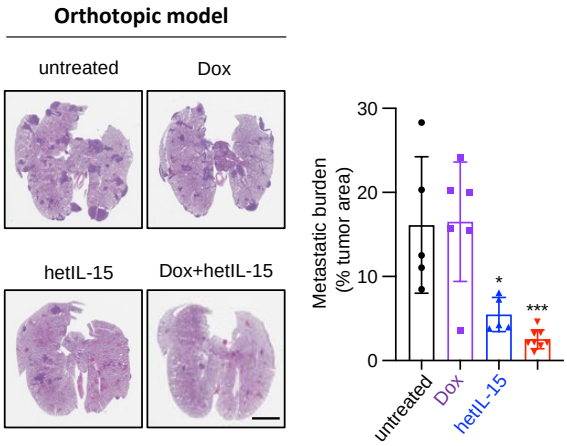


Figure 17: Representative images from H&E-stained lung sections (**left panel**), and the percentage of tumor areas present in each mouse lungs (**right panel**, asterisks show the significance of difference from the untreated group) presented as mean±SD for each group.

1.1.1.2.1 Lungs

To evaluate this observation in more depth, the anti-metastatic effect in the lungs was further examined by clonogenic assays. On day 22, whole lung homogenates from each animal were cultured in a selection medium to allow for tumor colony formation. Based on the number of tumor colonies per mouse (represented by each plate), the mice were classified into 4 groups: (i) 0 (no colonies), (ii) 1-50, (iii) 50-500 and (iv) >500 colonies. We found that 62.5% (10/16) of the untreated control mice had more than 500 metastatic colonies, and we observed a decrease in these numbers in the therapeutic groups: 2/16 in the doxorubicin group (12.5%), 2/16 in the hetIL-15 group (12.5%) and 1/16 in the combination group (6.25%) (**Fig. 18**). Importantly, only mice in the hetIL-15 (1 mouse) and combination group (4 mice) were found to have lungs completely free of metastatic cells, revealing the strong anti-metastatic effects of these treatments.

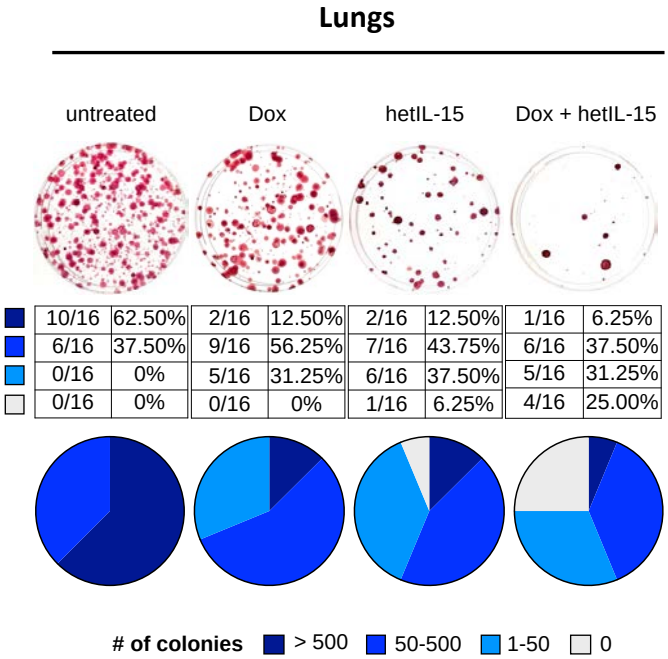


Figure 18: Representative images (**upper panels**) from clonogenic assays showing SRB-stained tumor colonies derived from lungs and tables with the number and the percentage (plotted in pies) of mice with different number of tumor colonies (**lower panels**) in each group on day 22.

1.1.1.2.2 Blood

Next, we sought to evaluate the effect of the treatments on CTCs, which are cells capable of initiating metastatic lesions (Baccelli et al., 2013). The evaluation was performed by clonogenic assays in blood collected on day 22. The mice were classified into four groups based on the number of tumor colonies, as described above. Of the 19 untreated 4T1 tumor-bearing animals, 7 (36.84%) developed CTC colonies with 4 of these having more than 500 colonies (**Fig. 19**). In contrast, none

of the animals in hetIL-15 and combination groups had more than 500 CTC colonies. Interestingly, only 4 out of 18 mice (22.2%) developed CTC colonies in the hetIL-15 group, which however were minimal, as the 2 of these animals developed only 15 CTC colonies and the other 2, only 2 colonies. The most prominent effect though was observed in the combination group, where only 2 out of 19 mice (10.53%) were positive for CTC colonies with very low numbers (20 and 1 colonies, respectively). The rest of the mice in both hetIL-15 and combination groups did not develop any colony. Mice in the doxorubicin group also showed a reduction in the number of CTCs, however it was not as effective as in the other treated groups.

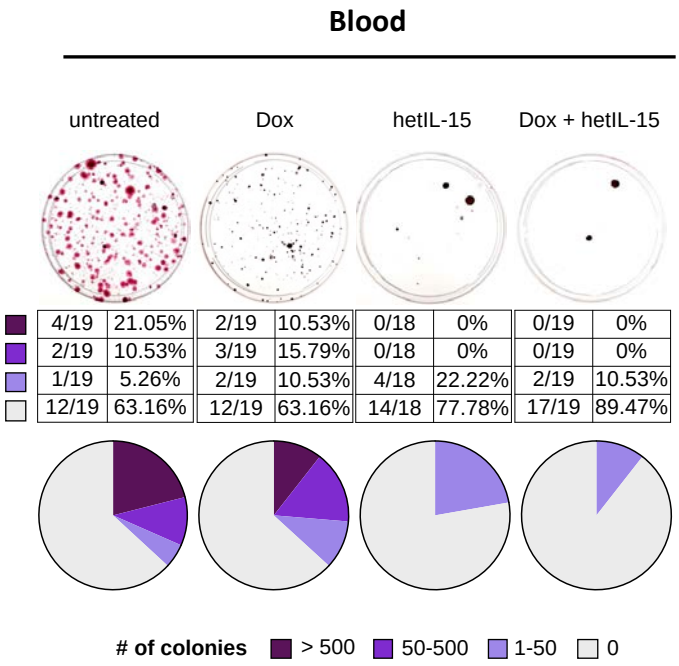


Figure 19: Representative images (**upper panels**) from clonogenic assays showing SRB-stained tumor colonies derived from blood and tables with the number and the percentage (plotted in pies) of mice with different number of tumor colonies (**lower panels**) in each group on day 22.

1.1.1.2.3 IV induced metastatic model

Given the observation that the treatments decreased the CTCs in the blood, we aimed to study whether the observed anti-metastatic effect in the lungs is a result of fewer cells reaching the tissue. For this purpose, we injected 4T1 tumor cells intravenously into the lateral tail-vein making the lungs the main site of tumor cells seeding. The treatment started 24 hours post cells injection following the schedule shown in **Fig. 20A**. On day 18, mice were sacrificed, and their lungs were analyzed by histology for the presence of metastatic foci (expressed as tumor areas). Mice in the untreated and doxorubicin groups showed approximately 30% of the lung area occupied by tumor cells. hetIL-15 treatment decreased the area to 7% and combination therapy to 4% (5-fold and 8-

fold decrease, respectively) (**Fig. 20B**). Of note, two mice in the combination group had less than 1% of lung surface occupied by tumor.

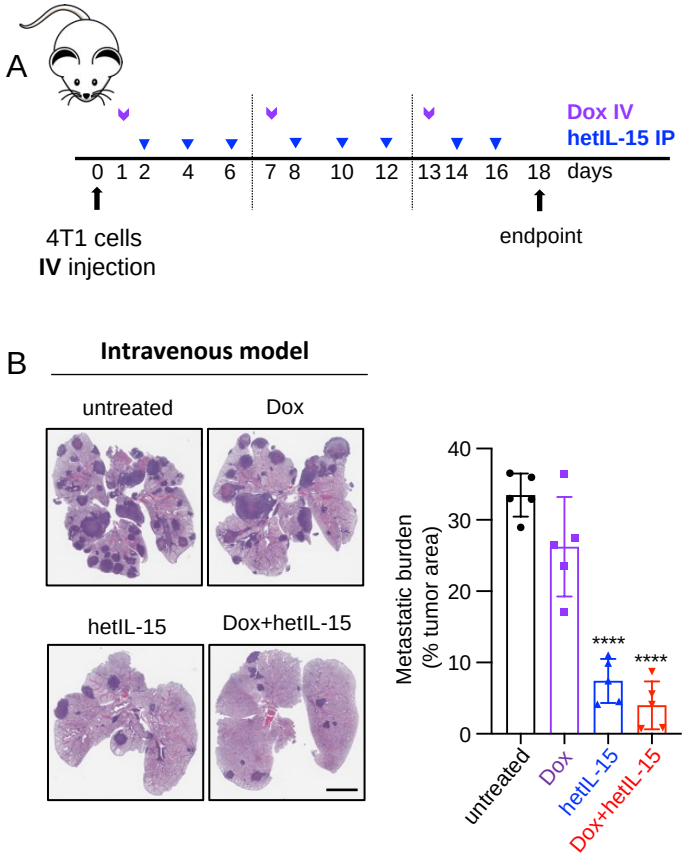


Figure 20: A. Treatment scheme of balb/c mice IV injected with 4T1 cells and treated with doxorubicin and hetIL-15 and **B.** Representative images of H&E-stained lung sections (left panel) and the metastatic burden expressed as percentage of tumor area (right panel) in each group on day 18. Asterisks show the significance of difference from the untreated group.

Taken together, these data indicate that hetIL-15 shows anti-tumor activity in the 4T1 breast cancer model, mainly by reducing the metastatic burden. hetIL-15 was found to exert its anti-metastatic activity by both inhibiting metastatic cell colonization in the lungs and decreasing the number of CTCs in the blood. This activity was found to improve the efficacy of doxorubicin, leading ultimately to the extended survival of the combination-treated mice.

1.1.1.3 hetIL-15 synergizes with doxorubicin to increase $CD8^+$T and NK cells and reduce PMN-MDSCs systemically

To identify the mechanisms of the observed anti-cancer effects, we sought to evaluate the immune profile of the 4T1 tumor-bearing mice upon treatment. We have previously reported the

role of hetIL-15 on expanding CD8$^+$T and NK cells in tumor-bearing mice (Ng et al., 2017, Bergamaschi et al., 2020), while others have shown that doxorubicin decreases the myeloid-derived suppressor cells (MDSCs) (Alizadeh et al., 2014). For these reasons, we analyzed by flow cytometry analysis the blood, spleens, lungs and tumors of tumor-bearing and age-matched tumor-free mice, to monitor changes in the frequencies of CD8$^+$T, NK, PMN (polymorphonuclear) - and M (monocytic) -MDSC cells. All the tissues were collected two days after the last hetIL-15 dose (day 16).

Comparison between tumor-bearing mice and tumor-free mice revealed that the disease causes a systemic reduction in the frequencies of both CD8$^+$T and NK cells, as flow cytometry analysis revealed. This reduction was prevented by hetIL-15 monotherapy, as the mice showed increased CD8$^+$T and NK cell frequencies in their lungs (**Fig. 21A**), blood (**Fig. 22A**) and spleen (**Fig. 23A**). Interestingly, co-administration of hetIL-15 with doxorubicin increased both populations to significantly higher levels than those observed in any of the other groups, which underlines that the two agents benefit from each other. Both hetIL-15 and combination treatment promoted CD8$^+$T and NK cells proliferation (measured by Ki67 expression) in the same tissues (**Figs 21B, 22B and 23B**). These data suggest that the observed increased cell frequencies are a result of increased proliferation within the tissues.

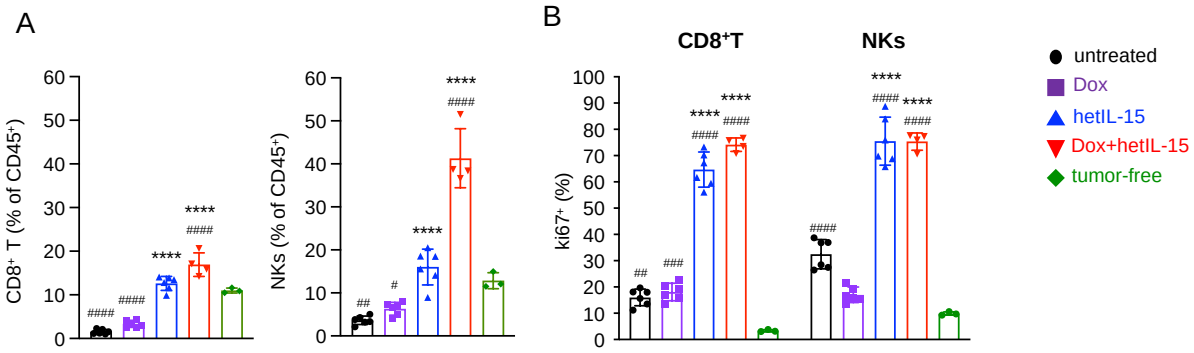


Figure 21: Frequencies (**A**) and Ki67 expression (**B**) of CD8$^+$T and NK cells in the lungs of each group. Data obtained from lungs of tumor-free mice are also included. Asterisks show the significance of difference from the untreated group and hashtags from the tumor-free group.

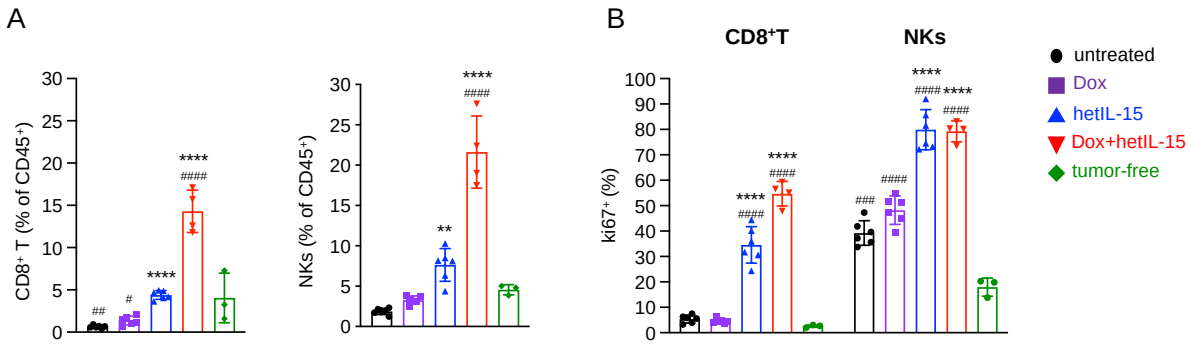


Figure 22: Frequencies (**A**) and Ki67 expression (**B**) of CD8$^+$T and NK cells in the blood of each group. Data obtained from blood of tumor-free mice are also included. Asterisks show the significance of difference from the untreated group and hashtags from the tumor-free group.

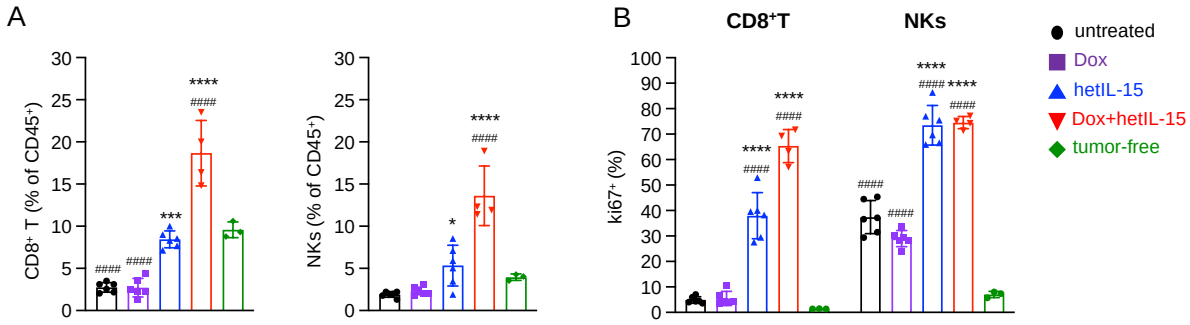


Figure 23: Frequencies (**A**) and Ki67 expression (**B**) of CD8$^+$T (**left panels**) and NK cells (**right panels**) in the spleens of each group. Data obtained from spleens of tumor-free mice are also included. Asterisks show the significance of difference from the untreated group and hashtags from the tumor-free group.

Within tumors, hetIL-15 treatment resulted in enhanced accumulation of NK cells, while the combination therapy increased both CD8$^+$T and NK cells, revealing that the treatments trigger the intratumoral infiltration of effector cells (**Fig. 24**). In contrast, doxorubicin as monotherapy did not affect either the frequency or the Ki67 expression in these two cell subsets, in any of the tissues.

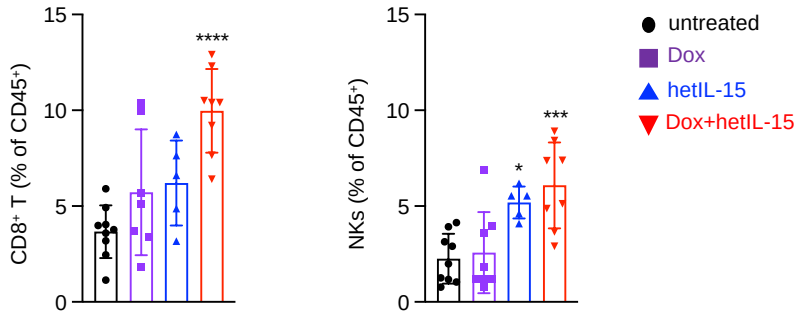


Figure 24: Frequencies of $CD8^+T$ (**left panel**) and NK cells (**right panel**) in the tumors of each group. Asterisks show the significance of difference from the untreated group.

Subsequently, we examined the frequencies of MDSCs as these cells are characterized by the ability to suppress T and NK cell functions (Veglia et al., 2021). Flow cytometry analysis revealed that tumor-bearing animals had increased frequencies of MDSCs compared to tumor-free counterparts. The population of PMN-MDSCs was found to be significantly expanded in the lungs (**Fig. 25, left panel**), blood (**Fig. 26, left panel**) and spleen (**Fig. 27, left panel**) while M-MDSCs were found to be increased mostly in the spleen (**Fig. 27, right panel**). Mice treated with hetIL-15 and combination therapy were found with significantly reduced PMN-MDSCs systemically (**Figs 25-27, left panels**). Combination therapy showed the most effective reduction, reducing the PMN-MDSCs frequencies close to normal levels, especially in the lungs (**Fig. 25, left panel**). Mice treated with doxorubicin monotherapy also had a lower frequency of PMN-MDSCs, although to a lesser extent as compared to the other two treated groups and only in lungs (**Fig. 25, left panel**). Interestingly, mice treated with doxorubicin monotherapy showed an increase in the frequency of M-MDSCs (**Figs 25-27, right panels**).

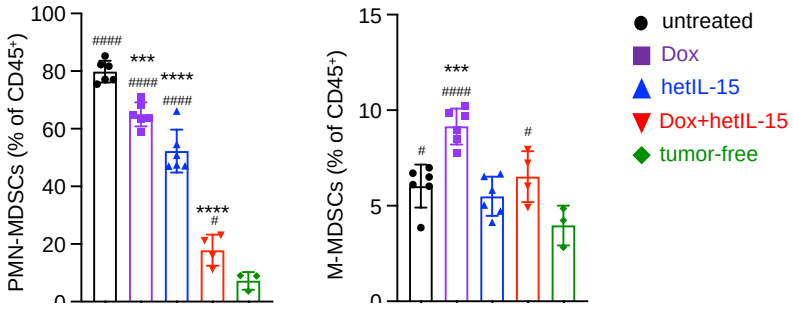


Figure 25: Frequencies of PMN-MDSCs (**left panel**) and M-MDSCs (**right panel**) in the lungs of each group. Data obtained from lungs of tumor-free mice are also included. Asterisks show the significance of difference from the untreated group and hashtags from the tumor-free group.

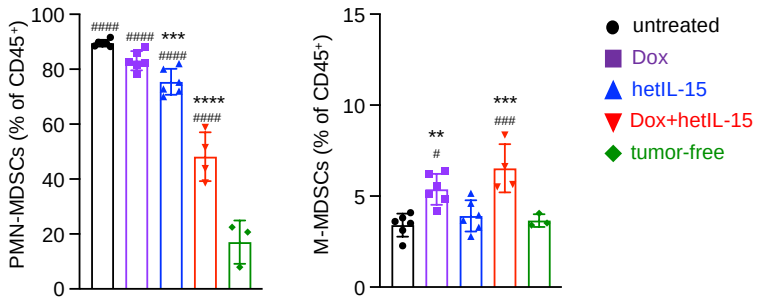


Figure 26: Frequencies of PMN-MDSCs (**left panel**) and M-MDSCs (**right panel**) in the blood of each group. Data obtained from blood of tumor-free mice are also included. Asterisks show the significance of difference from the untreated group and hashtags from the tumor-free group.

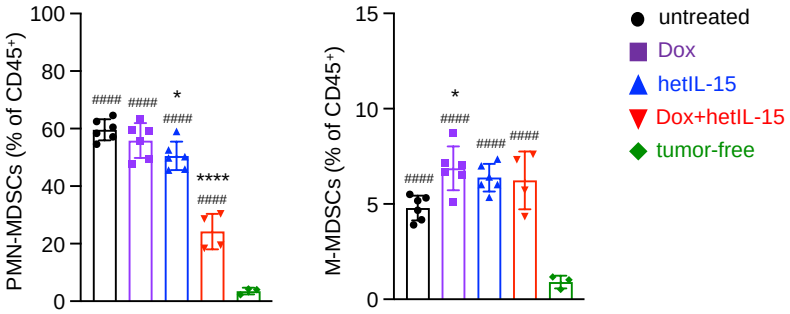


Figure 27: Frequencies of PMN-MDSCs (**left panel**) and M-MDSCs (**right panel**) in the spleens of each group. Data obtained from spleens of tumor-free mice are also included. Asterisks show the significance of difference from the untreated group and hashtags from the tumor-free group.

Finally, mice from all the groups that received therapy showed a reduction in the intratumoral PMN-MDSC population, which however reached statistical significance only in the mice from the combination group (**Fig 28, left panel**). In contrast, the tumor-infiltrating M-MDSC population was increased in all the therapeutic groups (**Fig 28, right panel**).

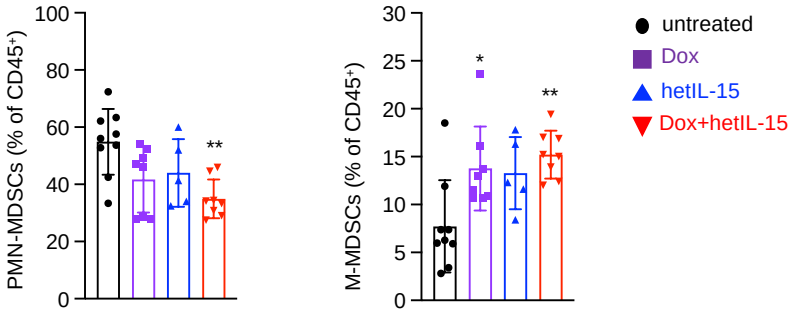


Figure 28: Frequencies of PMN-MDSCs (**left panel**) and M-MDSCs (**right panel**) in the tumors of each group. Asterisks show the significance of difference from the untreated group.

To confirm the findings from the flow cytometry analysis, the absolute number of $CD8^+$ and PMN-MDSC cells in the lungs was also evaluated by immunohistochemistry (IHC). Indeed, the numbers of $CD8^+$ cells were higher in mice from the hetIL-15 and combination groups (**Fig. 29**), while the number of PMN-MDSC cells was significantly reduced in all the therapeutic groups (**Fig. 30**). These results suggest that all the treatments not only affect the relative frequency of these two cell subsets, but also their absolute numbers.

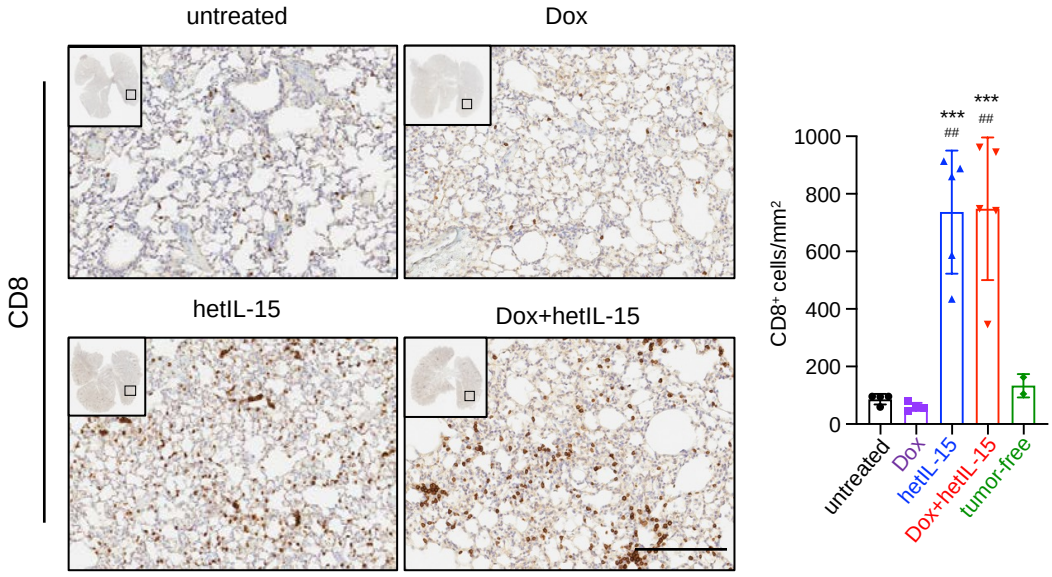


Figure 29: Representative IHC images of lung slides stained with anti-CD8 for each group (**left panels**). The bar graphs show the absolute numbers of $CD8^+$ cells/mm^2 (**right panel**) of the whole lung area in each group. Asterisks show the significance of difference from the untreated group and hashtags from the tumor-free group.

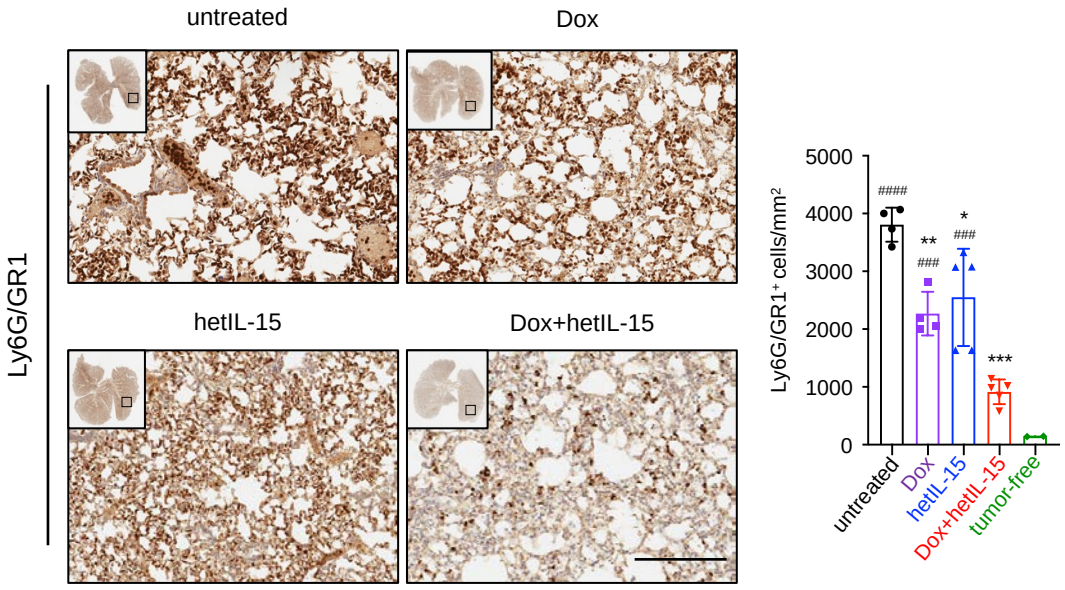


Figure 30: Representative IHC images of lung slides stained with anti-Ly6G/GR1 for each group (**left panels**). The bar graphs show the absolute numbers of Ly6G/GR1$^+$ cells/mm^2 (**right panel**) of the whole lung area in each group. Asterisks show the significance of difference from the untreated group and hashtags from the tumor-free group.

Overall, these data suggest that hetIL-15 and doxorubicin synergize towards an effective antitumor immunity by increasing the CD8$^+$T and NK cells and decreasing the PMN-MDSCs in the lungs, blood, and spleen. The intratumoral populations were found to be similarly affected, although to a lesser extent.

To further evaluate the impact of the different treatments on the immune population landscape, we calculated the ratios of MDSCs (both PMN-MDSCs and M-MDSCs) to CD8$^+$T and NK cells in blood, spleen, lungs, and tumors from the mice in the therapeutic groups and contrasted the results with data obtained from age-matched tumor-free mice.

All the ratios were found to be significantly increased in the examined tissues of the 4T1 tumor-bearing mice compared to tumor-free animals. Notably, the ratios of PMN-MDSCs to both CD8$^+$T and NK cells were more elevated compared to the ratios of M-MDSCs (**Figs 31, 32**). Combination treatment was the most effective at restoring the ratios back to the levels measured in tumor-free mice, in lungs (**Fig. 31**), blood (**Fig. 32A-B**) and spleen (**Fig. 32C-D**). hetIL-15 monotherapy showed a similar trend, although of lower magnitude, in reducing the ratios, but it was as efficient at reducing the M-MDSCs to effector ratios in lungs (**Fig. 31B**) and blood (**Fig. 32B**). Intratumorally, PMN-MDSCs to CD8$^+$T ratios were also significantly reduced in the tumor-bearing mice that received the combination therapy while hetIL-15 decreased only the PMN-MDSCs to NKs ratios (**Fig. 32E**). No significant changes were observed in any treated mice for the M-MDSCs to effector ratios within the tumors (**Fig. 32F**). Mice treated with doxorubicin alone

had also decreased ratios, mostly of PMN-MDSCs to effectors in lungs and blood (**Figs 31A, 68A**). However, an increase in M-MDSCs to effector ratios was observed as a trend in the spleen (**Fig. 32D**) and tumors (**Fig. 32F**) of doxorubicin-treated mice.

