



Role of dipeptidyl peptidase-4 in the regulation of leucocyte trafficking in hepatocellular carcinoma

Clémence Hollande

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Université Pierre et Marie Curie

École doctorale Physiologie, physiopathologie et thérapeutique (ED 394)

Immunobiologie des cellules dendritiques (Unité mixte Pasteur/Inserm U1223)

**ROLE DE DIPEPTIDYL PEPTIDASE-4 DANS LA
REGULATION DU TRAFIC LEUCOCYTAIRE AU COURS DU
CARCINOME HEPATOCELLULAIRE**

Par Clémence Hollande

Thèse de doctorat d'Immunologie

Dirigée par Matthew L. Albert et codirigée par Stanislas Pol

Présentée et soutenue publiquement le 29 septembre 2017

Devant un jury composé de :

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Jessica ZUCMAN-ROSSI, MD, PhD

Eric TARTOUR, MD, PhD

Olivier ROSMORDUC, MD, PhD

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Doctoral thesis in Immunology

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*« N'attends pas que les événements arrivent comme tu le souhaites ;
décide de vouloir ce qui arrive et tu seras heureux. »*
Epictète

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	9
TABLE OF CONTENTS	11
ABSTRACT	13
ABBREVIATIONS LIST	15
I. INTRODUCTION	19
HEPATOCELLULAR CARCINOMA	22
EPIDEMIOLOGY	22
RISK FACTORS	22
PHYSIOPATHOLOGY	24
DIAGNOSIS	25
STAGING	26
TREATMENT	27
PERSPECTIVES FOR PATIENTS	29
IMMUNOLOGY	29
MOUSE MODELS OF HCC	33
CANCER IMMUNOLOGY	35
ROLE OF T HELPER CELLS	35
EOSINOPHILS	39
IMMUNE CELL TRAFFICKING	45
INTERLEUKIN-33 (IL-33)	54
II. PRE-CLINICAL PROJECT	59
INTRODUCTION	59
RESULTS	59
DPP4 INHIBITION DELAYS GROWTH OF HCC AND ENHANCES EOSINOPHIL MIGRATION	59
DPP4 INHIBITION DELAYS HCC GROWTH IN AN EOSINOPHIL-DEPENDENT MANNER	63
DPP4 INHIBITION ACTS ON CCL11 TO ENHANCE CCR3-ACTIVATED EOSINOPHIL MIGRATION	66
DPP4 INHIBITION DELAYS GROWTH OF HCC INDEPENDENTLY OF THE ADAPTIVE IMMUNE SYSTEM AND ILC	67

TUMOR IL-33 EXPRESSION IS REQUIRED AND SUFFICIENT TO INDUCE EOSINOPHIL-MEDIATED TUMOR IMMUNITY UPON DPP4 INHIBITION	68
EOSINOPHILS CONTRIBUTE TO CHECKPOINT INHIBITOR EFFICACY IN IL-33-PRODUCING TUMORS, A MECHANISM THAT IS ENHANCED BY INHIBITION OF DPP4	72
DISCUSSION	75
DPP4 INHIBITION, ACTING ON CCL11, ENHANCES CCR3-ACTIVATED EOSINOPHIL MIGRATION AND DELAYS HCC TUMOR GROWTH	75
EOSINOPHILS ARE CYTOTOXIC CELLS ABLE TO DIRECTLY ACT ON TUMORS	75
IL-33 MEDIATES ENGAGEMENT OF EOSINOPHILS IN ANTITUMOR RESPONSE	77
EOSINOPHILS CONTRIBUTE TO CHECKPOINT INHIBITOR EFFICACY UPON DPP4I	78
PERSPECTIVES	79
VALIDATE OUR RESULTS IN AN ORTHOTOPIC MODEL OF HCC	79
III. CLINICAL PROJECT	83
INTRODUCTION	83
RESULTS	86
CHARACTERISTICS OF PATIENTS	86
SAFETY	86
IMMUNOLOGICAL EFFECTS OF DPP4 INHIBITION IN PATIENTS WITH HCC	86
DISCUSSION	89
SITAGLIPTIN TREATMENT MODIFIES CHEMOKINES AND CIRCULATING IMMUNE CELLS EXPRESSION IN HUMAN	89
PERSPECTIVES	90
CONDUCT A SAFETY PHASE Ib CLINICAL TRIAL WHERE DPP4I IS ADMINISTERED IN COMBINATION WITH ANTI-PD-(L)1.	90
APPENDIX	93
IV. CONCLUSION	97
V. MATERIALS AND METHODS	99
BIBLIOGRAPHY	105
MANUSCRIPT	123

ABSTRACT

Dipeptidyl peptidase-4 (DPP4 or CD26)–mediated post-translational modification of chemokines has been shown to negatively regulate lymphocyte trafficking, and its inhibition enhances T cell migration and tumor immunity by preserving functional CXCL10. In extending these initial findings to humans and pre-clinical hepatocellular carcinoma models, we discovered a new mechanism whereby DPP4 inhibition improves anti-tumor responses by eosinophil recruitment. Specifically, administration of DPP4 inhibitors (DPP4i) resulted in higher concentrations of CCL11 (or eotaxin) and increased CCR3-mediated eosinophil migration into mouse tumors. Enhanced tumor control was observed upon treatment with DPP4i, an effect strikingly preserved in *Rag2*^{−/−} mice, and abrogated only upon depletion of eosinophils or inhibition of their degranulation. We further demonstrated that tumor expression of IL-33 was necessary and sufficient for eosinophil-mediated anti-tumor responses, and that this mechanism contributed to checkpoint inhibitor efficacy. These findings provide new insight into IL-33- and eosinophil-mediated tumor control, revealed when endogenous mechanisms of DPP4 immune regulation are inhibited.

Keywords: dipeptidyl peptidase-4, eotaxin, hepatocellular carcinoma, eosinophils, IL-33, immune checkpoint inhibitor.

RESUME

La modification post-traductionnelle des chimiokines par la dipeptidyl peptidase-4 (DPP4 ou CD26) régule négativement le trafic des lymphocytes, et son inhibition améliore la migration des lymphocytes T et l'immunité anti-tumorale en préservant la forme fonctionnelle de CXCL10. En étendant ces résultats initiaux aux humains et à un modèle préclinique de carcinome hépatocellulaire, nous avons découvert un nouveau mécanisme par lequel l'inhibition de DPP4 améliore les réponses anti-tumorales par le recrutement des éosinophiles. Plus précisément, l'administration d'inhibiteurs de DPP4 (DPP4i) conduit à des concentrations tumorales plus élevées de CCL11 (ou eotaxine) et à une augmentation de la migration des éosinophiles exprimant CCR3 dans les tumeurs. Un meilleur contrôle de la croissance tumorale a été observé lors du traitement par DPP4i, un effet conservé chez les souris *Rag2*^{−/−} mais abrogé uniquement lors de la déplétion des éosinophiles ou de l'inhibition de leur dégranulation. Nous avons également démontré que l'expression tumorale d'IL-33 était nécessaire et suffisante pour une réponse anti-tumorale médiée par les éosinophiles et que ce mécanisme contribuait à l'efficacité des inhibiteurs de points de contrôle immunitaires. Ces résultats révèlent un nouveau mécanisme par lequel le contrôle tumoral est médiée par IL-33 et les éosinophiles, mécanisme ici révélé lorsque les mécanismes endogènes de régulation immunitaire par DPP4 sont inhibés.

Mots clés : dipeptidyl peptidase-4, eotaxine, carcinome hépatocellulaire, éosinophiles, IL-33, inhibiteur de point de contrôle immunitaire.

ABBREVIATIONS LIST

AAM	alternatively activated macrophages
AASLD	American Association for the Study of Liver Diseases
APC	antigen-presenting cell
BCLC	Barcelona Clinic Liver Cancer Classification
CAF	carcinoma-associated fibroblast
CCL ₄	carbon tetrachloride
CCR3	CC-chemokine receptor 3
CT-scan	computerized tomography scan
CTLA-4	cytotoxic T-lymphocyte antigen-4
CTLs	cytotoxic T cells
DAAs	direct-acting antivirals
DAMP	damaged-associated molecular pattern
DEN	diethylnitrosamine
DNA	deoxyribonucleic acid
DPP	dipeptidyl peptidase
EASL	European Association for the Study of Liver
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EPO/EPX	eosinophil peroxidase
EGF	epidermal growth factor
FAP	fibroblast activation protein
FDA	Food and Drug Administration
FGF	fibroblast growth factor
GAG	glycosaminoglycan
GMM	genetically modified model
GIP	glucose insulintropic peptide
GLP-1	glucagon-like peptide-1

GPCR	G protein-coupled seven transmembrane receptor
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HIV	human immunodeficiency virus
ICAM-1	intracellular adhesion molecule
IDO	indoleamine 2,3-dioxygenase
IFN- γ	interferon- γ
IL	interleukin
MBP	major basic protein
MHC	major histocompatibility complex
MRI	magnetic resonance imaging
mTOR	phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin
NK cells	natural killer cells
NASH	non-alcoholic steatohepatitis
NK- κ B	nuclear factor- κ B
PD-1	programmed cell death protein-1
PD-L1	programmed death-ligand 1
PDGF	platelet-derived growth factor
PGE2	prostaglandin E2
PPs	peroxisome proliferators
PSGL1	P-selectin glycoprotein 1
SCC	oral squamous cell carcinoma
TAA	tumor-associated antigens
TACE	transcatheter arterial chemoembolization
TGF- α/β	tumor growth factor alpha/beta
TAM	telomerase activating molecule
Tc1/2	type 1/2 CD8 ⁺ T cells
TIM	T-cell immunoglobulin domain and mucin domain

TNF- α	tumor necrosis factor alpha
TNM	classification of malignant tumor
T _{reg}	T regulatory cells
VEGF	vascular endothelial growth factor

I. INTRODUCTION

Cancer is one of the three leading causes of death in the world, with infectious and cardiovascular diseases.

Cancer is a disease with abnormal cell growth regulation; normal cells become immortal cancer cells with tissue invasion properties, when regulator cell growth genes are altered. Curing cancer requires that all malignant cells be destroyed without killing the patient. To achieve this, one possible approach is to induce an immune response against the tumor that would discern the tumor cells versus the host cells.

In the 1950s, the concept of immune surveillance, in which cells of the immune system would detect the expression of neoantigens at the surface of tumor cells and lead to their killing, was formulated. Since then, it has been discovered that the relationship between immune system and cancer is considerably more complex, and this first hypothesis has been modified to envisage three phases of tumor growth. The first phase (previously described as the immune surveillance phase) is the elimination phase, where the immune system recognizes and eliminates tumor cells. If eradication is not complete, an equilibrium phase follows, in which tumor cells incur mutations following the selection pressure inflicted by the immune system. This cancer immunoediting process allows tumor cells to survive. In the final escape phase, tumor cells accumulated enough mutations to escape the immune system and grow unhampered (1,2).

More recently, several necessary steps to effectively kill cancer cells have been described by Chen and Mellman as the Cancer–Immunity Cycle (3). Upon release of cancer cell antigens after tumor cell death, dendritic cells will present cancer antigens therefore prime and activate T cells in lymph nodes through co-stimulatory molecules. Tumors can avoid activating an immune response or can elude it, by several mechanisms. Some tumors have low immunogenicity and appear normal to the immune system; they lack mutations and do not produce neoantigens to be presented by major histocompatibility complex (MHC) molecules. Others can present neoantigens but in the absence of co-stimulatory signals, the responding T cells will become tolerant to these antigens. Tumors can also initially express neoantigens to which the immune system will respond, but they can lose them by antibody-induced internalization or antigenic variation. Tumors are also able to produce some

immunosuppressive molecules (tumor growth factor β (TGF- β), interleukin-10 (IL-10), programmed death-ligand 1 (PD-L1)) that abolish the immune responses or recruit T regulatory (T_{reg}) cells. Finally, tumor cells can produce molecules such as collagen that create a physical barrier around the tumor, preventing lymphocyte interaction.

Upon activation, and following gradients of chemokines, T cells will migrate from circulation towards tumor site thanks to adhesion molecules and infiltrate into tumor. Finally, T cells will recognize and kill cancer cells upon interferon- γ (IFN- γ) secretion.

Several therapies were developed to improve immune system efficacy at each of these steps. For example, the most recent immunotherapies developed are targeting negative immune modulators (PD1 and PD-L1). However, to date no drug aiming at improving chemokine-mediated trafficking of T cells into tumors are approved for cancer treatment (Figure 1, step 4).

Some proteins are known to post-translationally negatively regulate chemokines *in vivo*, contributing to a shorter life span of active chemokines leading to impaired leucocyte infiltration. Among these proteins is dipeptidyl peptidase-4 (DPP4), which was shown to delay tumor growth by improving T cell migration towards melanoma and colon carcinoma tumors (4). However, as in most of cancer research, this study focused mainly on the impact of DPP4 in T cell trafficking, while recent studies demonstrated the involvement of other leucocytes in tumor killing.

In this work, I studied the impact of DPP4 activity on leucocyte trafficking in a pre-clinical model of hepatocellular carcinoma (HCC) and evaluated the use of DPP4 inhibitors as an efficient therapy. Additionally, I assessed the safety of DPP4 inhibitors and their effect on immune cell trafficking in hepatocellular carcinoma patients.

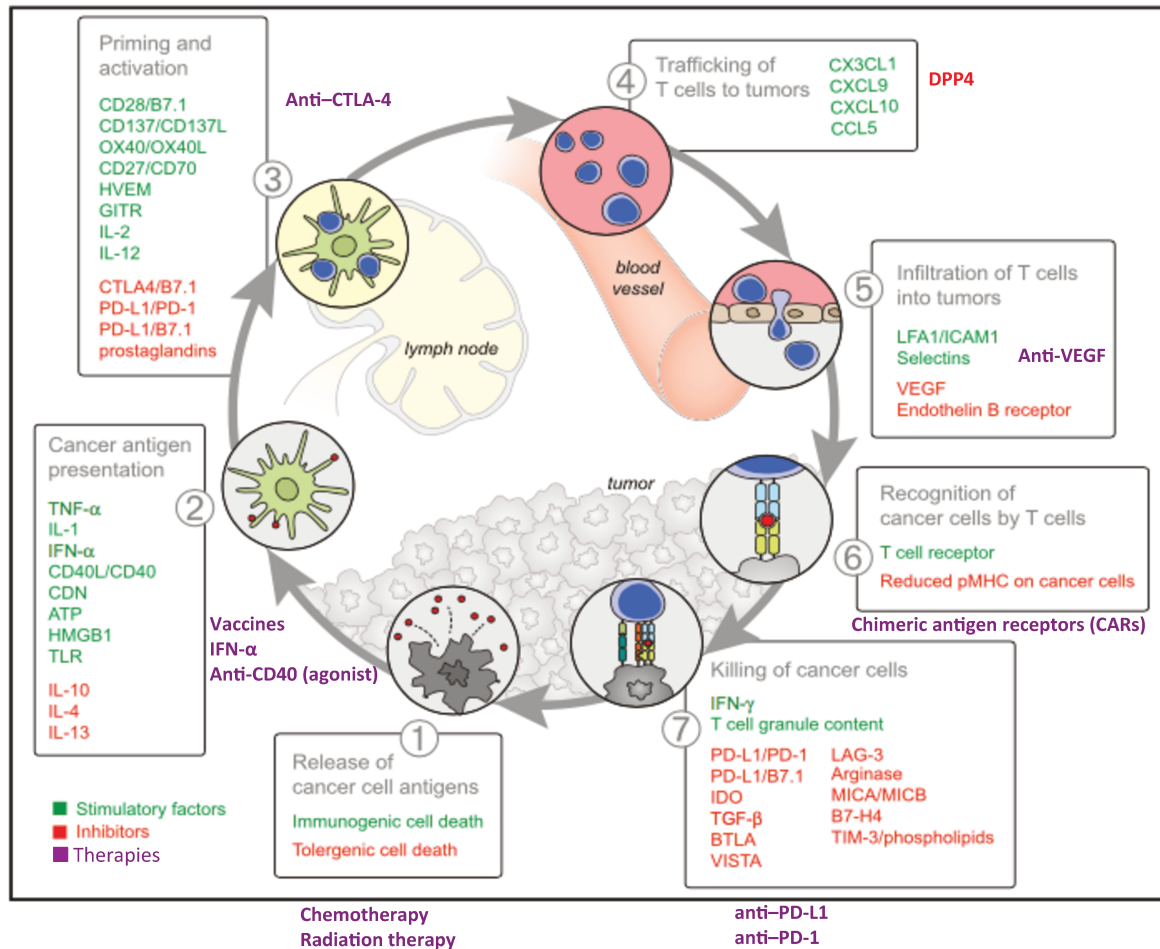


Figure 1: The Cancer–Immunity Cycle with stimulatory, inhibitory factors and existing therapies. Seven steps are necessary to generate immunity to cancer. We focused on the 4th step, the trafficking of T cells to tumors due to the secretion of chemokines. We identified a negative regulator of chemokines, DPP4, and tried to use DPP4 inhibitor as a therapy improving leucocyte trafficking into tumors. IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; CDN, cyclic dinucleotide; ATP, adenosine triphosphate; HMGB1, high-mobility group protein B1; TLR, Toll-like receptor; HVEM, herpes virus entry mediator; GITR, glucocorticoid-induced TNFR family-related gene; CTLA4, cytotoxic T-lymphocyte antigen-4; PD-L1, programmed death-ligand 1; CXCL/CCL, chemokine motif ligands; LFA1, lymphocyte function-associated antigen-1; ICAM1, intracellular adhesion molecule 1; VEGF, vascular endothelial growth factor; IDO, indoleamine 2,3-dioxygenase; TGF, transforming growth factor; BTLA, B- and T-lymphocyte attenuator; VISTA, V-domain Ig suppressor of T cell activation; LAG-3, lymphocyte-activation gene 3 protein; MIC, MHC class I polypeptide-related sequence protein; TIM-3, T cell immunoglobulin domain and mucin domain-3. Adapted from ref. (3).

Hepatocellular carcinoma

Epidemiology

Liver cancer is the fifth most common cancer in men worldwide, and the seventh cause of cancer in women, representing about 7% of the total number of cancer diagnoses (approximately 850,000 new cases each year) and this incidence is still growing (Figure 2). It is the second leading cause of cancer-related deaths (approximately 800,000 per year) (5-7). The annual incidence of liver cancer is very close to the number of deaths per year that it generates, which points out the aggressiveness of this disease (5).

Hepatocellular carcinoma (HCC) represents 85–90% of all primary liver cancers. The incidence of HCC is highest in men, with a male to female ratio of 3 (8).

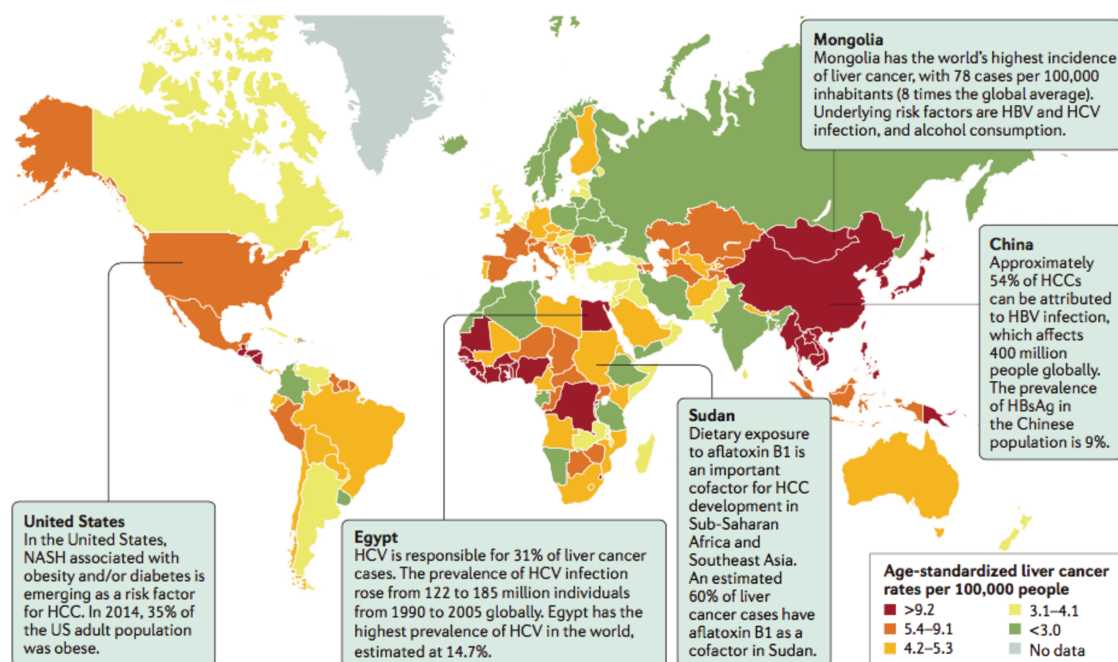


Figure 2: The incidence of HCC. The main risk factors for HCC development are hepatitis C virus (HCV) infection (for example, Egypt), hepatitis B virus (HBV) infection (China), alcohol intake, non-alcoholic steatohepatitis (NASH; United States) and aflatoxin B1 ingestion (Sudan). Mongolia has the highest incidence of HCC globally. HBsAg, hepatitis B surface antigen. Adapted from ref. (6)

Risk factors

In contrast to other human malignancies, the risk factors for HCC are well known. Indeed, HCC occurs in patients with advanced hepatic fibrosis or cirrhosis due to chronic

liver disease, mainly caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) infections, alcohol abuse, aflatoxin B1 exposure, non-alcoholic steatohepatitis (NASH), α 1-antitrypsin deficiency and hemochromatosis (9).

The highest incidence rates of HCC are in Asia (China, Mongolia) and Sub-Saharan Africa, owing to the high prevalence of HBV infection (5). In North America and Europe, HCV is the leading cause of HCC. Overall, 54% of cases could be attributed to HBV infection, 31% to HCV infection and 15% to other causes (10).

NASH together with HBV infection may be the two causes that can lead to HCC development without association with cirrhosis (11). A recent study suggests that around 40% of patients with HCC and NASH might not have cirrhosis (12).

Cirrhosis results from different mechanisms of liver injury that lead to inflammation and fibrogenesis (13). Histologically, it is characterized by diffuse nodular regeneration surrounded by fibrotic septa with parenchymal extinction that causes distortion of hepatic vascular architecture (13,14). It results in portal hypertension and in hepatic dysfunction. The Child-Turcotte-Pugh classification is widely used to characterize the degree of liver injury and to predict the prognosis of patients with cirrhosis (14,15) (Figure 3).

Child-Turcotte-Pugh Classification for Severity of Cirrhosis			
	Points*		
	1	2	3
Encephalopathy	None	Grade 1-2 (or precipitant induced)	Grade 3-4 (or chronic)
Ascites	None	Mild to moderate (diuretic responsive)	Severe (diuretic refractory)
Bilirubin (mg/dL)	< 2	2-3	>3
Albumin (g/dL)	> 3.5	2.8-3.5	<2.8
INR	<1.7	1.7-2.3	>2.3
*Child-Turcotte-Pugh Class obtained by adding score for each parameter (total points)			
Class A = 5 to 6 points (least severe liver disease)			
Class B = 7 to 9 points (moderately severe liver disease)			
Class C = 10 to 15 points (most severe liver disease)			

Figure 3: Child-Turcotte-Pugh Classification for severity of cirrhosis. The Child-Turcotte-Pugh (CTP) classification system utilizes two clinical parameters (encephalopathy and ascites) and three laboratory values (bilirubin, albumin, and prothrombin time). Patients are classified as class A, B, or C based on their total points. Adapted from ref. (16).

Cirrhosis is the principal risk factor for the development of HCC and about 30 to 35% of cirrhotic patients will develop HCC with an annual risk of ~1–8% depending on the etiology (17). HCC can develop in all stages of cirrhosis and of all causes (18). Guidelines recommend 6-monthly ultrasonographic screening because treatment of smaller HCCs is more efficient.

Metabolic syndrome (19), tobacco consumption (20) and oral contraceptive use (21) seem to be co-factors of risk of liver cancer.

Some protective factors have been described, such as medications (statin, antidiabetic, aspirin) or dietary agents (coffee, vitamin E) (22).

Physiopathology

The natural history of HCC in cirrhosis follows a chain of events from the development of pre-cancerous cirrhotic nodules to high-grade dysplastic nodules that can lead to early-stage HCC (stages 0 and A) and progress to more advanced HCC (stages B and C) (6). HCC is the result of the accumulation of somatic mutations in driver cancer genes. In HCC nodules, an accumulation of mutations in coding regions of around 40 functional somatic genes can be observed, uncovering the important diversity of HCC (23).

Some discriminant markers between pre-cancerous tumor and HCC have been identified: WNT– β -catenin pathway activation, re-expression of fetal genes, deregulation of protein-folding machinery, response to oxidative stress and telomerase maintenance (24,25).

Genetic and epigenetic changes underlie oncogenic transformation (26). They can deregulate the expression of oncofetal antigens that constitute tumor-associated antigens (TAAs) capable of inducing immune responses (27). Two of them are α -fetoprotein (AFP) and glypican-3 (GPC3) (Figure 4).

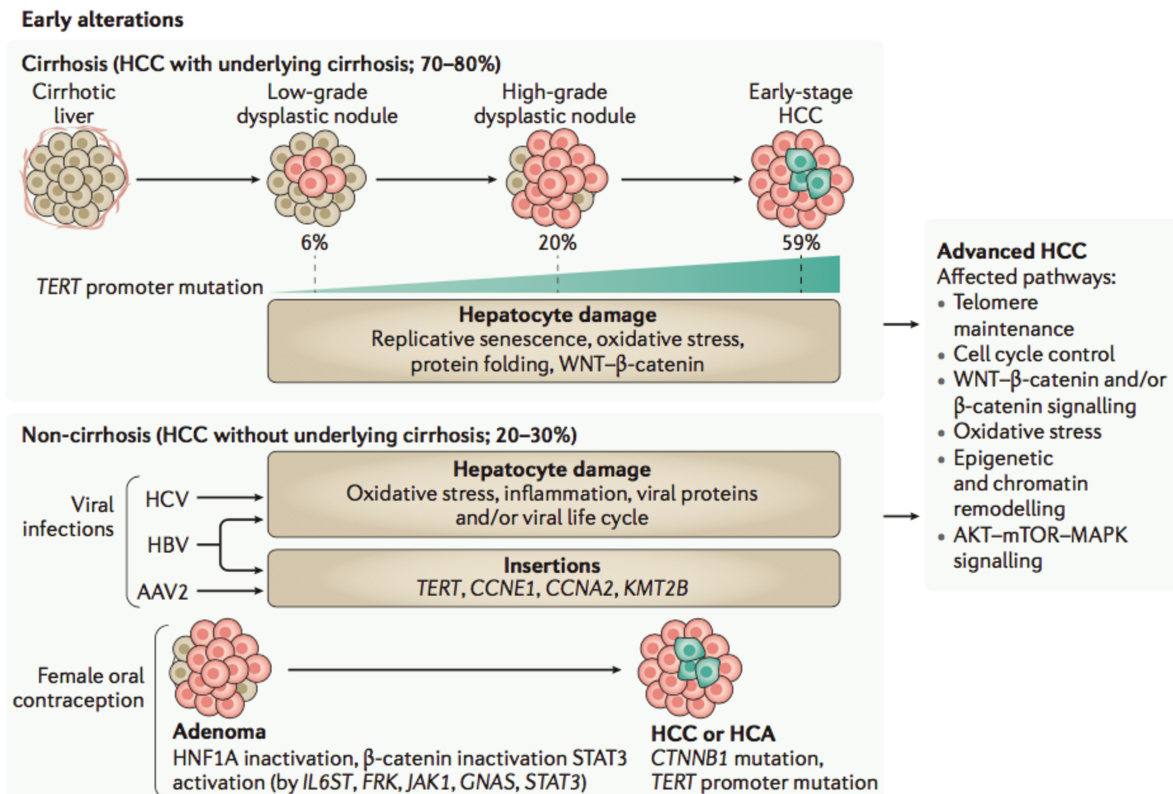


Figure 4. Driver genes in cancer progression. Major recurrent molecular defects observed early in liver carcinogenesis. CCN, cyclin; *FRK*, fyn-related Src family tyrosine kinase; *GNAS*, GNAS complex locus; HCV, hepatitis C virus; HNF1A, hepatocyte nuclear factor 1 α ; *IL6ST*, interleukin 6 signal transducer; *JAK1*, Janus kinase 1; *KMT2B*, lysine (K)-specific methyltransferase 2B; MAPK, MAP kinase; mTOR, mammalian target of rapamycin. Adapted from ref. (6).

HCC is an inflammation-associated cancer with a very high (90%) association with prolonged hepatitis due to viral infection, alcohol abuse or NASH, indicating that the immune microenvironment has a major role in the pathogenesis of the disease (28). Yet, although the presence of immune infiltrate is associated with a better prognosis (29), activation of nuclear factor- κ B (NF- κ B), epidermal growth factor (EGF) and interleukin-6 (IL-6) in the liver parenchyma but not in tumor cells is linked to a poor prognosis (30).

Diagnosis

A diagnosis of HCC can be made by non-invasive (radiological) or invasive (biopsy) methods. Radiological diagnosis is reached with a high degree of confidence if the lesion occurs in a patient with cirrhosis. Using contrast imaging, the lesion has to fit with the radiological hallmarks of HCC, i.e. a hypervascularity in the arterial phase (using computerized tomography (CT)-scan or magnetic resonance imaging (MRI)) and a decrease

of signal compared with the rest of the liver parenchyma in the portal venous (wash-out) (31). When these typical features are detected, the diagnosis is confirmed and a biopsy is usually not needed (9,32). A biopsy is required for patients without any particular risks for HCC, i.e. patients without cirrhosis. The recommended algorithm for investigation of lesions in at-risk patients is: for nodules <1 cm in size, ultrasound follow-up at 3 months is recommended; for lesions >1 cm, the radiological hallmarks of HCC define diagnosis; if the radiology is not typical in at least one of the two imaging techniques (CT scan and MRI), a liver biopsy is recommended (32,33).

Staging

The main prognosis predictors of survival in patients with HCC are liver function, tumor burden (size and number of HCC nodules, vascular invasion), serum alpha-fetoprotein level and performance status (7). The most widely and accepted staging system in oncology is the classification of malignant tumors (TNM). The problem of this system is that it does not take account of liver function reserve, which is critical in HCC. The current European Association for the Study of Liver (EASL)-EORTC guidelines and the American Association for the Study of Liver Diseases (AASLD) guidelines approve the Barcelona clinic liver cancer classification (BCLC) classification and recommend the use of this staging system for prognosis prediction and treatment allocation (32,33). The BCLC classification divides HCC patients in five stages (0, A, B, C, D) according to pre-established prognosis variables: size and number of nodules, vascular invasion, performance status and Child-Pugh stage (Figure 6).

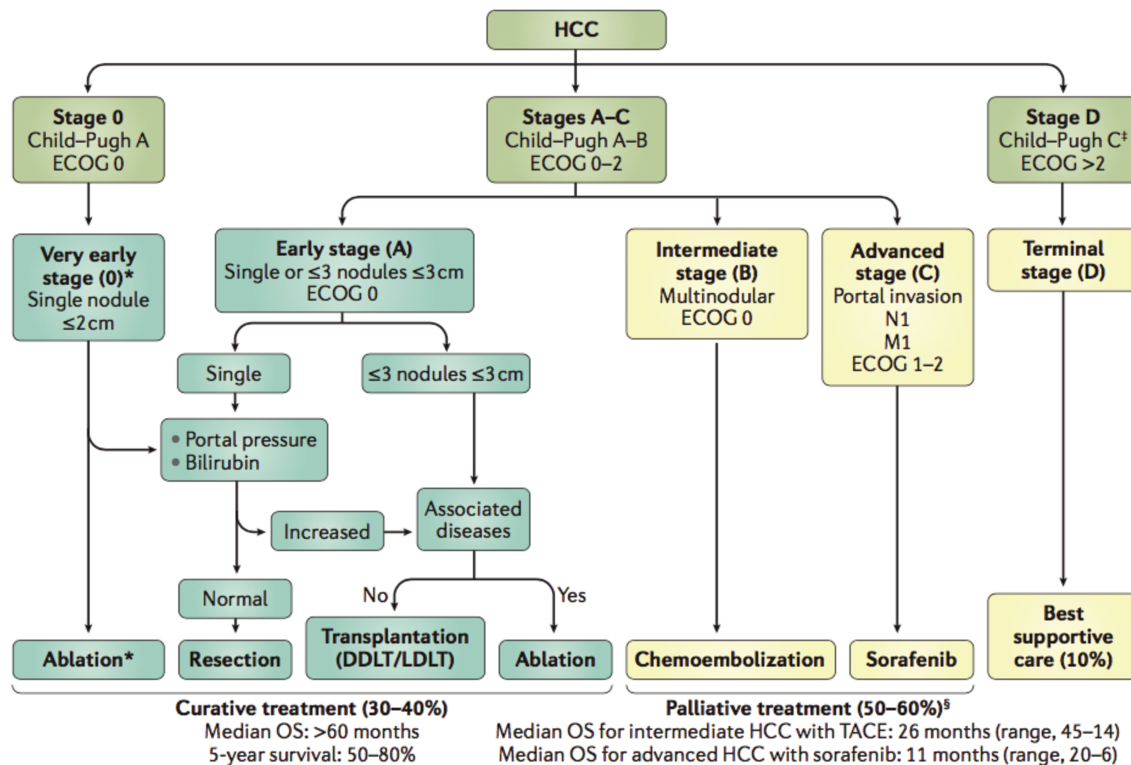


Figure 6: BCLC staging system and therapeutic strategy. The Barcelona Clinic Liver Cancer (BCLC) classification consists of five stages to select the best candidates for the best therapies currently available. Patients with asymptomatic early tumors (stages 0–A) are candidates for radical therapies (resection, transplantation or local ablation). Asymptomatic patients with multinodular hepatocellular carcinoma (HCC; stage B) are suitable for transcatheter arterial chemoembolization (TACE), whereas patients with advanced symptomatic tumors and/or an invasive tumoral pattern (stage C) are candidates to receive sorafenib. End-stage disease (stage D) includes patients with poor prognoses who should be given the best supportive care possible. DDLT, deceased donor liver transplantation; ECOG, Eastern Cooperative Oncology Group; LDLT, living donor liver transplantation; M1, M1 metastasis; N1, N1 lymph node; OS, overall survival. *Patients at very early stages (stage 0) can be considered first for ablation in case they have contraindications for liver transplantation. †Patients in Child–Pugh class C should be first considered for liver transplantation. §Treatment stage migration (consider the next efficacious treatment in the algorithm when previous therapies fail). Adapted from ref. (6).

Treatment

For early-stage disease (BCLC 0 and A), surgical resection is the first-line option in case of solitary nodule and confers 70% of 5-year survival rate (32–35). Complications and recurrence have been reduced with restrictive selection of candidates: single nodule, absence of portal hypertension, and preserved liver function (32). Metastases and *de novo* HCC account for 70% of 5-year recurrence after resection (36), and no adjuvant therapies can prevent it (37). For BCLC A patients within Milan criteria (single nodule of ≤5 cm in size or

up to three nodules of ≤ 3 cm in size, without vascular invasion) and unsuitable for resection, liver transplantation is the best first-line treatment (32,38) with a 5-year survival rate of 70% with a recurrence rate of around 10%, and a 10-year survival rate $>50\%$ (32,39,40). Finally, in patients with early-stage tumors and not suitable for surgery or transplantation, local ablation using radiofrequency is the standard of care with 5-year survival rates of 50–70% (32).