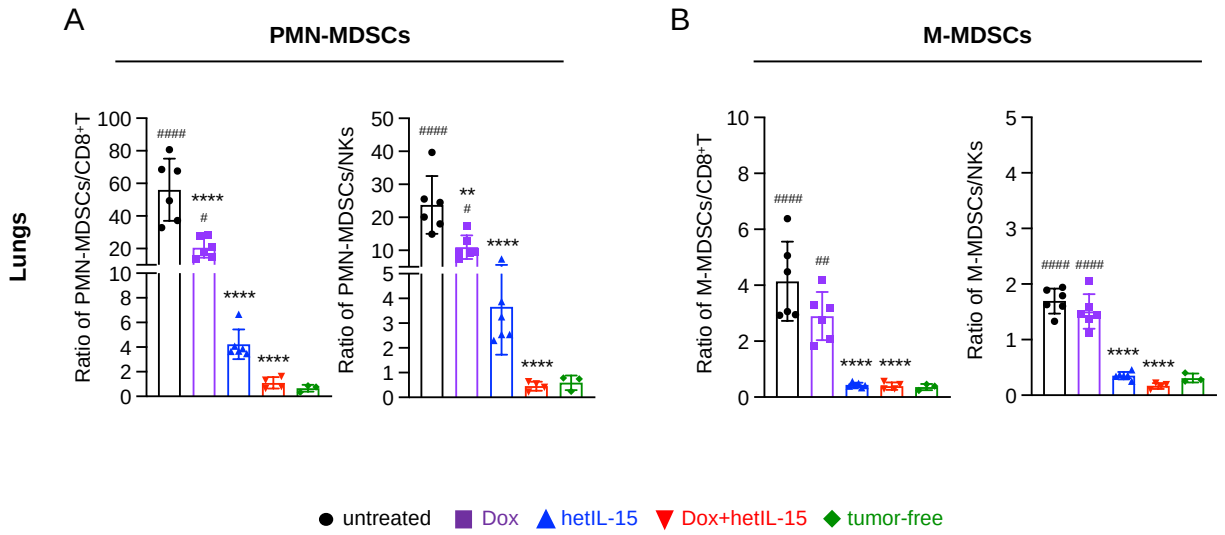


Figure 31: hetIL-15 alone and in combination with doxorubicin decreases the suppressor/effector ratios in the lungs. Ratio of (**A**) PMN-MDSCs to $CD8^+T$ (left) or NKs (right) and (**B**) M-MDSCs to $CD8^+T$ (left) or NKs (right) in the lungs of each group. Asterisks show the significance of difference from the untreated group and hashtags from the tumor-free group.

Taken together, these data show that hetIL-15 treatment alone and most effectively in combination with doxorubicin decreases the suppressor to effector cell ratios restoring the imbalance induced by the disease, which suggests an enhanced antitumor response.

1.1.1.4 The pre- and post-surgery administration of hetIL-15 monotherapy or combination therapy eradicate metastatic burden curing the animals

It has been previously reported that 4T1 tumor resection reduces MDSCs in the lungs leading to a better control of metastasis (Bosiljcic et al., 2019). Taking this into account and given the observed effects in PMN-MDSCs reduction and metastatic control upon hetIL-15 monotherapy or combination with doxorubicin, we explored the effects of the treatments together with tumor resection. 4T1 tumor cells were orthotopically inoculated in Balb/c mice and the formed tumors were resected one week later. To imitate the neoadjuvant and adjuvant settings (pre- and post-surgery) of the clinical therapeutic schemes (Bianchini et al., 2016), the treatments started before surgery and continued afterwards following the administration schedule shown in **Fig. 33**.

The frequency of circulating PMN-MDSCs was analyzed by flow cytometry on day 16 (**Fig. 34**). Mice in the groups that underwent surgery showed approximately 2.5 – 3.5-fold reduction of

PMN-MDSCs compared to mice from the unresected group, regardless of the treatment type. Additionally, in all the mice that underwent surgery we found a decrease in the number of Ly6G/GR1 positive cells in their lungs on day 22, indicating a reduction in the absolute numbers of PMN-MDSCs that was again independent of the treatment (**Fig. 35**). These results show that surgery alone results in the depletion of PMN-MDSCs in blood and lungs.

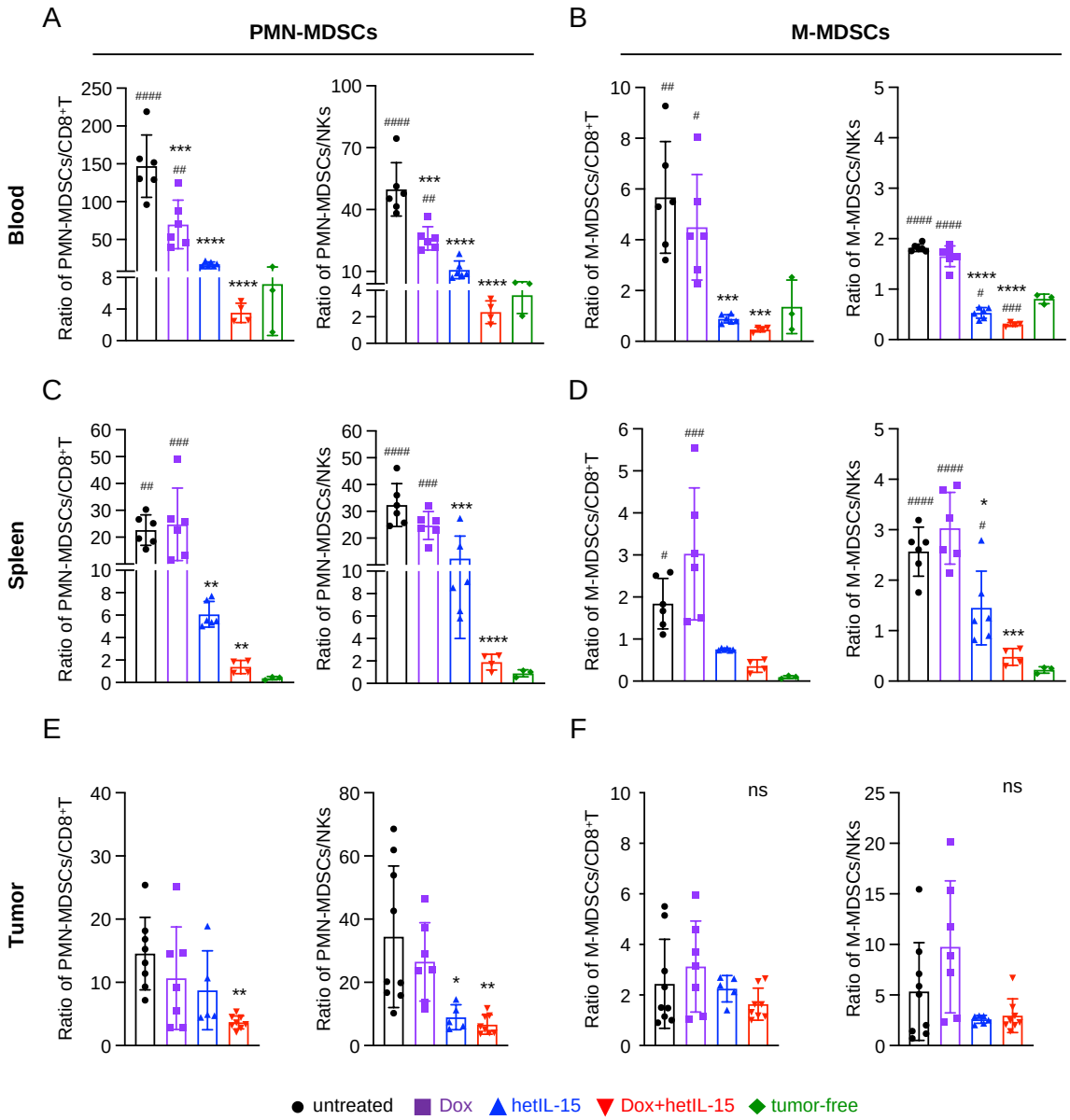


Figure 32: hetIL-15 alone or in combination with doxorubicin decreases the suppressor/effector ratios in blood and spleens and partially in tumors. Ratio of PMN-MDSCs to $CD8^+T$ (left) or NKs (right) and M-MDSCs to $CD8^+T$ (**left**) or NKs (**right**) in blood (**A-B**), spleen (**C-D**) and tumors (**E-F**) of each group. Asterisks show the significance of difference from the untreated group and hashtags from the tumor-free group (ns, non-significant)

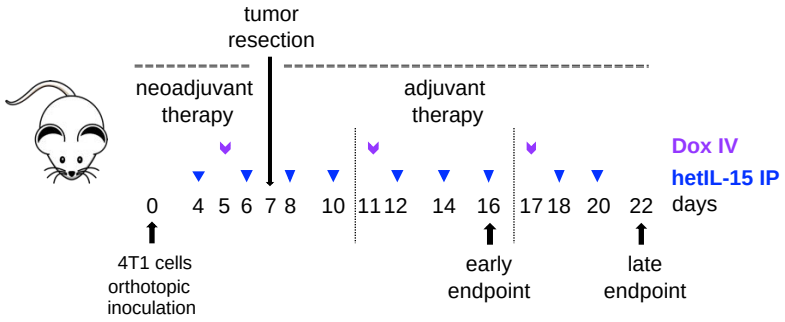


Figure 33: Therapeutic scheme of Balb/c mice that underwent surgical excision of the 4T1 primary tumor and were treated with doxorubicin and hetIL-15 in neoadjuvant and adjuvant setting.

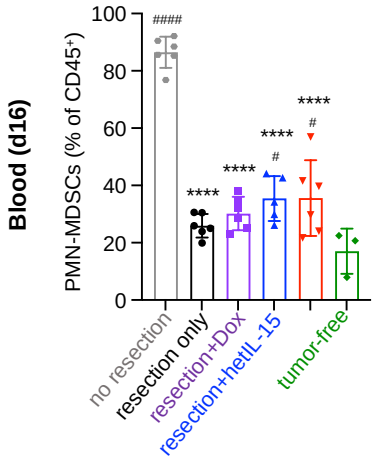


Figure 34: PMN-MDSCS frequencies in blood of each group on day 16. Data obtained from tumor-free mice are also included. Asterisks show the significance of difference from the untreated group and hashtags from the tumor-free group.

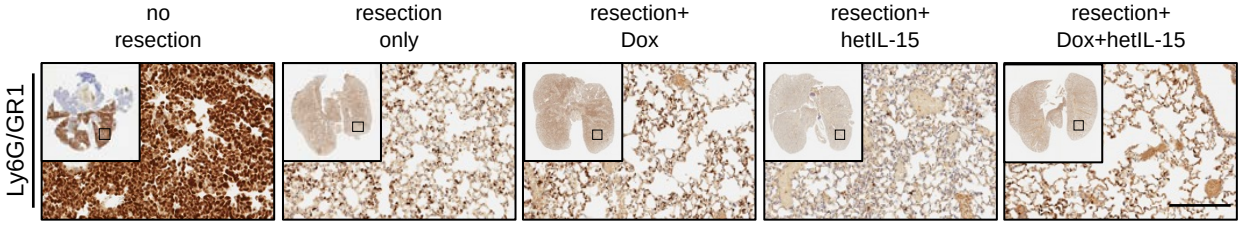


Figure 35: Representative IHC images of Ly6G/GR1-stained lungs from one mouse in each group on day 22.

Metastatic disease was evaluated by India Ink staining of the lungs for the detection of pulmonary nodules on day 22 (**Fig. 36, left and upper panel**). Primary tumor resection alone was effective in decreasing the number of metastatic nodules in comparison to no resection (mean of 21±3 and 78±4 nodules, respectively) (**Fig. 36, right panel**). Remarkably, no metastatic foci were

found in the lungs of the mice that underwent surgery and received hetIL-15, either alone or in combination with doxorubicin, revealing that the treatments can eradicate the metastatic disease in the absence of the primary tumor. Mice treated with doxorubicin showed significant foci reduction compared to both non-resected and resected only mice. Similar results were obtained with H&E staining of the lungs (**Fig. 36, left and bottom panel**).

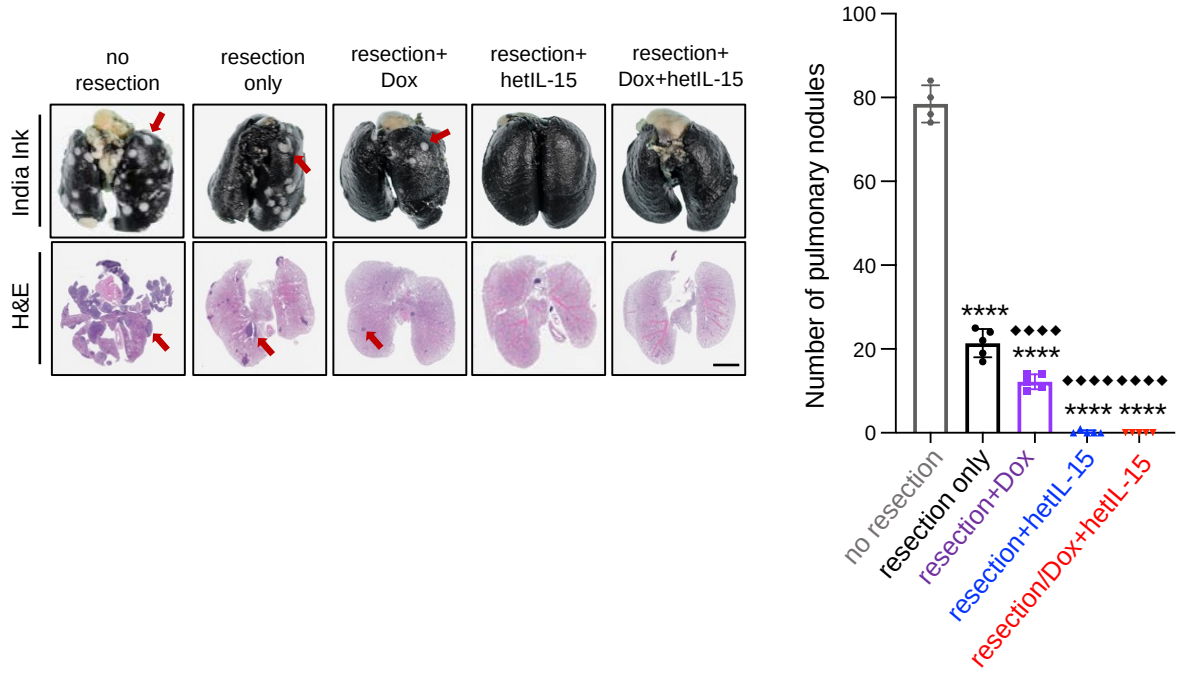


Figure 36: Representative images from India ink- and H&E-stained lungs (**left panels**) and number of white tumor nodules (from India ink staining evaluation) in lungs (**right panel**) are shown as mean±SD for each group. Asterisks show the significance of difference from the non-resected group and rhombuses from the resection-only group.

1.1.1.5 hetIL-15 alone or in combination with doxorubicin cures the resected mice and facilitates the development of tumor-specific immunological memory

This therapeutic surgery scheme was also evaluated regarding animal survival (**Fig. 37**). Pre- and post-surgery administration of hetIL-15 monotherapy led to 70% (9 out of 13) cures as the mice did not show any signs of morbidity up to day 80 when the studies were terminated. Similarly, combination treatment led to 45% (4 out of 9) cures of the resected mice, while doxorubicin monotherapy resulted in just 1 cure and marginally extended the median survival to 33 days. None of the non-resected or resected-only mice were cured, and the median survival was similar for both groups (approximately 28 days). Mice that were considered long-term survivors from hetIL-15 (n=7) and combination (n=2) groups were rechallenged with the 4T1 tumor cells. The rechallenge was performed 90 days after the first challenge and the tumor growth was monitored up to day 19 in the absence of any treatment. Survivor animals significantly controlled tumor growth compared

to age-matched control animals (challenged for the first time) (**Fig. 38**), indicating the presence of anti-tumor immunity elicited during the first challenge.

Taken together, these data show that pre- and post-surgery administration of hetIL-15 as monotherapy or in combination with doxorubicin can eradicate the metastatic disease which leads to complete cures and allows the development of effective immunological memory against 4T1 tumor cells.

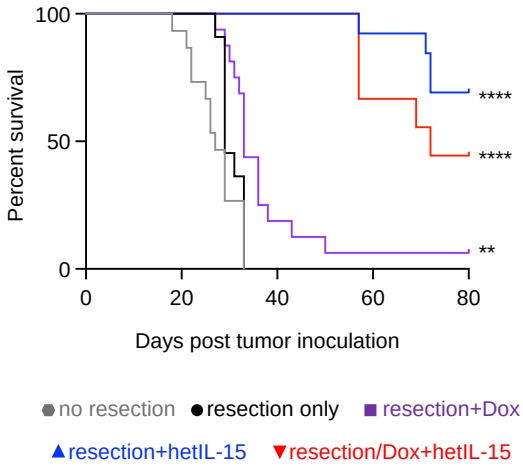


Figure 37: Survival curves for the animals in each experimental group. Mice were resected and treated following the therapeutic schedule shown in **Fig. 33**.

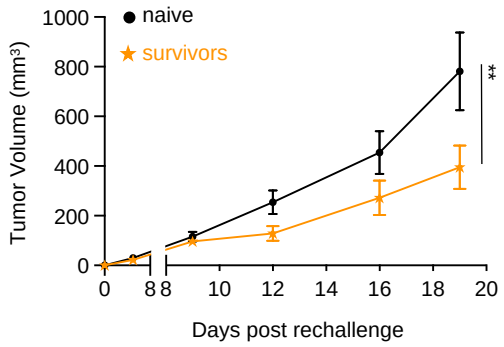


Figure 38: Tumor growth curves of naive and survivor animals (hetIL-15 and combination group mice merged from **Fig. 37** after rechallenging with 4T1 cells without any treatment until day 19.

1.1.2 EO771 murine breast cancer model

1.1.2.1 hetIL-15 monotherapy eradicates EO771 tumors

EO771 model of TNBC was the other breast cancer model that was used for evaluating hetIL-15 anti-tumor effects. C57BL/6 mice were orthotopically inoculated with EO771 tumor cells in the fourth mammary fat pad and the treatment was initiated when tumors reached ~20 mm^3. The hetIL-15 molecule used in these studies was the hetIL-15FC, which contains two IL-15/IL-15Ra molecules fused to the Fc region of human IgG1, from now on referred as hetIL-15. hetIL-15 was provided every 4 days locoregionally (in proximity to the tumor) at a dose of 5μg/injection (**Fig. 39A**). 5 hetIL-15 injections completely eradicated 40% of the tumors (**Fig. 39B**) and cured the animals (**Fig. 39C**) as they did not develop tumor regrowth or signs of morbidity that could implicate metastatic disease.

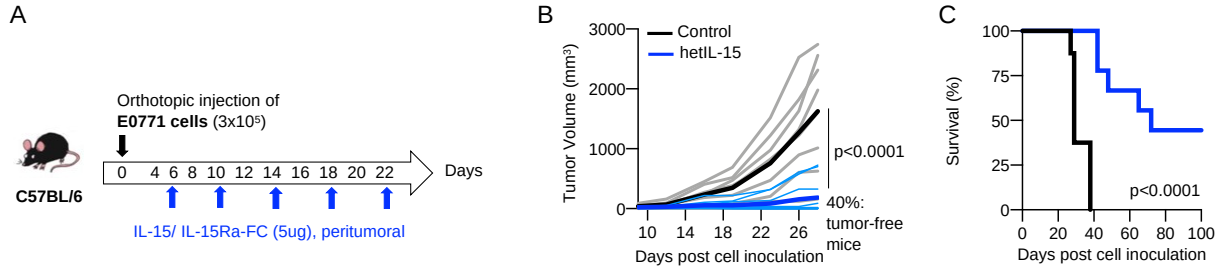


Figure 39: A. Therapeutic scheme of C57BL/6 mice that were orthotopically injected with EO771 cells and treated with peritumoral administration of hetIL-15, **B.** Tumor growth curve (bold lines represent average values) overtime up to day 27 and **C.** Kaplan-Meier survival curve, of the mice in control and hetIL-15-treated groups.

1.1.2.2 hetIL-15 monotherapy increases $CD8^+$ T and NK cells infiltration into the EO771 tumor

EO771 tumors were analyzed by flow cytometry and immunohistochemistry (IHC) to explore the changes in the tumor microenvironment upon hetIL-15 treatment. The tumors were collected in an earlier time point at day 16 and 48h after the last injection. Flow cytometric analysis revealed significant accumulation of both $CD8^+$T and NK cells (**Figs 40 and 41, left panels**) in the hetIL-15-treated tumors. Tumor-infiltrating $CD8^+$T and NK cells were characterized by higher content of the cytotoxic marker Granzyme B and increased expression of the proliferation marker Ki67 (**Figs 40 and 41, right panels**). The results were also verified by IHC analysis. IHC staining for CD8 and NKs showed increased accumulation of the populations (**Fig. 42**) in the hetIL-15-treated tumor sections. Overall, hetIL-15 administration altered the tumor microenvironment by promoting the intratumoral infiltration of activated cytotoxic T and NK cells.

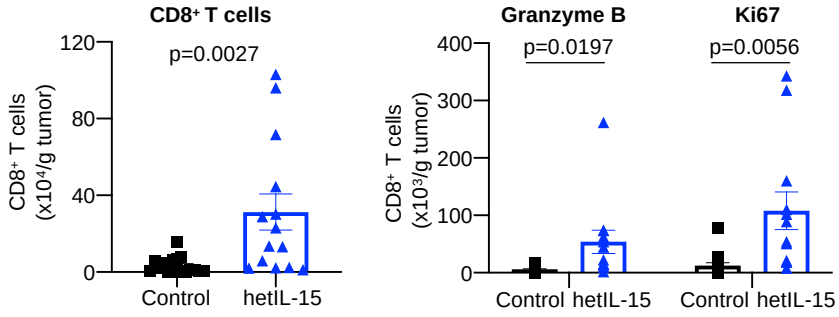


Figure 40: Frequencies of intratumoral CD8$^+$ T cells (**left panel**) and their Granzyme B and Ki67 expression (**right panel**) expressed as counts per gram of tumor in control and hetIL-15-treated groups of EO771-tumor bearing mice.

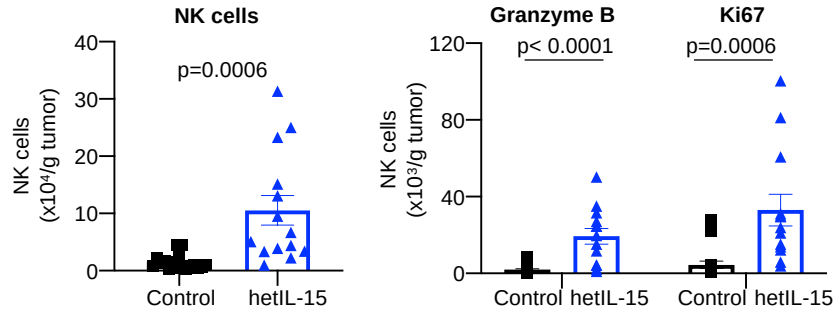


Figure 41: Frequencies of intratumoral NKs cells (**left panel**) and their Granzyme B and Ki67 expression (**right panel**) expressed as counts per gram of tumor in control and hetIL-15-treated groups of EO771-tumor bearing mice.

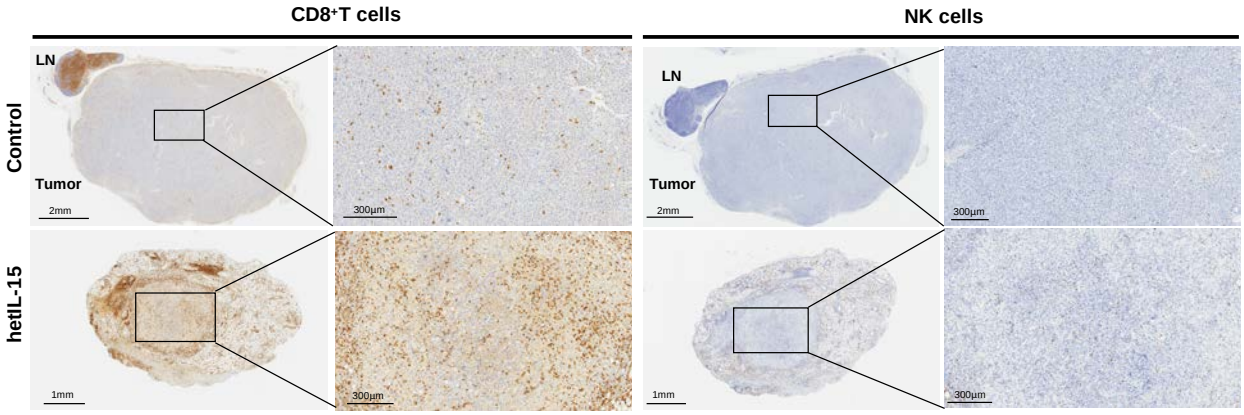


Figure 42: Representative IHC EO771 tumor sections after staining with antibodies specific for CD8$^+$ (**left panel**) or NK.1.1 cells (**right panel**) of control and hetIL-15-treated groups.

1.1.2.3 Tumor control by hetIL-15 monotherapy requires the presence of T lymphocytes

To better understand the contribution of the innate and adaptive immunity in hetIL-15-anti-tumor effect, the treatment was evaluated in RAG-1 knock out (ko) (**Fig. 43**) and NK cell-depleted C57BL/6 mice (**Fig. 44**). 6 hetIL-15 injections resulted in significant tumor growth delay compared to the control group in RAG-1 ko mice, but none of them achieved complete tumor regression (**Fig. 43**). In contrast, treatment with the same number of hetIL-15 injections in NK cell-depleted mice resulted in 20% complete tumor regression and significant tumor growth control in the rest of the animals (**Fig. 44**).

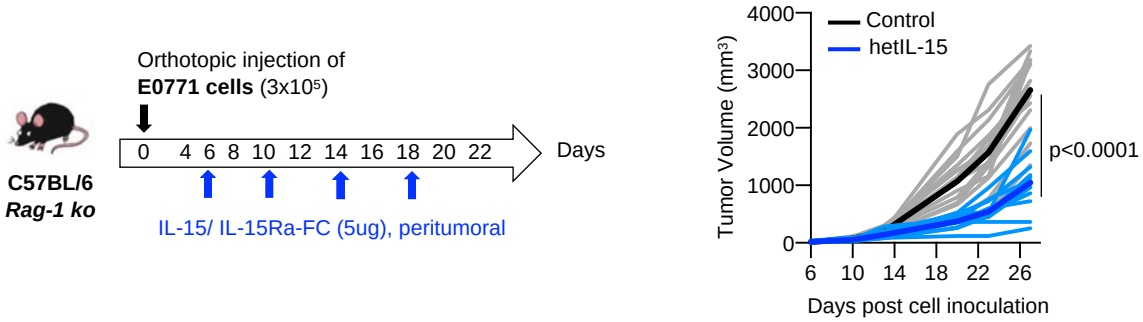


Figure 43: Therapeutic scheme of C57BL/6 RAG-1ko mice that were orthotopically injected with EO771 cells and treated with peritumoral administration of hetIL-15 (**left panel**) and tumor growth curve (bold lines represent average values) overtime up to day 27 (**right panel**) of the mice in control and hetIL-15-treated groups.

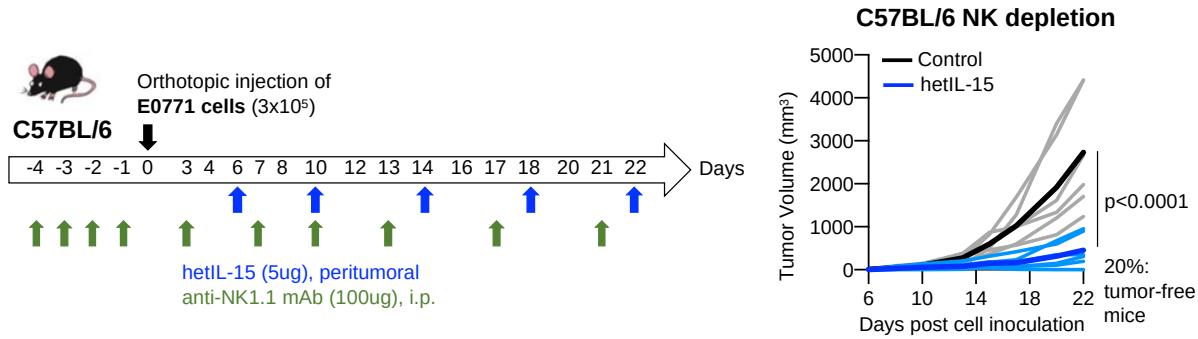


Figure 44: Therapeutic scheme of NK-depleted C57BL/6 mice that were orthotopically injected with EO771 cells and treated with peritumoral administration of hetIL-15FC (**left panel**) and tumor growth curve (bold lines represent average values) overtime up to day 27 (**right panel**) of mice in control and hetIL-15-treated groups.

1.1.2.4 hetIL-15 monotherapy decreases metastasis through both T and NK cells

hetIL-15 therapeutic effects were also evaluated regarding metastatic disease. The examination of the lungs of hetIL-15 treated mice revealed significant reduction in the number of metastatic foci, as was shown by H&E histological analysis (**Fig. 45A**), supporting a beneficial

role of hetIL-15 also in the control of metastatic burden. These anti-metastatic effects of hetIL-15 treatment were also observed in both *Rag-1 ko* (**Fig. 45B**) and NK cell-depleted mice (**Fig. 45C**).

Thus, both T and NK cells contributed to the anti-tumor effect of hetIL-15 on tumor growth delay and metastatic disease in the EO771 model, however tumor eradication required the presence of T cells.

1.1.2.5 hetIL-15 treatment enhanced the expression of genes associated with lymphocyte migration, activation, and cytotoxicity in tumors and dLNs

To gain more detailed understanding on the function of tumor-infiltrating lymphocytes (TILs), we performed gene expression analysis of EO771 tumors excised 48hrs after either the 1st, 2nd, and 3rd hetIL-15 administration (**Fig. 46**), using a panel of 780 immune-oncology related gene probes (Nanostring Technology). We identified ~300 differentially expressed genes (log2 fold-change > 1, adjusted $p<0.05$) in hetIL-15-treated tumors in comparison to control, at all three analyzed time points (**Fig. 46**). Genes associated with a cytotoxic phenotype, such as *Gzmb*, *Gzma*, *Prf1*, *Ctsw* and *Klrg1* (red dots), were among the most significantly overexpressed genes in hetIL-15-treated mice. In addition, expression of *Zap70*, *Cd247* (CD3ζ), *Cd3d* and *Ifng* (green dots), as well as *Cxcr3*, *Ccl9*, *Ccl19* (blue dots) was also increased, highlighting the stimulation of pathways related to T cell activation/TCR signaling and leukocyte migration. GO (gene ontology) pathway enrichment analysis of the Nanostring data showed that the T cell co-stimulation (GO:0031295), the antigen receptor mediated signaling (GO:0050851) and the positive regulation of T cell activation (GO:0050870) pathways ranked in the top 10 canonical pathways upregulated upon hetIL-15 treatment. The upregulated genes that are associated with these pathways are depicted in **Fig. 47A-C.**

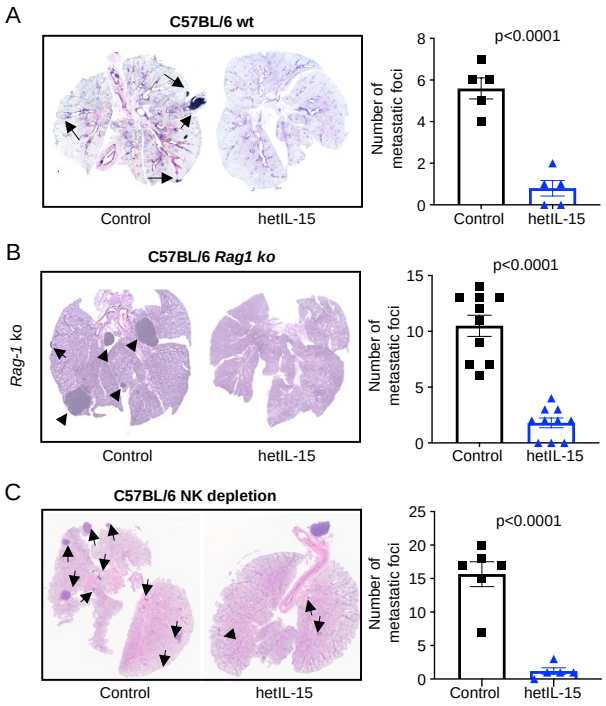


Figure 45: H&E representative staining images of EO771 lung metastases (**left panel**) and the number of metastatic foci found in whole lung section area (**right panel**) in control and hetIL-15-treated C57BL/6 (**A**) C57BL/6 Rag-1 ko (**B**) or C57BL/6 NK-depleted (**C**) mice.