Patients with intermediate stage HCC (BCLC B) are characterized by multinodular disease, preserved liver function and the absence of tumor-related symptoms, vascular invasion and extrahepatic disease. Chemoembolization in the form of transcatheter arterial chemoembolization (TACE), a technique that combines local delivery of beads to restrict tumor blood supply with local administration of chemotherapy, is recommended (41). The current median of survival is 26–40 months (42,43).

In patients with advanced-stage HCC (BCLC C), sorafenib—a multiple tyrosine kinase inhibitor—was able to increase survival from 7.9 months to 10.7 months in a randomized clinical trial (44). On the basis of these data and since no other molecule showed survival benefit, sorafenib became the standard of care for this setting. The lack of a better treatment can be explained by the toxicity of some agents linked to the underlying cirrhosis, the lack of stratification of patients according to prospective biomarkers and a relative resistance to conventional chemotherapy. Nevertheless, several drugs are currently tested in Phase III clinical trials, such as anti-angiogenic agents, cell cycle inhibitors, receptor tyrosine kinase inhibitors and checkpoint inhibitors.

Patients with end-stage disease (BCLC D) have to benefit of best supportive care.

In parallel, treatment of the cause of the underlying liver chronic disease is often associated to treatment of HCC, in order to reduce the level of fibrosis and improve liver function: alcohol discontinuance, HBV viral suppression, HCV eradication. However, there is a controversy towards HCV eradication and risk of occurrence or recurrence of HCC. Until recently, viral eradication with interferon-based regimens was associated with a reduced risk of HCC occurrence (45). Currently, rates of sustained virological response increase thanks to direct-acting antivirals (DAAs) (46). Surprisingly, a higher level of HCC recurrence after antiviral treatment of HCV with DAAs was reported in several studies (47,48). Qualitative and quantitative immune alterations occur during chronic HCV infection, and viral eradication can lead to their restoration (49), however the persistence of a cytokine unbalance may reduce immune control of tumoral clones (50,51). The hypothesis for this higher level of

HCC recurrence after DAAs treatment was that the rapid control of inflammatory state might affect antitumor immune control of tumor niches. Nevertheless, these data were not validated in three French prospective cohorts, where no higher risk of HCC recurrence after DAAs treatment was observed (52).

Perspectives for patients

The global burden of HCC is increasing and there remain extensive challenges to improve the understanding and treatment of this complex disease. Notably, integration of molecular subclasses of HCC into the clinical staging system could be helpful to allocate the best treatment. The development of new drugs for advanced HCC has to become a priority, with addressing new targets or improving tumor immunity. Recent drugs have been tested in combination with sorafenib treatment, or in second line after progression. Neither inhibitor of epidermal growth factor receptor (anti-EGFR, erlotinib) (53), nor inhibitor of phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (mTOR) pathway (everolimus) (54), nor inhibitor of MET (tivantinib) (55), demonstrated better efficacy than reference treatment. Yet, the multikinase inhibitor, regorafenib, was shown to be the first treatment providing survival benefit in HCC patients progressing on sorafenib treatment (56). Finally, the most promising therapy seems to be immunotherapies that inhibit the immune checkpoint interaction between programmed cell death protein-1 (PD1) and programmed death-ligand 1 (PD-L1), which have survival benefit in patients with metastatic carcinomas of multiple tissue origins (57,58). In HCC patients, high level of serum PD-L1 was a pejorative prognostic factor (59). Phase I/II trial showed potential beneficial use of nivolumab in advanced HCC, with a 20% of objective response rate (60), supporting the promise of a new effective treatment with acceptable side effects.

Finally, it would be profitable to identify other biomarkers predicting treatment response and risk of recurrence/progression.

Immunology

HCC immunogenicity is indicated by the presence of tumor-infiltrating lymphocytes (61). Observable tumor-associated antigens (TAA)-specific CD8⁺ lymphocytes from peripheral blood of ~50% of patients with HCC are indicators of the immunogenicity of this

cancer (27). These TAA-specific CD8⁺ lymphocytes produce IFN- γ upon stimulation but tumor-infiltrating lymphocytes fail to do so, suggesting the exhaustion of intratumoral CD8⁺ T cells (27). In HCC, tumor elimination requires a T_H1 type of response. It is supported by the expression of T_H1 cytokines, such as IL-1 α , IL-1 β , IL-2 and IFN- γ , is associated with a good prognosis (62). On the contrary, it seems that the expression of T_H2 cytokines, such as IL-4, IL-5 and IL-10, is upregulated in advanced and metastatic HCC (62). Finally, recent studies showed the involvement of T_H17 response in HCC tumor progression, with the presence of IL-17-producing T cells (63,64) and high level of IL-22 expression (65). T cells are interacting with stroma cells carrying immunosuppressive, cytoprotective and proangiogenic properties (26). Immune-inhibitory activities coupled with permanent exposure to tumor antigens cause T cell exhaustion, a mechanism partially driven by intratumoral expression of immune-inhibitory checkpoint molecules and immunosuppressive factors (26) (Figure 5). HCC cells escape innate and adaptive immunity by producing a large number of immunosuppressive molecules including TGF- β , IL-10, IL-8, adenosine, VEGF, PDGF, EGFR ligands, regulatory T (T_{reg}) cell-attracting chemokines and immunoinhibitory checkpoint molecules (26,66,67). Stroma cells can also participate in immune evasion. For instance, the presence of cancer-associated fibroblasts in the HCC microenvironment inhibit NK cell function by releasing the immunosuppressive molecules prostaglandin E2 (PGE2) and indoleamine 2,3-dioxygenase (IDO) (68). Furthermore, the presence of tumor-associated macrophages (TAMs) is associated with poor outcomes in HCC; TAMs producing IL-6 which promotes expansion of cancer stem cells and tumorigenesis (69). Finally, HCC cells cross-talk with activated hepatic stellate cells, and engender a permissive inflammatory microenvironment (induction of VEGF and metalloproteinase 9 expression), driving HCC progression (70).

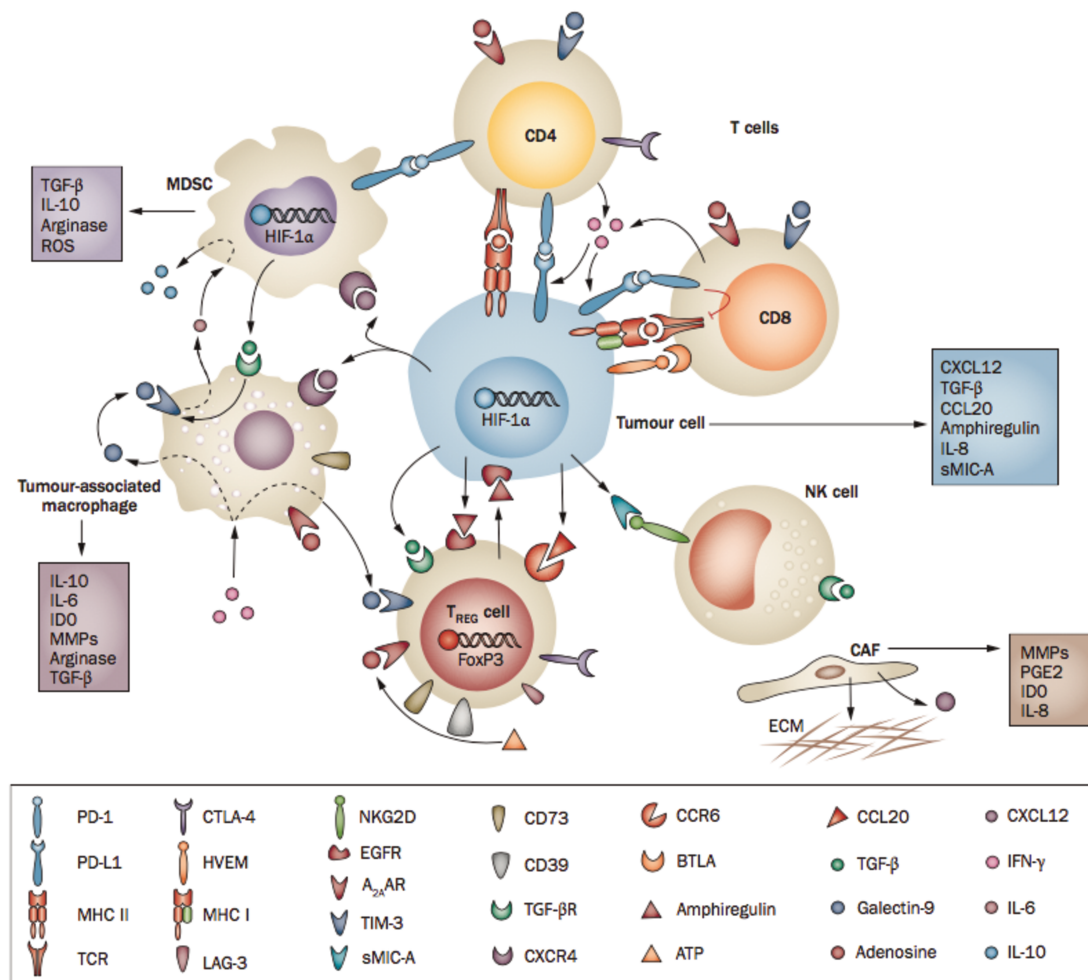


Figure 5: Inhibition of NK and T cell effector functions by the HCC microenvironment. Upon antigen recognition, IFN- γ released by CD4⁺ and CD8⁺ T cells induces PD-L1 on both APCs and tumor cells. PD1–PD-L1 interactions result in T cell exhaustion and prevent tumor cell killing. Both HCC cells and T_{reg} cells secrete and respond to the EGFR ligand amphiregulin, which stimulates HCC cell growth and T_{reg} cell activity. Tumor cells release CXCL12, which attracts myeloid and lymphoid cells via CXCR4. MDSCs abrogate NK and T cell activity via TGF- β and other mechanisms. Myeloid cells mediate immunosuppression by releasing ROS, TGF- β , IL-10, PGE2, MMPs and via the enzymatic action of arginase and IDO. Hypoxia induces PD-L1 in HCC and myeloid cells in the tumor microenvironment. Adenosine acting via its receptor blocks CD4⁺ and CD8⁺ T cell effector functions and inhibits macrophage activation. IFN- γ stimulates TAMs to secrete galectin-9, which upon binding to TIM-3 enhances T_{reg} cell activity and promotes IL-6 secretion by TAMs, which in turn induces IL-10 production by MDSCs. Adapted from ref. (26).

Various studies have broadened our understanding of the role of chemokines in HCC, and there are to date 23 chemokines and 15 chemokine receptors reportedly linked to HCC (71) (Table 1). One study reported a 14 immune-gene signature predicting patient survival; this signature included the chemokine genes CXCL10, CCL5 and CCL2, which drive tumor infiltration by CD8⁺ T cells and NK cells, leading to increase HCC cell death (72). Chemokines in HCC exert pleiotropic effects on immune cells and stroma cells in the

microenvironment bringing both anti- and pro-tumor effects. Also, HCC tumor cells themselves express chemokine receptors; chemokines can thus directly modulate the behaviors of tumor cells including migration, invasion, growth and survival (71).

Categories	Chemokines	Chemokine origins	Receptors participated	Functions
Inflammation	CXCL8 CXCL16 CCL3 CCL5	HCC cells HCC cells HCC cells and HSC HCC cells and HSC	Not clarified CXCR6 CCR3 CCR5	Increasing inflammatory cytokines (IL-6, IL-8, etc.) and recruiting leukocytes (macrophages, neutrophils, etc.)
Influences on immune cells	CXCL5 CXCL9 CXCL10 CXCL16 CCL2 CCL5 CCL20 CCL22	HCC cells HCC cells and endothelial cells HCC cells, macrophages and TILs HCC cells HCC cells, macrophages and TILs HCC cells, macrophages and TILs HCC cells HCC cells and DCs	Not clarified CXCR3 CXCR3 CXCR6 CCR2 CCR5 CCR6 CCR4	Chemotaxis of neutrophils Chemotaxis of T cells Chemotaxis of T cells and NK cells Chemotaxis of neutrophils Chemotaxis of HSC, macrophages, MDSC, and T cells Chemotaxis of T cells and NK cells Chemotaxis of Th17 cells and Tregs Chemotaxis of Tregs
Angiogenesis	CXCL3, CXCL5 CXCL8 CXCL9 CXCL12 CXCL16 CCL2 CCL3	HCC cells CSCs Not clarified Endothelial cells HCC cells HCC cells and endothelial cells HCC cells and endothelial cells	Not clarified Not clarified CXCR3 CXCR4, CXCR7 CXCR6 CCR2 CCR1, CCR5	Promoting angiogenesis <i>via</i> mechanisms not clarified Promoting endothelial cell tube formation Inhibiting angiogenesis by abrogation of VEGF effects Enhancing angiogenesis through VEGF Promoting angiogenesis <i>via</i> mechanisms not clarified Enhancing the proliferation of endothelial cells Enhancing the proliferation of endothelial cells
Direct effects on HCC cells	CXCL1, CXCL2, CXCL16 CXCL5 CXCL8 CXCL10 CXCL12 CCL2 CCL3, CCL5 CCL15 CCL20	Not clarified HCC cells Not clarified hepatocytes HCC cells, HSC WAT, CAF Not clarified HCC cells HCC cells	Not clarified Not clarified CXCR2 Not clarified CXCR4, CXCR7 CCR2 CCR1 Not clarified CCR6	Enhancing the growth of HCC cells Enhancing the migration, invasion, and growth of HCC cells Enhancing the migration of HCC cells Enhancing the survival of hepatocytes Enhancing the migration, invasion, growth and survival of HCC cells Enhancing the migration, invasion, and growth of HCC cells Enhancing the migration and invasion of HCC cells Enhancing the migration, invasion, and growth of HCC cells

HSC: Hepatic stellate cells; TILs: Tumor-infiltrating leucocytes; MDSC: Myeloid derived suppressor cells; Tregs: Regulatory T cells; DCs: Dendritic cells; VEGF: Vascular endothelial growth factor; CSCs: Cancer stem cells; WAT: White adipose tissue; CAF: Cancer-associated fibroblast; HCC: Hepatocellular carcinoma.

Table 1: Pleiotropic functions of chemokines in hepatocellular carcinoma. Adapted from ref. (71)

In sum, HCC is an immunogenic liver lesion but despite HCC antigenicity and intratumoral accumulation of effector T cells, the antitumor immune response is abolished by a variety of immunoinhibitory mechanisms.

Mouse models of HCC

Numerous experimental models have been developed to define the pathogenesis of HCC and to test novel drugs candidates (73).

Chemically induced models. Several chemical reagents induce tumor formation after high doses and time span administration. There are two types of carcinogenic compounds: first, genotoxic compounds with the capacity to induce structural DNA changes and second, compounds which lack direct genotoxic capability but enhance tumor formation after initiation by a hepatotoxic drug (73,74). The advantage of chemically induced models is the similarity with the injury–fibrosis–malignancy cycle seen in humans. Induction models are used to identify chemicals that might be carcinogenic to humans and understand multistep development and characteristics of human liver cancer by toxic or virus. The drawback of such models are the latency of liver cancer (2–5 months), the high rate of mortality, the uncertain time of cancer formation and the wide variation in location and number of lesions (75). These models are more suitable for hepatocarcinogenesis study (75). The different carcinogenic reagents used are diethylnitrosamine (DEN), which alkylates DNA structures, peroxisome proliferators (PPs), aflatoxin B₁, which causes DNA mutations, and carbon tetrachloride (CCl₄), which is cytotoxic.

Genetically modified models (GMM). Those are engineered to mimic pathophysiological and molecular features of HCC (76). It is a unique model for assessing the effects of oncogenes either alone or in combination with other oncogenes or carcinogenic agents (73). This approach allows research towards the role of oncogenes in tumor maintenance and the influence of age on the carcinogenesis. There are transgenic models expressing viral genes (HBV, HCV), transgenic models over-expressing oncogenes (Myc protein, β -catenin) or growth factors (transforming growth factor- α (TGF- α), epidermal growth factor (EGF), fibroblast growth factor 19 (FGF19)). The advantages of GMM include: histological similarity to human tumors—tumors are generally nonimmunogenic but arise in immunocompetent mice—metastatic distribution similar to clinical situation, relevant host immune cell infiltration and tumor microenvironment (75). However, GMM has several disadvantages: they are expensive and difficult to purchase, they require hard labor to screen the transgene, tumor type can be affected by the genetic background of the mouse, and it takes time to induce and develop tumors (75). Potential uses of GMM are: therapy of overt tumors; chemoprevention studies, application of genetic and genomic approaches in mice for

understanding cancer susceptibility, development and maintenance; exploring cancer biology (75).

Transplantable models of HCC. The aim of these models is to provide suitable conditions for the viability of the tumor samples so that they can grow and establish an interaction with the host that features the situation observed in the donor (77). Differences among these models include the type of sample (cancer cell suspension in culture or fresh tumor pieces from surgery) and the anatomical site of implantation (78). The latter factor determines if the tumor grows as a discrete nodule (subcutaneous or orthotopic), or if it will be disseminated from the beginning (intraportal or intrasplenic injection of cells). Also, transplantable models can usually be divided into two groups, syngeneic models and xenograft models. Syngeneic transplantable models refer to mouse cell lines or tissues that result in tumor inbred animals of the same genetic background as the derived cell line or tissue. These models have intact immune system host. Xenograft models refer to human tumor fragments or cells transplanted to immunodeficient mice (79). The most commonly employed model for liver is xenograft models which advantages are human origin of tumors, rapid tumor growth and metastasis formation (79). However one major downside is the lack of interaction between the host and the tumor because the impaired cellular immune system of the animal does not display the mechanism of immune surveillance and immune escape with development of tumor (75). In transplantable models, two ways of implementation are possible. Subcutaneous models present some benefits: the rapid tumor growth, the relatively inexpensive and simple procedure with low mortality, the reproducibility and availability. Drawbacks include are the irrelevance of the site for the study of host–tumor interaction and microenvironment, and the absence of metastasis. Orthotopic models have the great advantages to be relevant to study site-specific dependence of therapy; host–tumor interaction and microenvironment; organ-specific expression of genes and proteins; and metastasis. Unfortunately, they are relatively expensive and much more complex.

In our work, we chose to use syngeneic transplantable with subcutaneous implantation model for the following reasons: intact immune system host, rapidity of tumor growth and reproducibility.

Cancer Immunology

Both innate and adaptive immune systems play a critical role in recognizing and eliminating tumor cells. The innate immune system acts as a first line of defense and responds over a short period. It has a variety of components, including physical barriers (skin epithelium), effector cells (natural killer cells, innate lymphoid cells, dendritic cells, eosinophils among others), mechanisms of pattern recognition (Toll-like receptors), and humoral mechanisms (complement proteins, cytokines). It is also characterized by tissue inflammation that helps to stimulate adaptive immune system and to initiate tissue repair. Adaptive immune system consists of B and T cells recognizing antigens and undergoing selective activation and proliferation. T cells detect antigens in the form of small peptides presented by antigen-presenting cells (APC) in the context of MHC molecules on the cell surface. There are two major classes of T cells, $CD4^+$ T cells that bind to MHC class II molecules expressed by APC; and $CD8^+$ T cells that bind to MHC class I molecules expressed on every cell. After engagement by an APC, $CD4^+$ naïve T cells can differentiate for instance into T helper cells or regulatory (T_{reg}) cells. In contrast, most $CD8^+$ T cells are responsible of direct cell-mediated cytotoxicity following APC activation; others become long-lived memory cells (80).

In this section, we will focus on the role of T helper cells in the activation of effector cells involved in antitumor immunity (steps 2 and 3 of the Cancer–Immunity cycle, Figure 1) and will describe the potential role of eosinophils in antitumor responses. Second, we will detail the mechanisms that allow immune cells trafficking and infiltration into tumor and their regulation (steps 4 and 5, Figure 1).

Role of T helper cells

$CD4^+$ T cells, also known as T helper (T_H) cells, play critical roles in the adaptive immune response (81-83) (Figure 7). They help B cells to produce antibody and they recruit and activate $CD8^+$ T cells, macrophages, neutrophils, eosinophils and other effector cells. T_H cells can be activated by either antigen-presenting cells (APCs) in the lymph node or directly by MHC class II–expressing tumors (step 3 of the Cancer–Immunity Cycle, Figure 1).

There are two predominant T_H cell subtypes, T_H1 and T_H2 cells. T_H1 cells, characterized by the secretion of $IFN-\gamma$ and $TNF-\alpha$, are responsible for activating and regulating the development of cytotoxic T cells (CTLs). T_H1 cells also activate APCs. T_H2 cells produce IL-4, IL-5, IL-10 and IL-13, critical for IgE production and eosinophil recruitment. The cytokine environment at the site of antigen deposition or in the local lymph node is particularly important during T_H differentiation: T_H1 commitment relies on the local production of IL-12, and T_H2 development is promoted by IL-4 in the absence of IL-12 (83).

T_H1 cells can directly activate CTLs through cytokines, such as IL-2 (84), but they can also enhance CTL activity indirectly by activating other cell types. For instance, T_H1 cells release $IFN-\gamma$, which can activate APC to upregulate molecules (such as MHC class I) increasing antigen presentation to CTLs (85). In addition, direct tumor cell-killing effects of T_H1 cells are suggested in some studies where T_H cells mediate cell death through direct contact with tumor cells (86). The interaction of T_H1 cells and tumor cells through MHC class II can impair tumor cell survival through upregulation of death-inducing receptors (Fas/FasL pathway) (87) or through toxic secretions. T_H1 cells, by producing $IFN-\gamma$, can activate macrophages, which produce nitric oxide and super-oxide, playing an important role in tumor killing. In total, specific T_H1 cells can mediate anti-tumor effect via a variety of mechanisms: creation of an immune environment through cytokine secretion, stimulation of the recruitment of CTLs, cytotoxic effect to the tumor, and orchestration the activation of innate immune cells (83).

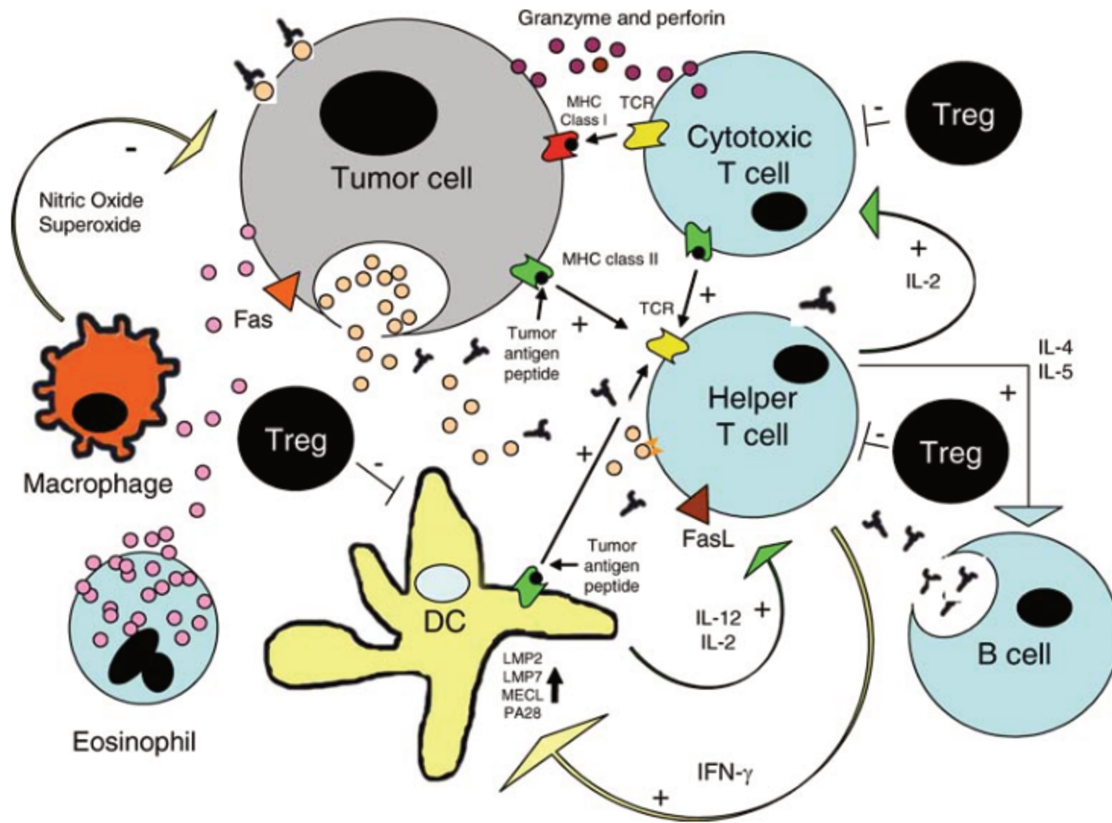


Figure 7: The role of T helper cell in the tumor microenvironment. The T_H cell plays an extensive role and is able to interact with the tumor cell and number of immune effectors through contact-dependent or contact-independent mechanisms. A *plus sign* indicates a potentially positive impact on the antitumor response while a *minus sign* indicates a detrimental effect. Adapted from ref. (83).

In contrast, T_H2 -mediated immunity, known to be mainly involved in allergy and infection by parasites, has been classically considered as favoring tumor growth, by promoting angiogenesis and by impeding cell-mediated immunity and thereby tumor cell killing (88). It is true that components of type 2 immunity like B cells or IL-10 are promoting tumor growth, yet many studies showed the anti-tumor activity of $CD4^+$ T_H2 cells, notably with the involvement of tumor-infiltrating granulocytes such as eosinophils (88) (Figure 8). In one study, a critical role for antigen-specific $CD4^+$ T cells with a T_H2 -cytokine profile has been shown in the clearance of B16 melanoma metastasis (89). Recent studies suggest that $CD4^+$ T cells can induce either type 1 (90) or type 2 (91) or both (92). Whether a type 1 or type 2 immune response is required seems to differ depending of the tumor type and the model system used (88). T_H2 cells can intensify the innate anti-tumor response and recruit tumoricidal myeloid cells into the tumor, such as eosinophils, often in collaboration with macrophages (89,93). This T_H2 -mediated anti-tumor immunity seems to be under the

dependence of IL-4 (94). IL-4 is the archetypal T_H2 cytokine (95) and several studies showed its role in tumor clearance; indeed, it recruits eosinophils, macrophages, neutrophils and lymphocytes into tumors (93). Using neutralizing anti-IL-4 antibody resulted in absence of infiltrating eosinophils and macrophages, coinciding with loss of antitumor immunity (94). The presence of $CD8^+$ T cells has been showed either unnecessary (94) or crucial (93) for IL-4-mediated tumor clearance and might depend on the tumor model. However, $CD8^+$ T cells involvement is not related to CTL activity but could induce direct or indirect interactions with eosinophils (93) or macrophages (96). In opposition to type 1-mediated anti-tumor immunity that promotes tumor cell apoptosis, type 2-mediated immunity appears to result in tumor necrosis (94). For instance, IL-4 has been showed to possess anti-angiogenic properties (97). Beside IL-4, IL-13 is also upregulated during T_H2 response, and both can bind the same receptor (the type 2 IL-4R) and so have a similar biological activity (98). As IL-4, overexpression of IL-13 appeared to induce anti-tumor immune response mediated by myeloid cells, such as neutrophils and macrophages (99).

Similarly to $CD4^+$ T cells, $CD8^+$ T cells can be classified into two types based on their cytokine secretion profile (100,101): type 1 $CD8^+$ T cells (Tc1) produce IFN- γ , IL-2 and TNF- α , and type 2 $CD8^+$ T cells (Tc2) secrete IL-4, IL-5 and IL-10. While the role of Tc1 in tumor clearance is well demonstrated, notably through the production of IFN- γ and the Fas pathway (102,103), the role of Tc2 cells is less understood. Some studies reported that the adoptive transfer of antigen-specific Tc2 cells induced eradication of B16 melanoma and EG7 thymoma in mice (102,104). However, in comparison with Tc1 cells, Tc2 cells were (5- to 25-fold) less effective in controlling tumor growth than Tc1 cells. In summary, some components of the T_H2 -mediated immune response appear to have an antitumoral activity, particularly eosinophils which could induce tumor cell death.

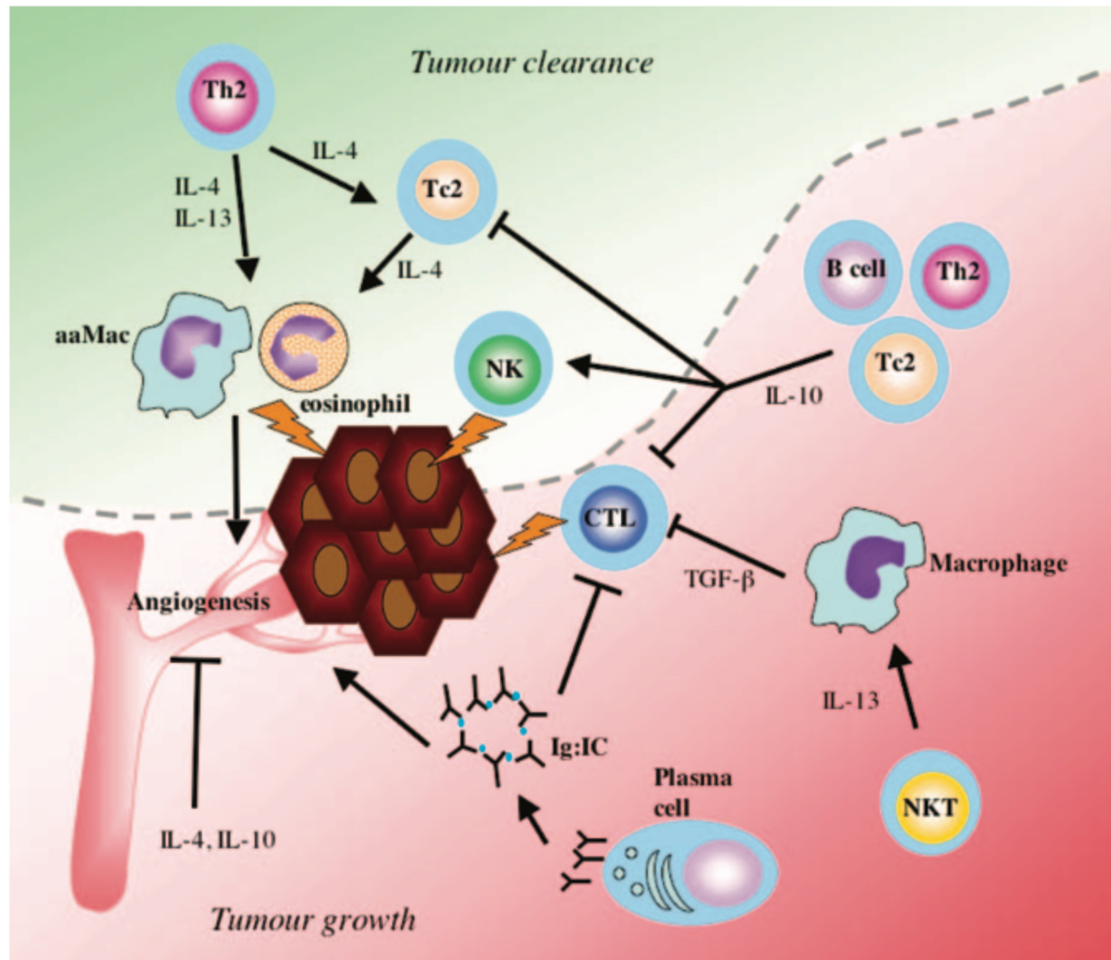


Figure 8: Balancing the good and the bad in T_H2 -mediated inflammation within tumors. The T_H2 -mediated inflammation enhances tumor clearance (green zone) through the secretion of IL-4 and IL-13 by T_H2 and Tc2 cells. These cytokines activate eosinophils and alternatively macrophages (aaMac) that can reach the tumor and promote anti-tumor cytotoxic activity. On the contrary, T_H2 -mediated inflammation promotes tumor growth (red zone) via the secretion of IL-10 (by T_H2 , Tc2 cells and B cells) and TGF- β (by macrophages), which inhibits CTL-mediated anti-tumor activity by interfering with antigen processing and presentation. Adapted from ref. (88).

Eosinophils

Eosinophils are multifunctional leucocytes involved in the pathogenesis of inflammatory processes, such as parasitic helminthes infections or allergic diseases (105,106). After some stimuli, eosinophils are recruited from the circulation into inflammatory sites, where they regulate immune responses through diverse mechanisms. They are produced in bone marrow from pluripotent stem cells, which differentiate into a first precursor, common to basophils, and then separate to the eosinophil lineage, by the interplay of transcription factors (GATA-1, PU.1 and C/EBP) (107). Three cytokines are especially important in the

regulation of eosinophil development: IL-3, IL-5 and GM-CSF. They support proliferative and differentiation signals. Among them, IL-5 is the most specific for eosinophils; it is responsible for their differentiation, their activation, their recruitment from bone marrow to peripheral circulation, and their survival. They traffic mostly into the gastrointestinal tract but also home into the thymus, mammary gland and uterus under homeostatic conditions. This recruitment is also regulated by eotaxin-1 (also known as CCL11). Two additional chemokines with eosinophil chemoattractant activity, eotaxin-2 (CCL24) and eotaxin-3 (CCL26) have also been identified. The activity of all eotaxins is mediated by the expression of the transmembrane G protein-coupled receptor CC-chemokine receptor 3 (CCR3), expressed on eosinophils (108). IL-5 augments the number of eotaxin-responsive cells and primes eosinophils to respond to CCR3 ligands. Besides the two main receptors, IL-5R α and CCR3, the sialic acid-binding immunoglobulin-like lectin 8 (Siglec-8) in humans (Siglec-F in mice) is also eosinophil specific (Figure 9).

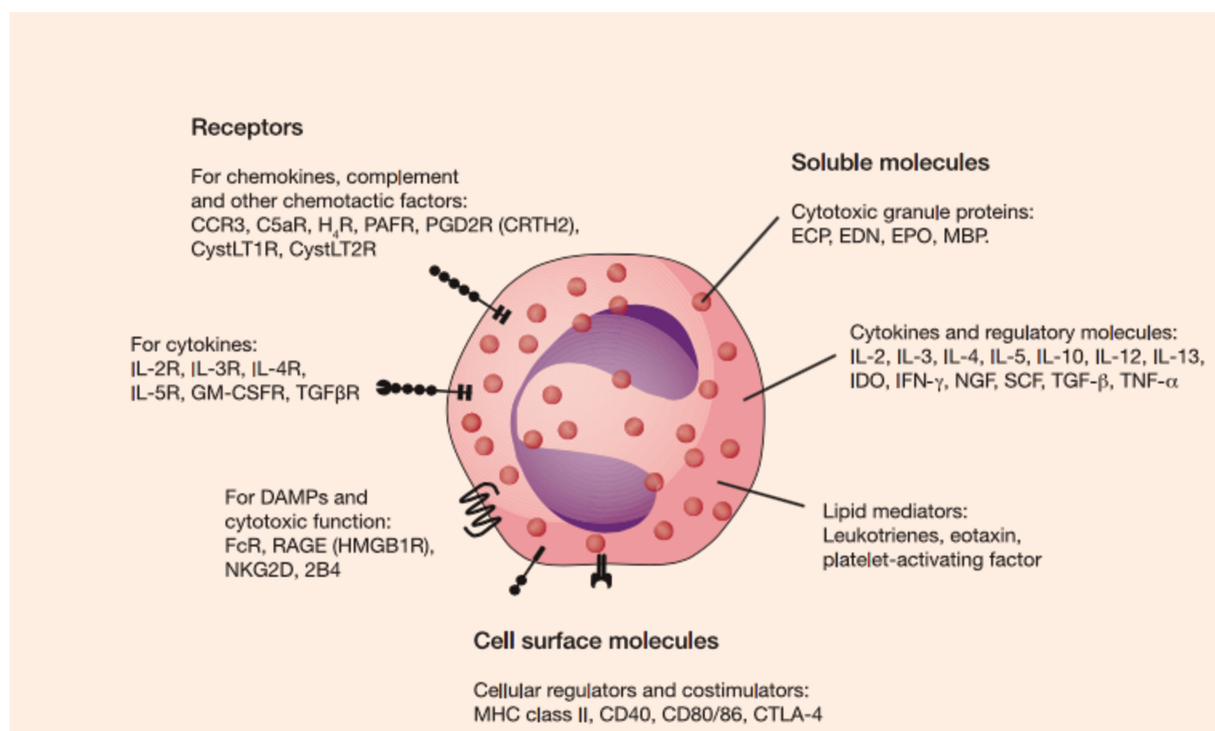


Figure 9: Surface and secreted molecules of eosinophils. Eosinophils express cell surface molecules important for adhesion and antigen presentation and receptors for immunoglobulin, stress-induced pattern recognition, chemokines, and cytokine signaling. Eosinophils also secrete a wide variety of molecules important for their immune function, including cytotoxic granule proteins, cytokines, chemokines, lipid mediators, neurotransmitters, and DNA traps. Adapted from ref. (109).