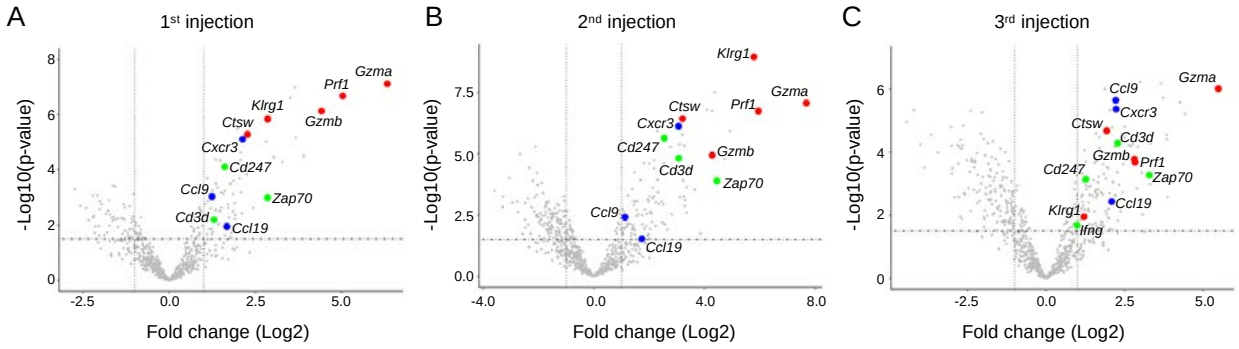


Figure 46: Volcano plots of differentially expressed genes between control and hetIL-15-treated groups in tumors, after the 1st (**A**), 2nd (**B**) and 3rd (**C**) hetIL-15 injection. The genes marked in red, green and blue are associated with T and NK cell cytotoxicity, enhanced T cell activation/TCR signaling and lymphocyte migration, respectively. Dashed line represents adjusted p-value=0.05 and dotted lines represent log2(FC)=1 and log2(FC)=-1.

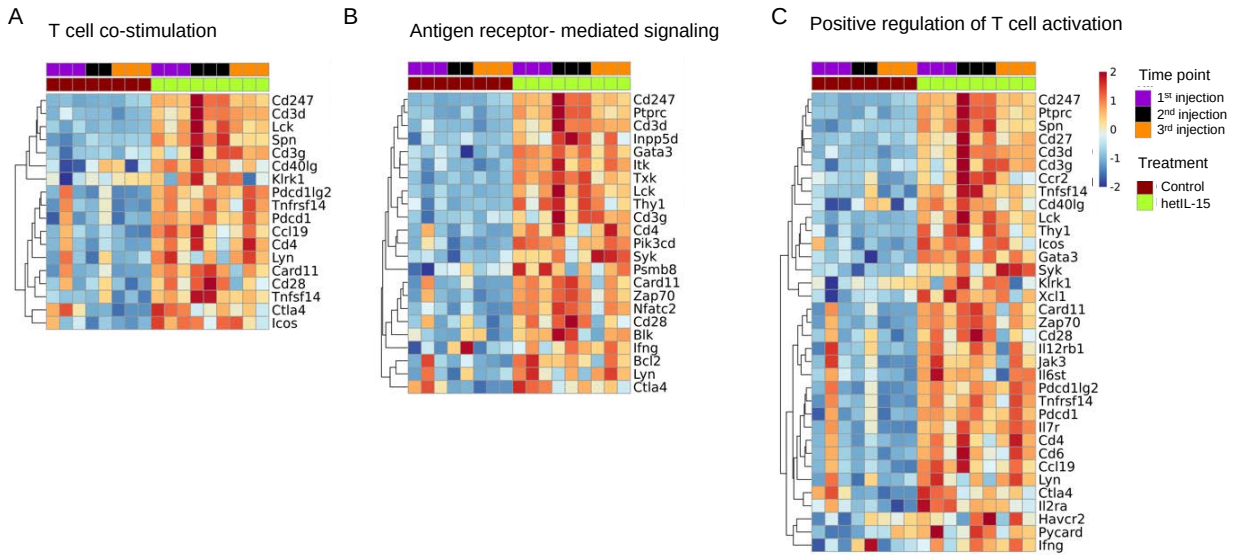


Figure 47: Heatmaps of differentially expressed genes of T cell co-stimulation (**A**), antigen receptor-mediated signaling (**B**) and positive regulation of T cell activation (**C**) pathways in control and hetIL-15-treated tumors after the 1st, 2nd and 3rd hetIL-15 injection.

To analyze the systemic effects of locoregional hetIL-15 treatment, we also evaluated the gene expression pattern in draining lymph nodes (dLN) 48hrs after the 1st, 2nd, and 3rd hetIL-15 injection (**Fig. 48A-C**). Transcriptomic analysis from the dLN further supported the findings that hetIL-15 enhanced T cell cytotoxicity (Gzmb, Gzma, Prf1, Ctsw and Klrg1), TCR activation (Zap70, Ifng) and immune cells chemotaxis (Cxcr3, Ccr5, Cxcl9, Ccl9). GO pathway enrichment analysis revealed that leukocyte migration (GO:0050900) and T cell activation (GO:0002286) ranked among the top upregulated canonical pathways (**Fig. 49A-B**). Flow cytometric analysis of dLNs also showed increased frequency of $CD8^+$ T and NK cells (**Fig. 49C-D**).

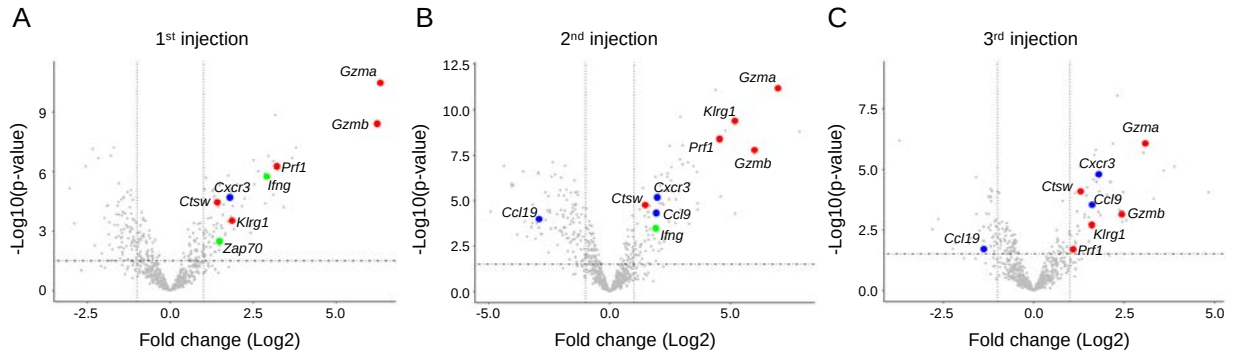


Figure 48: Volcano plots of differentially expressed genes between control and hetIL-15-treated groups in dLNs, after the 1st (**A**), 2nd (**B**) and 3rd (**C**) hetIL-15 injection. The genes marked in red, green and blue are associated with T and NK cell cytotoxicity, enhanced T cell activation/TCR signaling and lymphocyte migration, respectively. Dashed line represents adjusted p-value=0.05 and dotted lines represent log2(FC)=1 and log2(FC)=-1.

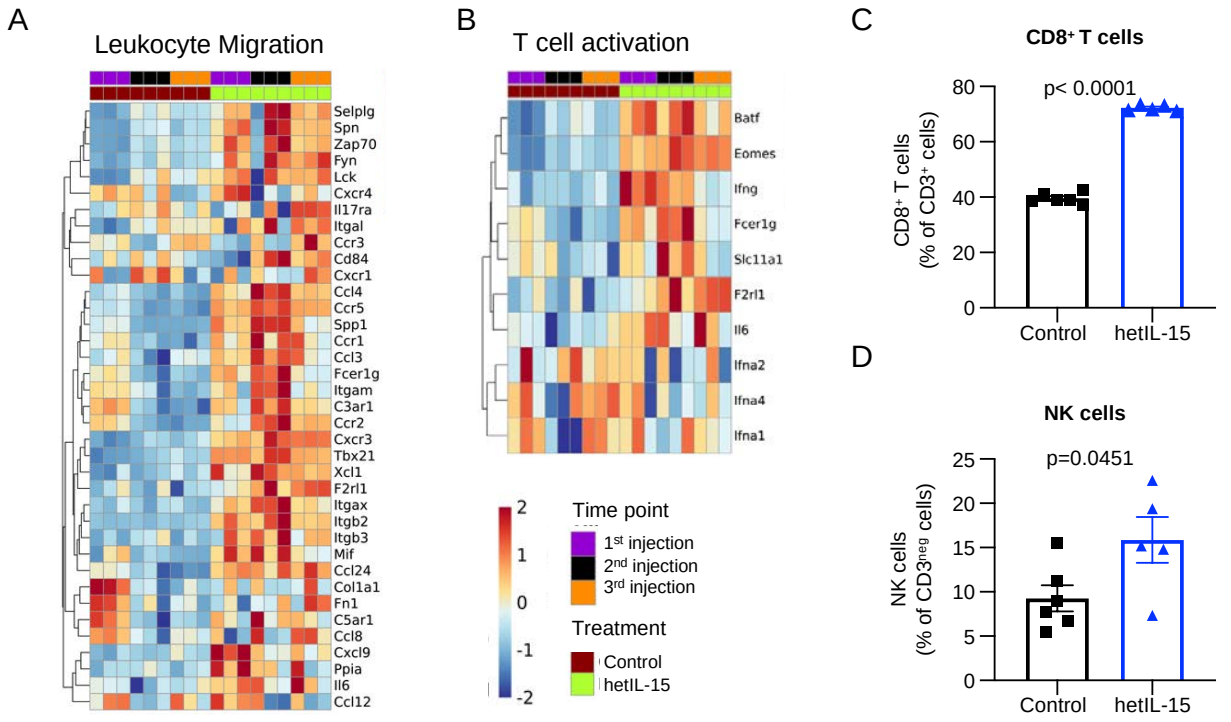


Figure 49: Heatmaps of differentially expressed genes of lymphocyte migration (**A**) and T cell activation (**B**) pathways after the 1st, 2nd and 3rd injection and frequencies of $CD8^+$ T (**C**) and NK (**D**) cells after the 3rd injection, in control and hetIL-15 treated dLNs.

Overall, these data demonstrate that hetIL-15 induced a cascade of transcriptional events triggering the cytotoxic capacity, activation and migration of T and NK cells, as well as their accumulation within the tumors and dLNs.

1.1.2.6 hetIL-15 peritumoral administration induced the accumulation of a novel $CD103^{int}CD11b^+$ population of dendritic cells in tumors

Next, we decided to explore the effect of hetIL-15 on the myeloid cell composition of the tumors. For this purpose, we established a flow cytometric staining protocol (**Fig. 50**) that allowed distinction of the different myeloid cell populations based on published markers (Guilliams et al., 2016, Bottcher et al., 2018). $CD103^+$cDC1s were defined as Lineage(NK1.1, CD19, B220, $CD3)^{neg}CD64^-MHCII^+CD11c^+CD11b^-CD103^+$; $CD11b^+$cDC2s were defined as Lineage(NK1.1, CD19, B220, $CD3)^{neg}CD64^-MHCII^+CD11c^+CD11b^+CD103^-$ and macrophages were defined as Lineage(NK1.1, CD19, B220, $CD3)^{neg}CD64^+F4/80^+$. Indeed, locoregional hetIL-15 treatment resulted in increased tumor infiltration of $CD103^+$cDC1s (**Fig. 51**), whereas no significant difference was found in the number of $CD11b^+$cDC2s (**Fig. 51**). Surprisingly, flow cytometry

analysis revealed a novel DC population that was prominent only upon hetIL-15 treatment (**Fig. 51**) and appeared to be distinct from the DC subsets previously reported in tumor mouse models. This new DC population showed a unique phenotypic expression of the CD103 and CD11b markers that used to name the population as CD103^{int}CD11b^{+}DCs. Importantly, tumor infiltration by both CD103^{+}cDC1s and CD103^{int}CD11b^{+}DCs inversely correlated with the EO771 tumor size in hetIL-15-treated animals 48hrs after the 3rd hetIL-15 injection (**Fig. 52**). In contrast, no correlation between intratumoral CD11b^{+}cDC2s and tumor size was observed (**Fig. 52**).

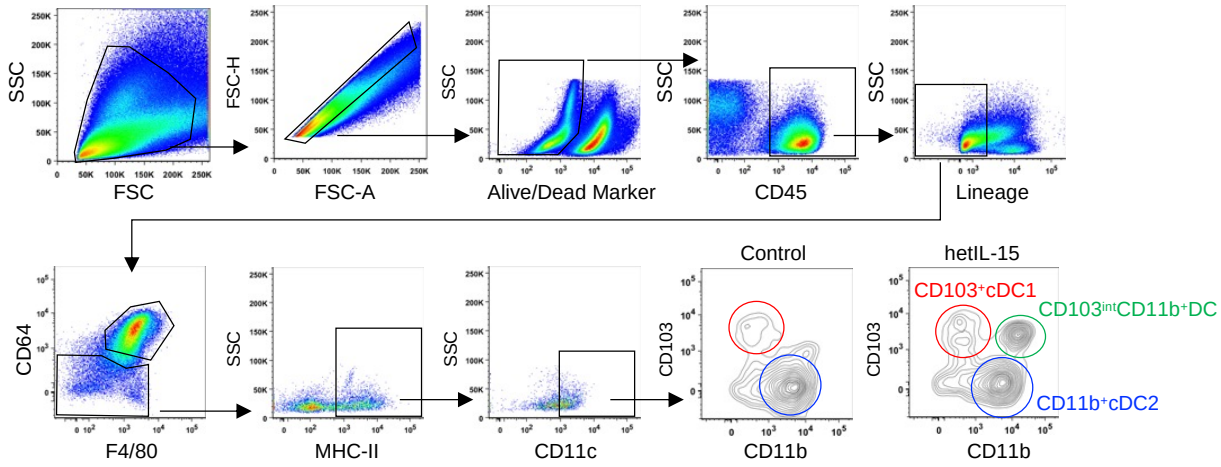


Figure 50: Gating strategy performed to identify the distinct DC population in the EO771 tumors. The CD103^{+} cDC1 (red), CD11b^{+} cDC2 (blue) and the novel population CD103^{int}CD11b^{+} DC (green) are indicated in the contour plots.

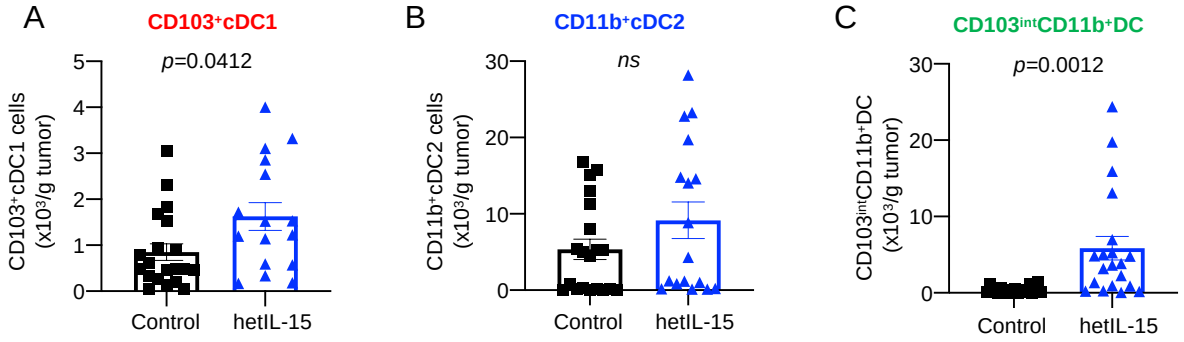


Figure 51: Frequencies of intratumoral CD103^{+} cDC1 (**A**), CD11b^{+} cDC2 (**B**) and CD103^{int}CD11b^{+} DC (**C**) populations in control and hetIL-15-treated mice, expressed as absolute numbers of cells per gram of tumor and represented as mean ±SEM.

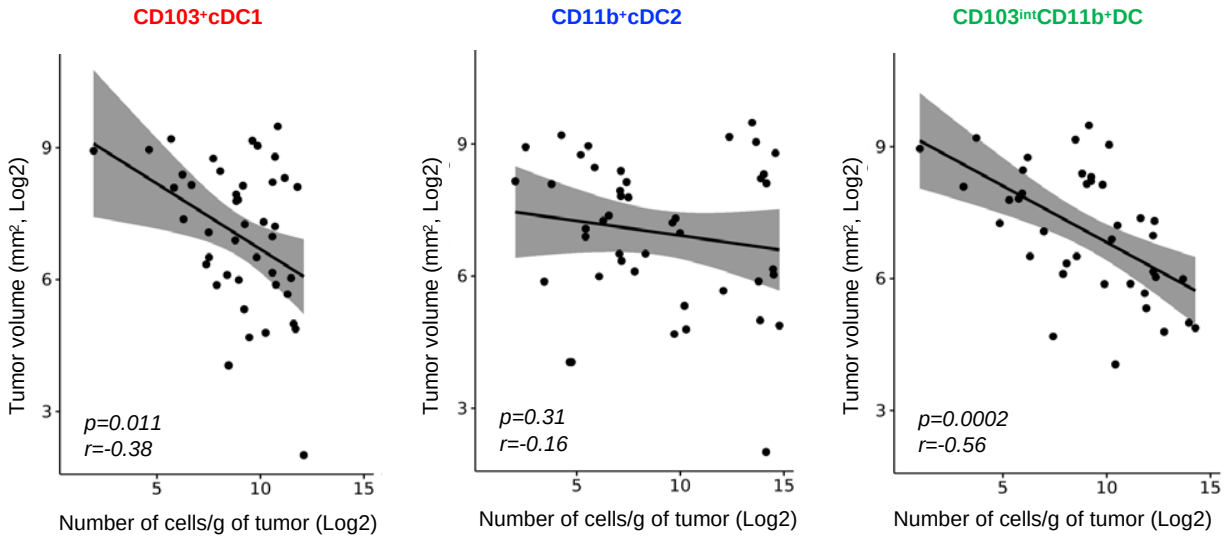


Figure 52: Pearson correlation analysis between tumor volume (mm^3) and number of tumor-infiltrating DCs per gram of tissue.

1.1.2.7 CD103^{int}CD11b^{+} DCs displayed a gene transcriptional signature similar to monocyte-derived DCs (moDCs)

To better characterize the properties of the different DC subsets localized in tumors, we performed RNA-sequencing (RNA-seq) analysis on sorted tumor-infiltrating myeloid cell subsets. Principal component analysis (PCA) of the different sorted populations (CD103^{+} cDC1, CD11b^{+} cDC2, CD103^{int}CD11b^{+} DC and macrophages) based on their transcriptome, revealed segregation of CD103^{int}CD11b^{+} DCs; they showed a transcriptomic profile close to CD11b^{+}cDC2s and mapped away from the macrophages in PCA space (**Fig. 53**). Comparison with immune cell transcriptome profiles reported by Brown et al. (Brown et al., 2019) confirmed that infiltrating CD103^{int}CD11b^{+}DCs showed low expression of the key macrophage genes Fcgr1, Cx3cr1, Siglec1, Ly6c1, Ly6c2, whereas DC-expressed markers *CD24a, Xcr1*, *Itgae*, *Itgam*, *Itgax, Sirpa*, *Irf4, Cd207* and *CD209a* (**Fig. 54**) were highly or intermediately expressed in tumor-infiltrating CD103^{int}CD11b^{+}DCs. This cell population also has increased *Rbpj and Batf3* gene expression but low expression of *Flt3* and *CD8a*. Furthermore, a heatmap of the antigen presentation pathway, using reference genes from Kaczanowska et al. (Kaczanowska et al., 2021), revealed that many genes implicated in antigen processing and presentation (e.g., *Naaa, Ciita*, *Batf3, H2-DMa, H2-Aa*, *H2-Aa*, *CD74*) (Theisen et al., 2018, Santambrogio et al., 2019, Kaczanowska et al., 2021) were upregulated in CD103^{int}CD11b^{+}DCs compared to macrophages (**Fig. 55**). Overall, these results showed that tumor-infiltrating CD103^{int}CD11b^{+}DCs have a unique transcriptome profile that differs from macrophages and shows similarities with DCs including genes that are involved in antigen presentation.

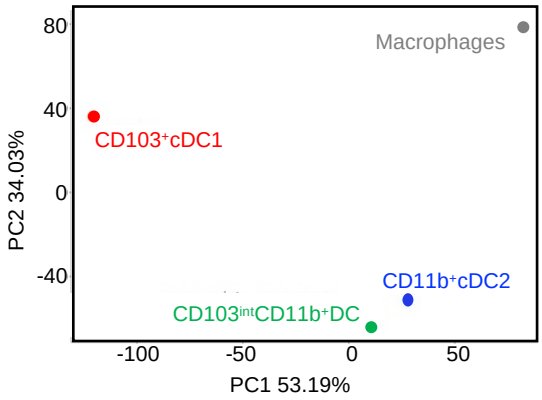


Figure 53: PCA of CD103^{+} cDC1s, CD11b^{+} cDC2s, CD103^{int}CD11b^{+} DCs and macrophages based on RNA-seq global transcriptional profiles.

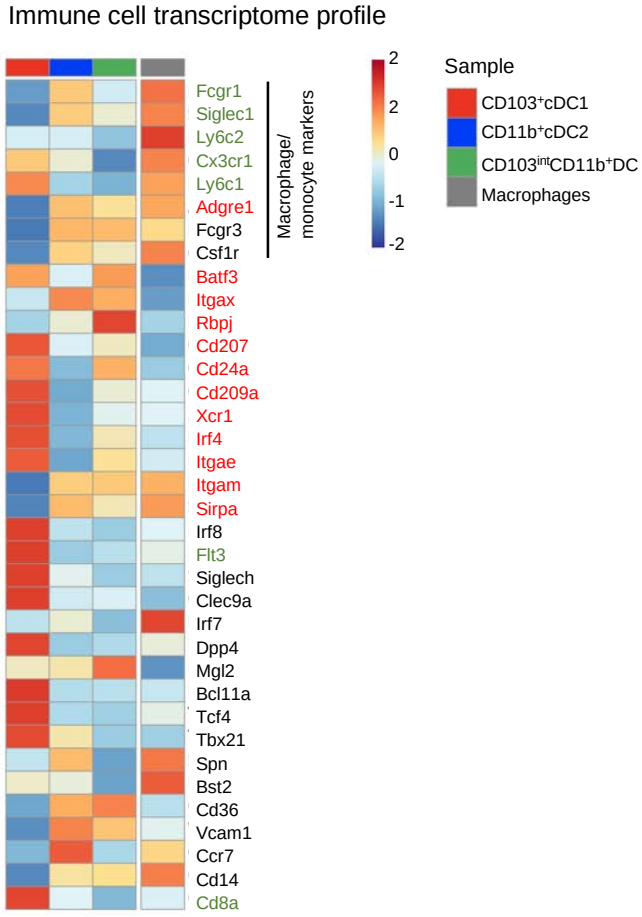


Figure 54: Heatmap of differential expression of DC and macrophage/monocyte canonical markers across the DC populations and macrophages by RNA-sequencing analysis. Red and green gene names indicate genes that are upregulated and downregulated, respectively.

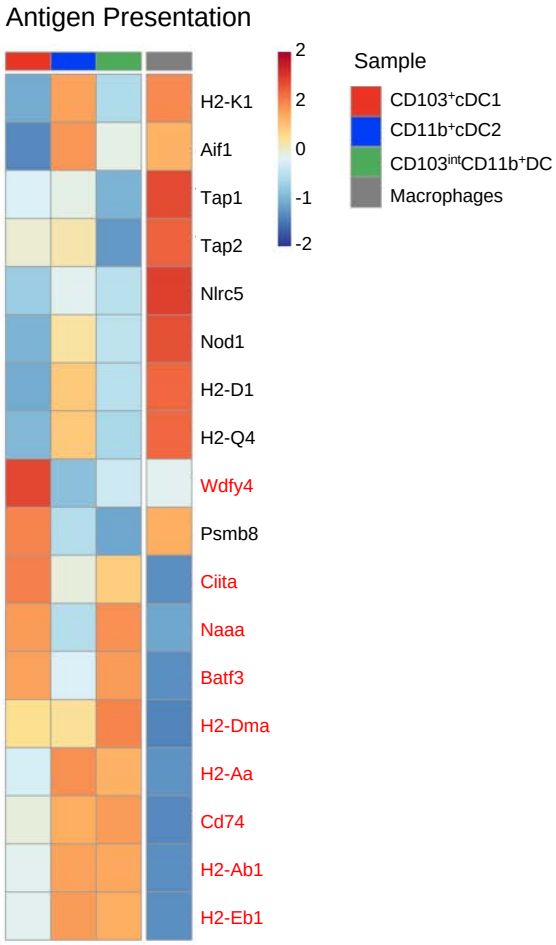


Figure 55: Heatmap of differential expression of genes in the antigen presentation pathway across the DC populations and macrophages by RNA-sequencing analysis.

To further characterize the tumor infiltrating CD103^{int}CD11b^{+}DCs, we performed single-cell RNA sequencing (scRNA-seq) on sorted CD11c^{+} cells obtained from EO771 tumors of control or hetIL-15-treated animals. After removal of cell-cycle signals, scRNA-seq of the CD11c^{+}CD64^{neg} cells identified 7 distinct clusters visualized using UMAP (**Fig. 56**). The CD103^{int}CD11b^{+}DC population in hetIL-15 treated tumors was enriched in the sample density UMAP plot (**Fig. 56, right panel**). In addition, the CD103^{int}CD11b^{+}DC population expressed a unique gene signature. Shared gene expression among individual clusters (**Fig. 57**) revealed that CD103^{int}CD11b^{+}DCs possess a gene profile with similarities to monocytes (monocyte 1 and monocyte 2), with several highly expressed (mo)DC/DC markers (e.g., *Mgl2*, *Ccl17, Plet1, Clec4n* (Dectin2), *CD24a*, *mmp12, clec4b1* (DCAR), and *Anxa1* (Annexin1)) (Tzelepis et al., 2015, Toyonaga et al., 2016, Qu et al., 2014, Napoletano et al., 2007, Kis-Toth et al., 2013, Bonnardel

et al., 2015), suggesting a possible monocytic origin for this DC subset. CD103^{int}CD11b^{+}DCs expressed the highest levels of *Mgl2* and *Ccl17* among the different DC subtypes.

Based on our scRNAseq results demonstrating that CD103^{int}CD11b^{+}DCs have the highest expression levels of *Mgl2* and *Ccl17* among the different DC subtypes and monocytes and also express CD24a, we performed in situ RNA hybridization (RNAscope) using probes that target these markers. RNAscope analysis confirmed the presence of CD103^{int}CD11b^{+}DCs in the tumors of the hetIL-15-treated mice (**Fig. 58**) whereas in control tumors was very limited (data not shown).

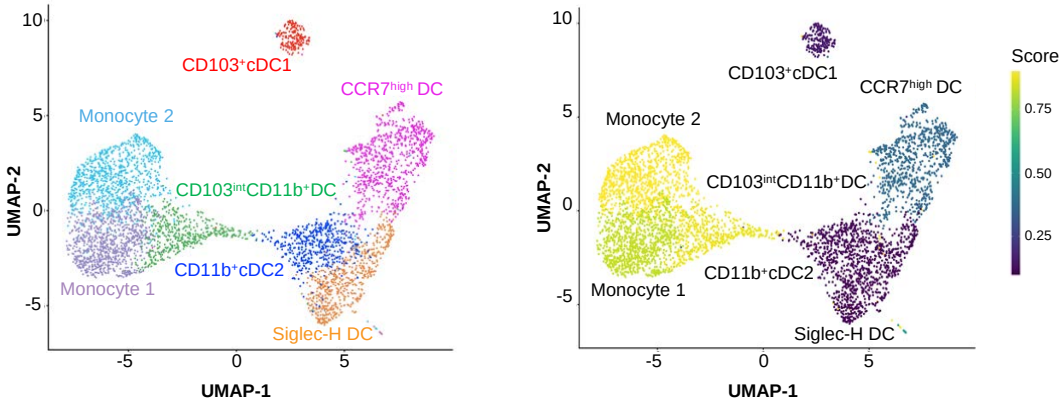


Figure 56: UMAP plot of scRNA-seq analysis of CD11c^{+} tumor-infiltrating cells (**left panel**) and scaled density UMAP plot (**right panel**) (0 to 1 indicates 100% of cells originating from control or hetIL15-treated sample, respectively) shows sample origin of cells in each cluster.

Overall, our RNA-seq data demonstrated that CD103^{int}CD11b^{+}DCs form a distinct cluster with a transcriptional profile characteristic of moDCs that express genes associated with antigen presentation.

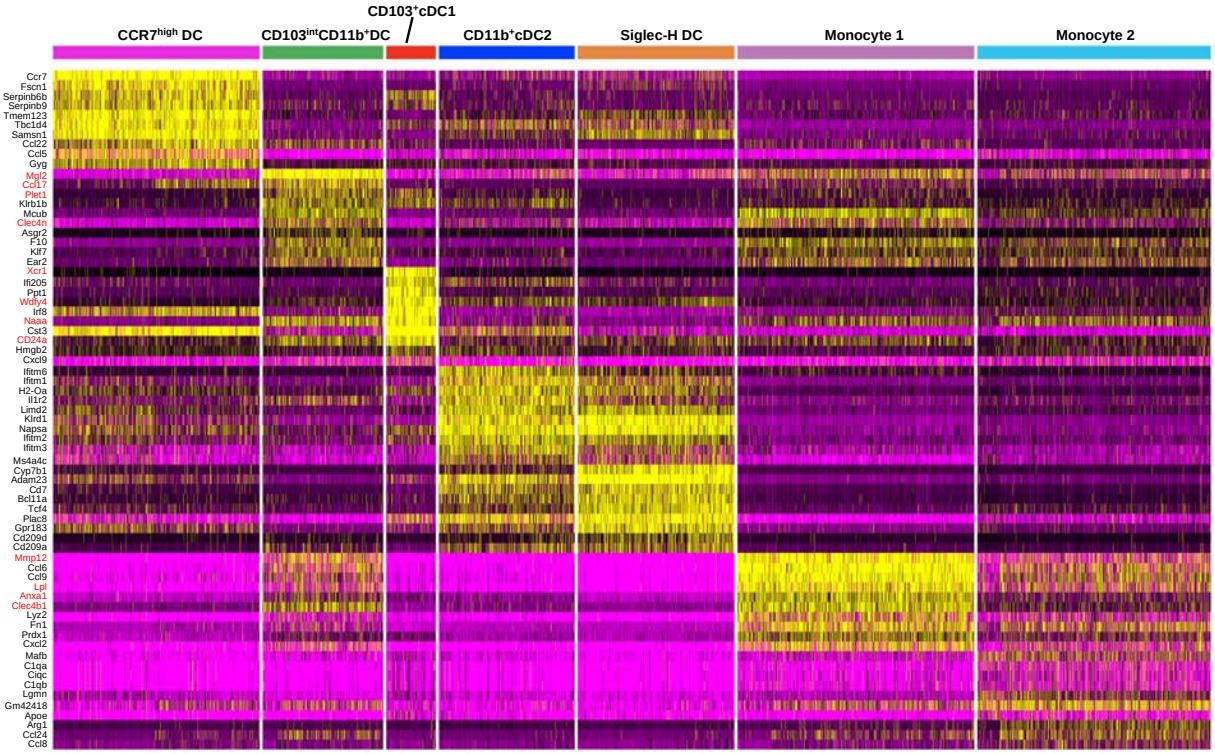


Figure 57: Heatmap reporting scaled expression of the top differentially expressed genes for each cluster across all cells, identified in Fig. 42. Genes of interest are shown in red.

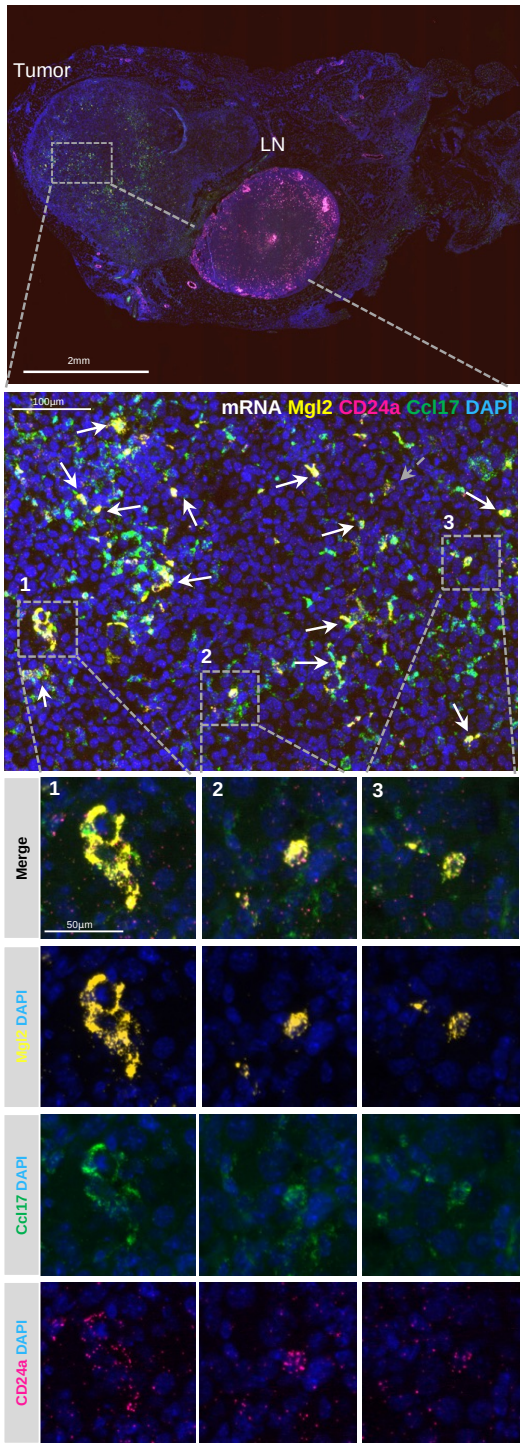


Figure 58: RNAscope analysis from a representative hetIL-15-treated tumor showing the colocalization of CD24a, Ccl17 and Mgl2 markers which are highly expressed in intratumoral CD103^{int}CD11b^{+}DCs.

1.1.2.8 hetIL-15 locoregional administration resulted in a long-lasting specific anti-tumor immunity

We also examined the development of anti-tumor memory T cells upon locoregional hetIL-15 administration. Mice that had previously eradicated EO771 tumors were re-challenged with the same tumor cell line 68 and 158 days after the last hetIL-15 dose (**Fig. 59**). Age-matched control mice developed EO771 tumors as expected, whereas tumors failed to be established in mice with a previous history of tumor eradication after hetIL-15 therapy, suggesting development of protective anti-tumor immunity (**Fig. 60A**). To verify the specificity of the anti-tumor immune response, mice were also challenged using the syngeneic pancreatic KPC tumor cells (challenge #2, **Fig. 59**). KPC tumors developed at the same rate in both groups (**Fig. 60B**), supporting the conclusion that hetIL-15 treated mice were able to develop and maintain specific immunity against EO771 tumor.

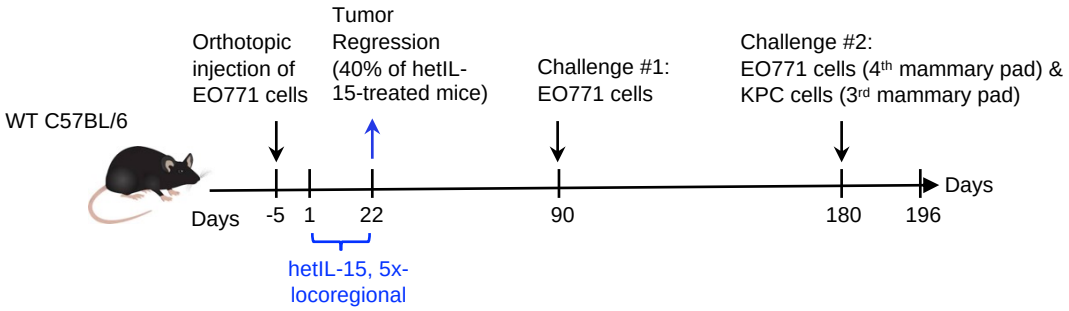


Figure 59: Scheme with the timeline of the tumor rechallenge experiments. C57BL/6 mice were inoculated with EO771 cells and subsequently treated with peritumoral hetIL-15 injections. hetIL-15-treated mice that completely eliminated EO771 tumors were rechallenged on day 90 (challenge #1) with the EO771 cancer cell line, and on day 180 (challenge #2) with both the EO771 and KPC cells lines without any treatment.

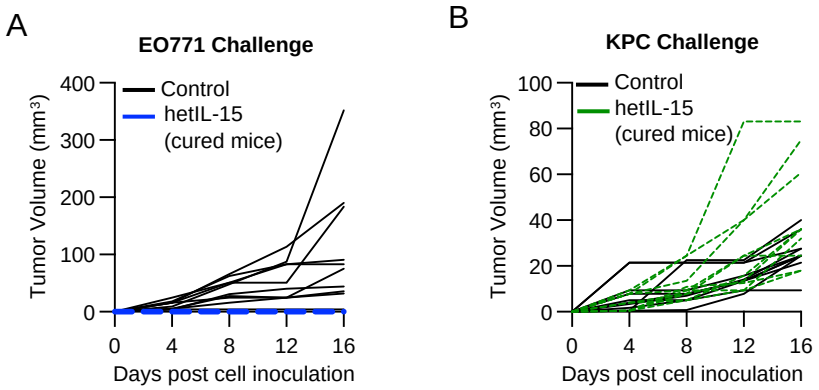


Figure 60: Growth curves of EO771 (**A**) and KPC (**B**) tumors of individual mouse were monitored from day 180 (challenge #2) until the endpoint.

Next, we performed adoptive cell transfer of purified $CD8^+$T cells from hetIL-15-treated mice that had previously eradicated EO771 tumors and successfully rejected EO771 tumors upon subsequent re-challenge of **Fig. 60A** (**Fig. 61**). Recipients were treated with hetIL-15 every 2 days to support the adoptively transferred $CD8^+$T cells. Transfer of the $CD8^+$T cells into lymphodepleted EO771 tumor-bearing mice reduced tumor growth (**Fig. 62, left panel**) and increased the survival of the recipient mice (**Fig. 62, right panel**) compared to mice receiving $CD8^+$T cells from mice never exposed to EO771 tumor cells. We also monitored the development of lung metastasis in mice that underwent adoptive cell transfer of $CD8^+$T cells. The number of lung tumor foci in mice that received $CD8^+$T cells from donors previously cured from EO771 was significantly reduced (**Fig. 63**), suggesting that hetIL-15-induced memory $CD8^+$T cells could reduce or control metastatic disease in the lungs. Overall, these findings indicated that monotherapy using locoregional hetIL-15 administration induced the development of specific long-lasting anti-tumor immunity, which resulted in complete tumor eradication and protection from subsequent exposure.

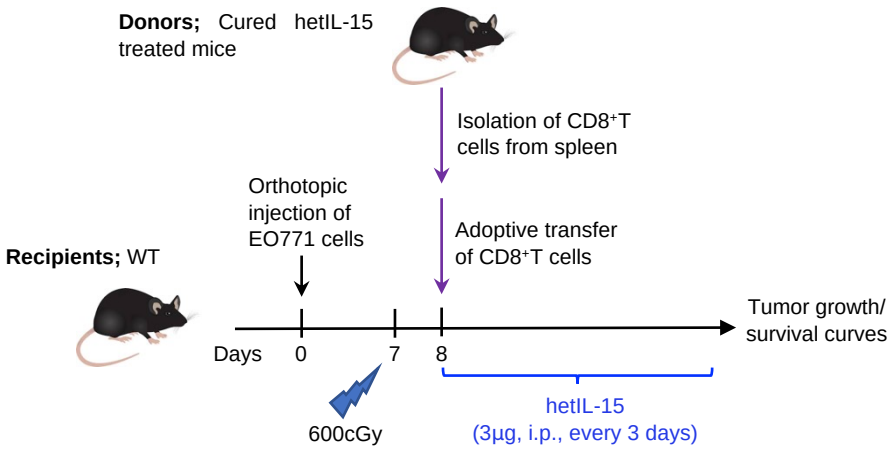


Figure 61: Schematic representation of adoptive cell transfer. Recipient mice were inoculated with EO771 cells and 6 days later were irradiated with 600cGy. The next day, $CD8^+$T cells from the spleens of naïve or previously hetIL-15-treated and rechallenged mice from Fig. 47A were isolated and injected into the EO771 tumor-bearing mice. Recipient mice were then boosted with IP hetIL-15 injections every 2 days until the end point.

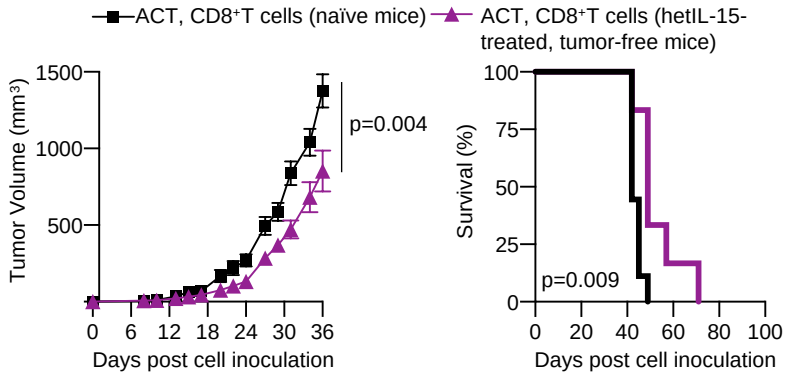


Figure 62: EO771 tumor growth (**left panel**) and survival curve (**right panel**) following ACT of either CD8^{+}T cells from naïve or rechallenged mice. Both groups were administered with hetIL-15 treatment during the experiment.

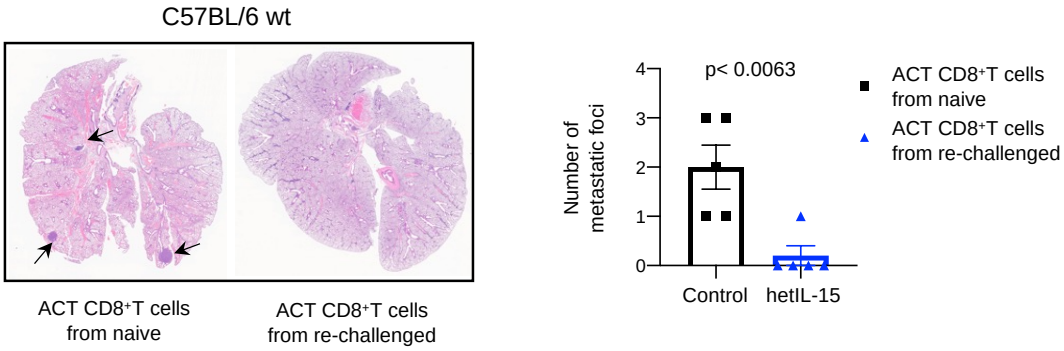


Figure 63: Representative section of H&E-stained lungs of the ACT experiment (**left panel**) and the corresponding numbers of metastatic foci found in the whole lung area of each individual mouse (**right panel**) from the two groups.

SECTION 2

1. Pancreatic cancer immunotherapy

1.1 hetIL-15 monotherapy does not show delay of the pancreatic tumors and does not synergize with gemcitabine or MSLN-targeted agents

hetIL-15 anti-tumor activity was also tested in different models of pancreatic cancer. We used both the orthotopic model and the KPC (Pdx1-Cre; KrasG12D; Trp53R172H) transgenic model to evaluate hetIL-15 as single agent and upon combination with targeted therapy and chemotherapy.

The pancreatic cell line that was used for the orthotopic model was a KPC pancreatic cell line (KPC223481+hMSLN-H06) that has been genetically modified to express the human version of the tumor associated antigen mesothelin (MSLN). The mouse model that was used for this study was the TPO which has intrinsic tolerance for human mesothelin to avoid the cell line rejection. TPO C57BL/6 mice were orthotopically injected with 10^4 KPC cells with the human MSLN. We used two different agents that target mesothelin, the immunotoxin LMB100 and the anti-mesothelin antibody MB (more information provided in the materials and methods section). The treatment schedules are shown in **Figs 64** and **66**. Tumor growth was monitored via ultrasound imaging and when the tumor reached a size of 20-80 mm^3 the mice were randomized in four groups: (i) control (PBS), (ii) LMB100 or MB, (i) hetIL-15 and (iv) LMB100 or MB plus hetIL-15. Tumor growth was measured overtime until day 19. hetIL-15 and LMB100 monotherapies showed delay of the KPC primary tumor, but the combination did not exhibit any benefit (**Fig. 65**). However, repeat of hetIL-15 monotherapy treatment in a second experiment did not reproduce the results (**Fig. 67**). In this second experiment, it was also tested the MN antibody as single agent, that also did not show any tumor delay. Similar results were obtained with the combination treatment of hetIL-15 and MN antibody suggesting that the two agents do not have additive anti-tumor activity against the primary KPC tumor.

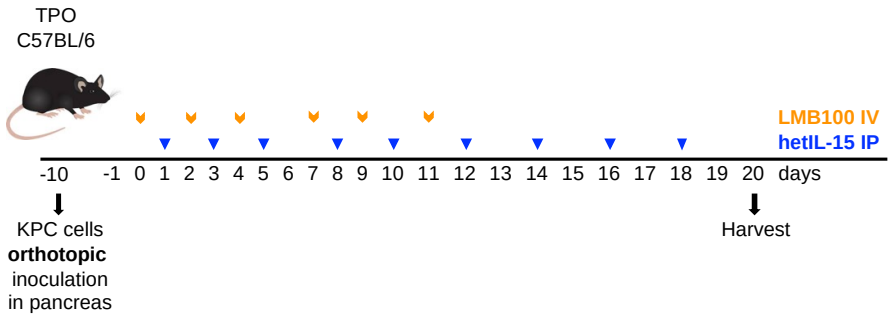


Figure 64: Treatment scheme of TPO C57BL/6 mice that orthotopically injected with KPC cells and treated with LMB100 and hetIL-15.

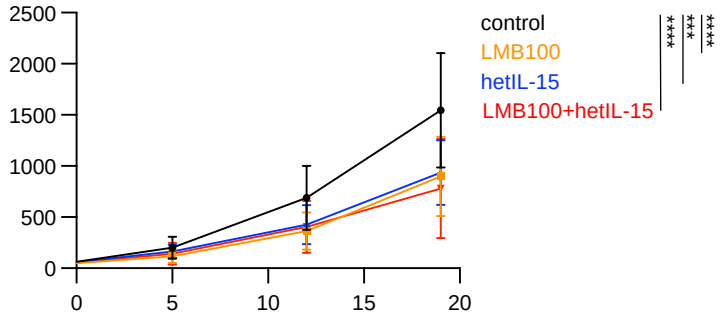


Figure 65: Mean of the pancreatic tumor growth overtime up to day 19 of the KPC tumor-bearing mice in the four groups.

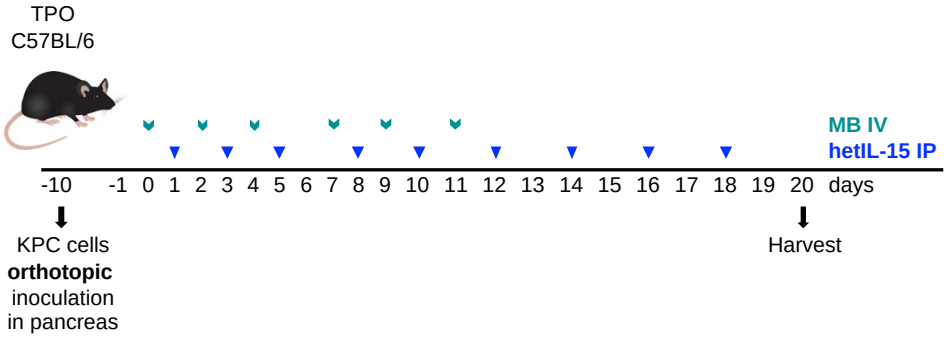


Figure 66: Treatment scheme of TPO C57BL/6 mice that orthotopically injected with KPC cells and treated with MB and hetIL-15.

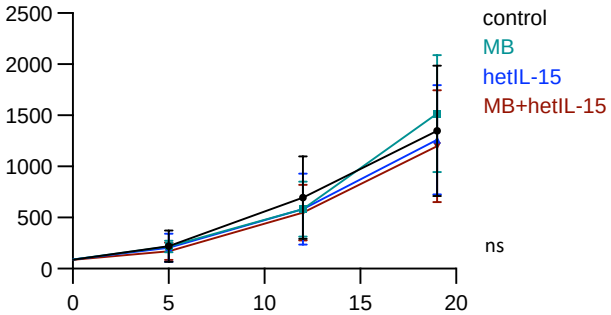


Figure 67: Mean of the pancreatic tumor growth overtime up to day 19 of the KPC tumor-bearing mice in the four groups.

We also used the genetically engineered KPC mouse model of pancreatic cancer to test hetIL-15 anti-tumor activity as single agent and in combination with the chemotherapeutic agent gemcitabine. GEMM (genetically engineered mouse model) KPC mice develop pancreatic tumors at the age of around 15-weeks-old. Tumor growth was measured via ultrasound imaging and when

the tumor reached the size of around 40mm^3, the mice were randomized in four groups: (i) control (PBS), (ii) gemcitabine (100mg/kg), (iii) hetIL-15 (3ug) and (iv) gemcitabine plus hetIL-15. The treatment schedule is shown in **Fig. 68**. Gemcitabine monotherapy was given sequentially as this treatment scheme is followed in the clinic. We monitored both the tumor growth and survival of the mice, which are shown in **Fig. 69**. Only gemcitabine showed delay of the primary pancreatic tumor, but it did not reach significance due to the high standard deviation (SD) of the tumor sizes (**Fig. 69, left panel**). One of the reasons for the high SD is that the KPC model expresses the Pdx1 promoter in all the pancreatic cells (ductal, acinar and cells in islets), which leads to the development of multiple tumors at different times during the tumorigenesis. The different tumors could potentially merge giving divergent tumor measurements. Gemcitabine also reached significance in extending animal survival (**Fig. 69, right panel**). hetIL-15 monotherapy did not show any effect in delaying the primary tumor or in extending animal survival and the combination showed only a modest benefit in survival, which however was significant compared to the control group (**Fig. 69**).

Unfortunately, we did not find any benefit in the chosen combination schemes of hetIL-15 with targeted therapy or chemotherapy regarding tumor delay. However, we decided to evaluate the effects of hetIL-15 monotherapy in tumor necrosis, tumor infiltration of immune cell populations and metastasis.

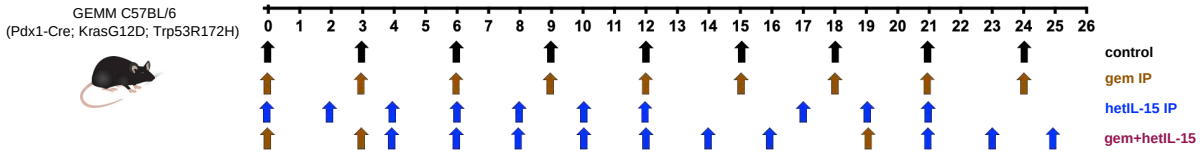


Figure 68: Treatment scheme of the GEMM KPC C57BL/6 mice that treated with gemcitabine and hetIL-15.

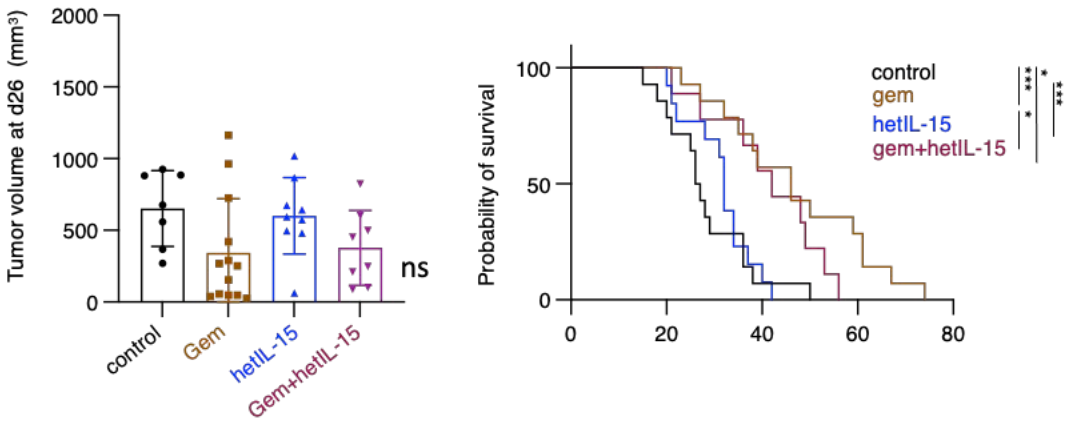


Figure 69: Mean of the pancreatic tumor size on day 26 (**left panel**) and Kaplan-Meier survival curve (**right panel**) of the pancreatic tumor-bearing GEMM KPC mice in the four groups.

1.2 hetIL-15 monotherapy shows extensive primary tumor necrosis in both transgenic and orthotopic models

Although we did not observe any major effect on the primary pancreatic tumor control by hetIL-15 treatment, histology analysis revealed differences in necrosis. Tumors from both the transgenic and orthotopic KPC models were H&E-stained and the areas of necrosis was calculated for each group. The evaluation of necrosis with H&E staining is possible as the necrotic areas are depicted with a paler pink derived from the eosin-stained proteins that are released by the necrotic cells (**Figs 70, 71**). Both the orthotopic (**Fig. 70**) and transgenic (**Fig. 71**) (similar size tumors comparison of mice with endpoint from day 26-50) KPC models showed extensive intratumoral necrosis upon hetIL-15 monotherapy.

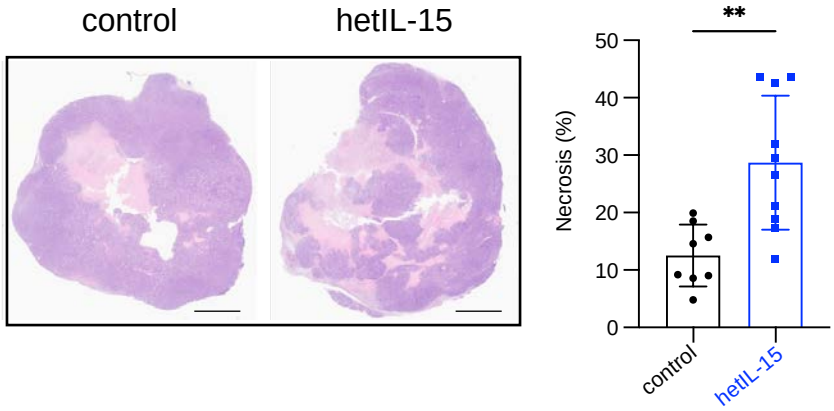


Figure 70: H&E staining of tumors from mice in control and hetIL-15-treated groups at day 19 (**left**) and the percentage of total necrotic area per mouse in each group (**right**) of the orthotopic KPC model. scale bar, 3000μm

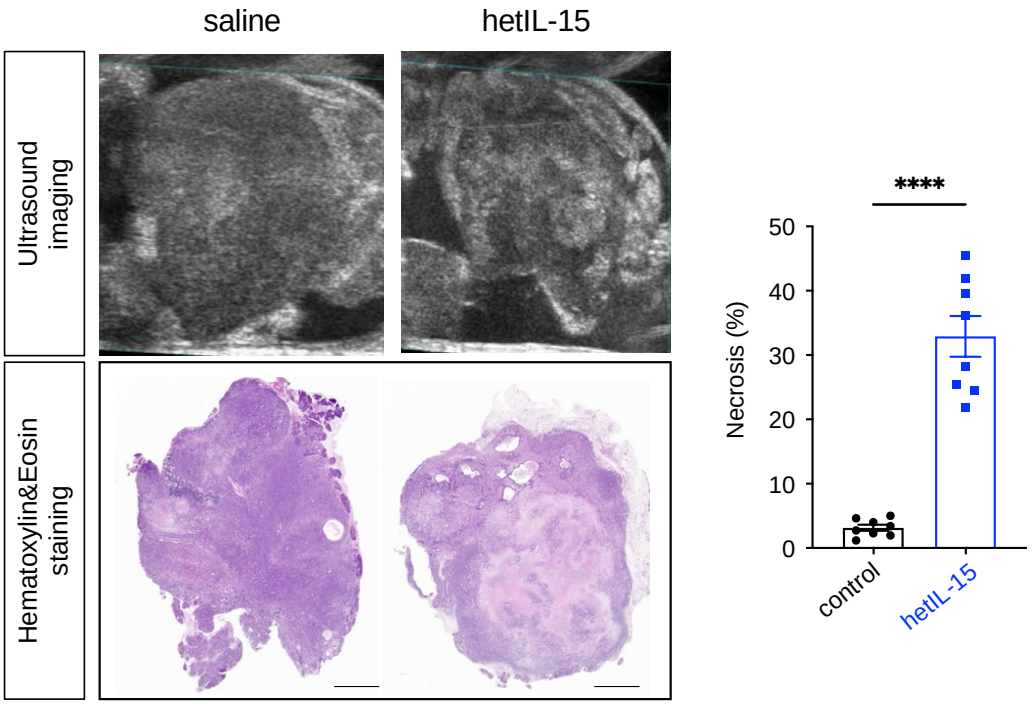


Figure 71: Ultrasound imaging and H&E staining of tumors from mice in control and hetIL-15-treated groups with endpoints from day 26-50 (**left**) and the percentage of total necrotic area per mouse in each group (**right**) of the transgenic KPC model. scale bar, 3000μm

1.3 hetIL-15 monotherapy induces the accumulation of $CD8^+$ T and NK cells intratumorally in both transgenic and orthotopic models

As it was mentioned, hetIL-15 has been found to induce the intratumoral accumulation of the cytotoxic lymphocytes in previous studies (Ng et al., 2017, Bergamaschi et al., 2020) and the TNBC mouse models that were presented in section 1.1.1.3 and 1.1.2.2 (at lesser extent in 4T1 model – **Fig. 24** and mostly in EO771 – **Figs 40 and 41**). For that reason, we also evaluated the pancreatic tumors of both the transgenic and orthotopic models upon hetIL-15 treatment for the presence of increased intratumoral $CD8^+$T and NK frequencies by flow cytometry. Both models were found with increased frequencies of intratumoral $CD8^+$T cells upon hetIL-15 treatment (**Figs 72, 73**), whereas NKs were increased only in orthotopic hetIL-15-treated tumors (**Fig. 73**) (NK cell frequencies in transgenic model are not shown).

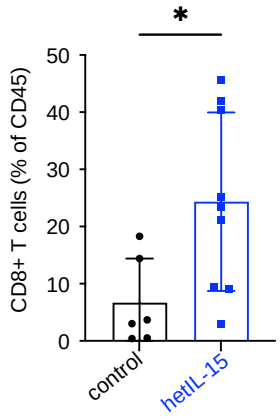


Figure 72: Frequencies of intratumoral CD8$^+$ T cells in control and hetIL-15-treated groups of transgenic KPC-tumor bearing mice.

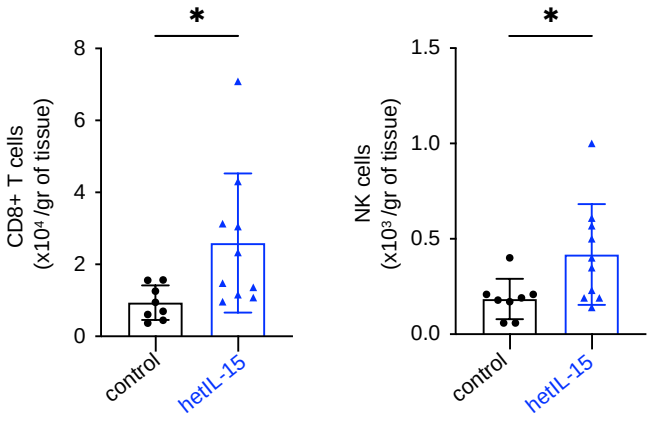


Figure 73: Frequencies of intratumoral CD8$^+$ T (**left**) and NK (**right**) cells in control and hetIL-15-treated groups of orthotopic KPC-tumor bearing mice.

The findings were also verified in the orthotopic model by IHC staining of tumor sections using anti-CD8 and anti-CD161c/NK1.1 antibodies. Both stainings showed increased accumulation of CD8$^+$T and NK cells into the tumor upon hetIL-15 treatment (**Fig. 74**). The most profound increase was of CD8$^+$T cells.