Eosinophils secrete four cytotoxic granules cationic proteins: eosinophil cationic protein (ECP), eosinophil peroxidase (EPO or EPX), major basic protein (MBP) and eosinophil-derived neurotoxin (EDN). These granules can induce tissue damage and dysfunction (110). ECP and EDN are ribonucleases, MBP increases membrane permeability, ECP causes ion-selective toxic pores in the membrane of target cells and EPO catalyzes the oxidation of nitric oxide to form reactive oxygen species and reactive nitrogen metabolites, promoting oxidative stress and cell death. Eosinophils are capable to undergo piecemeal degranulation in response to CCL11, a mechanism where they release specific mediators (pieces of their granules) while remaining otherwise intact, viable and fully responsiveness to subsequent stimuli (111,112).

Eosinophils interact with other leucocytes (Figure 10). They respond to signals provided by T cells but also T cells can respond to signals provided by eosinophils (113). Eosinophils express MHC class II and co-stimulatory molecules (CD40, CD80/86, CTLA-4) and they can process antigens to CD4⁺ T cells, thereby stimulating T cell proliferation (114). They secrete many cytokines favoring T_H1 response (IL-2, IL-12) or, more frequently T_H2 response (IL-4, IL-5, IL-6, IL-10, IL-13) able to regulate T cell proliferation, activation and T_H1/T_H2 polarization. Eosinophils interact also with innate immune cells such as macrophages and mast cells.

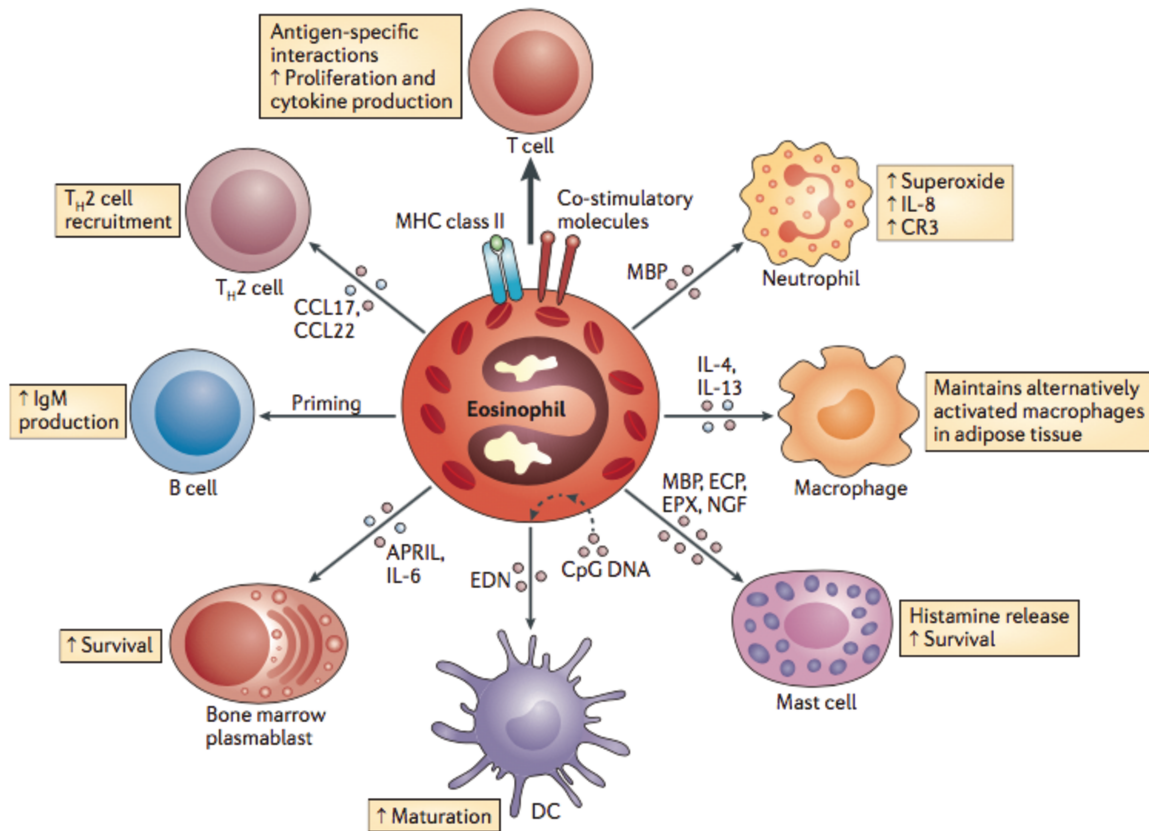


Figure 10: Eosinophils modulate the function of other leucocytes. Eosinophils can express MHC class II and co-stimulatory molecules, process antigens and stimulate T cells to proliferate and produce cytokines in an antigen-specific manner. Furthermore, acting together with dendritic cells (DCs), eosinophils regulate the recruitment of T_H2 cells in response to allergen sensitization and challenge by producing CC-chemokine ligand 17 (CCL17) and CCL22. Eosinophils also prime B cells for antigen-specific IgM production and sustain long-lived plasma cells in mouse bone marrow via the production of a proliferation-inducing ligand (APRIL) and IL-6. Eosinophils that are stimulated by CpG DNA induce DC maturation. Indeed, the eosinophil granule protein eosinophil-derived neurotoxin (EDN) promotes the maturation and activation of DCs. Major basic protein (MBP) released from eosinophils activates neutrophils, causing them to release superoxide and IL-8 and increase their expression of the cell-surface integrin complement receptor 3 (CR3). Eosinophils also maintain alternatively activated macrophages in adipose tissue by producing IL-4 and IL-13. The eosinophil granule proteins MBP, eosinophil cationic protein (ECP) and eosinophil peroxidase (EPX) activate mast cells, resulting in the release of histamine. Likewise, eosinophil-derived nerve growth factor (NGF) prolongs mast cell survival. Adapted from ref. (112).

Eosinophils are known to be involved in host defense against parasites. They can mediate antibody-dependent cellular cytotoxicity against helminthes. But they have also an antiviral action through the ribonucleases activity of ECP and EDN granules.

On the other hand, eosinophils are implicated in some inflammatory fibrotic diseases. Eosinophil granules are toxic for many tissues and hypereosinophilia can damage tissues, such as brain, lung, skin, heart (112). Notably, eosinophils participate in the development of

asthma-associated airway hyperresponsiveness (AHR), particularly because they are the main source of leukotrienes, responsible of mucus hypersecretion, AHR and edema (115).

Eosinophils infiltrate various solid tumors. Tumor-associated tissue eosinophilia and evidence of eosinophil degranulation were associated with better prognosis in several human solid tumors such as oral squamous cell carcinoma (SCC) (116), pulmonary adenocarcinoma, bladder cancer, prostate cancer and colon carcinoma (117,118). The recruitment of eosinophils at tumor site has not been clearly identified. Some tumor cells are source of IL-5 and CCL11, which can act on eosinophils activation and migration (Figure 11). The accumulation of eosinophils in tumors such as SCC (119), colorectal (120) and lung cancers, appears to be dependent of the IL-5 receptor–CCR3 signaling pathway (121). Additionally, eosinophils can be activated by danger signals released from necrotic tumor cell death (122), and one of these alarmins is IL-33 (123), for which eosinophil express the specific receptor ST2. Chemotactic factors that pilot the eosinophils trafficking into the tumor microenvironment seem to be dependent on the tumor type, the anatomical location of the tumor and the stage of the disease.

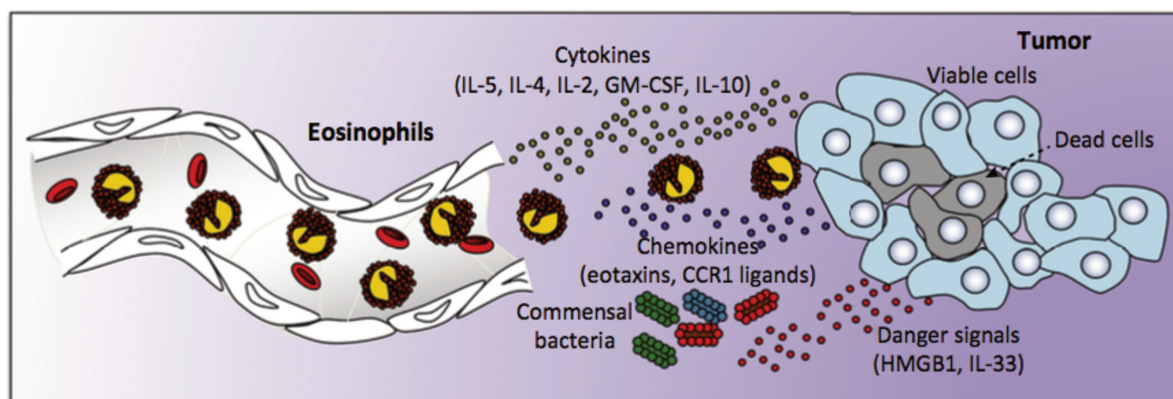


Figure 11: Mechanisms of eosinophil recruitment and retention in the tumor microenvironment. Mobilization of eosinophils from the peripheral blood into sites of tumor growth and/or metastasis may be directed by various mechanisms including the presence of chemokines (CCR3 and CCR1 ligands), T_H2 cell-derived cytokines (IL-5, IL-4), immunotherapy (GM-CSF, IL-4, and IL-2), as well as danger signals (HMGB1 and IL-33). Specifically, IL-5 potentiates CCR3-ligand–induced responses in eosinophils, thus stimulating their migration and retention. Additional factors regulate eosinophil migration and retention, such as commensal bacteria and IL-10. Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin. Adapted from ref. (124)

Eosinophils possess antitumorigenic activities that can impact tumor cell growth directly and indirectly. Eosinophils can directly cause tissue damages through their cytotoxic

granules, such as MBP, which can disrupt the integrity of lipid bilayers of tumor cells (125). Numerous *in vitro* studies reported killing of tumor cells in presence of eosinophils, notably in colorectal cancer (126) where interaction between eosinophils and tumor cells is mediated by adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) (127). We however lack *in vivo* demonstration of direct eosinophil-mediated tumor killing. Nonetheless, various *in vivo* studies suggested that eosinophils are an important effector arm of the antitumor immunity orchestrated by T_H2 cells and their cytokines (IL-4, IL-5). For example, injection of IL-4-expressing tumor cell lines into mice resulted in antitumor activity mediated by infiltrating eosinophils (128). This antitumor effect was associated with production of eotaxin. Another study showed that eradication of melanoma lung-metastasis was dependent on IL-5 secretion by T_H2 cells, eotaxin expression and eosinophils (89). Finally, the use of hepatocellular carcinoma cells overexpressing eotaxin revealed eosinophil-mediated tumor immunity in the presence of high levels of IL-5 (129).

Recently, some studies showed that eosinophils orchestrate antitumor immunity via indirect mechanisms. In a model of melanoma in T regulatory cell-depleted mice, eosinophils improved the recruitment of CD8⁺ T cells, the vascular healing and the polarization of macrophages into a proinflammatory phenotype (130). Eosinophil depletion with anti-Siglec-F antibody caused a decrease of CD8⁺ T cell recruitment and following reduced survival.

The importance of immune system in the tumor microenvironment is illustrated by the current use of immune checkpoint blockers (anti-PD-(L)1 and anti-CTLA4) to reactivate antitumor activity (131). The main targets of these therapies are T cells, but myeloid cells have started to be studied with more attention (132). Indeed, it has been shown that an early increase in eosinophil counts during the treatment with ipilimumab (anti-CTLA4) was associated with better clinical response and improved overall survival in patients with melanoma (133,134). The development of new pharmacological strategies to improve eosinophils activities in cancer is a new challenge, and deciphering the conditions that contribute to tumor cell susceptibility to the cytotoxic effects of eosinophils could pave the way for new therapies.

Immune cell trafficking

Adhesion molecules

Leucocyte–endothelial cell adhesion is required for leucocyte recruitment from the blood circulation to the inflammatory sites (step 5 of the Cancer–Immunity cycle, Figure 1). Three steps of this process have been described: capture, rolling and firm adhesion before allowing trans- or para-endothelial migration (135) (Figure 12). All these steps require the participation of specific adhesion glycoproteins at the surface of leucocytes and endothelial cells.

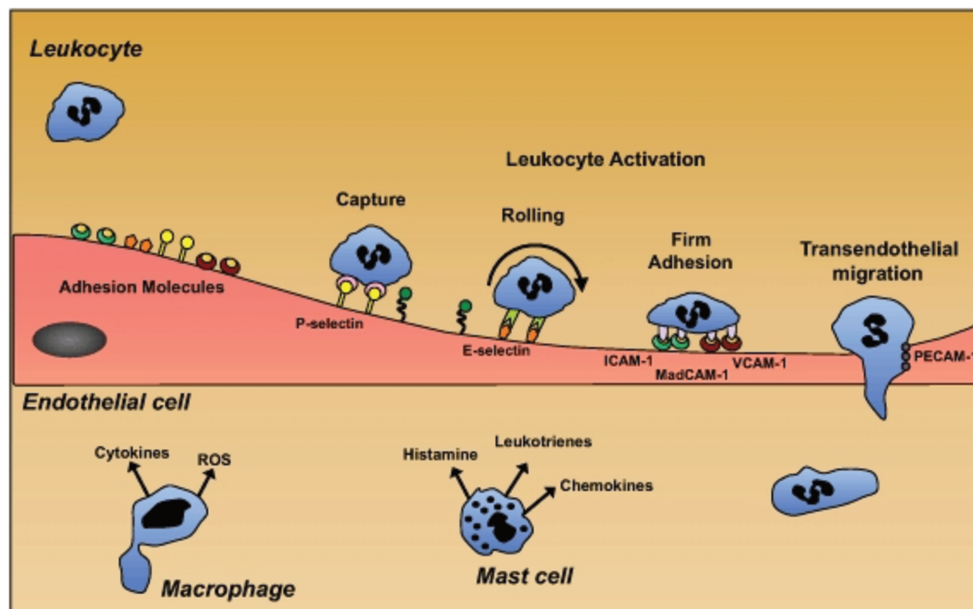


Figure 12: Multistep adhesion cascade and the major molecular contributors to leukocyte recruitment during an inflammatory response. Following endothelial cell activation and the increased expression of P- and E-selectins, low-affinity adhesive interactions (capture and rolling) are elicited that subsequently lead to leukocyte activation, followed by firm adhesion and transendothelial migration. Adapted from ref. (135).

Selectins are lectin-like adhesion glycoproteins that mediate capture and rolling of leucocytes (136,137). They are useful to reduce the velocity of leucocyte movement along endothelial cells to then allow firm adhesion. L-selectin is expressed by leucocytes whereas P-selectin is expressed by endothelial cells and platelets and E-selectin only by endothelial cells (138). All these selectins interact with P-selectin glycoprotein ligand 1 (PSGL1), expressed by most leucocytes (139).

Leucocyte integrins are glycoproteins complexes with α - and β -subunits and participate to the step of arrest. The two most studied integrins are the β_1 -integrin VLA4 and the β_2 -integrin LFA1. They bind to endothelial cells immunoglobulin-like adhesion molecules such as ICAM-1, ICAM-2, and VCAM-1. Finally, transmigration through venular walls is the last step and some endothelial junctional molecules actively mediate leucocyte transendothelial migration, like PECAM-1 and ICAM-2 (140) (Table 2).

Leukocyte adhesion receptor	Endothelial ligand	Function(s)
PSGL-1	P-selectin	Capture
		Rolling
L-selectin	P-selectin	Capture
	Peripheral node addressin (PNAd)	Rolling
	E-selectin	
	MadCAM-1	
$\alpha_4\beta_7$ (unactivated)	MadCAM-1	Rolling
$\alpha_4\beta_7$ (activated)	VCAM-1/MAdCAM-1	Firm adhesion
$\alpha_4\beta_1$ (unactivated)	VCAM-1	Rolling
$\alpha_4\beta_1$ (activated)	VCAM-1	Firm adhesion
CD11a/CD18 (LFA-1)	ICAM-1, ICAM-2	Firm adhesion
		Emigration
CD11b/CD18 (Mac-1)	ICAM-1	Firm adhesion
		Emigration
PECAM-1	PECAM-1	Emigration

Table 2: Leukocyte adhesion receptors and their ligands on activated endothelial cells. Adapted from ref. (135).

This whole process is initiated and regulated by a variety of chemical mediators (cytokines and chemokines) produced by inflamed tissues.

Chemokines

Chemokines are a family of around 50 low molecular weight (8–15 kDa) chemotactic cytokines that can regulate leucocyte trafficking by mediating the adhesion of leucocytes to endothelial cells, the initiation of transendothelial migration and tissue invasion (141-143). They also participate in embryogenesis, angiogenesis, hematopoiesis, atherosclerosis, tumor growth and metastasis, as well as HIV infection (144,145). They are classified first in two

groups based on their function: homeostatic and inflammatory chemokines. Homeostatic chemokines are constitutively expressed in some cell types and tissues and have a major role in the development and maintenance (homeostasis) of the immune system. On the contrary, inflammatory chemokines are inducible and upregulated upon inflammatory stimuli. Chemokines are then subclassified into four classes, depending on the positioning of the first two cysteine residues in the aminoterminal sequence (X represents any amino acid): CXC, CC, CX₃C, and C chemokine ligands (143). We so far know 24 human CC chemokines (CCL1–CCL28), 15 human CXC chemokines (CXCL1–CXCL16), one CX₃C (CX3CL1) and one C chemokine (lymphotactin or XCL1) (143).