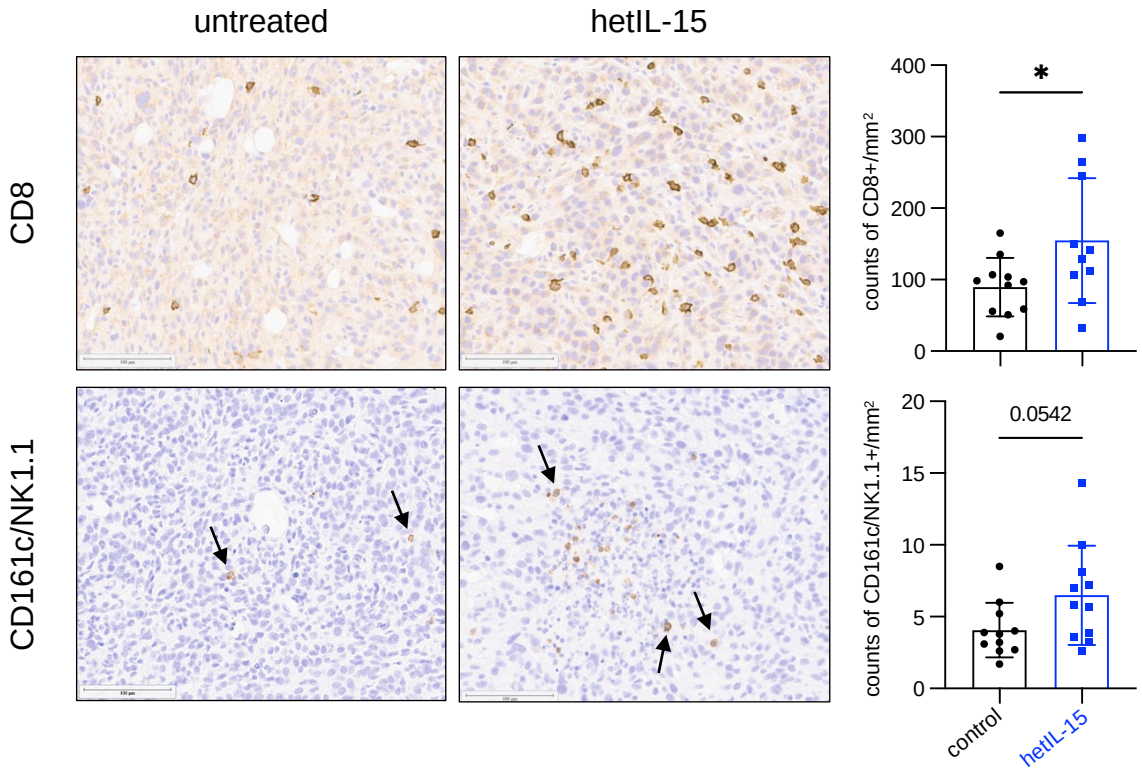


Figure 74: Representative IHC anti-CD8- and anti-CD161c/NK1.1 stainings (**left**) and the counts/mm^2 (**right**) of tumors in control and hetIL-15 group of orthotopic KPC-tumor bearing mice.

1.4 hetIL-15 monotherapy induces the intratumoral accumulation of the novel CD103^{int}CD11b$^+$ DC population in the orthotopic KPC model

We examined the orthotopic KPC pancreatic tumors for the frequencies of cDC1s, cDC2s and the novel CD103^{int}CD11b$^+$ DCs by flow cytometry. cDC2s and CD103^{int}CD11b$^+$ DCs were found to be increased in the tumors upon hetIL-15 treatment, but not cDC1s (**Fig. 75**). However, the difference of cDC2s frequencies between the two groups did not reach significance.

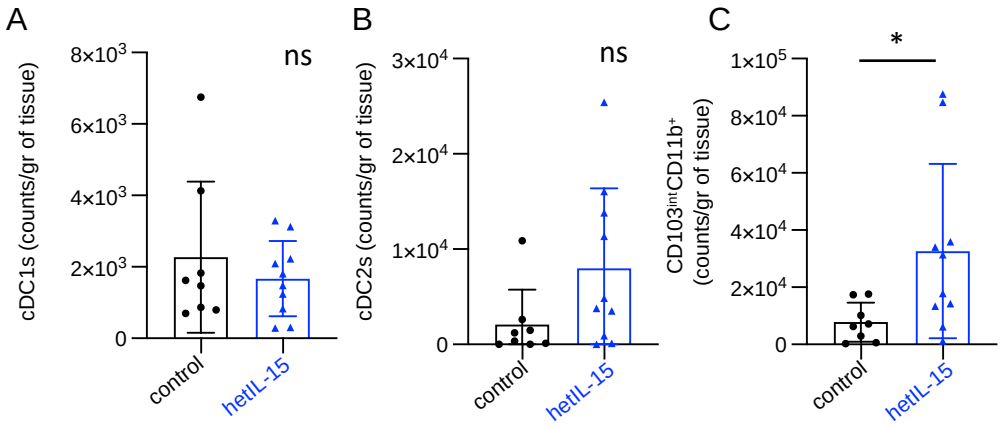


Figure 75: Frequencies of intratumoral cDC1s (**A**), cDC2s (**B**) and CD103intCD11b^{+} DCs (**C**) expressed as absolute numbers of cells per gram of tissue in orthotopic KPC-tumor bearing mice.

Next, we stained the tumors with RNAscope probes of the specific markers *Mgl2, CCL17* and *CD24a* that we found in single-seq RNA sequencing analysis (section 1.1.2.7) to verify the presence of CD103^{int}CD11b^{+} DCs. Indeed, we were able to identify cells expressing all the three markers in the tumors of both control and hetIL-15 groups (**Fig. 76**). The number of the cells was elevated upon hetIL-15 treatment compared to control groups (data not shown).

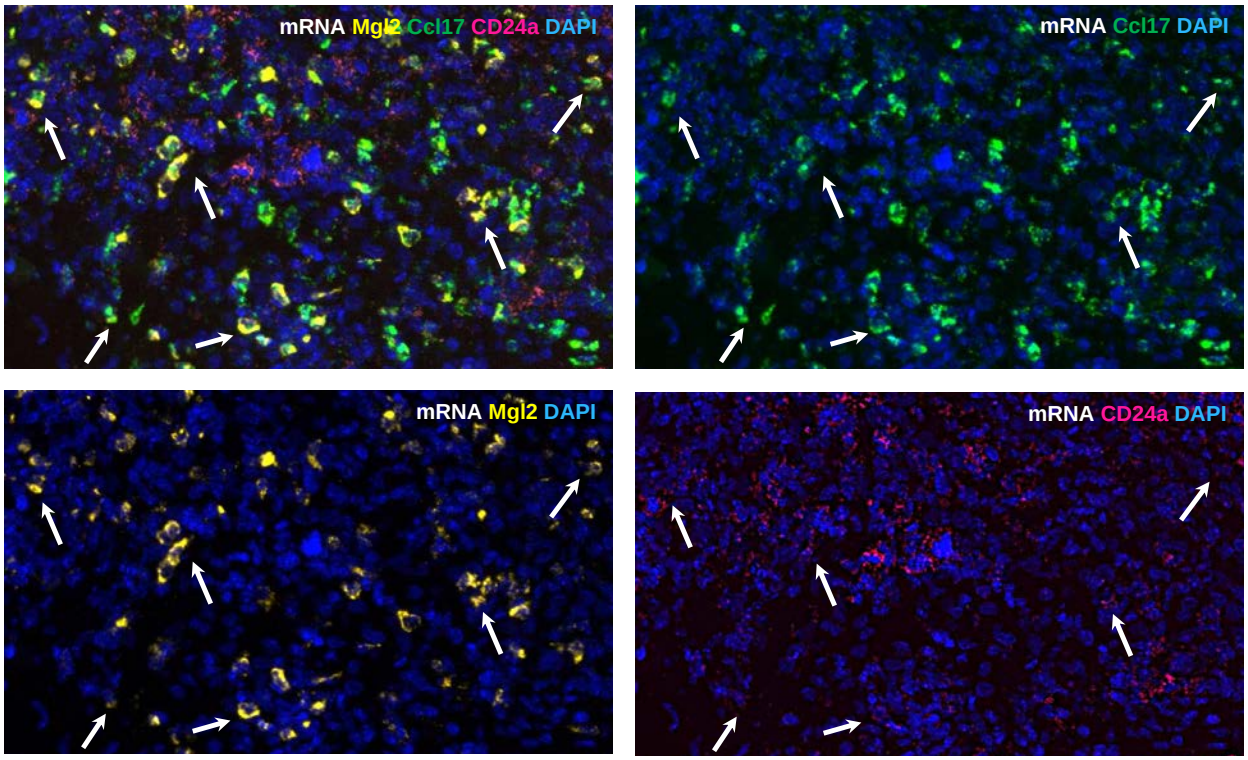


Figure 76: Representative RNAscope images of an orthotopic hetIL-15-treated KPC tumor stained with the probes Mgl2 (yellow), CCL17 (green) and CD24a (pink) as merged and individual-stained.

1.5 hetIL-15 monotherapy shows potent anti-metastatic effect in both transgenic and orthotopic models of pancreatic cancer

Given the major effects of hetIL-15 in metastasis in both EO771 and 4T1 TNBC mouse models (sections 1.1.1.2 and 1.1.2.4), we also wanted to evaluate the anti-metastatic activity of hetIL-15 in the pancreatic cancer model. For this purpose, H&E-stained lungs of control and hetIL-15-treated mice of both orthotopic (day 19) and transgenic (endpoints up to day 50) models were evaluated for the presence of pulmonary metastatic foci. Similarly, to what we observed in the other cancer models, hetIL-15 treatment reduced the metastatic burden in the lungs of the mice bearing KPC tumors (**Fig. 77, 78**).

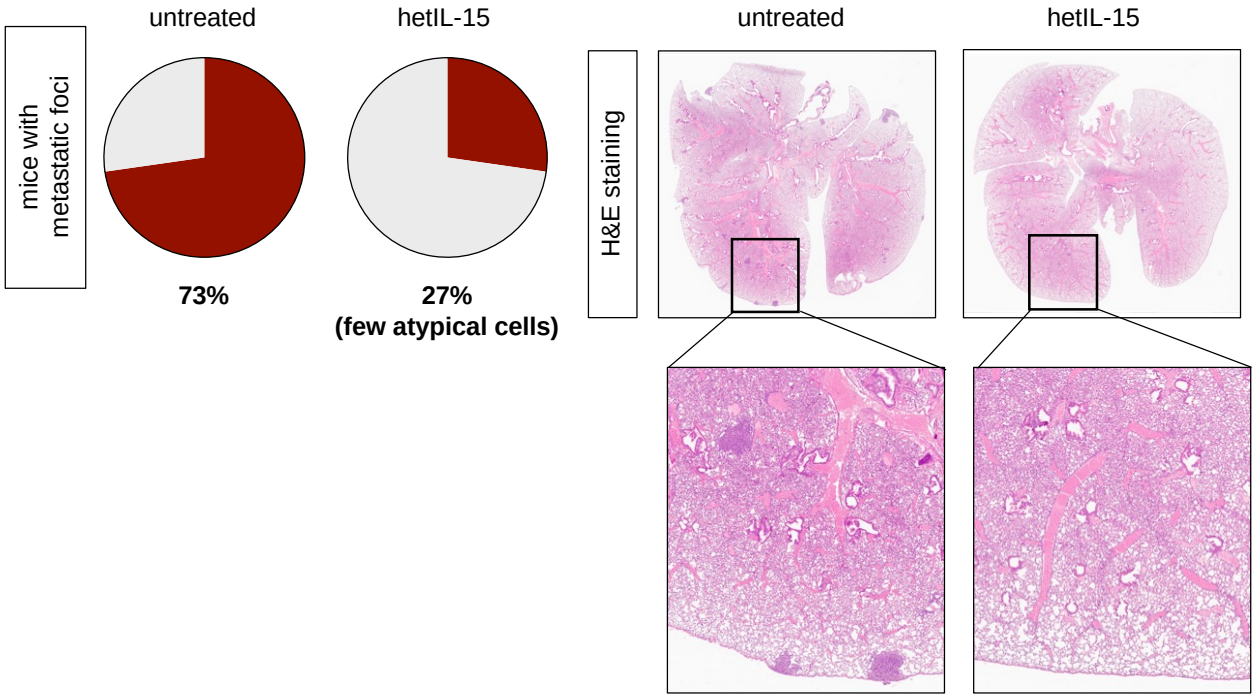


Figure 77: Pies with the percentage of the mice with metastatic foci in lungs (**left**) and representative images of H&E-stained lungs sections (**right**) in control and hetIL-15 groups of the orthotopic KPC model.

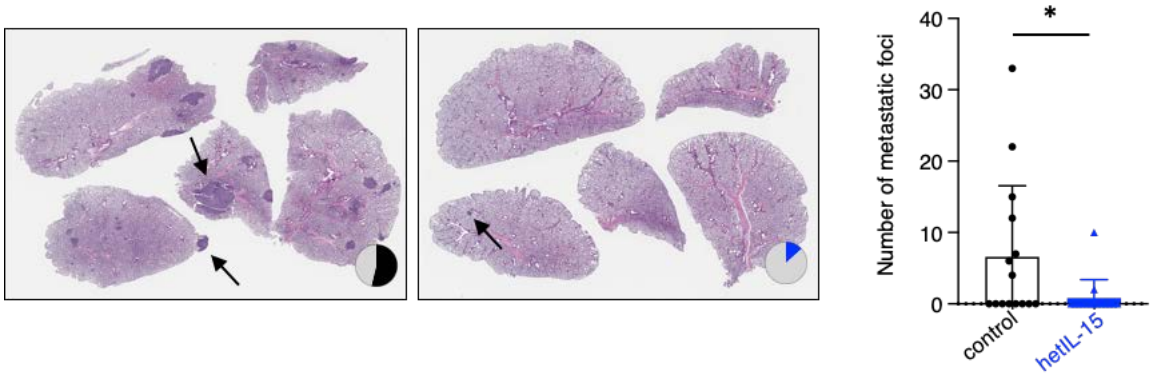


Figure 78: Representative images of H&E-stained lung sections (**left**) and the number of metastatic foci in lungs (**right**) of control and hetIL-15-treated animals in the transgenic KPC model. Pies show the percentage of the mice that found with metastasis in each group.

1.6 hetIL-15 monotherapy increases the infiltration of $CD8^+$ T and NKs in the lungs of the orthotopic and IV model

To further evaluate the anti-metastatic effect of hetIL-15 treatment, we performed IHC analysis in the lungs of the mice using the anti-CD8 and anti-CD161c/NK1.1 antibodies. The animal models that we used were the orthotopic and induced metastatic IV KPC pancreatic cancer models. The IV model is used in metastasis evaluation as the cancer cells that are inoculated through the tail vain preferably colonize the lungs. As IHC revealed, hetIL-15 therapy increased CD8 positive cells in the intra-metastatic foci area in the lungs of the mice in both the orthotopic (**Fig. 79**) and IV KPC models (**Figs 80 and 81**). CD161c/NK1.1 positive cells were found increased only in the hetIL-15-treated mice in the IV model (**Fig. 80 and 81**) (data not shown for the orthotopic model). The counts of CD8 and CD161c/NK1.1 positive cells refer to the intra-metastatic foci area only (**Fig. 80 and 81**). The significant increase of both effector cells revealed that hetIL-15 treatment induces the accumulation of these cells also in the metastatic foci. Finally, hetIL-15 monotherapy decreased the total number of the metastatic foci also in the IV KPC model (**Fig. 80**).

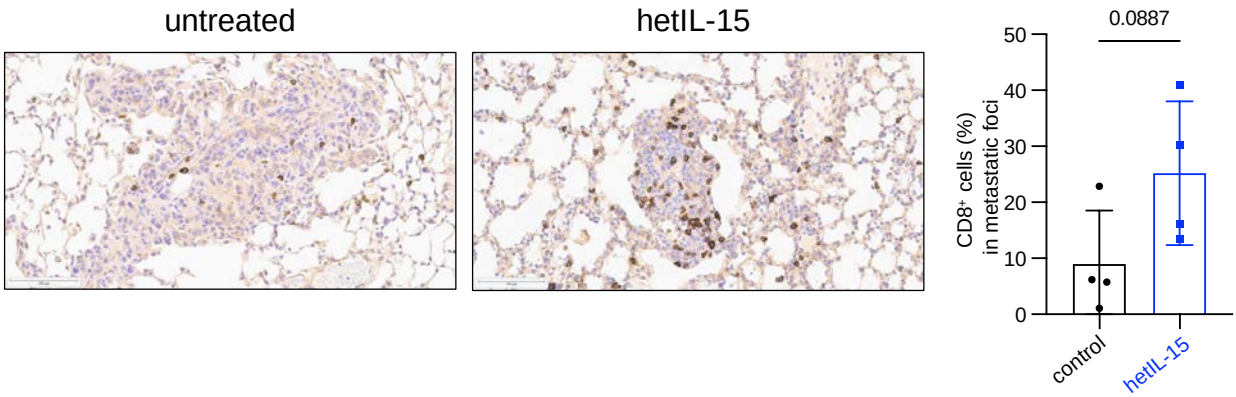


Figure 79: Representative images of anti-CD8-stained lung sections of the individual foci (**left**) and counts of $CD8^+$ cells found in the intra-metastatic area (**right**) of control and hetIL-15-treated animals in the orthotopic KPC model.

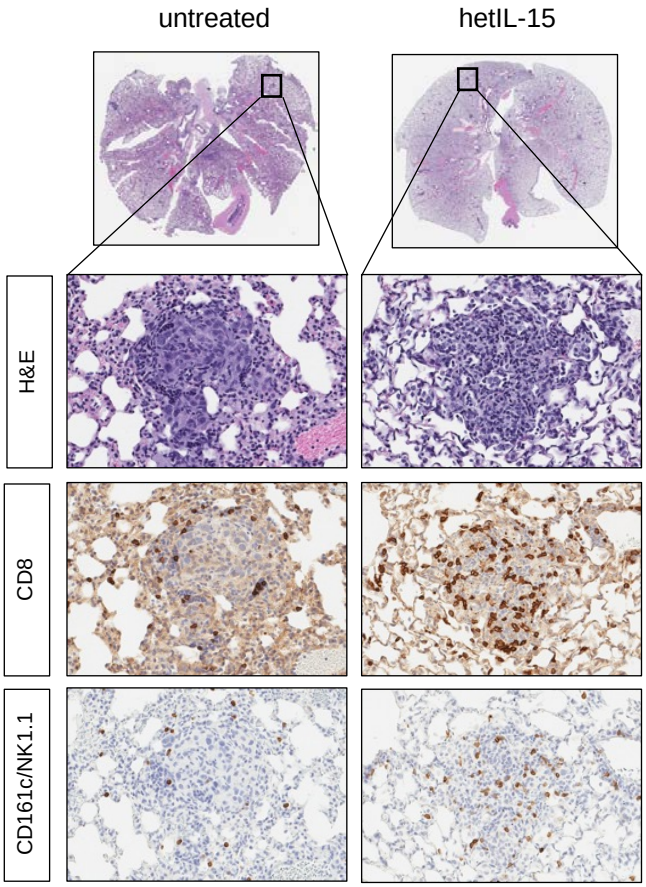


Figure 80: Representative images of H&E-stained whole lung sections and individual foci (**first and second panels**), and anti-CD8 and -CD161c/NK1.1 stainings of the individual foci (**third and fourth panels**) of control and hetIL-15 treated animals in the IV KPC model.

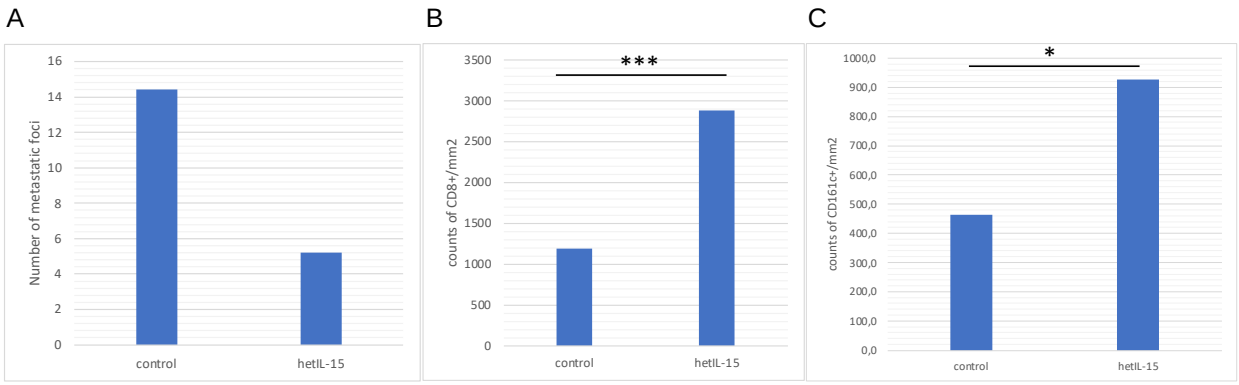


Figure 81: Number of metastatic foci (**A**) and counts of $CD8^+$ (**B**) and $CD161c/NK1.1^+$ (**C**) cells in lungs of control and hetIL-15-treated animals in the IV KPC model.

Discussion

This dissertation evaluates the anti-cancer activity of the immunotherapeutic drug hetIL-15 in mouse models of breast and pancreatic cancer. Several IL-15 variants have been investigated as potential anti-cancer agents in mouse cancer models (Bergamaschi et al., 2020, Mathios et al., 2016, Xu et al., 2013, Yu et al., 2012, Yu et al., 2010), and they are currently being tested in several clinical trials (Conlon et al., 2021b, Conlon et al., 2015, Conlon et al., 2019, Cooley et al., 2019, Margolin et al., 2018, Miller et al., 2018, Romee et al., 2018). Similarly, hetIL-15 has advanced in clinical trials either alone or in combination with the anti-PD-1 antibodies Spartalizumab or Tislelizumab (NCT02452268, NCT04261439). Results from the first-in-human study were recently published reporting that hetIL-15 monotherapy showed stable disease as the best clinical response in 21% of the patients with metastatic or unresectable cancer (Conlon et al., 2021a). The data agree with the results obtained in clinical studies using the other IL-15 variants (Miller et al., 2018, Margolin et al., 2018). Here, hetIL-15 anti-tumor effects were evaluated in three mouse cancer models: EO771 and 4T1 models of triple negative breast cancer and KPC-derived models of pancreatic cancer. hetIL-15 was tested as monotherapy and in combination with targeted therapy, chemotherapy and surgery. The evaluation of the treatment included the effect on the primary tumor delay, infiltration of immune cells intratumorally and in other organs, induction of immunological memory, inhibition of metastasis (primarily in lungs) and extension of animal survival.

hetIL-15 showed potent anti-metastatic activity in all the three mouse cancer models that were evaluated. hetIL-15, either as a single agent or in combination with doxorubicin, significantly decreased CTCs and metastatic foci in the lungs of the 4T1-tumor bearing mice. The immunochemotherapeutic regimen exhibited systemic expansion of $CD8^{+}$T and NK cells and reduction of PMN-MDSCs. When the regimen tested upon surgical excision of the 4T1 primary tumor, half of the treated animals cured and developed immunological memory against 4T1 tumor cells. hetIL-15 showed potent anti-metastatic effects also in the lungs of both the EO771 and pancreatic cancer models. Although the effect on the metastasis inhibition was very prominent, the effect on the primary tumors was not consistent among the cancer models. hetIL-15 as monotherapy showed the strongest anti-tumor effect on the EO771 breast cancer model leading to the complete tumor regression in 40% of the mice and building long-term immunological memory. Immune phenotypic analysis of the hetIL-15-treated EO771 primary tumor revealed increased frequencies of the cytotoxic effector cells ($CD8^{+}$T and NK cells) and dendritic cells (cDC1s). Interestingly, another dendritic cell population was found increased intratumorally and only upon hetIL-15 administration. The population, that we named $CD103^{int}CD11b^{+}$DCs, had the

characteristics of monocytic DCs and correlated with EO771 tumor regression. hetIL-15 monotherapy had a moderate effect on the 4T1 primary tumor and the combination with chemotherapy did not show synergy. hetIL-15 treatment did not show delay of the primary tumor in either the orthotopic or the transgenic KPC model of pancreatic cancer and did not synergize with MSLN-targeted agents or chemotherapy. However, the distinct population of $CD103^{int}CD11b^{+}$DCs were found increased in both 4T1 and KPC primary tumors upon hetIL-15 therapy.

IL-15 anti-metastatic activity has been reported in preclinical studies using the 4T1 model, as single agent (rhIL-15 (Yu et al., 2010, Tang et al., 2008), N-803 (Kim et al., 2016) and RLI (Receptor-Linker-IL-15) (Desbois et al., 2020, Bessard et al., 2009)) and in combination (N-803 with PD-L1) (Knudson et al., 2019) or fusion with ICIs [N-809 (N-803 fused to two αPD-L1 domains)] (Knudson et al., 2020). In these studies, the use of IL-15 variants as single agents reduced the pulmonary metastatic foci (Kim et al., 2016, Desbois et al., 2020, Bessard et al., 2009, Yu et al., 2010, Tang et al., 2008) and co-administration or fusion with the ICIs increased the anti-metastatic efficacy of IL-15 (Knudson et al., 2019, Knudson et al., 2020). Here, hetIL-15 monotherapy significantly reduces metastatic disease in the lungs of EO771, 4T1 and KPC-derived models and co-administration with chemotherapy enhances the efficacy. These results suggest that hetIL-15 anti-metastatic mechanism is ubiquitous and raise the necessity to explore it. The effect could be related to the higher $CD8^{+}$T and NK cell frequencies found in the lungs of hetIL-15-treated mice in 4T1 and KPC models. This finding has also been reported in previous preclinical studies by our group (Bergamaschi et al., 2008) and others (Desbois et al., 2020). hetIL-15 monotherapy also reduced the frequencies of the immunosuppressive PMN-MDSCs in the blood and lungs of the 4T1-tumor bearing mice. Reduced frequencies of PMN-MDSCs in blood, spleen and lungs could contribute to the anti-metastatic effect as studies have shown that PMN-MDSCs are key regulators of metastasis (Ouzounova et al., 2017). Others have also reported that MDSC frequencies are reduced upon IL-15/IL-15Ra administration in the $Her2/neu^{+}$ mammary carcinoma (Guo et al., 2021). We also show that hetIL-15 decreases the absolute number (not only the frequency) of $Ly6G/GR1^{+}$ cells in the lungs, suggesting that their infiltration is prevented by the cytokine treatment. In agreement with these data, Desbois et al. reported that the $CD11b^{+}$ $Ly6G^{+}$ $Ly6C^{low}$ cells in the lungs (frequency and absolute counts) were reduced in the 4T1 model upon IL-15-based treatment (Desbois et al., 2020). In line with other studies (Knudson et al., 2019), we did not find major changes in M-MDSCs frequencies, but the M-MDSCs to effectors ratios were restored to normal levels upon hetIL-15 treatment.

Co-administration of hetIL-15 with doxorubicin showed beneficial anti-tumoral effects by increasing the effector populations ($CD8^{+}$T and NK cells) and by reducing the immunosuppressive

PMN-MDSCs in all the tissues of the 4T1 model that were analyzed. Doxorubicin induces immunogenic cell death facilitating cytotoxic T cells stimulation (Casares et al., 2005), and also depletes MDSCs (Alizadeh et al., 2014). This dual function of Doxorubicin helps the immune system to mount effective anti-tumor responses and provides the rationale for combining doxorubicin with IL-15. Here, the combination of these two agents successfully controlled metastatic disease in the lungs and blood resulting in better animal survival. In earlier studies, IL-15 combined with cyclophosphamide showed better survival and control of metastatic disease in an IV model of rhabdomyosarcoma (Chapoval et al., 1998) providing the initial supportive findings of these therapeutic modalities. PMN-MDSCs depletion by doxorubicin may facilitate the expansion of effectors cells mediated by hetIL-15, suggesting a mechanism for the observed enhanced anti-metastatic activity. Furthermore, the effector cells expressed high levels of the Ki67 marker after combination treatment, indicating that doxorubicin does not negatively affect them even if they actively proliferate upon hetIL-15 administration. Overall, these findings support the notion of combining doxorubicin, and potentially other anthracyclines, with cytokines like hetIL-15 that stimulate the activation and proliferation of cytotoxic immune cells to improve the treatment outcome.

The effect in the 4T1 model of breast cancer showed that the modified cytokine, not only reduces the metastatic disease in the lungs, but also diminishes the number of circulating tumor cells (CTCs). CTCs are the precursors of metastatic lesions (Baccelli et al., 2013) and there is an increasing interest in their biology and in therapeutic strategies to target them (Zhong et al., 2020). However, CTCs detection is challenging as they are present in low numbers in the blood (Hattori et al., 2019). Here, using clonogenic assays, we detected CTCs in 36.84% of the untreated mice, with more than half of them forming more than 500 CTC colonies. In contrast, the frequency of mice with detectable CTCs was reduced in both hetIL-15- and combination-treated groups. This finding could be the result of several effects targeting different steps of the metastatic process. Reduction of the shedding from the primary site and increased immunosurveillance in the parenchyma or vasculature of the target organs may independently contribute to this protection. It has been suggested that NK cells patrol the circulation in search of malignant cells (Barlozzari et al., 1983, Lambert et al., 2017) and here it is shown that they are increased in animals receiving hetIL-15 or combination treatment with doxorubicin. Furthermore, the reduction of PMN-MDSCs upon these treatments may also contribute to the protection as a study has shown that they inhibit NK cytotoxic activity against tumor cells trapped in pulmonary microvessels (Spiegel et al., 2016). Additionally, it was recently reported that IL-15 renders NK cells less susceptible to the oxidative stress induced by myeloid cells in the tumor microenvironment (Yang et al., 2020). Thus, it is

possible that hetIL-15 not only expands NKs but also shields them against PMN-MDSCs, helping them to maintain their cytotoxic activity. Further studies are needed to unfold these mechanisms.

To develop an additional clinically relevant approach, we surgically removed the orthotopically implanted 4T1 tumors and treated the animals pre- and post-surgery. Lumpectomy or mastectomy is the standard treatment of TNBC upon clinical detection, and this is followed by chemotherapy. In our experimental model, tumor resection supported by neoadjuvant and adjuvant administration of hetIL-15, either alone or combined with doxorubicin, resulted in the cure of approximately half of the treated mice. Other studies have shown that adjuvant administration of the IL-15 agonist N-803 significantly prolongs animal survival (Kim et al., 2016, Knudson et al., 2019). Interestingly, Liu et al. demonstrated that neoadjuvant immunotherapy is superior to adjuvant in controlling metastatic disease in two different murine models of TNBC (including the 4T1 model) (Liu et al., 2016). We hypothesize that hetIL-15 administration as neoadjuvant (in addition to adjuvant) increases the effector cells and systemically protects the tissues from the cancer cells dissemination associated with surgery. A therapeutic scheme that combines pembrolizumab (anti-PD-1) as neoadjuvant therapy with chemotherapy has been already approved by the FDA for early-stage patients with TNBC (Schmid et al., 2020). Additionally, our data indicate that hetIL-15 treatment facilitates the development of tumor-specific immunological memory. This could represent an effective defense against future relapse caused by dormant cancer cells resistant to chemotherapy (Sosa et al., 2014). Furthermore, it has been shown that tumor resection leads to decreased metastatic burden in the lungs by depleting MDSCs (Bosiljcic et al., 2019). We also observed reduced numbers of PMN-MDSCs in the periphery and lung sections after surgery in all the resected groups. This reduction might contribute to the enhanced anti-metastatic effect of hetIL-15 suggesting that the cytokine can cure metastatic disease when tumor-derived factors are removed. Interestingly, MDSCs have been reported to differentiate into macrophages and mature DCs in the absence of tumor-derived factors (Narita et al., 2009). The phenotypic analysis of MDSCs upon surgery and treatment would be of interest for future studies.