Chemokines activate leucocytes through binding to G protein-coupled seven transmembrane receptors (GPCR) labeled CXCR or CCR. Chemokines use the residues following the CC/CXC motif (the “N-loop” region) for initial binding of their receptors and use the residues preceding the CC/CXC motif (the chemokine “N-terminal” region) for receptor activation. This phenomenon is called the “two-site” model (146). There are around 20 chemokine receptors identified and there is a certain redundancy in the chemokine family, many ligands can bind different receptors and vice versa. After chemokine binding to its receptor, leucocytes transendothelial migration occurs through interactions with selectins and integrins. Chemokines attract most leucocytes cell types: monocytes, macrophages, dendritic cells, B cells, T cells, NK cells, basophils, eosinophils and mast cells (Table 3). Furthermore, chemokines can impact leucocyte cell survival and effector functions such as degranulation (147).

Leukocyte	Chemokine receptor	Corresponding ligand(s)
Monocytes	CCR1 CCR2 CCR5 CXCR4 CX ₃ CR1	CCL3, 5 CCL2 CCL3–5 CXCL12 CX ₃ CL1
Monocyte-derived DC		
Immature	CCR1 CCR5 CCR9 CXCR4	CCL3, 5 CCL3–5 CCL25 CXCL12
Mature	CCR7 CCR9 CXCR4	CCL19, 21 CCL25 CXCL12
CD34-derived DC		
Immature	CCR1 CCR5 CCR6 CCR9 CXCR4	CCL3, 5 CCL3–5 CCL20 CCL25 CCL12
Mature	CCR7 CCR9 CXCR4	CCL19, 21 CCL25 CXCL12
CD4⁺ T cell		
Naïve	CCR7	CCL19, 21
T _H 1	CCR2 (CCR5) CXCR3	CCL2, 7, 13 CCL3–5 CXCL9–11
T _H 2	CCR2 (CCR3) CCR4 CCR8	CCL2, 7, 13 CCL5, 7, 8, 11, 13, 24, 26 CCL24 CCL1
CD8⁺ T cell		
Naïve	CCR7	CCL19, 21
CTL	CXCR3	CXCL9–11
NK cell		
	CCR1 CCR2 CCR4 CCR5 CCR7 CCR8 CXCR1 CXCR2 CXCR3 CXCR4 XCR1	CCL3, 5 CCL2, 7, 13 CCL17, 22 CCL3–5 CCL19, 21 CCL1 CXCL8 CXCL1–3, CXCL5–8 CXCL9–11 CXCL12 XCL1
B cell		
	CCR6 CXCR4 CXCR5	CCL20 CXCL12 CXCL15

CTL, cytotoxic T lymphocyte; DC, dendritic cell; NK, natural killer cell; T_H1, T helper 1 cell; T_H2, T helper 2 cell.

Table 3: Chemokine receptor expression by leucocytes. Adapted from ref. (141).

Leucocytes infiltrate the tissue according to a gradient of chemokines produced by the inflammatory site (Step 4 of the cancer Immunity–Cycle, Figure 1). Tumor cells, stroma cells and tumor-associated leucocytes collaborate to the local production of chemokines inside the

tumor (148). Besides, tumor cells can adopt the chemokine-mediated mechanisms used for leucocytes transport to form metastasis (149). Additionally, chemokines can alter tumor development by their angiogenic or angiostatic activity. Angiogenesis is the formation of new vessels from existing ones and it is an essential condition for physiological and pathological processes (150). Notably, growth of tumor happens when the balance between angiostatic and angiogenic factors is in favor of angiogenic ones. In sum, chemokines alter tumor development by impacting angiogenesis, tumor–leucocyte interactions and by influencing tumor transformation, survival and growth, invasion and metastasis (145). Chemokines thus play a dual role, some of them favoring tumor growth and progression and others enhancing anti-tumor immunity (Figure 13).

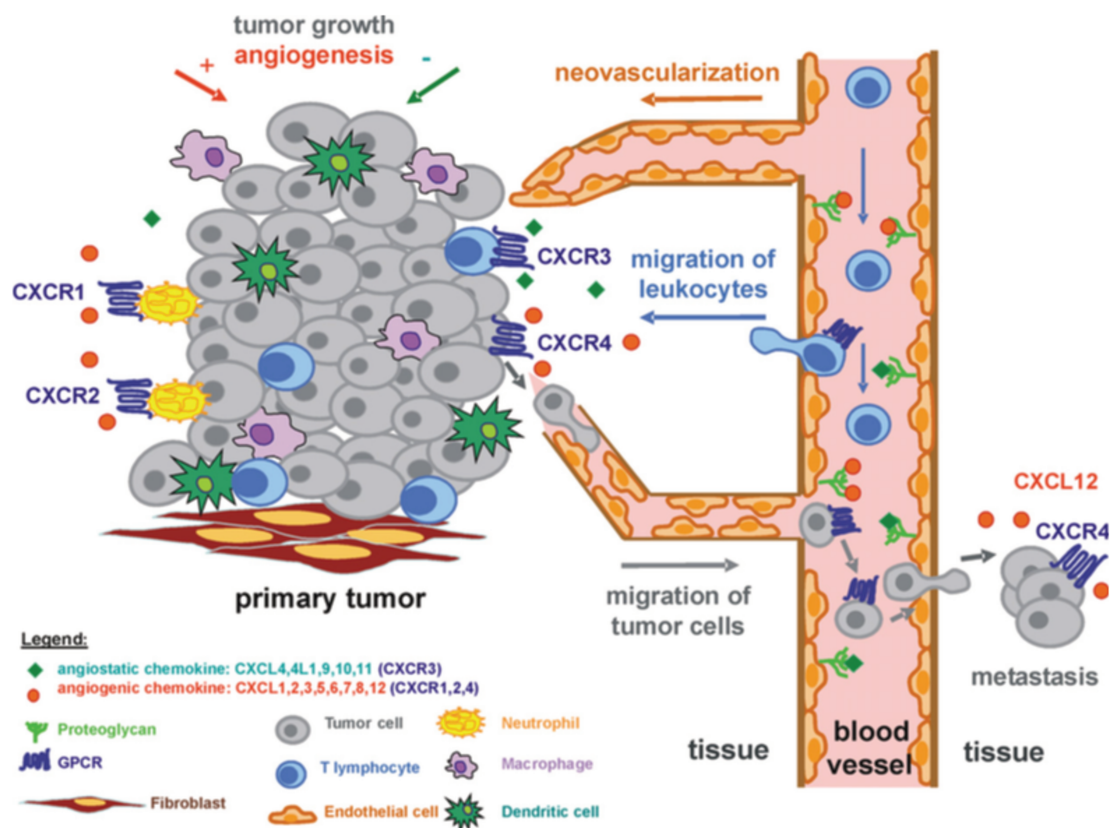


Figure 13: Role of chemokines and their receptors in the tumor environment. Solid tumors contain various types of stromal cells, such as fibroblasts and endothelial cells. All these cell types are good producers of chemokines, regulating the migration of leucocytes. Leucocyte traffic is a highly coordinated multistep process. Briefly, leucocytes roll along the endothelium and chemokines bind leucocytes via their G protein-coupled receptor. As a result, leucocytes firmly attach to the endothelial cell layer. Finally, they transgress the endothelium and migrate into the underlying

chemokine producing tissue in response to the chemokine gradient. As a result, inflammatory cells, including neutrophils, macrophages, T lymphocytes and dendritic cells infiltrate the tumor. Adapted from ref. (145).

Chemokine regulation

Appropriate recruitment of inflammatory leucocytes by chemokines towards inflammatory sites is subject to tight regulation. The prompt up-regulation of chemokine and chemokine receptor expression is crucial to achieve a rapid influx of leucocytes. However, this influx has to be transient to avoid accumulation of leucocytes, which may lead to chronic inflammation and tissue damage (151). Several mechanisms contribute to regulate chemokine availability and chemokine function. The study of these mechanisms of positive and negative regulation is relevant in cancerology to design therapies aiming to upregulate anti-tumor chemokines and downregulate pro-tumor chemokines.

Several regulation mechanisms modulate chemokine availability. For example, NF- κ B can induce the transcription of genes encoding inflammatory chemokines and cytokines (147). The induced cytokines in turn stimulate cells to produce inflammatory chemokines and so amplifying chemokine responses. In contrast, some endogenous anti-inflammatory mediators, such as IL-10 and TGF- β , downregulate chemokine gene expression. Another mechanism of regulation playing with chemokine availability is the glycosaminoglycan (GAG) binding. Chemokines interact with GAG component of proteoglycans in the glycocalyx and the extracellular matrix (152). Six classes of GAGs are known: heparin, heparin sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate and hyaluronic acid. These GAGs permit the localization and concentration of the secreted chemokines in specific tissue compartments by producing a haptotactic gradient and avoiding their fast diffusion into blood. Moreover, GAGs could participate to the transcytosis of chemokines from basolateral to apical side of endothelial cells to be presented to circulating leucocytes (153).

Other regulation mechanisms modulate chemokine function. The most important of them is the posttranslational modification. Inflammatory sites are rich of proteases either expressed on the cell membrane or stored in intracellular granules, or present in biological fluids (147). Different types of posttranslational modifications have been described: glycosylation, proteolytic processing and citrullination.

Among these proteases is the dipeptidyl peptidase (DPP) family with four prolyl-specific peptidases: DPP4 (also called CD26), fibroblast activation protein α (FAP), DPP8

and DPP9. The most studied of them is DPP4, which has a variety of roles in metabolism, immunity, endocrinology and cancer biology.

Dipeptidyl peptidase-4 (DPP4)

DPP4 is a serine-type protease that NH₂-terminally cleaves off dipeptides with proline or alanine at the penultimate position (154). It is a homodimer and a type II glycoprotein attached to the membrane by its signal peptide. The primary structure consists of a short 6-amino-acid cytoplasmic tail, a 22-amino-acid transmembrane, and a 738-amino-acid extracellular part composed of a flexible stalk with glycosylation-rich region, cysteine-rich region and catalytic region (Figure 14).

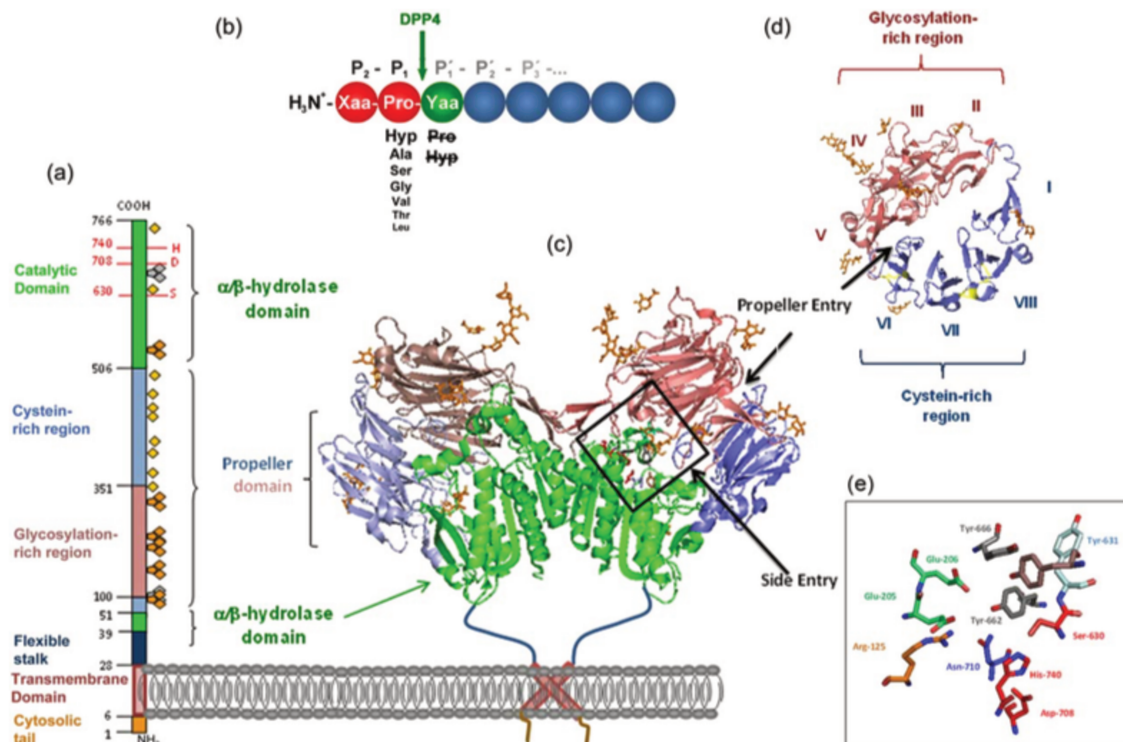


Figure 14. Primary and quaternary structure of human dipeptidyl peptidase-4 (DPP4). (a) Primary structure of DPP4 subunit, consisting of an intracellular tail (aa 1–6), transmembrane region (aa 7–28), flexible stalk (aa 29–39), glycosylated region (aa 101–350), cysteine-rich region (aa 55–100, 351–497), and catalytic region (aa 506–766). (b) Substrate specificity of DPP4. Xaa and Yaa indicate any amino acid. Arrow indicates site of cleavage. (c) Quaternary structure of homodimeric human recombinant DPP4 showing the α/β-hydrolase domain (aa 39–51 and 506–766) in green and propeller domain (aa 55–497) with the glycosylation-rich subdomain (red) and the cysteine-rich subdomain (blue). (d) Propeller domain viewed from the top, illustrating the eight propeller blades designated with roman numbers and two subdomains. (e) Active site zoomed in, depicting the

residues involved in catalysis. S–S bridges are illustrated in yellow and carbohydrates in orange. Adapted from ref. (154)

The human gene is located on chromosome 2q24.3, spans 81.8 kb and include 26 exons. Its expression is regulated at the RNA level and is organ specific (154). DPP4 is ubiquitously expressed by epithelial cells in many organs (liver, gut, kidney, pancreas, uterus), by endothelial cells, and by immune organs (thymus, spleen and lymph node) and immune cells (155) (Figure 15). Among immune cells, DPP4 is expressed in up to 70% of peripheral blood lymphocytes but has been described as a negative marker for human regulatory T (T_{reg}) cells. NK and B cells express low amount of DPP4 but surface expression increases after cytokine stimulation.


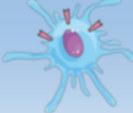






INNATE				
	Neutrophil	Dendritic Cell	Macrophage	NK cell
	CD26 expression low to absent	CD26 expression	CD26 expression low to absent	Low CD26 expression. Activation-induced.
	(154)	(135)	(82,135-137)	(158,160)
ADAPTIVE				
	T_H1 cell	T_H17 cell	T_{reg} cell	B cell
	High CD26 expression. Activation-induced.	Very high CD26 expression. All cells positive.	Very low CD26 expression.	Low CD26 expression. Activation-induced.
	(165-174)	(176)	(176-178)	(162)

Figure 15: DPP4 Expression in immune cells. Adapted from ref. (156).

DPP4 has many substrates: hormones, growth factors, neuropeptides and chemokines sharing the X-Proline or -Alanine motif at their N-terminus.

	Peptide	N-terminus	# Amino acids
Inactivation/Alteration <i>in vivo</i> and <i>in vitro</i>	<i>Pancreatic polypeptides:</i>		
	Peptide YY	YP↓IKPE...	36
	Neuropeptide Y	YP↓SKPD...	36
	<i>Chemokines:</i>		
	SDF-1α	KP↓VSL...	68
	MDC	GP↓YG↓AN	69
	I-TAC	FP↓MFKR...	73
	IP-10	VP↓LSRT...	77
	Mig	TP↓VVRK...	10
	RANTES	SP↓YSSD...	68
	Eotaxin	GP↓ASVP...	74
	LD78β	AP↓LAAD...	70
	<i>PACAP/glucagon family:</i>		
	GLP-1	HA↓EG↓TF...	30
	GIP	YA↓EGTF...	42
	PACAP38/PACAP27	HS↓EG↓IF...	38/27
	Glucagon	HS↓QGTF...	29
	GLP-2	HA↓DG↓SF...	33
	<i>Neuropeptides/Peptides:</i>		
	Substance P	RP↓KP↓Q...	11
	Endomorphin-2	YP↓WF-NH ₂	4
	GRP	VP↓LP↓AG...	27
	Procalcitonin	AP↓FRSA...	116

Table 4: Known DPP4 substrates. Adapted from ref. (154).

DPP4 is well known for its enzymatic ability to inactivate the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose insulinotropic peptide (GIP) thereby its involvement in pathogenesis of type 2 diabetes. These incretins are released upon glucose absorption and enhance the insulin secretion from pancreatic beta cells (154). Once released, GLP-1 and GIP are rapidly degraded by DPP4. Thus, DPP4 competitive inhibitors have been developed to prolong GLP-1/GIP half-life. The first DPP4 inhibitor approved by the Food and Drug Administration (FDA) was sitagliptin (Januvia) in 2006. Then, other eight DPP4 inhibitors have been commercially available, named gliptins (157). All are orally administrated, taken daily and reduce approximately 70–90% of DPP4 baseline with an overall favorable adverse-side effect profile (158).

Another substrate of DPP4 is the chemokine CXCL10 (also called IP-10), responsible of CXCR3-receptor expressing T cell trafficking towards inflammatory sites (159). Some *in vitro* and *in vivo* studies showed that DPP4-mediated N-terminal truncation of CXCL10 led to the generation of an antagonist form, still capable to bind its receptor but without inducing lymphocytes migration anymore (160,161), in line with the “two-site” model of binding. Notably, it has been first shown in the laboratory that elevated levels of cleaved form of CXCL10 were correlated with an increase of DPP4 activity, both associated with viral clearance failure of chronic hepatitis C in patients (162). Next, Barreira et al. demonstrated

that the cleavage of CXCL10 also impaired lymphocytes trafficking limiting their infiltration in the tumor parenchyma, in a mouse model of melanoma and colon cancer (4). Moreover, in this work, they provide evidence that DPP4 inhibitors (sitagliptin) indeed protect active form of CXCL10, enhancing lymphocyte recruitment in tumors. They established preclinical support for the application of DPP4 inhibitors as tumor immunotherapy (4). DPP4 was found to be highly expressed in numerous tumors, notably in HCC (163), and clinical data suggest that elevated DPP4 expression within tumors correlated with worse overall survival in colorectal cancer (164), bladder cancer (165) and gastrointestinal tumor (166) patients. In the same context, DPP4 inhibitors can protect other immune cell activator chemokines that can also participate in antitumor immunity, such as CCL11/eotaxin, which activates eosinophils. Therefore, the use of DPP4 inhibitor could be a therapeutic option to improve leucocyte trafficking in multiple cancers playing through different pathways.

In the following work, we focused on the cytokine IL-33 that can drive T_H2 response and stimulate IL-5 that can in turn activate CCL11. This cytokine could thus initiate eosinophil-mediated antitumor response.

Interleukin-33 (IL-33)

IL-33 is a member of the IL-1 family, a group of 11 pro-inflammatory cytokines which stimulate integrin expression on leucocytes and endothelial cells and regulate inflammatory responses (167). IL-33 acts as an alarmin (also called damaged-associated molecular pattern (DAMP)); it is released by necrotic cells and promotes activation of innate immune system (168).

The human *IL33* gene has eight exons that span around 42 kb of DNA (169) (Figure 16). Protein possesses 270 residues (266 in mice) and the carboxy-terminal region (amino acids 112–270) corresponds to the IL-1–like domain. IL-33 binds to its specific receptor ST2 (170), leading to IL-1RAcP co-receptor recruitment to form a heterodimeric signaling complex.

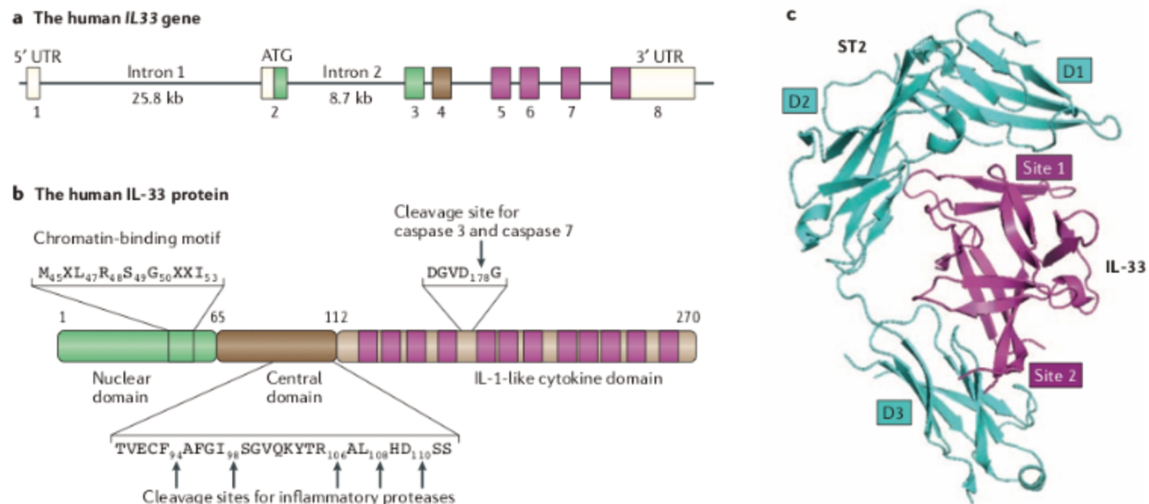


Figure 16. The interleukin-33 gene and protein structure. a) The human interleukin-33 (*IL33*) gene is located on the short arm of chromosome 9 at 9p24.1, whereas its mouse counterpart is found on the syntenic chromosome 19qC1 region1,4. A large intron (>25 kb; intron 1) separates the first non-coding exon (exon 1) from the first coding exon (exon 2). **b)** IL-33 protein is composed of two evolutionary conserved domains (the nuclear domain and the IL-1-like cytokine domain) that are separated by a highly divergent linker region in the center (the central domain). Chromatin-binding motif and cleavage sites for caspases and inflammatory proteases are indicated. **c)** The crystal structure of the IL-33–ST2 complex is shown. Two ST2-binding sites were identified in IL-33 and acidic residues Glu148 and Asp149 at site 1 and Glu165 at site 2 were found to have a crucial role in high-affinity binding by forming specific salt bridge interactions with basic residues of ST2. UTR, untranslated region. Adapted from ref. (167).

IL-33 is localized in nucleus of cells due to its amino-terminal domain (amino acids 1–65) allowing a chromatin association (171). It does not have a signal sequence, but functions as an endogenous danger signal or nuclear alarmin that is delivered after cell injury. IL-33 is constitutively expressed in the nuclei of many cell types such as endothelial, epithelial and fibroblast-like cells and in almost all organs. In context of inflammation, the expression of IL-33 can increase; for example, IL-33 expression is accrued in alveolar type II pneumocystis after exposition to cigarette smoke (172).

Neutrophil and mast cell proteases can process full-length IL-33 to short mature forms (18–21 kDa) that are 10- to 30-fold more effective than the full-length protein (173) (Figure 17a). The processing sites occur in a central domain. Cleaved forms of IL-33 have been detected *in vivo*, e.g. in acute lung injury or airway inflammation caused by allergen, suggesting that this activating cleavage may be determinant for the induction of ST2-dependent responses (173).

Some mechanisms limit IL-33 activity (Figure 17b). The nuclear localization of IL-33 is crucial for immune homeostasis. Besides, IL-33 processing by caspases during apoptosis, through the presence of a consensus site for cleavage by caspases 3 and 7 generate two biologically inactive forms of IL-33 (174). Finally, soluble ST2 receptor may function as a decoy receptor neutralizing IL-33 and oxidation of cysteine residues in extracellular environment may lead to IL-33 inactivation (175).

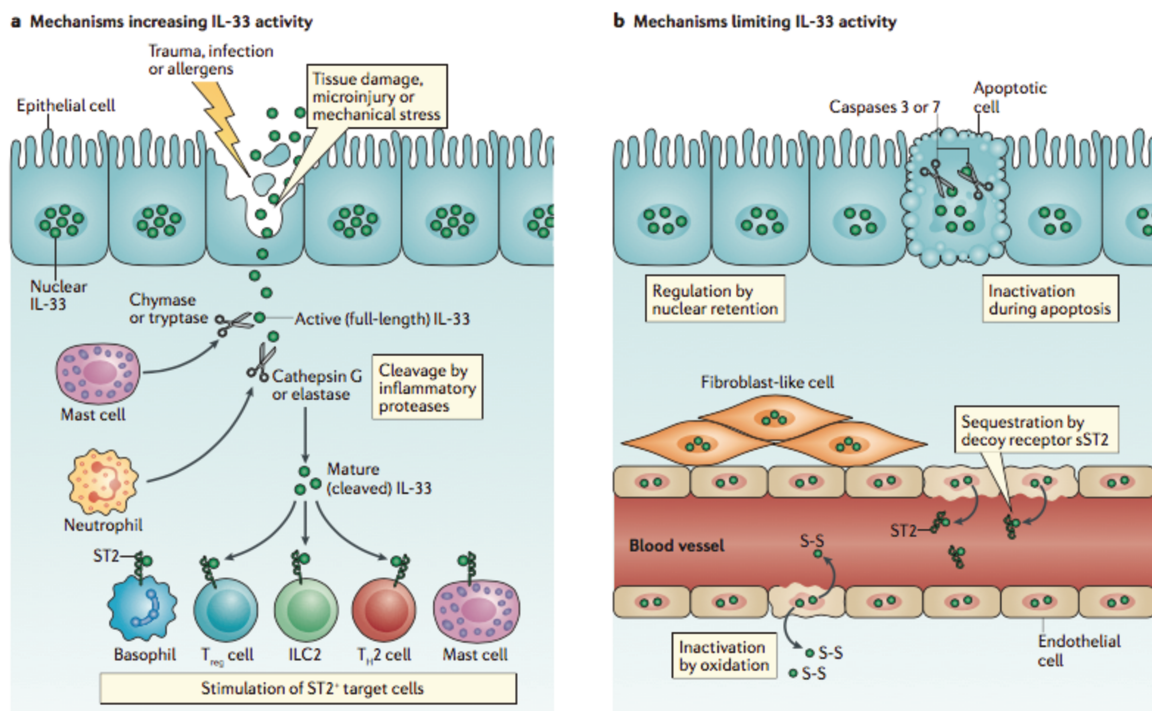


Figure 17: Regulation of IL-33 activity. **a)** IL-33 functions as an alarmin, being released after cell or tissue damage. Full-length IL-33 protein is active. However, processing by inflammatory proteases can generate mature forms that have greatly increased (up to 30-fold) cytokine activity. The mature forms of IL-33 (IL-33 95–270 and IL-33 109–270) can be generated by both neutrophil and mast cell proteases and therefore might be the major bioactive forms of IL-33 in diseased tissues. **b)** IL-33 is a potent pro-inflammatory cytokine and its activity needs to be regulated. Retention of IL-33 in the nucleus of producing cells during homeostasis is crucial to avoid its constitutive release and lethal multi-organ inflammation by signaling through suppression of tumorigenicity 2 (ST2). Cleavage by caspases in the IL-1-like cytokine domain could be an important mechanism of IL-33 inactivation during apoptosis. Extracellular mechanisms are also involved in the inhibition of IL-33 cytokine activity. Sequestration by the decoy receptor soluble ST2 (sST2) is likely to be crucial. Rapid oxidation and formation of disulfide bridges (S-S) in IL-33 following its extracellular release could be another crucial mechanism that limits the range and duration of ST2-dependent responses *in vivo*. ILC2, group 2 innate lymphoid cell; TH2, T helper 2; Treg, regulatory T. Adapted from ref. (167)

IL-33 stimulates both myeloid and lymphoid cells by activating their proliferation and their migration and by triggering production of type 2 immune mediators, notably IL-5 and IL-13, during infection. ST2⁺ lymphoid cells are T_H2 cells, T_{reg} cells, and type 2 innate lymphoid cells (ILC2s). Macrophages and dendritic cells (176) also express ST2, as well as eosinophils (177), which are effector immune cells during parasite infections. The stimulation of T_{reg} cells and ILC2s would allow the repair of tissues after elimination of pathogen. IL-33 can also activate T_H1 immune responses by stimulating ST2⁺ T_H1 cells, CD8⁺ T cells and NK cells (178). This expression of ST2 in these cells implicated in T_H1 responses may be a mechanism to redirect the default type 2-inducing capacity of IL33 to alternative effectors (179). Beneficial role of IL-33 in infectious diseases is known in helminth infection (180) and fungal candida albicans infection (181) in mice through the activation of mast cells, neutrophils and macrophages. Its involvement in bacterial and viral infections appears very variable and pathogen-dependent.

On the other hand, IL-33 can be deleterious in some inflammatory diseases where it can induce excessive inflammatory responses leading to tissues damages. For example, many studies showed the implication of IL-33 in airway inflammatory diseases (168,179), in particularly in asthma, where IL-33 level is correlated with severity (182). The airway inflammation is driven by ILC2s producing type 2 cytokines that mobilize eosinophils (183) and polarize M2 macrophages.

IL-33 is involved in tumor-associated inflammation (184). Many studies showed that serum IL-33 levels are increased in many cancers, e.g. hepatocellular carcinoma (185), breast cancer (186), gastric cancer (187) and lung cancer. IL-33 is also detected in epithelial tumor cells, especially in well-differentiated carcinomas. Tumor development results in IL-33 downregulation in epithelial cells but upregulation in the stroma and serum. Notably, IL-33 acts on tumor stromal cells by activating carcinoma-associated fibroblasts (CAF) and alternatively activated macrophages (AAM) in an autocrine and paracrine manner (188). IL-33 can also directly cooperate with oncogenes and promote tumor cell proliferation and metastasis (189). On the other hand, downregulation of IL-33 in tumor epithelial cells is correlated with tumorigenesis, suggesting that maintaining the level of IL-33 in these cells might suppress cancer progression. Remarkably, some studies reported that ST2 is expressed on CD8⁺ T cells, T_H1 cells, NK and NKT cells, indicating a role for IL-33/ST2 in type 1 immune responses (190-192). Furthermore, one study reported that the expression of IL-33 in murine B16-F10 tumor cells inhibited tumor growth and metastasis, by increasing the number

of IFN- γ -producing CD8⁺ T cells and NK cells (193). Finally, systemic administration of IL-33 inhibited growth of established tumors in several mouse models via promotion of antitumor CD8⁺ T cells (194).

In human HCC, immuno-histochemical analyses indicated higher levels of IL-33-positive tissue in HCC compared to normal liver (195). Particularly, HCC analyses showed only cytoplasmic IL-33 whereas normal liver samples showed both nuclear and cytoplasmic IL-33 in hepatocytes, suggesting a potential role of that cytoplasmic/non-nuclear IL-33 in HCC development (196). Furthermore it was observed higher amount of serum sST2 in patients with cirrhosis or HCC compared to healthy controls, with a correlation with overall survival (185). At least, in resected HCC, infiltration tissue with IL33⁺ cells was correlated with prolonged patient survival, due to the activation of cytotoxic IL-33-producing effector-memory CD8⁺ T cells (197).

In sum, IL-33 is an alarmin, capable of inducing innate immune response and modulating tumorigenesis. This modulation can be pro- or anti-tumorigenic, depending on the localization of expression (tumor cells or stroma cells), the type and the stage of cancers, and maybe other parameters to be determined.

II. PRE-CLINICAL PROJECT

Introduction

Tumor growth and immune evasion have been associated with poor cytotoxic T cell responses. This may occur as a result of the failure to prime tumor antigen-specific T cells, or due to the presence of tumor-associated immunosuppressive cells (198). Additionally, it has been described that lymphocyte migration may be perturbed by the post-translational modification (PTM) of CXCR3 ligands (159). Previous work in the lab established that DPP4-mediated N-terminal cleavage of CXCL10 limits T and NK cell migration in pre-clinical models of melanoma and colorectal carcinoma (CRC) (4). DPP4 inhibitor (DPP4i)-treated mice showed both improved naturally occurring tumor immunity and enhanced responses to T cell-mediated immunotherapy. Our aim was to study the impact of DPP4 in a model of hepatocellular carcinoma and to study whether DPP4 inhibition improved leucocyte migration into HCC tumor.

Results

DPP4 inhibition delays growth of HCC and enhances eosinophil migration

To explore the role of DPP4 in the context of HCC, we used an experimental mouse model of Hepa 1-6 inoculated in syngeneic wild type (WT) C57BL/6 mice. Animals were fed with control chow or with chow containing DPP4i (1.1% sitagliptin) (4). The level of tumor-associated DPP4 enzymatic activity was diminished upon DPP4i administration, confirming the expected pharmacodynamics effects of this treatment (Fig. 1A). Next, we studied the development of the tumors, and observed significantly reduced growth (Fig. 1B) and tumor mass (Fig. 1C) in DPP4i-treated mice. Of note, DPP4i did not alter the growth of the tumor cells *in vitro*, thus excluding a direct effect on tumor cell proliferation or metabolic activity (Fig. 1D).

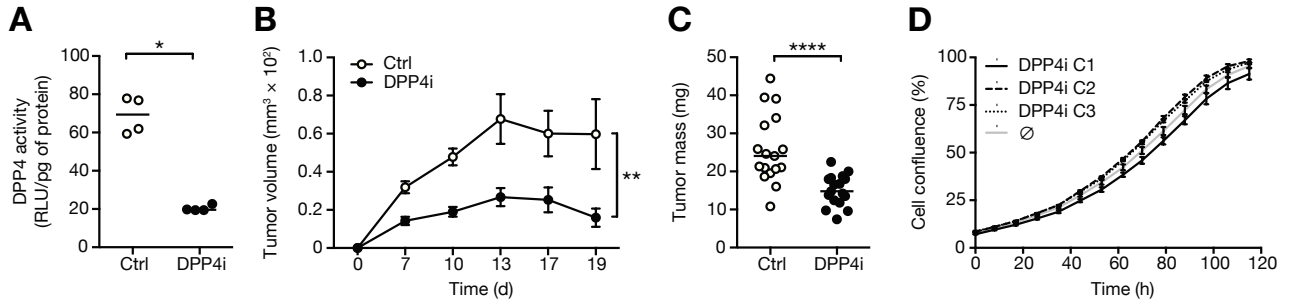


Figure 1: DPP4i delays growth of HCC. (A) Wild type (WT) C57BL/6 mice were fed with chow containing 1.1% sitagliptin (DPP4i) or with control (Ctrl) chow and subcutaneously injected with Hepa1-6 cells. Eight days after tumor cell inoculation, tumors were collected and homogenates were prepared. DPP4 activity per tumor mass was determined and normalized to the amount of DPP4 protein (RLU, relative luminescence units). (B) Mice were treated as in described in A and tumor volumes were measured over time (mean \pm s.e.m $n \geq 16$ mice per group, d = days). (C) Tumors were collected at day 10 post-inoculation and tumor mass was determined. (D) Hepa1-6 cells were cultured in the presence of 5 (C1), 0.05 (C2) or 0.005 (C3) $\mu\text{g/mL}$ of DPP4i, or left untreated (\emptyset). Cell confluence was measured over time using the Incucyte (mean \pm s.e.m $n = 3$ technical replicates). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significance was determined using Mann–Whitney test (A and C) or two-way analysis of variance (B).