The strong anti-cancer activity of hetIL-15 treatment in the EO771 breast cancer model, that included complete tumor regression in 40% of the mice, could be attributed to the hot profile of this tumor compared to the other two, and the route of administration that was performed. EO771 primary tumor is not characterized by strong immunosuppressive mechanisms as 4T1 and KPC tumors are, and in general it exhibits a less aggressive profile of growth and metastasis that could provide the appropriate therapeutic window for the treatment. There is an increase interest on exploring local delivery of immune modulators for the treatment of solid tumors. Several studies reviewed by Marabelle et al. (Marabelle et al., 2014) have shown that intratumoral or locoregional administration of immune-stimulating drugs allows for higher concentrations in the tumor

microenvironment than systemic infusions, resulting in improved therapeutic effects and lower toxicities. As a result, the number of trials investigating local administration of cancer therapies has experienced a rapid growth (Champiat et al., 2021). The growth of 4T1 and KPC primary tumors was not controlled by hetIL-15 monotherapy as efficiently as in EO771 model. hetIL-15 IP administration showed a moderate effect in delaying 4T1 primary tumor, while the combination with chemotherapy was more effective. Other IL-15 monotherapy variants have not shown any effect on delaying the primary tumor growth of 4T1 tumors (Kim et al., 2016, Desbois et al., 2020, Knudson et al., 2019), but they have been tested only via IP route. hetIL-15 monotherapy delayed the growth of the orthotopic KPC tumor, but the effect was not consistent. Others have published conflicted results regarding IL-15 treatment activity on the KPC pancreatic cancer. Emma Kurz et al. reported that high dose of hetIL-15 monotherapy shows anti-tumor activity (Kurz et al., 2022) and others that only the combination with chemotherapy (Hsu et al., 2022) or other immunomodulators (such as CD40 agonists) (Van Audenaerde et al., 2020) exhibits significant tumor delay. Overall, these data provide evidence that hetIL-15 administration in proximity to the primary tumor is a therapeutic approach with strong activity. Further improvements in drug delivery systems and combination treatments could improve the anti-tumor activity of hetIL-15 in "colder" tumors, such as in pancreatic cancer.

hetIL-15 monotherapy stimulated $CD8^+$T and NK cells trafficking into the tumors and promoted their proliferation and cytotoxicity, especially in the EO771 model. These findings were also verified by both protein and gene expression analysis of the hetIL-15-treated EO771 tumors showing the up-regulation of the cytotoxic genes *GZMA, GZMB, PRF1* and *IFNG*. The data agree with preclinical and clinical studies where the antitumor responses induced by IL-15 were linked to expansion and activation of the cytotoxic effector cells (Waldmann et al., 2011, Conlon et al., 2015, Berger et al., 2009, Bergamaschi et al., 2020). Furthermore, the depletion experiments, using *Rag-1* ko and NK cell-depleted C57BL/6 mice, suggested that the effects of hetIL-15 treatment in controlling tumor growth and metastasis were mediated through both T and NK cells, but that the adaptive immunity is required for the curative effect of hetIL-15 as *Rag-1* ko mice did not reach complete tumor regression. Additionally, the short-term hetIL-15 treatment schedule, that was performed in the EO771 model, provided T cell-dependent protection against the primary tumor and subsequent rechallenge showing the important role of adaptive immunity in achieving strong anti-tumor effects. Also, that hetIL-15, even in a short-term schedule, could elicit long-term T cell memory against tumor cells. The preserved T cell responses were specific for the EO711 cells because those animals fail to control challenge with an unrelated syngeneic pancreatic tumor line. Finally, experienced $CD8^+$ T cells from donor mice (with complete EO771 tumor regression) successfully reduced the EO771 tumor growth and increased the survival of the recipient mice

upon hetIL-15 treatment, supporting previous data (Ng et al., 2017) and concluding that hetIL-15 immunotherapy in combination with ACT can enhance anti-tumor treatment efficacy.

hetIL-15 affects the dendritic cell populations and, interestingly, increases $CD103^{int}CD11b^{+}$DCs intratumorally which is a distinct dendritic cell subset. Effects of IL-15 on DC phenotypic characteristics and functions have been previously reported. IL-15 enhances the ability of DCs to stimulate antigen-specific $CD8^{+}$T cell proliferation and the expression of their costimulatory markers (Mattei et al., 2001). Here, locoregional administration of hetIL-15 increased $CD103^{+}$cDC1s intratumorally (only in the EO771 model) which correlated with EO771 tumor regression. These results agree with a previous report in which systemic hetIL-15 delivery increased the intratumoral $CD103^{+}$cDC1s in MC-38 and TC-1 tumors (Bergamaschi et al., 2020). cDC2s only increased in the 4T1 and KPC hetIL-15-treated tumors. $CD103^{int}CD11b^{+}$DCs were found elevated in both TNBC models as well as in KPC model suggesting a universal effect of hetIL-15 on expanding this novel DC population. $CD103^{int}CD11b^{+}$DCs correlated with EO771 tumor regression while it was present in low numbers in the control group.

Combination of scRNA-seq, bulk RNA-seq and flow cytometry analysis showed that $CD103^{int}CD11b^{+}$DCs form a unique cell cluster which expresses canonical DC markers, but they are different from the most DC populations (inflammatory cDC2s (Bosteels et al., 2020), tumor moDC3s (Diao et al., 2018), PDAC-associated $CD11c^{+}$DCs (Kenkel et al., 2017) and the mouse dermal moDCs (Tamoutounour et al., 2013)) and macrophages that have been previously reported in the literature. The reason of this distinction is their unique expression pattern of key markers such as IRF8, SIRPA, XCR1, CD24, F4/80 and CD103. XCR1, IRF8 and SIRPA are expressed intermediately in $CD103^{int}CD11b^{+}$DCs which distinguish them from both cDC1 (apart from CD11b) and cDC2 populations. In addition, $CD103^{int}CD11b^{+}$ DCs have increased Rbpj *and* Batf3 gene expression. Although $CD103^{int}CD11b^{+}$ DCs express the common macrophage marker F4/80, they lack many key macrophage markers including Fcgr1 (CD64), CD169, CX3CR1, Ly6C and Siglec1. F4/80 has also been found in another non-macrophage population with similarities to cDC2s and functional DC features, named $F4/80^{high}$APCs (Sheng et al., 2017). Differences in the expression of CD64 or CD169 markers distinguishes $CD103^{int}CD11b^{+}$DCs from $F4/80^{high}$APCs. The types of moDCs that show similarities to hetIL-15-induced $CD103^{int}CD11b^{+}$DCs are the $CD64^{-}MHC^{+}CD11c^{+}Ly6C^{lo}CX3CR1^{int}$moDCs (Bain et al., 2013, Tamoutounour et al., 2012, Zigmond et al., 2012) and the $Ly6C^{lo}CD209a^{+}$moDCs (Cheong et al., 2010), which are migratory and antigen-presenting cells. Several reports have provided evidence that the immune system uses monocytes as DC precursors for efficient antigenic presentation in the periphery during inflammation (Wakim et al., 2008, Nakano et al., 2009, Le Borgne et al., 2006) suggesting that moDCs are important players in the development of an adaptive immune response (Qu et al.,

2014). Many genes associated with antigen processing and presentation were highly expressed in the novel intratumoral CD103^{int}CD11b^{+}DCs indicating the antigen-presenting properties of those cells. Their possible involvement in intratumoral antigen presentation relevant for tumor control needs to be further investigated.

Murine CXCL2 seems to be closely associated with DC maturation (Lee et al., 2009) and CCL17 is expressed in the most mature moDCs and cDCs (Alferink et al., 2003). CCL17 along with the CCL22 mainly recruit activated and memory T cells, as well as B lymphocytes (McColl, 2002, Eberlein et al., 2010, Plantinga et al., 2013). Transcriptomic cytokine profiling revealed high expression of *CXCL2*, *CCL17* and *CCL22* in CD103^{int}CD11b^{+}DCs implying that they may be activated or mature moDCs (Kobayashi, 2008). This was supported by the GSEA analysis using the scRNA-seq data since it revealed the upregulation of many cellular processes involved in the maturation and activation of DCs.

In summary, hetIL-15 exhibits potent anti-cancer effects in two preclinical TNBC models (EO771 and 4T1 mouse models) and one pancreatic cancer model (KPC orthotopic and transgenic models). hetIL-15 showed potent anti-metastatic effects in the lungs of all the preclinical models suggesting a ubiquitous mechanism of metastasis control. hetIL-15 monotherapy also reduced the dissemination of tumor cells in peripheral blood implying additional anti-metastatic mechanisms. The combination with chemotherapy and surgery resulted in improved therapeutic benefit in the 4T1 breast cancer model. The anti-cancer response could be achieved by the systemic expansion of CD8^{+}T and NK cells and the reduction of the immunosuppressive PMN-MDSCs caused by hetIL-15 combination with chemotherapy. Importantly, tumor resection accompanied by the pre- and post-surgery administration of hetIL-15 alone or in combination with doxorubicin led to the cure of the animals and the establishment of immunological memory against 4T1 tumor. The strongest effect on the primary tumor was observed in the EO771 breast cancer model in which hetIL-15 monotherapy led to complete tumor regression in 40% of the mice. Additionally, it prolonged survival and induced immunological memory against the EO771 cancer cells. Immune phenotyping revealed that hetIL-15 re-shapes the EO771 tumor microenvironment by promoting the intratumoral accumulation of cytotoxic lymphocytes, cDC1s and a distinct DC population, defined as CD103^{int}CD11b^{+}DCs. This novel DC population correlated with tumor regression while showed phenotypic and genetic similarities with both cDC1s and cDC2s and similar transcriptomic profiles to monocyte derived DCs (moDCs). CD103^{int}CD11b^{+}DCs were also found intratumorally in KPC models suggesting that hetIL-15 effect is not model specific. Overall, the potent anti-tumor effect of hetIL-15 in the EO771 primary tumor and the consistent anti-metastatic effect in all the tested models holds promises for the future clinical use of the cytokine as an anti-tumor and anti-metastatic agent for solid tumors.

Literature

AJCC 2017. AJCC CANCER STAGING MANUAL Seventh Edition.

ALFERINK, J., LIEBERAM, I., REINDL, W., BEHRENS, A., WEISS, S., HUSER, N., GERAUER, K., ROSS, R., RESKE-KUNZ, A. B., AHMAD-NEJAD, P., WAGNER, H. & FORSTER, I. 2003. Compartmentalized production of CCL17 in vivo: strong inducibility in peripheral dendritic cells contrasts selective absence from the spleen. *J Exp Med,* 197**,** 585-99.

ALIZADEH, D., TRAD, M., HANKE, N. T., LARMONIER, C. B., JANIKASHVILI, N., BONNOTTE, B., KATSANIS, E. & LARMONIER, N. 2014. Doxorubicin eliminates myeloid-derived suppressor cells and enhances the efficacy of adoptive T-cell transfer in breast cancer. *Cancer Res,* 74**,** 104-18.

ARANDA-GUTIERREZ, A. & DIAZ-PEREZ, H. M. 2022. Histology, Mammary Glands. *StatPearls.* Treasure Island (FL): StatPearls Publishing

Copyright © 2022, StatPearls Publishing LLC.

ASHWORTH, T. R. 1869. A Case of Cancer in Which Cells Similar to Those in the Tumours Were Seen in the Blood after Death.: The Medical Journal of Australia.

ASLAKSON, C. J. & MILLER, F. R. 1992. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Res,* 52**,** 1399-405.

BACCELLI, I., SCHNEEWEISS, A., RIETHDORF, S., STENZINGER, A., SCHILLERT, A., VOGEL, V., KLEIN, C., SAINI, M., BÄUERLE, T. & WALLWIENER, M. 2013. Identification of a population of blood circulating tumor cells from breast cancer patients that initiates metastasis in a xenograft assay. *Nature biotechnology,* 31**,** 539-544.

BAIN, C. C., SCOTT, C. L., URONEN-HANSSON, H., GUDJONSSON, S., JANSSON, O., GRIP, O., GUILLIAMS, M., MALISSEN, B., AGACE, W. W. & MOWAT, A. M. 2013. Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. *Mucosal Immunol,* 6**,** 498-510.

BARLOZZARI, T., REYNOLDS, C. W. & HERBERMAN, R. B. 1983. In vivo role of natural killer cells: involvement of large granular lymphocytes in the clearance of tumor cells in anti-asialo GM1-treated rats. *J Immunol,* 131**,** 1024-7.

BAUSS, F., LECHMANN, M., KRIPPENDORFF, B. F., STAACK, R., HERTING, F., FESTAG, M., IMHOF-JUNG, S., HESSE, F., POMPIATI, M., KOLLMORGEN, G., DA SILVA MATEUS SEIDL, R., BOSSENMAIER, B., LAU, W., SCHANTZ, C., STRACKE, J. O., BRINKMANN, U., ONDA, M., PASTAN, I., BOSSLET, K. & NIEDERFELLNER, G. 2016. Characterization of a re-engineered, mesothelin-targeted Pseudomonas exotoxin fusion protein for lung cancer therapy. *Mol Oncol,* 10**,** 1317-29.

BEGER, H. G. 2018. *The pancreas : an integrated textbook of basic science, medicine, and surgery,* Hoboken, New Jersey, Wiley Blackwell.

BERGAMASCHI, C., PANDIT, H., NAGY, B. A., STELLAS, D., JENSEN, S. M., BEAR, J., CAM, M., VALENTIN, A., FOX, B. A., FELBER, B. K. & PAVLAKIS, G. N. 2020. Heterodimeric IL-15 delays tumor growth and promotes intratumoral CTL and dendritic cell accumulation by a cytokine network involving XCL1, IFN-gamma, CXCL9 and CXCL10. *J Immunother Cancer,* 8.

BERGAMASCHI, C., ROSATI, M., JALAH, R., VALENTIN, A., KULKARNI, V., ALICEA, C., ZHANG, G. M., PATEL, V., FELBER, B. K. & PAVLAKIS, G. N. 2008. Intracellular interaction of interleukin-15 with its receptor alpha during production leads to mutual stabilization and increased bioactivity. *J Biol Chem,* 283**,** 4189-99.

BERGAMASCHI, C., STRAVOKEFALOU, V., STELLAS, D., KARALIOTA, S., FELBER, B. K. & PAVLAKIS, G. N. 2021. Heterodimeric IL-15 in Cancer Immunotherapy. *Cancers (Basel),* 13.

BERGER, C., BERGER, M., HACKMAN, R. C., GOUGH, M., ELLIOTT, C., JENSEN, M. C. & RIDDELL, S. R. 2009. Safety and immunologic effects of IL-15 administration in nonhuman primates. *Blood,* 114**,** 2417-26.

BERRAONDO, P., SANMAMED, M. F., OCHOA, M. C., ETXEBERRIA, I., AZNAR, M. A., PÉREZ-GRACIA, J. L., RODRÍGUEZ-RUIZ, M. E., PONZ-SARVISE, M., CASTAÑÓN, E. & MELERO, I. 2019. Cytokines in clinical cancer immunotherapy. *British Journal of Cancer,* 120**,** 6-15.

BESSARD, A., SOLÉ, V., BOUCHAUD, G., QUÉMÉNER, A. & JACQUES, Y. 2009. High antitumor activity of RLI, an interleukin-15 (IL-15)–IL-15 receptor α fusion protein, in metastatic melanoma and colorectal cancer. *Molecular Cancer Therapeutics,* 8**,** 2736-2745.

BIANCHINI, G., BALKO, J. M., MAYER, I. A., SANDERS, M. E. & GIANNI, L. 2016. Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. *Nature Reviews Clinical Oncology,* 13**,** 674-690.

BONNARDEL, J., DA SILVA, C., HENRI, S., TAMOUTOUNOUR, S., CHASSON, L., MONTANANA-SANCHIS, F., GORVEL, J. P. & LELOUARD, H. 2015. Innate and adaptive immune functions of peyer's patch monocyte-derived cells. *Cell Rep,* 11**,** 770-84.

BORISH, L. C. & STEINKE, J. W. 2003. 2. Cytokines and chemokines. *J Allergy Clin Immunol,* 111**,** S460-75.

BORREGAARD, N. 2010. Neutrophils, from Marrow to Microbes. *Immunity,* 33**,** 657-670.

BOSILJCIC, M., CEDERBERG, R. A., HAMILTON, M. J., LEPARD, N. E., HARBOURNE, B. T., COLLIER, J. L., HALVORSEN, E. C., SHI, R., FRANKS, S. E., KIM, A. Y., BANÁTH, J. P., HAMER, M., ROSSI, F. M. & BENNEWITH, K. L. 2019. Targeting myeloid-derived suppressor cells in combination with primary mammary tumor resection reduces metastatic growth in the lungs. *Breast Cancer Research,* 21**,** 103.

BOSTEELS, C., NEYT, K., VANHEERSWYNGHELS, M., VAN HELDEN, M. J., SICHIEN, D., DEBEUF, N., DE PRIJCK, S., BOSTEELS, V., VANDAMME, N., MARTENS, L., SAEYS, Y., LOUAGIE, E., LESAGE, M., WILLIAMS, D. L., TANG, S. C., MAYER, J. U., RONCHESE, F., SCOTT, C. L., HAMMAD, H., GUILLIAMS, M. & LAMBRECHT, B. N. 2020. Inflammatory Type 2 cDCs Acquire Features of cDC1s and Macrophages to Orchestrate Immunity to Respiratory Virus Infection. *Immunity,* 52**,** 1039-1056 e9.

BOTTCHER, J. P., BONAVITA, E., CHAKRAVARTY, P., BLEES, H., CABEZA-CABRERIZO, M., SAMMICHELI, S., ROGERS, N. C., SAHAI, E., ZELENAY, S. & REIS E SOUSA, C. 2018. NK Cells Stimulate Recruitment of cDC1 into the Tumor Microenvironment Promoting Cancer Immune Control. *Cell,* 172**,** 1022-1037 e14.

BOZZONE, D. M. 2009. The biology of cancer: Leukemia. New York, Chelsea House pub.

BRONTE, V., BRANDAU, S., CHEN, S. H., COLOMBO, M. P., FREY, A. B., GRETEN, T. F., MANDRUZZATO, S., MURRAY, P. J., OCHOA, A., OSTRAND-ROSENBERG, S., RODRIGUEZ, P. C., SICA, A., UMANSKY, V., VONDERHEIDE, R. H. & GABRILOVICH, D. I. 2016. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat Commun,* 7**,** 12150.

BRONTE, V., SERAFINI, P., MAZZONI, A., SEGAL, D. M. & ZANOVELLO, P. 2003. L-arginine metabolism in myeloid cells controls T-lymphocyte functions. *Trends Immunol,* 24**,** 302-6.

BROWN, C. C., GUDJONSON, H., PRITYKIN, Y., DEEP, D., LAVALLEE, V. P., MENDOZA, A., FROMME, R., MAZUTIS, L., ARIYAN, C., LESLIE, C., PE'ER, D. & RUDENSKY, A. Y. 2019. Transcriptional Basis of Mouse and Human Dendritic Cell Heterogeneity. *Cell,* 179**,** 846-863 e24.

BRUGER, A. M., DORHOI, A., ESENDAGLI, G., BARCZYK-KAHLERT, K., VAN DER BRUGGEN, P., LIPOLDOVA, M., PERECKO, T., SANTIBANEZ, J., SARAIVA, M., VAN GINDERACHTER, J. A. & BRANDAU, S. 2019. How to measure the immunosuppressive activity of MDSC: assays, problems and potential solutions. *Cancer Immunol Immunother,* 68**,** 631-644.

BRUNET, J. F., DENIZOT, F., LUCIANI, M. F., ROUX-DOSSETO, M., SUZAN, M., MATTEI, M. G. & GOLSTEIN, P. 1987. A new member of the immunoglobulin superfamily--CTLA-4. *Nature,* 328**,** 267-70.

BURNET, F. M. 1970. The concept of immunological surveillance. *Prog Exp Tumor Res,* 13**,** 1-27.

BURNET, M. 1957. Cancer—A Biological Approach. *I. The Processes Of Control. II. The Significance of Somatic Mutation,* 1**,** 779-786.

BY CAUSE, G. D. & AGE, S. 2020. by Country and by Region, 2000-2019. *World Health Organization*.

CAILLEAU, R., YOUNG, R., OLIVÉ, M. & REEVES, W. J., JR. 1974. Breast tumor cell lines from pleural effusions. *J Natl Cancer Inst,* 53**,** 661-74.

CASARES , N., PEQUIGNOT , M. O., TESNIERE , A., GHIRINGHELLI , F. O., ROUX , S. P., CHAPUT , N., SCHMITT , E., HAMAI , A., HERVAS-STUBBS , S., OBEID , M., COUTANT , F. D. R., MÉTIVIER , D., PICHARD , E., AUCOUTURIER , P., PIERRON , G. R., GARRIDO , C., ZITVOGEL , L. & KROEMER , G. 2005. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *Journal of Experimental Medicine,* 202**,** 1691-1701.

CHAFFER, C. L. & WEINBERG, R. A. 2011. A perspective on cancer cell metastasis. *Science,* 331**,** 1559-64.

CHAMBERS, A. F., GROOM, A. C. & MACDONALD, I. C. 2002. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer,* 2**,** 563-72.

CHAMPIAT, S., TSELIKAS, L., FARHANE, S., RAOULT, T., TEXIER, M., LANOY, E., MASSARD, C., ROBERT, C., AMMARI, S., DE BAERE, T. & MARABELLE, A. 2021. Intratumoral Immunotherapy: From Trial Design to Clinical Practice. *Clin Cancer Res,* 27**,** 665-679.

CHAPLIN, D. D. 2010. Overview of the immune response. *J Allergy Clin Immunol,* 125**,** S3-23.

CHAPOVAL, A. I., FULLER, J. A., KREMLEV, S. G., KAMDAR, S. J. & EVANS, R. 1998. Combination chemotherapy and IL-15 administration induce permanent tumor regression in a mouse lung tumor model: NK and T cell-mediated effects antagonized by B cells. *J Immunol,* 161**,** 6977-84.

CHEN, DANIEL S. & MELLMAN, I. 2013. Oncology Meets Immunology: The Cancer-Immunity Cycle. *Immunity,* 39**,** 1-10.

CHEN, D. S. & MELLMAN, I. 2017. Elements of cancer immunity and the cancer-immune set point. *Nature,* 541**,** 321-330.

CHEONG, C., MATOS, I., CHOI, J. H., DANDAMUDI, D. B., SHRESTHA, E., LONGHI, M. P., JEFFREY, K. L., ANTHONY, R. M., KLUGER, C., NCHINDA, G., KOH, H., RODRIGUEZ, A., IDOYAGA, J., PACK, M., VELINZON, K., PARK, C. G. & STEINMAN, R. M. 2010. Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas. *Cell,* 143**,** 416-29.

COLLISSON, E. A., BAILEY, P., CHANG, D. K. & BIANKIN, A. V. 2019. Molecular subtypes of pancreatic cancer. *Nat Rev Gastroenterol Hepatol,* 16**,** 207-220.

CONDAMINE, T., RAMACHANDRAN, I., YOUN, J. I. & GABRILOVICH, D. I. 2015. Regulation of tumor metastasis by myeloid-derived suppressor cells. *Annu Rev Med,* 66**,** 97-110.

CONLON, K., WATSON, D. C., WALDMANN, T. A., VALENTIN, A., BERGAMASCHI, C., FELBER, B. K., PEER, C. J., FIGG, W. D., POTTER, E. L., ROEDERER, M., MCNEEL, D. G., THOMPSON, J. A., GUPTA, S., LEIDNER, R., WANG-GILLAM, A., PARIKH, N. S., LONG, D., KURTULUS, S., HO LEE, L., CHOWDHURY, N. R., BENDER, F. & PAVLAKIS, G. N. 2021a. Phase I study of single agent NIZ985, a recombinant heterodimeric IL-15 agonist, in adult patients with metastatic or unresectable solid tumors. *Journal for ImmunoTherapy of Cancer,* 9**,** e003388.

CONLON, K., WATSON, D. C., WALDMANN, T. A., VALENTIN, A., BERGAMASCHI, C., FELBER, B. K., PEER, C. J., FIGG, W. D., POTTER, E. L., ROEDERER, M., MCNEEL, D. G., THOMPSON, J. A., GUPTA, S., LEIDNER, R., WANG-GILLAM, A., PARIKH, N. S., LONG, D., KURTULUS, S., HO LEE, L., CHOWDHURY, N. R., BENDER, F. & PAVLAKIS, G. N. 2021b. Phase I study of single agent NIZ985, a recombinant heterodimeric IL-15 agonist, in adult patients with metastatic or unresectable solid tumors. *J Immunother Cancer,* 9.

CONLON, K. C., LUGLI, E., WELLES, H. C., ROSENBERG, S. A., FOJO, A. T., MORRIS, J. C., FLEISHER, T. A., DUBOIS, S. P., PERERA, L. P.,

STEWART, D. M., GOLDMAN, C. K., BRYANT, B. R., DECKER, J. M., CHEN, J., WORTHY, T. A., FIGG, W. D., SR., PEER, C. J., SNELLER, M. C., LANE, H. C., YOVANDICH, J. L., CREEKMORE, S. P., ROEDERER, M. & WALDMANN, T. A. 2015. Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. *J Clin Oncol,* 33**,** 74-82.

CONLON, K. C., POTTER, E. L., PITTALUGA, S., LEE, C. R., MILJKOVIC, M. D., FLEISHER, T. A., DUBOIS, S., BRYANT, B. R., PETRUS, M., PERERA, L. P., HSU, J., FIGG, W. D., PEER, C. J., SHIH, J. H., YOVANDICH, J. L., CREEKMORE, S. P., ROEDERER, M. & WALDMANN, T. A. 2019. IL15 by Continuous Intravenous Infusion to Adult Patients with Solid Tumors in a Phase I Trial Induced Dramatic NK-Cell Subset Expansion. *Clin Cancer Res,* 25**,** 4945-4954.

CONROY, T., DESSEIGNE, F., YCHOU, M., BOUCHÉ, O., GUIMBAUD, R., BÉCOUARN, Y., ADENIS, A., RAOUL, J.-L., GOURGOU-BOURGADE, S., DE LA FOUCHARDIÈRE, C., BENNOUNA, J., BACHET, J.-B., KHEMISSA-AKOUZ, F., PÉRÉ-VERGÉ, D., DELBALDO, C., ASSENAT, E., CHAUFFERT, B., MICHEL, P., MONTOTO-GRILLOT, C. & DUCREUX, M. 2011. FOLFIRINOX versus Gemcitabine for Metastatic Pancreatic Cancer. *New England Journal of Medicine,* 364**,** 1817-1825.

COOLEY, S., HE, F., BACHANOVA, V., VERCELLOTTI, G. M., DEFOR, T. E., CURTSINGER, J. M., ROBERTSON, P., GRZYWACZ, B., CONLON, K. C., WALDMANN, T. A., MCKENNA, D. H., BLAZAR, B. R., WEISDORF, D. J. & MILLER, J. S. 2019. First-in-human trial of rhIL-15 and haploidentical natural killer cell therapy for advanced acute myeloid leukemia. *Blood Adv,* 3**,** 1970-1980.

COUZIN-FRANKEL, J. 2013. Breakthrough of the year 2013. Cancer immunotherapy. *Science,* 342**,** 1432-3.

DESBOIS, M., BÉAL, C., CHARRIER, M., BESSE, B., MEURICE, G., CAGNARD, N., JACQUES, Y., BÉCHARD, D., CASSARD, L. & CHAPUT, N. 2020. IL-15 superagonist RLI has potent immunostimulatory properties on NK cells: implications for antimetastatic treatment. *Journal for ImmunoTherapy of Cancer,* 8**,** e000632.

DIAO, J., GU, H., TANG, M., ZHAO, J. & CATTRAL, M. S. 2018. Tumor Dendritic Cells (DCs) Derived from Precursors of Conventional DCs Are Dispensable for Intratumor CTL Responses. *J Immunol,* 201**,** 1306-1314.

DIGHE, A. S., RICHARDS, E., OLD, L. J. & SCHREIBER, R. D. 1994. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. *Immunity,* 1**,** 447-56.

DRAYTON, D. L., LIAO, S., MOUNZER, R. H. & RUDDLE, N. H. 2006. Lymphoid organ development: from ontogeny to neogenesis. *Nature Immunology,* 7**,** 344-353.

DUDLEY, J. P., GOLOVKINA, T. V. & ROSS, S. R. 2016. Lessons Learned from Mouse Mammary Tumor Virus in Animal Models. *Ilar j,* 57**,** 12-23.

EBERLEIN, J., NGUYEN, T. T., VICTORINO, F., GOLDEN-MASON, L., ROSEN, H. R. & HOMANN, D. 2010. Comprehensive assessment of chemokine expression profiles by flow cytometry. *J Clin Invest,* 120**,** 907-23.

ENGEL, A. M., SVANE, I. M., MOURITSEN, S., RYGAARD, J., CLAUSEN, J. & WERDELIN, O. 1996. Methylcholanthrene-induced sarcomas in nude mice have short induction times and relatively low levels of surface MHC class I expression. *Apmis,* 104**,** 629-39.

ENGEL, A. M., SVANE, I. M., RYGAARD, J. & WERDELIN, O. 1997. MCA sarcomas induced in scid mice are more immunogenic than MCA sarcomas induced in congenic, immunocompetent mice. *Scand J Immunol,* 45**,** 463-70.

ESHHAR, Z., WAKS, T., GROSS, G. & SCHINDLER, D. G. 1993. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci U S A,* 90**,** 720-4.

EWENS, A., MIHICH, E. & EHRKE, M. J. 2005. Distant metastasis from subcutaneously grown E0771 medullary breast adenocarcinoma. *Anticancer Res,* 25**,** 3905-15.

FESNAK, A. D., JUNE, C. H. & LEVINE, B. L. 2016. Engineered T cells: the promise and challenges of cancer immunotherapy. *Nat Rev Cancer,* 16**,** 566-81.

GARRIDO-NAVAS, C., DE MIGUEL-PEREZ, D., EXPOSITO-HERNANDEZ, J., BAYARRI, C., AMEZCUA, V., ORTIGOSA, A., VALDIVIA, J., GUERRERO, R., GARCIA PUCHE, J. L., LORENTE, J. A. & SERRANO, M. J. 2019. Cooperative and Escaping Mechanisms between Circulating Tumor Cells and Blood Constituents. *Cells,* 8.

GINHOUX, F., GRETER, M., LEBOEUF, M., NANDI, S., SEE, P., GOKHAN, S., MEHLER, M. F., CONWAY, S. J., NG, L. G. & STANLEY, E. R. 2010. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science,* 330**,** 841-845.

GODFREY, D. I., ULDRICH, A. P., MCCLUSKEY, J., ROSSJOHN, J. & MOODY, D. B. 2015. The burgeoning family of unconventional T cells. *Nature Immunology,* 16**,** 1114-1123.

GUILLIAMS, M., DUTERTRE, C. A., SCOTT, C. L., MCGOVERN, N., SICHIEN, D., CHAKAROV, S., VAN GASSEN, S., CHEN, J., POIDINGER, M., DE PRIJCK, S., TAVERNIER, S. J., LOW, I., IRAC, S. E., MATTAR, C. N., SUMATOH, H. R., LOW, G. H. L., CHUNG, T. J. K., CHAN, D. K. H., TAN, K. K., HON, T. L. K., FOSSUM, E., BOGEN, B., CHOOLANI, M., CHAN, J. K. Y., LARBI, A., LUCHE, H., HENRI, S., SAEYS, Y., NEWELL, E. W., LAMBRECHT, B. N., MALISSEN, B. & GINHOUX, F. 2016. Unsupervised High-Dimensional Analysis Aligns Dendritic Cells across Tissues and Species. *Immunity,* 45**,** 669-684.

GUO, S., SMELTZ, R. B., NANAJIAN, A. & HELLER, R. 2021. IL-15/IL-15Rα Heterodimeric Complex as Cancer Immunotherapy in Murine Breast Cancer Models. *Frontiers in Immunology,* 11.

GUO, Y., LUAN, L., PATIL, N. K. & SHERWOOD, E. R. 2017. Immunobiology of the IL-15/IL-15Ralpha complex as an antitumor and antiviral agent. *Cytokine Growth Factor Rev*.

HAGERTY, B., O'SULLIVAN, T. N., ZHANG, X., COLLINS, N. K., CUSTER LAWRENCE, W., BASSEL, L. L., PATE, N., XU, J., GUERIN, T. M., KOZLOV, S. & ALEWINE, C. 2021. Novel Humanized Mesothelin-Expressing Genetically Engineered Mouse Models Underscore Challenges in Delivery of Complex Therapeutics to Pancreatic Cancers. *Molecular Cancer Therapeutics*.

HALLE, S., HALLE, O. & FÖRSTER, R. 2017. Mechanisms and Dynamics of T Cell-Mediated Cytotoxicity In Vivo. *Trends in Immunology,* 38**,** 432-443.

HANAHAN, D. 2022. Hallmarks of Cancer: New Dimensions. *Cancer Discovery,* 12**,** 31-46.

HANAHAN, D. & WEINBERG, R. A. 2000. The hallmarks of cancer. *Cell,* 100**,** 57-70.

HANAHAN, D. & WEINBERG, R. A. 2011. Hallmarks of cancer: the next generation. *Cell,* 144**,** 646-74.

HARBECK, N., PENAULT-LLORCA, F., CORTES, J., GNANT, M., HOUSSAMI, N., POORTMANS, P., RUDDY, K., TSANG, J. & CARDOSO, F. 2019. Breast cancer. *Nature Reviews Disease Primers,* 5**,** 66.

HATTORI, M., NAKANISHI, H., YOSHIMURA, M., IWASE, M., YOSHIMURA, A., ADACHI, Y., GONDO, N., KOTANI, H., SAWAKI, M., FUJITA, N., YATABE, Y. & IWATA, H. 2019. Circulating tumor cells

detection in tumor draining vein of breast cancer patients. *Scientific Reports,* 9**,** 18195.

HINGORANI, S. R., WANG, L., MULTANI, A. S., COMBS, C., DERAMAUDT, T. B., HRUBAN, R. H., RUSTGI, A. K., CHANG, S. & TUVESON, D. A. 2005. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell,* 7**,** 469-83.

HODI, F. S., O'DAY, S. J., MCDERMOTT, D. F., WEBER, R. W., SOSMAN, J. A., HAANEN, J. B., GONZALEZ, R., ROBERT, C., SCHADENDORF, D., HASSEL, J. C., AKERLEY, W., VAN DEN EERTWEGH, A. J., LUTZKY, J., LORIGAN, P., VAUBEL, J. M., LINETTE, G. P., HOGG, D., OTTENSMEIER, C. H., LEBBÉ, C., PESCHEL, C., QUIRT, I., CLARK, J. I., WOLCHOK, J. D., WEBER, J. S., TIAN, J., YELLIN, M. J., NICHOL, G. M., HOOS, A. & URBA, W. J. 2010. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med,* 363**,** 711-23.

HSU, F. T., TSAI, C. L., CHIANG, I. T., LAN, K. H., YUEH, P. F., LIANG, W. Y., LIN, C. S., CHAO, Y. & LAN, K. L. 2022. Synergistic effect of Abraxane that combines human IL15 fused with an albumin-binding domain on murine models of pancreatic ductal adenocarcinoma. *J Cell Mol Med,* 26**,** 1955-1968.

HTTPS://PATHOLOGY.JHU.EDU/BREAST/OVERVIEW.

HTTPS://WWW.OLIVELAB.ORG/THE-PANCREAS-OVERVIEW.HTML.

HWANG, C. I., BOJ, S. F., CLEVERS, H. & TUVESON, D. A. 2016. Preclinical models of pancreatic ductal adenocarcinoma. *J Pathol,* 238**,** 197-204.

IACOBUZIO-DONAHUE, C. A. 2012. Genetic evolution of pancreatic cancer: lessons learnt from the pancreatic cancer genome sequencing project. *Gut,* 61**,** 1085-94.

ISHIDA, Y., AGATA, Y., SHIBAHARA, K. & HONJO, T. 1992. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *Embo j,* 11**,** 3887-95.

KACZANOWSKA, S., BEURY, D. W., GOPALAN, V., TYCKO, A. K., QIN, H., CLEMENTS, M. E., DRAKE, J., NWANZE, C., MURGAI, M., RAE, Z., JU, W., ALEXANDER, K. A., KLINE, J., CONTRERAS, C. F., WESSEL, K. M., PATEL, S., HANNENHALLI, S., KELLY, M. C. & KAPLAN, R. N. 2021. Genetically engineered myeloid cells rebalance the core immune suppression program in metastasis. *Cell,* 184**,** 2033-2052 e21.

KALOS, M. & JUNE, C. H. 2013. Adoptive T cell transfer for cancer immunotherapy in the era of synthetic biology. *Immunity,* 39**,** 49-60.

KANTOFF, P. W., HIGANO, C. S., SHORE, N. D., BERGER, E. R., SMALL, E. J., PENSON, D. F., REDFERN, C. H., FERRARI, A. C., DREICER, R., SIMS, R. B., XU, Y., FROHLICH, M. W. & SCHELLHAMMER, P. F. 2010. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med,* 363**,** 411-22.

KAPLAN, D. H., SHANKARAN, V., DIGHE, A. S., STOCKERT, E., AGUET, M., OLD, L. J. & SCHREIBER, R. D. 1998. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci U S A,* 95**,** 7556-61.

KENKEL, J. A., TSENG, W. W., DAVIDSON, M. G., TOLENTINO, L. L., CHOI, O., BHATTACHARYA, N., SEELEY, E. S., WINER, D. A., RETICKER-FLYNN, N. E. & ENGLEMAN, E. G. 2017. An Immunosuppressive Dendritic Cell Subset Accumulates at Secondary Sites and Promotes Metastasis in Pancreatic Cancer. *Cancer Res,* 77**,** 4158-4170.

KHALIL, D. N., SMITH, E. L., BRENTJENS, R. J. & WOLCHOK, J. D. 2016. The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. *Nat Rev Clin Oncol,* 13**,** 273-90.

KIM, P. S., KWILAS, A. R., XU, W., ALTER, S., JENG, E. K., WONG, H. C., SCHLOM, J. & HODGE, J. W. 2016. IL-15 superagonist/IL-15RαSushi-Fc fusion complex (IL-15SA/IL-15RαSu-Fc; ALT-803) markedly enhances specific subpopulations of NK and memory CD8+ T cells, and mediates potent anti-tumor activity against murine breast and colon carcinomas. *Oncotarget,* 7**,** 16130-45.

KIM, R., EMI, M. & TANABE, K. 2007. Cancer immunoediting from immune surveillance to immune escape. *Immunology,* 121**,** 1-14.

KIS-TOTH, K., BACSKAI, I., GOGOLAK, P., MAZLO, A., SZATMARI, I. & RAJNAVOLGYI, E. 2013. Monocyte-derived dendritic cell subpopulations use different types of matrix metalloproteinases inhibited by GM6001. *Immunobiology,* 218**,** 1361-9.

KLEEFF, J., KORC, M., APTE, M., LA VECCHIA, C., JOHNSON, C. D., BIANKIN, A. V., NEALE, R. E., TEMPERO, M., TUVESON, D. A., HRUBAN, R. H. & NEOPTOLEMOS, J. P. 2016. Pancreatic cancer. *Nat Rev Dis Primers,* 2**,** 16022.

KLEINMAN, H. K. & MARTIN, G. R. 2005. Matrigel: basement membrane matrix with biological activity. *Semin Cancer Biol,* 15**,** 378-86.

KNUDSON, K. M., HICKS, K. C., ALTER, S., SCHLOM, J. & GAMEIRO, S. R. 2019. Mechanisms involved in IL-15 superagonist enhancement of anti-PD-L1 therapy. *J Immunother Cancer,* 7**,** 82.

KNUDSON, K. M., HICKS, K. C., OZAWA, Y., SCHLOM, J. & GAMEIRO, S. R. 2020. Functional and mechanistic advantage of the use of a bifunctional anti-PD-L1/IL-15 superagonist. *Journal for ImmunoTherapy of Cancer,* 8**,** e000493.

KOBAYASHI, Y. 2008. The role of chemokines in neutrophil biology. *Front Biosci,* 13**,** 2400-7.

KOCHENDERFER, J. N., WILSON, W. H., JANIK, J. E., DUDLEY, M. E., STETLER-STEVENSON, M., FELDMAN, S. A., MARIC, I., RAFFELD, M., NATHAN, D. A., LANIER, B. J., MORGAN, R. A. & ROSENBERG, S. A. 2010. Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood,* 116**,** 4099-102.

KOWANETZ, M., WU, X., LEE, J., TAN, M., HAGENBEEK, T., QU, X., YU, L., ROSS, J., KORSISAARI, N., CAO, T., BOU-RESLAN, H., KALLOP, D., WEIMER, R., LUDLAM, M. J., KAMINKER, J. S., MODRUSAN, Z., VAN BRUGGEN, N., PEALE, F. V., CARANO, R., MENG, Y. G. & FERRARA, N. 2010. Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes. *Proc Natl Acad Sci U S A,* 107**,** 21248-55.

KRUMMEL, M. F. & ALLISON, J. P. 1995. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med,* 182**,** 459-65.

KURZ, E., HIRSCH, C. A., DALTON, T., SHADALOEY, S. A., KHODADADI-JAMAYRAN, A., MILLER, G., PAREEK, S., RAJAEI, H., MOHINDROO, C., BAYDOGAN, S., NGO-HUANG, A., PARKER, N., KATZ, M. H. G., PETZEL, M., VUCIC, E., MCALLISTER, F., SCHADLER, K., WINOGRAD, R. & BAR-SAGI, D. 2022. Exercise-induced engagement of the IL-15/IL-15Rα axis promotes anti-tumor immunity in pancreatic cancer. *Cancer Cell,* 40**,** 720-737.e5.

LAMBERT, A. W., PATTABIRAMAN, D. R. & WEINBERG, R. A. 2017. Emerging Biological Principles of Metastasis. *Cell,* 168**,** 670-691.

LE BORGNE, M., ETCHART, N., GOUBIER, A., LIRA, S. A., SIRARD, J. C., VAN ROOIJEN, N., CAUX, C., AIT-YAHIA, S., VICARI, A., KAISERLIAN, D. & DUBOIS, B. 2006. Dendritic cells rapidly recruited into epithelial tissues via CCR6/CCL20 are responsible for CD8+ T cell crosspriming in vivo. *Immunity,* 24**,** 191-201.

LEE, J. Y., KIM, H., CHA, M. Y., PARK, H. G., KIM, Y. J., KIM, I. Y. & KIM, J. M. 2009. Clostridium difficile toxin A promotes dendritic cell maturation and chemokine CXCL2 expression through p38, IKK, and the NF-kappaB signaling pathway. *J Mol Med (Berl),* 87**,** 169-80.

LEUNG, P. S. 2010. Overview of the Pancreas. *In:* LEUNG, P. S. (ed.) *The Renin-Angiotensin System: Current Research Progress in The Pancreas: The RAS in the Pancreas.* Dordrecht: Springer Netherlands.

LIM, H. X., KIM, T. S. & POH, C. L. 2020. Understanding the Differentiation, Expansion, Recruitment and Suppressive Activities of Myeloid-Derived Suppressor Cells in Cancers. *Int J Mol Sci,* 21.

LIN, D., SHEN, L., LUO, M., ZHANG, K., LI, J., YANG, Q., ZHU, F., ZHOU, D., ZHENG, S., CHEN, Y. & ZHOU, J. 2021. Circulating tumor cells: biology and clinical significance. *Signal Transduct Target Ther,* 6**,** 404.

LIN, J. X. & LEONARD, W. J. 2019. Fine-Tuning Cytokine Signals. *Annu Rev Immunol,* 37**,** 295-324.

LIU, J., BLAKE, S. J., YONG, M. C., HARJUNPÄÄ, H., NGIOW, S. F., TAKEDA, K., YOUNG, A., O'DONNELL, J. S., ALLEN, S., SMYTH, M. J. & TENG, M. W. 2016. Improved Efficacy of Neoadjuvant Compared to Adjuvant Immunotherapy to Eradicate Metastatic Disease. *Cancer Discov,* 6**,** 1382-1399.

MAGOULIOTIS, D. E., LAFAZANIS, K., KOUTSOUGIANNI, F., SAKELLARIDIS, N., IOANNOU, M., ZACHAROULIS, D. & DIMAS, K. 2022. Establishment of Patient-derived Orthotopic Xenografts (PDX) as Models for Pancreatic Ductal Adenocarcinoma. *In Vivo,* 36**,** 1114-1119.

MAKOHON-MOORE, A. & IACOBUZIO-DONAHUE, C. A. 2016. Pancreatic cancer biology and genetics from an evolutionary perspective. *Nat Rev Cancer,* 16**,** 553-65.

MARABELLE, A., KOHRT, H., CAUX, C. & LEVY, R. 2014. Intratumoral immunization: a new paradigm for cancer therapy. *Clin Cancer Res,* 20**,** 1747-56.

MARGOLIN, K., MORISHIMA, C., VELCHETI, V., MILLER, J. S., LEE, S. M., SILK, A. W., HOLTAN, S. G., LACROIX, A. M., FLING, S. P., KAISER, J. C., EGAN, J. O., JONES, M., RHODE, P. R., ROCK, A. D., CHEEVER, M. A., WONG, H. C. & ERNSTOFF, M. S. 2018. Phase I Trial of ALT-803, A Novel Recombinant IL15 Complex, in Patients with Advanced Solid Tumors. *Clin Cancer Res,* 24**,** 5552-5561.

MATHIOS, D., PARK, C. K., MARCUS, W. D., ALTER, S., RHODE, P. R., JENG, E. K., WONG, H. C., PARDOLL, D. M. & LIM, M. 2016. Therapeutic administration of IL-15 superagonist complex ALT-803 leads to long-term survival and durable antitumor immune response in a murine glioblastoma model. *Int J Cancer,* 138**,** 187-94.

MATTEI, F., SCHIAVONI, G., BELARDELLI, F. & TOUGH, D. F. 2001. IL-15 is expressed by dendritic cells in response to type I IFN, double-stranded

RNA, or lipopolysaccharide and promotes dendritic cell activation. *J Immunol,* 167**,** 1179-87.
MCCOLL, S. R. 2002. Chemokines and dendritic cells: a crucial alliance. *Immunol Cell Biol,* 80**,** 489-96.
MCGUIRE, K. P. 2019. Breast Anatomy and Physiology. *In:* AYDINER, A., IGCI, A. & SORAN, A. (eds.) *Breast Disease: Diagnosis and Pathology, Volume 1.* Cham: Springer International Publishing.
MELLMAN, I., COUKOS, G. & DRANOFF, G. 2011. Cancer immunotherapy comes of age. *Nature,* 480**,** 480-9.
MILLER, J. S., MORISHIMA, C., MCNEEL, D. G., PATEL, M. R., KOHRT, H. E. K., THOMPSON, J. A., SONDEL, P. M., WAKELEE, H. A., DISIS, M. L., KAISER, J. C., CHEEVER, M. A., STREICHER, H., CREEKMORE, S. P., WALDMANN, T. A. & CONLON, K. C. 2018. A First-in-Human Phase I Study of Subcutaneous Outpatient Recombinant Human IL15 (rhIL15) in Adults with Advanced Solid Tumors. *Clin Cancer Res,* 24**,** 1525-1535.
MOORE, M. J., GOLDSTEIN, D., HAMM, J., FIGER, A., HECHT, J. R., GALLINGER, S., AU, H. J., MURAWA, P., WALDE, D., WOLFF, R. A., CAMPOS, D., LIM, R., DING, K., CLARK, G., VOSKOGLOU-NOMIKOS, T., PTASYNSKI, M. & PARULEKAR, W. 2007. Erlotinib Plus Gemcitabine Compared With Gemcitabine Alone in Patients With Advanced Pancreatic Cancer: A Phase III Trial of the National Cancer Institute of Canada Clinical Trials Group. *Journal of Clinical Oncology,* 25**,** 1960-1966.
MOSSER, D. M. & EDWARDS, J. P. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol,* 8**,** 958-69.
MUNGER, W., DEJOY, S. Q., JEYASEELAN, R., TORLEY, L. W., GRABSTEIN, K. H., EISENMANN, J., PAXTON, R., COX, T., WICK, M. M. & KERWAR, S. S. 1995. Studies Evaluating the Antitumor Activity and Toxicity of Interleukin-15, a New T Cell Growth Factor: Comparison with Interleukin-2. *Cellular Immunology,* 165**,** 289-293.
MURPHY, K. & WEAVER, C. 2016. *Janeway's immunobiology*, Garland science.
NAING, A. & HAJJAR, J. 2017. *Immunotherapy*, Springer.
NAKANO, H., LIN, K. L., YANAGITA, M., CHARBONNEAU, C., COOK, D. N., KAKIUCHI, T. & GUNN, M. D. 2009. Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute T helper type 1 immune responses. *Nat Immunol,* 10**,** 394-402.
NAPOLETANO, C., RUGHETTI, A., AGERVIG TARP, M. P., COLEMAN, J., BENNETT, E. P., PICCO, G., SALE, P., DENDA-NAGAI, K., IRIMURA, T., MANDEL, U., CLAUSEN, H., FRATI, L., TAYLOR-

PAPADIMITRIOU, J., BURCHELL, J. & NUTI, M. 2007. Tumor-associated Tn-MUC1 glycoform is internalized through the macrophage galactose-type C-type lectin and delivered to the HLA class I and II compartments in dendritic cells. *Cancer Res,* 67**,** 8358-67.

NARITA, Y., WAKITA, D., OHKUR, T., CHAMOTO, K. & NISHIMURA, T. 2009. Potential differentiation of tumor bearing mouse CD11b+Gr-1+ immature myeloid cells into both suppressor macrophages and immunostimulatory dendritic cells. *Biomed Res,* 30**,** 7-15.

NASCIMENTO 2020. Histological and molecular classification of breast cancer: what do we know? *In:* OTONI, K. M. (ed.).

NG, S. S. M., NAGY, B. A., JENSEN, S. M., HU, X., ALICEA, C., FOX, B. A., FELBER, B. K., BERGAMASCHI, C. & PAVLAKIS, G. N. 2017. Heterodimeric IL15 Treatment Enhances Tumor Infiltration, Persistence, and Effector Functions of Adoptively Transferred Tumor-specific T Cells in the Absence of Lymphodepletion. *Clin Cancer Res,* 23**,** 2817-2830.

O'DONNELL, J. S., TENG, M. W. L. & SMYTH, M. J. 2018. Cancer immunoediting and resistance to T cell-based immunotherapy. *Nat Rev Clin Oncol*.

ORELLANA, E. A. & KASINSKI, A. L. 2016. Sulforhodamine B (SRB) Assay in Cell Culture to Investigate Cell Proliferation. *Bio-protocol,* 6.

ORTH, M., METZGER, P., GERUM, S., MAYERLE, J., SCHNEIDER, G., BELKA, C., SCHNURR, M. & LAUBER, K. 2019. Pancreatic ductal adenocarcinoma: biological hallmarks, current status, and future perspectives of combined modality treatment approaches. *Radiat Oncol,* 14**,** 141.

OUZOUNOVA, M., LEE, E., PIRANLIOGLU, R., EL ANDALOUSSI, A., KOLHE, R., DEMIRCI, M. F., MARASCO, D., ASM, I., CHADLI, A., HASSAN, K. A., THANGARAJU, M., ZHOU, G., ARBAB, A. S., COWELL, J. K. & KORKAYA, H. 2017. Monocytic and granulocytic myeloid derived suppressor cells differentially regulate spatiotemporal tumour plasticity during metastatic cascade. *Nat Commun,* 8**,** 14979.

O'DONNELL, J. S., TENG, M. W. L. & SMYTH, M. J. 2019. Cancer immunoediting and resistance to T cell-based immunotherapy. *Nature Reviews Clinical Oncology,* 16**,** 151-167.

PALMER, A. H. 1934. The preparation of a crystalline globulin from 3. biol.

PANDOL, S. J. 2010. Colloquium Series on Integrated Systems Physiology: From Molecule to Function to Disease. *The Exocrine Pancreas.* San Rafael (CA): Morgan & Claypool Life Sciences

PARKIN, J. & COHEN, B. 2001. An overview of the immune system. *Lancet,* 357**,** 1777-89.

PEROU, C. M., SØRLIE, T., EISEN, M. B., VAN DE RIJN, M., JEFFREY, S. S., REES, C. A., POLLACK, J. R., ROSS, D. T., JOHNSEN, H., AKSLEN, L. A., FLUGE, Ø., PERGAMENSCHIKOV, A., WILLIAMS, C., ZHU, S. X., LØNNING, P. E., BØRRESEN-DALE, A.-L., BROWN, P. O. & BOTSTEIN, D. 2000. Molecular portraits of human breast tumours. *Nature,* 406**,** 747-752.

PHAM, T. N. D., SHIELDS, M. A., SPAULDING, C., PRINCIPE, D. R., LI, B., UNDERWOOD, P. W., TREVINO, J. G., BENTREM, D. J. & MUNSHI, H. G. 2021. Preclinical Models of Pancreatic Ductal Adenocarcinoma and Their Utility in Immunotherapy Studies. *Cancers (Basel),* 13.

PLANTINGA, M., GUILLIAMS, M., VANHEERSWYNGHELS, M., DESWARTE, K., BRANCO-MADEIRA, F., TOUSSAINT, W., VANHOUTTE, L., NEYT, K., KILLEEN, N., MALISSEN, B., HAMMAD, H. & LAMBRECHT, B. N. 2013. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity,* 38**,** 322-35.

PORTER, D. L., LEVINE, B. L., KALOS, M., BAGG, A. & JUNE, C. H. 2011. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med,* 365**,** 725-33.

PRAT, A. & PEROU, C. M. 2011. Deconstructing the molecular portraits of breast cancer. *Mol Oncol,* 5**,** 5-23.

PROPPER, D. J. & BALKWILL, F. R. 2022. Harnessing cytokines and chemokines for cancer therapy. *Nat Rev Clin Oncol,* 19**,** 237-253.

PULASKI, B. A. & OSTRAND-ROSENBERG, S. 2001. Mouse 4T1 breast tumor model. *Curr Protoc Immunol,* Chapter 20**,** Unit 20.2.

QU, C., BRINCK-JENSEN, N. S., ZANG, M. & CHEN, K. 2014. Monocyte-derived dendritic cells: targets as potent antigen-presenting cells for the design of vaccines against infectious diseases. *Int J Infect Dis,* 19**,** 1-5.

RANDOLPH, G. J., ANGELI, V. & SWARTZ, M. A. 2005. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat Rev Immunol,* 5**,** 617-28.

RANGANATHAN, S., SIMPSON, K. J., SHAW, D. C. & NICHOLAS, K. R. 1999. The whey acidic protein family: a new signature motif and three-dimensional structure by comparative modeling. *J Mol Graph Model,* 17**,** 106-13, 134-6.

RAO, D. S. 2018. Chapter 19 - Overview and Compartmentalization of the Immune System. *In:* HOFFMAN, R., BENZ, E. J., SILBERSTEIN, L. E., HESLOP, H. E., WEITZ, J. I., ANASTASI, J., SALAMA, M. E. & ABUTALIB, S. A. (eds.) *Hematology (Seventh Edition)*. Elsevier.

REJNIAK, K. A. 2016. Circulating Tumor Cells: When a Solid Tumor Meets a Fluid Microenvironment. *Adv Exp Med Biol,* 936**,** 93-106.

RESTIFO, N. P., DUDLEY, M. E. & ROSENBERG, S. A. 2012. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nat Rev Immunol,* 12**,** 269-81.

ROBERT, C. A decade of immune-checkpoint inhibitors in cancer therapy.

ROBERT, C. 2020. A decade of immune-checkpoint inhibitors in cancer therapy.

ROBINSON, T. O. & SCHLUNS, K. S. 2017. The potential and promise of IL-15 in immuno-oncogenic therapies. *Immunol Lett,* 190**,** 159-168.

ROMEE, R., COOLEY, S., BERRIEN-ELLIOTT, M. M., WESTERVELT, P., VERNERIS, M. R., WAGNER, J. E., WEISDORF, D. J., BLAZAR, B. R., USTUN, C., DEFOR, T. E., VIVEK, S., PECK, L., DIPERSIO, J. F., CASHEN, A. F., KYLLO, R., MUSIEK, A., SCHAFFER, A., ANADKAT, M. J., ROSMAN, I., MILLER, D., EGAN, J. O., JENG, E. K., ROCK, A., WONG, H. C., FEHNIGER, T. A. & MILLER, J. S. 2018. First-in-human phase 1 clinical study of the IL-15 superagonist complex ALT-803 to treat relapse after transplantation. *Blood,* 131**,** 2515-2527.

ROSENBERG, S. A. 2014. IL-2: the first effective immunotherapy for human cancer. *J Immunol,* 192**,** 5451-8.

ROSENBERG, S. A., LOTZE, M. T., YANG, J. C., AEBERSOLD, P. M., LINEHAN, W. M., SEIPP, C. A. & WHITE, D. E. 1989. Experience with the use of high-dose interleukin-2 in the treatment of 652 cancer patients. *Ann Surg,* 210**,** 474-84; discussion 484-5.

ROSENBERG, S. A., PACKARD, B. S., AEBERSOLD, P. M., SOLOMON, D., TOPALIAN, S. L., TOY, S. T., SIMON, P., LOTZE, M. T., YANG, J. C., SEIPP, C. A. & ET AL. 1988. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. *N Engl J Med,* 319**,** 1676-80.

ROSENBERG, S. A. & RESTIFO, N. P. 2015. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science,* 348**,** 62-8.

RUTTER, N. 2000. RB Strassler: The Landmark Thucydides: a Comprehensive Guide to the Peloponnesian War. Pp. xxxiii+ 711, ills. New York, etc.: The Free Press, 1996. Cased, $45. ISBN: 0-684-82815-4. *The Classical Review,* 50.

SANTAMBROGIO, L., BERENDAM, S. J. & ENGELHARD, V. H. 2019. The Antigen Processing and Presentation Machinery in Lymphatic Endothelial Cells. *Front Immunol,* 10**,** 1033.

SCHMID, P., CORTES, J., PUSZTAI, L., MCARTHUR, H., KÜMMEL, S., BERGH, J., DENKERT, C., PARK, Y. H., HUI, R., HARBECK, N., TAKAHASHI, M., FOUKAKIS, T., FASCHING, P. A., CARDOSO, F., UNTCH, M., JIA, L., KARANTZA, V., ZHAO, J., AKTAN, G., DENT, R. & O'SHAUGHNESSY, J. 2020. Pembrolizumab for Early Triple-Negative Breast Cancer. *New England Journal of Medicine,* 382**,** 810-821.

SCHREIBER, R. D., OLD, L. J. & SMYTH, M. J. 2011. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science,* 331**,** 1565-70.

SEER TRAINING MODULES, U. S. N. N.

SERETI, E., KARAGIANELLOU, T., KOTSONI, I., MAGOULIOTIS, D., KAMPOSIORAS, K., ULUKAYA, E., SAKELLARIDIS, N., ZACHAROULIS, D. & DIMAS, K. 2018. Patient Derived Xenografts (PDX) for personalized treatment of pancreatic cancer: emerging allies in the war on a devastating cancer? *Journal of proteomics,* 188**,** 107-118.

SERETI, E., PAPAPOSTOLOU, I. & DIMAS, K. 2023. Pancreatic Cancer Organoids: An Emerging Platform for Precision Medicine? *Biomedicines,* 11.

SHANKARAN, V., IKEDA, H., BRUCE, A. T., WHITE, J. M., SWANSON, P. E., OLD, L. J. & SCHREIBER, R. D. 2001. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature,* 410**,** 1107-11.

SHENG, J., CHEN, Q., SONCIN, I., NG, S. L., KARJALAINEN, K. & RUEDL, C. 2017. A Discrete Subset of Monocyte-Derived Cells among Typical Conventional Type 2 Dendritic Cells Can Efficiently Cross-Present. *Cell Rep,* 21**,** 1203-1214.

SOSA, M. S., BRAGADO, P. & AGUIRRE-GHISO, J. A. 2014. Mechanisms of disseminated cancer cell dormancy: an awakening field. *Nat Rev Cancer,* 14**,** 611-22.

SOULE, H. D., VAZGUEZ, J., LONG, A., ALBERT, S. & BRENNAN, M. 1973. A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst,* 51**,** 1409-16.

SPIEGEL, A., BROOKS, M. W., HOUSHYAR, S., REINHARDT, F., ARDOLINO, M., FESSLER, E., CHEN, M. B., KRALL, J. A., DECOCK, J., ZERVANTONAKIS, I. K., IANNELLO, A., IWAMOTO, Y., CORTEZ-RETAMOZO, V., KAMM, R. D., PITTET, M. J., RAULET, D. H. &

WEINBERG, R. A. 2016. Neutrophils Suppress Intraluminal NK Cell-Mediated Tumor Cell Clearance and Enhance Extravasation of Disseminated Carcinoma Cells. *Cancer Discov,* 6**,** 630-49.

STARNES, C. O. 1992. Coley's toxins in perspective. *Nature,* 357**,** 11-2.

STELLAS, D., KARALIOTA, S., STRAVOKEFALOU, V., NAGY, B. A., FELBER, B. K. & PAVLAKIS, G. N. 2019. Abstract 3259: heterodimeric IL-15 monotherapy results in complete regression of EO771 murine breast tumors through cDC1-lymphocyte interactions and induction of antitumor immunity. *Cancer Research,* 79**,** 3259-3259.

SUNG, H., FERLAY, J., SIEGEL, R. L., LAVERSANNE, M., SOERJOMATARAM, I., JEMAL, A. & BRAY, F. 2021. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians,* 71**,** 209-249.

TAKEUCHI, O. & AKIRA, S. 2010. Pattern recognition receptors and inflammation. *Cell,* 140**,** 805-20.

TALPAZ, M., MCCREDIE, K. B., MAVLIGIT, G. M. & GUTTERMAN, J. U. 1983. Leukocyte interferon-induced myeloid cytoreduction in chronic myelogenous leukemia. *Blood,* 62**,** 689-92.

TAMOUTOUNOUR, S., GUILLIAMS, M., MONTANANA SANCHIS, F., LIU, H., TERHORST, D., MALOSSE, C., POLLET, E., ARDOUIN, L., LUCHE, H., SANCHEZ, C., DALOD, M., MALISSEN, B. & HENRI, S. 2013. Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin. *Immunity,* 39**,** 925-38.

TAMOUTOUNOUR, S., HENRI, S., LELOUARD, H., DE BOVIS, B., DE HAAR, C., VAN DER WOUDE, C. J., WOLTMAN, A. M., REYAL, Y., BONNET, D., SICHIEN, D., BAIN, C. C., MOWAT, A. M., REIS E SOUSA, C., POULIN, L. F., MALISSEN, B. & GUILLIAMS, M. 2012. CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis. *Eur J Immunol,* 42**,** 3150-66.

TANG, F., ZHAO, L., JIANG, Y., BA, D., CUI, L. & HE, W. 2008. Activity of Recombinant Human Interleukin-15 against Tumor Recurrence and Metastasis in Mice. *Cellular & Molecular Immunology,* 5**,** 189-196.

TAY, R. E., RICHARDSON, E. K. & TOH, H. C. 2021. Revisiting the role of CD4(+) T cells in cancer immunotherapy-new insights into old paradigms. *Cancer Gene Ther,* 28**,** 5-17.

TERME, M., ULLRICH, E., DELAHAYE, N. F., CHAPUT, N. & ZITVOGEL, L. 2008. Natural killer cell-directed therapies: moving from unexpected results to successful strategies. *Nat Immunol,* 9**,** 486-94.

THEISEN, D. J., DAVIDSON, J. T. T., BRISENO, C. G., GARGARO, M., LAURON, E. J., WANG, Q., DESAI, P., DURAI, V., BAGADIA, P., BRICKNER, J. R., BEATTY, W. L., VIRGIN, H. W., GILLANDERS, W. E., MOSAMMAPARAST, N., DIAMOND, M. S., SIBLEY, L. D., YOKOYAMA, W., SCHREIBER, R. D., MURPHY, T. L. & MURPHY, K. M. 2018. WDFY4 is required for cross-presentation in response to viral and tumor antigens. *Science,* 362**,** 694-699.

THOMAS, L. 1982. On immunosurveillance in human cancer. *Yale J Biol Med,* 55**,** 329-33.

TOPALIAN, S. L., HODI, F. S., BRAHMER, J. R., GETTINGER, S. N., SMITH, D. C., MCDERMOTT, D. F., POWDERLY, J. D., CARVAJAL, R. D., SOSMAN, J. A., ATKINS, M. B., LEMING, P. D., SPIGEL, D. R., ANTONIA, S. J., HORN, L., DRAKE, C. G., PARDOLL, D. M., CHEN, L., SHARFMAN, W. H., ANDERS, R. A., TAUBE, J. M., MCMILLER, T. L., XU, H., KORMAN, A. J., JURE-KUNKEL, M., AGRAWAL, S., MCDONALD, D., KOLLIA, G. D., GUPTA, A., WIGGINTON, J. M. & SZNOL, M. 2012. Safety, Activity, and Immune Correlates of Anti–PD-1 Antibody in Cancer. *New England Journal of Medicine,* 366**,** 2443-2454.

TOYONAGA, K., TORIGOE, S., MOTOMURA, Y., KAMICHI, T., HAYASHI, J. M., MORITA, Y. S., NOGUCHI, N., CHUMA, Y., KIYOHARA, H., MATSUO, K., TANAKA, H., NAKAGAWA, Y., SAKUMA, T., OHMURAYA, M., YAMAMOTO, T., UMEMURA, M., MATSUZAKI, G., YOSHIKAI, Y., YANO, I., MIYAMOTO, T. & YAMASAKI, S. 2016. C-Type Lectin Receptor DCAR Recognizes Mycobacterial Phosphatidyl-Inositol Mannosides to Promote a Th1 Response during Infection. *Immunity,* 45**,** 1245-1257.

TZELEPIS, F., VERWAY, M., DAOUD, J., GILLARD, J., HASSANI-ARDAKANI, K., DUNN, J., DOWNEY, J., GENTILE, M. E., JAWORSKA, J., SANCHEZ, A. M., NEDELEC, Y., VALI, H., TABRIZIAN, M., KRISTOF, A. S., KING, I. L., BARREIRO, L. B. & DIVANGAHI, M. 2015. Annexin1 regulates DC efferocytosis and cross-presentation during Mycobacterium tuberculosis infection. *J Clin Invest,* 125**,** 752-68.

VAN AUDENAERDE, J. R., MARCQ, E., VON SCHEIDT, B., DAVEY, A. S., OLIVER, A. J., DE WAELE, J., QUATANNENS, D., VAN LOENHOUT, J., PAUWELS, P., ROEYEN, G., LARDON, F., SLANEY, C. Y.,

PEETERS, M., KERSHAW, M. H., DARCY, P. K. & SMITS, E. L. 2020. Novel combination immunotherapy for pancreatic cancer: potent anti-tumor effects with CD40 agonist and interleukin-15 treatment. *Clin Transl Immunology,* 9**,** e1165.

VAN DER LEUN, A. M., THOMMEN, D. S. & SCHUMACHER, T. N. 2020. CD8+ T cell states in human cancer: insights from single-cell analysis. *Nature Reviews Cancer,* 20**,** 218-232.

VEGLIA, F., SANSEVIERO, E. & GABRILOVICH, D. I. 2021. Myeloid-derived suppressor cells in the era of increasing myeloid cell diversity. *Nature Reviews Immunology,* 21**,** 485-498.

VESELY, M. D., KERSHAW, M. H., SCHREIBER, R. D. & SMYTH, M. J. 2011. Natural innate and adaptive immunity to cancer. *Annu Rev Immunol,* 29**,** 235-71.

VESELY, M. D. & SCHREIBER, R. D. 2013. Cancer immunoediting: antigens, mechanisms, and implications to cancer immunotherapy. *Annals of the New York Academy of Sciences,* 1284**,** 1-5.

VIRCHOW 1863. Cellular Pathology as Based Upon Physiological and Pathological Histology.

VIVIER, E., TOMASELLO, E., BARATIN, M., WALZER, T. & UGOLINI, S. 2008. Functions of natural killer cells. *Nat Immunol,* 9**,** 503-10.

VON HOFF, D. D., ERVIN, T., ARENA, F. P., CHIOREAN, E. G., INFANTE, J., MOORE, M., SEAY, T., TJULANDIN, S. A., MA, W. W., SALEH, M. N., HARRIS, M., RENI, M., DOWDEN, S., LAHERU, D., BAHARY, N., RAMANATHAN, R. K., TABERNERO, J., HIDALGO, M., GOLDSTEIN, D., VAN CUTSEM, E., WEI, X., IGLESIAS, J. & RENSCHLER, M. F. 2013. Increased Survival in Pancreatic Cancer with nab-Paclitaxel plus Gemcitabine. *New England Journal of Medicine,* 369**,** 1691-1703.

WAKIM, L. M., WAITHMAN, J., VAN ROOIJEN, N., HEATH, W. R. & CARBONE, F. R. 2008. Dendritic cell-induced memory T cell activation in nonlymphoid tissues. *Science,* 319**,** 198-202.

WALDMAN, A. D., FRITZ, J. M. & LENARDO, M. J. 2020. A guide to cancer immunotherapy: from T cell basic science to clinical practice.

WALDMANN, T. A. 2006. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat Rev Immunol,* 6**,** 595-601.

WALDMANN, T. A. 2018. Cytokines in Cancer Immunotherapy. *Cold Spring Harb Perspect Biol,* 10.

WALDMANN, T. A., LUGLI, E., ROEDERER, M., PERERA, L. P., SMEDLEY, J. V., MACALLISTER, R. P., GOLDMAN, C. K., BRYANT, B. R.,

DECKER, J. M., FLEISHER, T. A., LANE, H. C., SNELLER, M. C., KURLANDER, R. J., KLEINER, D. E., PLETCHER, J. M., FIGG, W. D., YOVANDICH, J. L. & CREEKMORE, S. P. 2011. Safety (toxicity), pharmacokinetics, immunogenicity, and impact on elements of the normal immune system of recombinant human IL-15 in rhesus macaques. *Blood,* 117**,** 4787-95.

WALDMANN, T. A., MILJKOVIC, M. D. & CONLON, K. C. 2020. Interleukin-15 (dys)regulation of lymphoid homeostasis: Implications for therapy of autoimmunity and cancer. *J Exp Med,* 217.

WANG, S., ZHENG, Y., YANG, F., ZHU, L., ZHU, X.-Q., WANG, Z.-F., WU, X.-L., ZHOU, C.-H., YAN, J.-Y., HU, B.-Y., KONG, B., FU, D.-L., BRUNS, C., ZHAO, Y., QIN, L.-X. & DONG, Q.-Z. 2021. The molecular biology of pancreatic adenocarcinoma: translational challenges and clinical perspectives.

WCULEK, S. K., CUETO, F. J., MUJAL, A. M., MELERO, I., KRUMMEL, M. F. & SANCHO, D. 2019. Dendritic cells in cancer immunology and immunotherapy. *Nat Rev Immunol*.

WELSH, J. 2013. Chapter 40 - Animal Models for Studying Prevention and Treatment of Breast Cancer. *In:* CONN, P. M. (ed.) *Animal Models for the Study of Human Disease.* Boston: Academic Press.

WOLCHOK, J. D., KLUGER, H., CALLAHAN, M. K., POSTOW, M. A., RIZVI, N. A., LESOKHIN, A. M., SEGAL, N. H., ARIYAN, C. E., GORDON, R. A., REED, K., BURKE, M. M., CALDWELL, A., KRONENBERG, S. A., AGUNWAMBA, B. U., ZHANG, X., LOWY, I., INZUNZA, H. D., FEELY, W., HORAK, C. E., HONG, Q., KORMAN, A. J., WIGGINTON, J. M., GUPTA, A. & SZNOL, M. 2013. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med,* 369**,** 122-33.

WU, J. & LANIER, L. L. 2003. Natural killer cells and cancer. *Adv Cancer Res,* 90**,** 127-56.

XU, W., JONES, M., LIU, B., ZHU, X., JOHNSON, C. B., EDWARDS, A. C., KONG, L., JENG, E. K., HAN, K., MARCUS, W. D., RUBINSTEIN, M. P., RHODE, P. R. & WONG, H. C. 2013. Efficacy and mechanism-of-action of a novel superagonist interleukin-15: interleukin-15 receptor alphaSu/Fc fusion complex in syngeneic murine models of multiple myeloma. *Cancer Res,* 73**,** 3075-86.

YANG, Y., NEO, S. Y., CHEN, Z., CUI, W., CHEN, Y., GUO, M., WANG, Y., XU, H., KURZAY, A., ALICI, E., HOLMGREN, L., HAGLUND, F., WANG, K. & LUNDQVIST, A. 2020. Thioredoxin activity confers

resistance against oxidative stress in tumor-infiltrating NK cells. *J Clin Invest,* 130**,** 5508-5522.

YU, P., STEEL, J. C., ZHANG, M., MORRIS, J. C., WAITZ, R., FASSO, M., ALLISON, J. P. & WALDMANN, T. A. 2012. Simultaneous inhibition of two regulatory T-cell subsets enhanced Interleukin-15 efficacy in a prostate tumor model. *Proc Natl Acad Sci U S A,* 109**,** 6187-92.

YU, P., STEEL, J. C., ZHANG, M., MORRIS, J. C. & WALDMANN, T. A. 2010. Simultaneous blockade of multiple immune system inhibitory checkpoints enhances antitumor activity mediated by interleukin-15 in a murine metastatic colon carcinoma model. *Clin Cancer Res,* 16**,** 6019-28.

YUZHALIN, A. & KUTIKHIN, A. 2014. *Interleukins in cancer biology: their heterogeneous role*, Academic Press.

ZHONG, X., ZHANG, H., ZHU, Y., LIANG, Y., YUAN, Z., LI, J., LI, J., LI, X., JIA, Y., HE, T., ZHU, J., SUN, Y., JIANG, W., ZHANG, H., WANG, C. & KE, Z. 2020. Circulating tumor cells in cancer patients: developments and clinical applications for immunotherapy. *Molecular Cancer,* 19**,** 15.

ZIGMOND, E., VAROL, C., FARACHE, J., ELMALIAH, E., SATPATHY, A. T., FRIEDLANDER, G., MACK, M., SHPIGEL, N., BONECA, I. G., MURPHY, K. M., SHAKHAR, G., HALPERN, Z. & JUNG, S. 2012. Ly6C hi monocytes in the inflamed colon give rise to proinflammatory effector cells and migratory antigen-presenting cells. *Immunity,* 37**,** 1076-90.

Resume

Vasiliki Stravokefalou
B.Sc., M.Sc., PhD Candidate

National Cancer Institute, NIH, USA
Faculty of Medicine, School of Health Sciences, University of Thessaly, Greece
Email: vasostravoke@gmail.com

Education

2017-present **Ph.D. Candidate – Cancer Immunotherapy**
National Cancer Institute at Frederick, NIH, USA - University of Thessaly, Greece
Thesis title: "*The study of cellular immunity on the treatment of cancer"*

2014-2017 **M.Sc. in Molecular Medicine**
National and Kapodistrian University of Athens, Faculty of Medicine and Biomedical Research Foundation of the Academy of Athens (BRFAA) Athens, Greece
Thesis title: "*The study of PIK3CA gain-of-function mutations on the development of chemoresistance in breast cancer mouse models*"

2008-2013 **B.Sc. in Biochemistry and Biotechnology**
University of Thessaly, Faculty of Biochemistry and biotechnology, Larissa, Greece
Thesis title: "*Identification of novel interacting partners of poly(A)-specific ribonuclease (PARN)*"

Research Experience

2017-present **Graduate Program Researcher, NCI, NIH, USA and Faculty of Medicine, School of Medicine, University of Thessaly, Greece**
Advisors: Dr. George N. Pavlakis (Cancer Immunotherapy) and Professor Konstantinos Dimas (Cancer Immunotherapy)

- Specialized in pre-clinical therapeutic experimental designs using cytokines (IL-15 based), inhibitors and chemotherapeutic agents in mouse models of breast and pancreatic cancer, tissue processing for flow cytometry, flow cytometry sample acquiring in Fortessa and Symphony instruments and analysis in FlowJo software

2014-2017 **Master student, Biomedical Research Foundation of the Academy of Athens (BRFAA) Athens, Greece**

Advisors: Drs. Konstantinos Vougas and Dimitris Stellas (Cancer Biology)
- Specialized in mouse models, immunohistochemistry, immunofluorescence, shRNA transfection assay for gene knockdown, cell viability assays, cell culture, Western blotting

2008-2013 **Undergraduate student, Faculty of Biochemistry and biotechnology, School of Health Sciences, University of Thessaly, Larissa, Greece**
Advisor: Professor Nikolaos Balatsos (Biochemistry)
- Specialized in cell culture, co-immunoprecipitation, SDS-PAGE electrophoresis, Western blotting

2010-2011 **Intern student, NeoLab company, Athens, Greece**
Advisor: Dr. Evangelos D Papakonstantinou (Biochemistry)
- Trained in techniques for detection of metabolic disorders; quantitative specification of acyl-carnitines from blood spots of newborns with LC-MS/MS analysis, determination of enzymatic efficiency of biotinidase and G6PD and quantitative specification of pyruvic acid, lactic acid and total galactose with enzymatic assays

Selected Courses

- **Basic Principles of Immunology and Hypersensitivity**
  Foundation for Advanced Education in the Sciences (FAES) at NIH, Organized by National Cancer Institute, Spring term
- **Cancer Immunotherapy**
  Foundation for Advanced Education in the Sciences (FAES) at NIH, Organized by National Cancer Institute, Winter term
- **Immunology Course**
  Experimental Immunology Branch of NCI at NIH

Competencies

- English (IELTS)
- Greek (native speaker)
- **Flow Cytometry Training**
  Experimental Immunology Branch of NCI at NIH
- **Introduction of R Programming Training**
  Foundation for Advanced Education in the Sciences (FAES) at NIH, Organized by National Cancer Institute, Summer term

Awards

2020 Fellows Award for Research Excellence (FARE), NCI of NIH, USA

Poster Presentations

2022 **American Association for Cancer Research (AACR) Annual Meeting, New Orleans, Louisiana, USA**
hetIL-15 decreases tumor cell dissemination and colonization and synergizes with chemotherapy and surgery to cure murine 4T1 breast tumors. **Stravokefalou V**, Stellas D, Karaliota S, Myers B, Bergamaschi C, Dimas K, Felber KB, Pavlakis NG
https://aacrjournals.org/cancerres/article/82/12_Supplement/2441/699661/Abstract-2441-hetIL-15-decreases-tumor-cell

2022 **American Association for Cancer Research (AACR) Annual Meeting, New Orleans, Louisiana, USA**
heterodimeric IL-15 (hetIL-15) immunotherapy reverses $CD8^{+}$T cell metabolic dysfunction in murine breast tumors. Stellas D, Karaliota S, **Stravokefalou V**, Myers B, Felber KB, Pavlakis NG
https://aacrjournals.org/cancerres/article/82/12_Supplement/5603/704526

2021 **American Association for Cancer Research (AACR) Annual Virtual Meeting, USA**
heterodimeric IL-15 (hetIL-15) affects conventional dendritic cells and a distinct novel dendritic cell population in different mouse cancer models of breast and pancreatic cancer. **Stravokefalou V**, Stellas D, Karaliota S, Nagy AB, Guerin T, Kozlov S, Felber KB, Pavlakis NG
https://aacrjournals.org/cancerres/article/81/13_Supplement/2727/668609/Abstract-2727-Heterodimeric-IL-15-hetIL-15-affects

2021 **Center for Cancer Research Fellows & Young Investigators (CCR-FYI) Colloquium at NCI Virtual Meeting, USA**
A novel dendritic cell population infiltrates murine breast cancer tumors along with convectional dendritic cells, enhancing the immune response after heterodimeric IL-15 (hetIL-15) monotherapy. Stellas D, **Stravokefalou V**, Karaliota S, Nagy BA, Bergamaschi C, Dimas K, Felber KB, Pavlakis NG

2020 **American Association for Cancer Research (AACR) Annual Virtual meeting, USA**
Complete regression of murine breast tumors by hetIL-15FC monotherapy correlates with infiltration by T, NK, cDC1 cells and a novel population of

dendritic cells. Karaliota S, Stellas D, **Stravokefalou V**, Nagy BA, Bergamaschi C, Felber KB, Pavlakis NG
https://aacrjournals.org/cancerres/article/80/16_Supplement/5694/644041/Abstract-5694-Complete-regression-of-murine-breast

2020 **American Association for Cancer Research (AACR) Annual Virtual meeting, USA**
Combination of hetIL-15 with chemotherapy in triple negative breast and pancreatic cancer mouse models increases tumor necrosis and alleviates metastatic disease. Stellas D, **Stravokefalou V**, Karaliota S, Nagy BA, Koslov S, Dimas K, Felber KB, Pavlakis NG
https://aacrjournals.org/cancerres/article/80/16_Supplement/6651/644586/Abstract-6651-Combination-of-hetIL-15-with

2019 **American Association for Cancer Research (AACR) Annual Meeting, Atlanta, Georgia, USA**
heterodimeric IL-15 monotherapy results in complete regression of EO771 murine breast tumors through cDC1-lymphocyte interactions and induction of antitumor immunity. Stellas D, Karaliota S, **Stravokefalou V**, Nagy BA, Felber KB, Pavlakis NG
https://aacrjournals.org/cancerres/article/79/13_Supplement/3259/635473/Abstract-3259-heterodimeric-IL-15-monotherapy

2019 **15th Annual NIH Graduate Student Research Symposium, Bethesda, Maryland, USA**
Dose sparing effect and prolonged survival effect of heterodimeric interleukin-15 (IL-15) in combination with gemcitabine in KPC GEM model of pancreatic cancer. Stellas D, **Stravokefalou V**, Karaliota S, Kozlov S, Kalen JD, Dimas K, Felber KB, Pavlakis NG

2014 **65th Conference of Hellenic Company of Biochemistry and Molecular Biology, Thessaloniki, Greece**
Cleavage and Polyadenylation Factor Im directly interacts with Poly(A)-Specific ribonuclease; emerging roles in miRNA biogenesis. Tsiporis A, **Stravokefalou V**, Samiotaki M, Scutelnic D, Kyritsis A, Panayotou G, Balatsos N.A.A

Talks

2022 **Center for Cancer Research Fellows & Young Investigators (CCR-FYI) Colloquium at NCI Virtual Meeting, USA**
hetIL-15 decreases tumor cell dissemination and colonization and augments the outcomes of chemotherapy and surgery to cure murine 4T1 breast tumors. **Stravokefalou V**, Stellas D, Karaliota S, Myers B, Bergamaschi C, Dimas K, Felber KB, Pavlakis NG

Publications (peer-reviewed)

Stravokefalou V, Stellas D, Karaliota S, Nagy BA, Valentin A, Bergamaschi C, Dimas K, Pavlakis GN. Heterodimeric IL-15 (hetIL-15) reduces circulating tumor cells and metastasis formation improving chemotherapy and surgery in 4T1 mouse model of TNBC. Frontiers in Immunology. 2022;13. https://doi.org/10.3389/fimmu.2022.1014802

Bergamaschi, C., **Stravokefalou, V.**, Stellas, D., Karaliota, S., Felber, B.K., and Pavlakis, G.N.: Heterodimeric IL-15 in Cancer Immunotherapy. *Cancers* 13: 837, 2021. https://doi.org/10.3390/cancers13040837

Stellas, D., Karaliota, S., **Stravokefalou**, V., Nagy, B.A., Matthew A., Bergamaschi C, Felber, B.K., and Pavlakis, G.N. Tumor eradication by hetIL-15 locoregional administration is associated with an induced intratumoral CD103intCD11b+ dendritic cell population. 10.1016/j.celrep.2023.112501

Publications

(The first page of each publication is presented)

cancers

MDPI

Review

Heterodimeric IL-15 in Cancer Immunotherapy

Cristina Bergamaschi [1], Vasiliki Stravokefalou [2], Dimitris Stellas [2], Sevasti Karaliota [2,3], Barbara K. Felber [1] and George N. Pavlakis [2,*]

[1] Human Retrovirus Pathogenesis Section, Vaccine Branch, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, MD 21702-1201, USA; cristina.bergamaschi@nih.gov (C.B.); barbara.felber@nih.gov (B.K.F.)
[2] Human Retrovirus Section, Vaccine Branch, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, MD 21702-1201, USA; vasiliki.stravokefalou@nih.gov (V.S.); dimitrios.stellas@nih.gov (D.S.); sevasti.karaliota@nih.gov (S.K.)
[3] Basic Science Program, Frederick National Laboratory for Cancer Research, Frederick, MD 21702-1201, USA
* Correspondence: george.pavlakis@nih.gov

Simple Summary: The rapidly expanding field of cancer immunotherapy uses diverse technologies, including cytokines, T cells, and antibody administration, with the aim to induce effective immune responses leading to tumor control. Interleukin-15 (IL-15), a cytokine discovered in 1994, supports the homeostasis of cytotoxic immune cells and shows promise as an anti-tumor agent. Many studies have elucidated IL-15 synthesis, regulation and biological function and explored its therapeutic efficacy in preclinical cancer models. *Escherichia coli*-derived single-chain IL-15 was tested in the first in-human trial in cancer patients. Its effects were limited by the biology of IL-15, which in vivo comprises a complex of the IL-15 chain with the IL-15 receptor alpha (IL-15Rα) chain, together forming the IL-15 heterodimer (hetIL-15). Currently, single-chain IL-15 and several heterodimeric IL-15:IL-15Rα variants (hetIL-15, N-803 and RLI) are being tested in clinical trials. This review presents a summary of contemporary preclinical and clinical research on IL-15.

Abstract: Immunotherapy has emerged as a valuable strategy for the treatment of many cancer types. Interleukin-15 (IL-15) promotes the growth and function of cytotoxic $CD8^+$ T and natural killer (NK) cells. It also enhances leukocyte trafficking and stimulates tumor-infiltrating lymphocytes expansion and activity. Bioactive IL-15 is produced in the body as a heterodimeric cytokine, comprising the IL-15 and the so-called IL-15 receptor alpha chain that are together termed "heterodimeric IL-15" (hetIL-15). hetIL-15, closely resembling the natural form of the cytokine produced in vivo, and IL-15:IL-15Rα complex variants, such as hetIL-15Fc, N-803 and RLI, are the currently available IL-15 agents. These molecules have showed favorable pharmacokinetics and biological function in vivo in comparison to single-chain recombinant IL-15. Preclinical animal studies have supported their anti-tumor activity, suggesting IL-15 as a general method to convert "cold" tumors into "hot", by promoting tumor lymphocyte infiltration. In clinical trials, IL-15-based therapies are overall well-tolerated and result in the expansion and activation of NK and memory $CD8^+$ T cells. Combinations with other immunotherapies are being investigated to improve the anti-tumor efficacy of IL-15 agents in the clinic.

Keywords: cytokine; IL-15; IL-15 receptor alpha (IL-15Rα); heterodimeric IL-15 (hetIL-15); cancer immunotherapy; cytotoxic cells; clinical trials

Citation: Bergamaschi, C.; Stravokefalou, V.; Stellas, D.; Karaliota, S.; Felber, B.K.; Pavlakis, G.N. Heterodimeric IL-15 in Cancer Immunotherapy. *Cancers* **2021**, *13*, 837. https://doi.org/10.3390/cancers13040837

Academic Editor: Leonidas C. Platanias
Received: 15 January 2021
Accepted: 10 February 2021
Published: 17 February 2021

1. Introduction

Interleukin-15 (IL-15) belongs to the γ-chain family of cytokines, that includes also IL-2, IL-4, IL-7, IL-9 and IL-21. These cytokines bind to the same γ-chain in their receptor complex and have unique and overlapping roles in regulating the development, maintenance, trafficking, and function of different lymphocyte subsets [1–5]. IL-15 is a growth,

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 13 January 2023
DOI 10.3389/fimmu.2022.1014802

OPEN ACCESS

EDITED BY
Ernesto T. A. Marques,
University of Pittsburgh, United States

REVIEWED BY
Samir N. Khleif,
Georgetown University, United States
Rafael Dhalia,
Fiocruz Pernambuco, Brazil

*CORRESPONDENCE
Konstantinos Dimas
kdimas@uth.gr
George N. Pavlakis
George.pavlakis@nih.gov

SPECIALTY SECTION
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

RECEIVED 09 August 2022
ACCEPTED 23 December 2022
PUBLISHED 13 January 2023

CITATION
Stravokefalou V, Stellas D, Karaliota S, Nagy BA, Valentin A, Bergamaschi C, Dimas K and Pavlakis GN (2023) Heterodimeric IL-15 (hetIL-15) reduces circulating tumor cells and metastasis formation improving chemotherapy and surgery in 4T1 mouse model of TNBC.
Front. Immunol. 13:1014802.
doi: 10.3389/fimmu.2022.1014802

Heterodimeric IL-15 (hetIL-15) reduces circulating tumor cells and metastasis formation improving chemotherapy and surgery in 4T1 mouse model of TNBC

Vasiliki Stravokefalou[1,2], Dimitris Stellas[1,3], Sevasti Karaliota[1,4], Bethany A. Nagy[5], Antonio Valentin[1], Cristina Bergamaschi[6], Konstantinos Dimas[2*] and George N. Pavlakis[1*]

[1]Human Retrovirus Section, Vaccine Branch, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, MD, United States, [2]Department of Pharmacology, Faculty of Medicine, University of Thessaly, Larissa, Greece, [3]Department of Chemical Biology, National Hellenic Research Foundation, Athens, Greece, [4]Basic Science Program, Frederick National Laboratory for Cancer Research, Leidos Biomedical Research, Frederick, MD, United States, [5]Laboratory Animal Sciences Program, Frederick National Laboratory for Cancer Research, Leidos Biomedical Research, Frederick, MD, United States, [6]Human Retrovirus Pathogenesis Section, Vaccine Branch, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, MD, United States

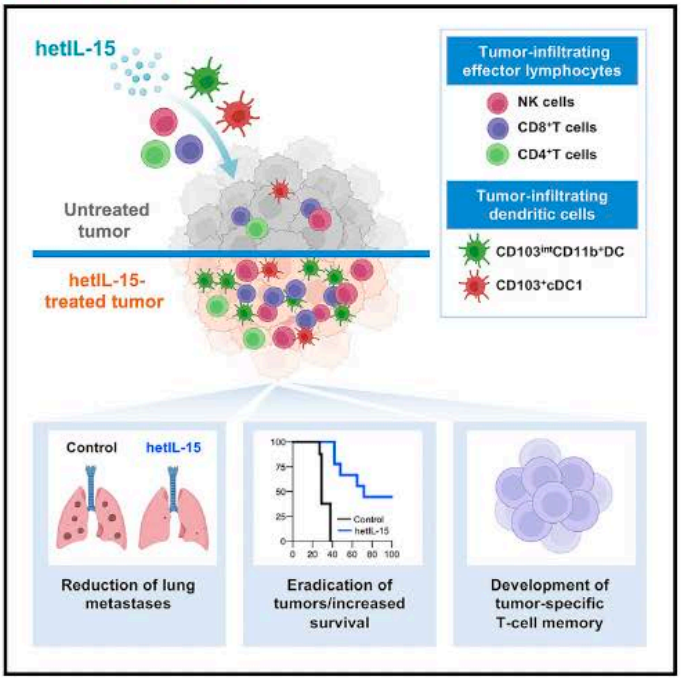
Immunotherapy has emerged as a viable approach in cancer therapy, with cytokines being of great interest. Interleukin IL-15 (IL-15), a cytokine that supports cytotoxic immune cells, has been successfully tested as an anti-cancer and anti-metastatic agent, but combinations with conventional chemotherapy and surgery protocols have not been extensively studied. We have produced heterodimeric IL-15 (hetIL-15), which has shown anti-tumor efficacy in several murine cancer models and is being evaluated in clinical trials for metastatic cancers. In this study, we examined the therapeutic effects of hetIL-15 in combination with chemotherapy and surgery in the 4T1 mouse model of metastatic triple negative breast cancer (TNBC). hetIL-15 monotherapy exhibited potent anti-metastatic effects by diminishing the number of circulating tumor cells (CTCs) and by controlling tumor cells colonization of the lungs. hetIL-15 treatment in combination with doxorubicin resulted in enhanced anti-metastatic activity and extended animal survival. Systemic immune phenotype analysis showed that the chemoimmunotherapeutic regimen shifted the tumor-induced imbalance of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) in favor of cytotoxic effector cells, by simultaneously decreasing PMN-MDSCs and increasing the frequency and activation of effector ($CD8^+$T and NK) cells. Tumor resection supported by neoadjuvant and adjuvant administration of hetIL-15, either alone or in combination with doxorubicin, resulted in the cure

Article

Cell Reports

Tumor eradication by hetIL-15 locoregional therapy correlates with an induced intratumoral CD103^{int}CD11b^{+} dendritic cell population

Graphical abstract



Authors

Dimitris Stellas, Sevasti Karaliota, Vasiliki Stravokefalou, ..., Cristina Bergamaschi, Barbara K. Felber, George N. Pavlakis

Correspondence

george.pavlakis@nih.gov (G.N.P.), dstellas@eie.gr (D.S.)

In brief

Stellas et al. show that hetIL-15 monotherapy orchestrates an interplay between lymphoid and myeloid immune cells leading to the tumor infiltration of cytotoxic lymphocytes, cDC1s, and of a distinct CD103^{int}CD11b^{+} DC subpopulation and resulting in eradication of TNBC tumors in mice, with generation of long-term immunological memory.

Highlights

- hetIL-15 locoregional administration eradicates EO771 tumors
- Locoregional hetIL-15 results in long-lasting specific anti-tumor immunity
- hetIL-15 induces a distinct CD103^{int}CD11b^{+} DC population in TNBC tumor models
- Transcriptional signature of CD103^{int}CD11b^{+}DCs is similar to monocyte-derived DCs

Stellas et al., 2023, Cell Reports 42, 112501
May 30, 2023
https://doi.org/10.1016/j.celrep.2023.112501

CellPress