When examining tumor-associated leucocytes, we observed no differential accumulation of T lymphocytes, NK cells, NKT cells or neutrophils between the control and treatment groups (Fig. 2A and B), but there was a significantly greater infiltration of eosinophils in tumors from mice receiving DPP4i (Fig. 2A and B). We also found a higher infiltrate of eosinophils in tumors derived from *Rag2*^{-/-} mice treated with DPP4i compared to control (Fig. 2C).

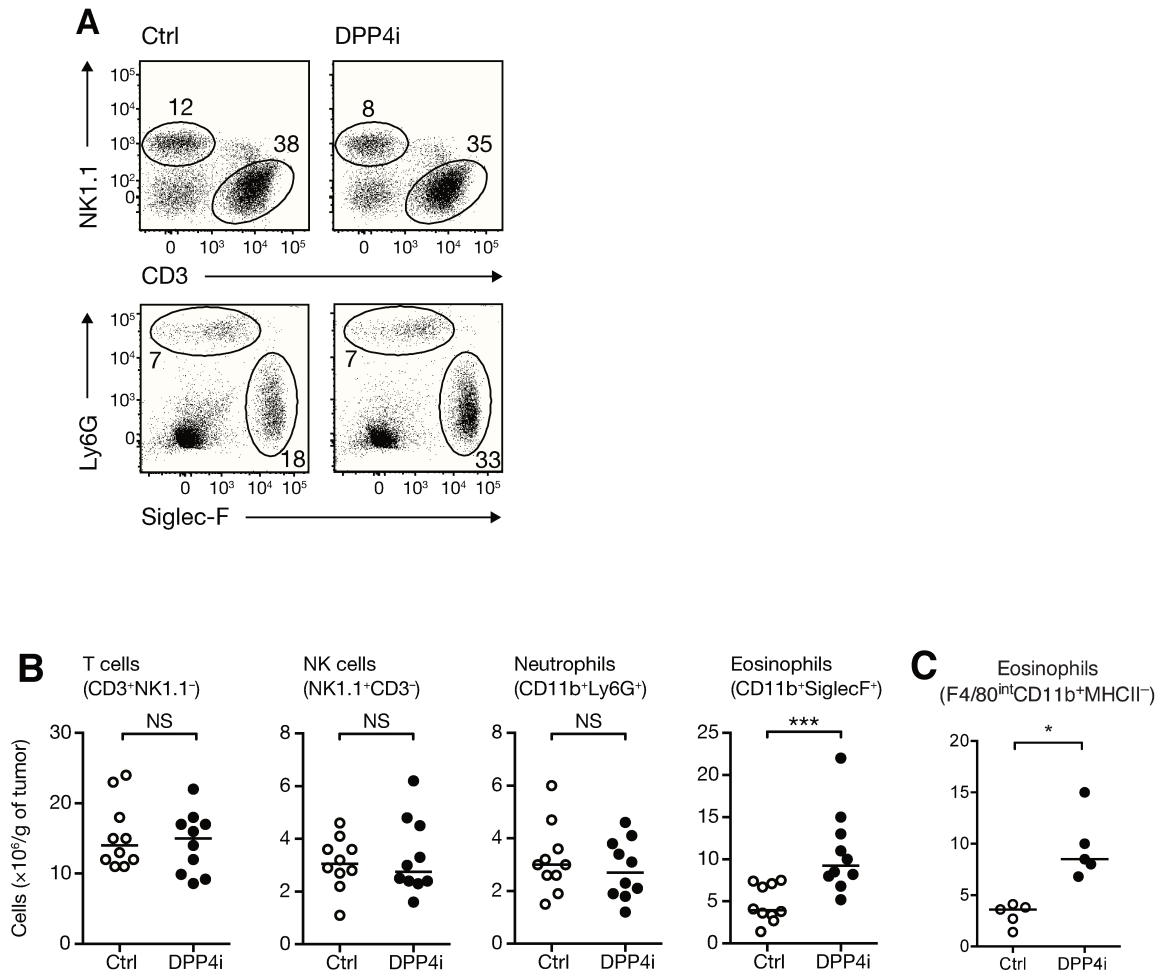
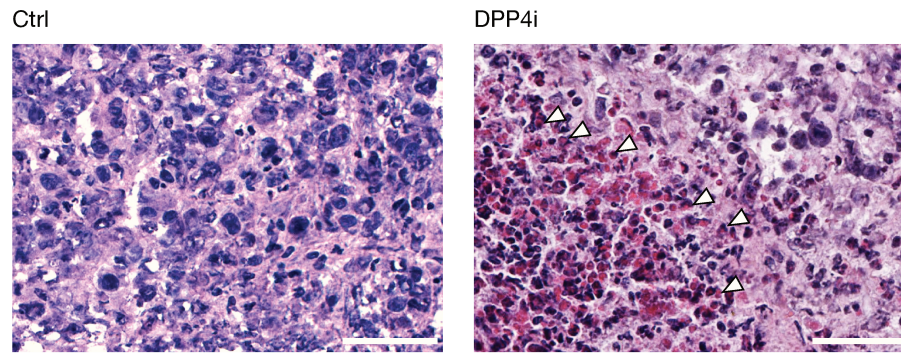


Figure 2: DPP4i enhances HCC eosinophil infiltration. (A) WT mice were treated as in Figure 1A. Tumors were collected 10 days after inoculation and tumor-associated leucocytes were analyzed by flow cytometry. Representative flow cytometry plots for the analysis of T cells (CD3⁺NK1.1⁻), NK cells (CD3⁻NK1.1⁺), neutrophils (CD11b⁺Ly6G⁺) and eosinophils (CD11b⁺Siglec-F⁺). (B) Number of tumor-associated leucocyte populations is shown. (C) *Rag2*^{-/-} mice were treated as in Figure 1A. Tumors were collected 17 days after inoculation and tumor-associated leucocytes were analyzed by flow cytometry. NS, not significant, **P* < 0.05. ****P* < 0.001. Significance was determined using the Mann-Whitney test.

Histological analysis confirmed the higher number of eosinophils infiltrating the Hepa 1-6 tumors in DPP4i-treated mice (Fig. 3).



Semi-quantitative analysis of eosinophils		
Mouse	Control	DPP4i
1	+	++
2	0	++
3	0	++
4	+	++
5	0	++
6	0	+

Figure 3: Histological analyses of Hepa 1-6 tumors. Representative illustration of eosinophils infiltration in Hepa1-6 tumors in mice receiving Ctrl (left) or DPP4i (right) after hematein and eosin staining. Arrows indicate eosinophils. Scale, 100 μ m. Semi-quantitative analysis of eosinophils. $n = 6$ mice per group. *Analysis performed by V. Paradis.*

Furthermore, we detected elevated levels of cytotoxic granule-associated eosinophil peroxidase (EPX) and eosinophil cationic protein (ECP) in tumors isolated from DPP4i-treated mice (Fig. 4). Together, these data suggest that DPP4i administration is beneficial for the control of Hepa1-6 tumor growth and indicate eosinophil accumulation and degranulation as a possible tumoricidal mechanism.

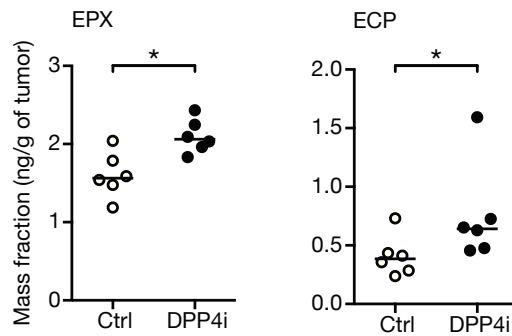


Figure 4: Eosinophil granules in HCC tumors. Eosinophil peroxidase (EPX) and eosinophil cationic protein (ECP) expression was quantified by ELISA in extracts from Hepa1-6 tumors collected 8 days after inoculation. Each dot corresponds to one mouse. * $P < 0.05$. Significance was determined using Mann–Whitney test.

DPP4 inhibition delays HCC growth in an eosinophil-dependent manner

Despite the unchanged number of lymphocytes, we tested the role of the adaptive immune system as mediator of anti-tumor responses in DPP4i-treated animals. Tumors were implanted in WT mice that received a combination of anti-CD4 and anti-CD8 depleting antibodies or alternatively, inoculation was done in *Rag2*^{-/-} mice (lacking T and B lymphocytes). While the beneficial effect of DPP4i treatment on B16F10 was lost if T cells are not present (4), the positive effects of DPP4i treatment on tumor growth remained evident in the Hepa 1-6 model (Fig. 5A and B). These findings supported DPP4 as a negative regulator of anti-tumor responses, but challenged enhanced T cell migration as the suggested mechanism of action in this model.

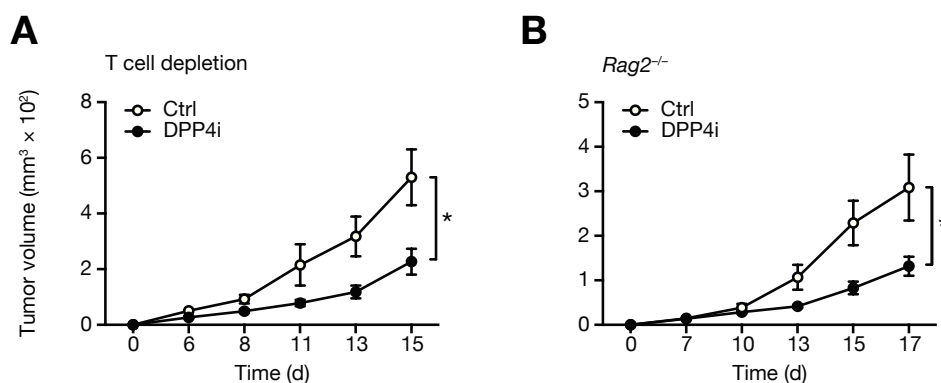


Figure 5: HCC tumor growth in T cell depleted models. (A) Wild type (WT) C57BL/6 mice were fed with chow containing 1.1% sitagliptin (DPP4i) or with control (Ctrl) chow and subcutaneously injected with Hepa 1-6 cells. Mice were dosed with anti-CD4 anti-CD8 antibodies depleting antibodies on the

day of tumor cell inoculation and every 6 days after. Tumor volumes were assessed; (mean \pm s.e.m, $n = 11$ mice per group). **(B)** Hepa 1-6 cells were inoculated in *Rag2*^{-/-} mice treated with Ctrl or DPP4i chow as described in A. Tumor volumes are shown; (mean \pm s.e.m $n = 5$ mice per group). * $P < 0.05$. Significance was determined using two-way analysis of variance.

To decipher the mechanisms behind DPP4i-mediated control of HCC, we performed protein analysis on extracts of tumors grown in WT mice fed with control or DPP4i chow. The amount of the T cell chemoattractant CXCL10 was similar between the different experimental groups (Fig. 6), providing an explanation for the unchanged lymphocyte accumulation. Furthermore, the levels of IFN- γ were significantly lower in tumor samples from DPP4i-treated mice (Fig. 6). By contrast, we noticed higher level of CCL11 and increased concentration of IL-4, IL-5 and IL-33 upon DPP4i treatment (Fig. 6).

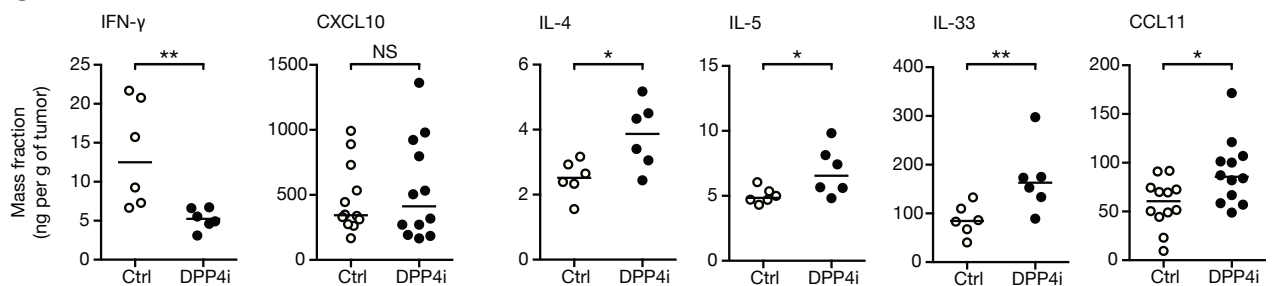


Figure 6: Cytokines and chemokines expression in HCC tumors. Quantification of cytokine and chemokine expression in extracts from Hepa1–6 tumors collected 8 d after inoculation. * $P < 0.05$, ** $P < 0.01$. Significance was determined using the Mann–Whitney test.

While CCL11 was previously shown to regulate eosinophil migration, IL-4, IL-5 and IL-33 play an important role in eosinophil homeostasis and activation (106,199). Therefore, we assessed the role of eosinophils in our HCC model by treating mice with anti-Siglec-F antibodies (previously shown to efficiently deplete Siglec-F-expressing eosinophils (200)) and studied tumor growth in the presence and absence of DPP4i. Strikingly, depletion of eosinophils in WT and *Rag2*^{-/-} mice abrogated the anti-tumor responses initiated by DPP4i treatment (Fig. 7), providing direct evidence for a role of eosinophils in this experimental model.

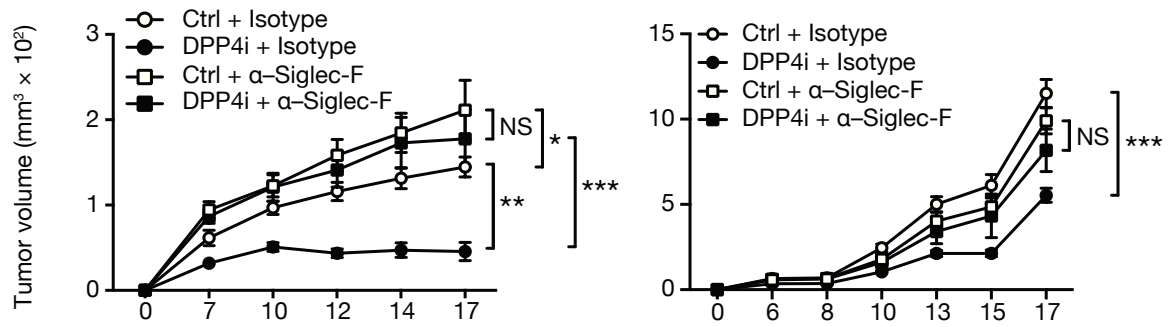


Figure 7: HCC tumor growth in eosinophil-depleted models. WT mice (left panel) and *Rag2*^{-/-} mice (right panel) were treated as in Figure 4A. Depletion of eosinophils was performed by intraperitoneal injection of α-Siglec-F antibody. Parallel groups were injected with isotype control. Tumor volumes were measured over time; (mean ± s.e.m, $n \geq 9$ mice per group). NS, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significance was determined using two-way analysis of variance.

In order to study if CCR3-mediated migration and eosinophil degranulation were important, we treated mice with anti-CCR3 blocking antibodies or with histamine H1 receptor antagonist (cetirizine) (201) and observed a loss of beneficial effect initiated by DPP4i (Fig. 8). Together, these data demonstrate that inhibition of DPP4 enhances anti-tumor responses in the manner that is dependent on CCR3 expression and eosinophil degranulation.

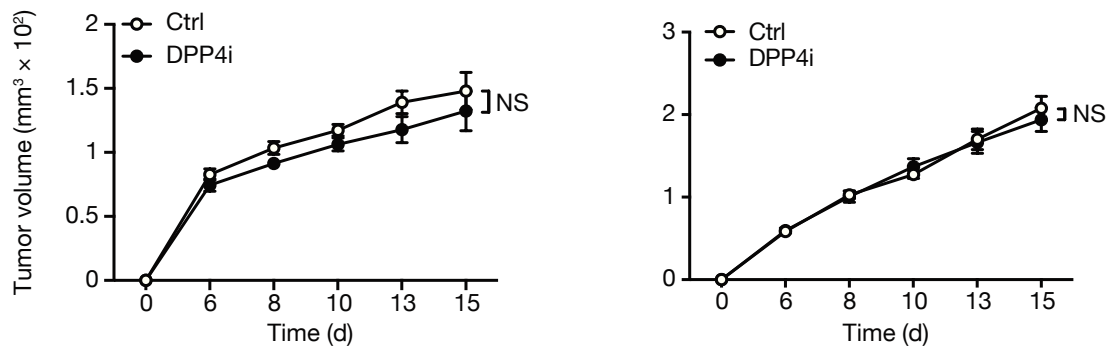


Figure 8: HCC tumor growth after CCR3⁺ eosinophil depletion and after eosinophil degranulation inhibition. WT mice fed with Ctrl or DPP4i chow were inoculated with Hepa 1-6 tumor cells and treated with α-CCR3 blocking antibody (left panel). Tumor volumes are shown; (mean ± s.e.m $n \geq 11$ mice per group). WT mice fed with Ctrl or DPP4i chow were dosed with cetirizine (α-H1) incorporated in the drinking water (right panel). Hepa 1-6 tumor cells were inoculated and tumor growth was assessed; (mean ± s.e.m, $n \geq 11$ mice per group).

DPP4 inhibition acts on CCL11 to enhance CCR3-activated eosinophil migration

To gain insight into DPP4 regulation of eosinophil migration, we examined its role on the main eosinophil chemoattractant in mouse, CCL11. As observed with human CCL11 (Fig. 9A), we detected N-terminal truncation of two amino acids from recombinant mouse CCL11 upon incubation with recombinant DPP4 (Fig. 9A and B). The cleaved form of CCL11 (referred to as CCL11₃₋₇₄) has been reported to have reduced chemoattractive properties (202). In order to access the *in vivo* role of DPP4 enzymatic activity on CCL11-mediated eosinophil recruitment, we fed WT mice with control chow or DPP4i and studied eosinophil mobilization after intravenous CCL11₁₋₇₄ administration. Strikingly, eosinophil recruitment was only evident upon treatment with DPP4i (Fig. 9C); furthermore, we observed downregulation of CCR3 expression on eosinophils (Fig. 9D) suggesting that inhibition of DPP4 enhances CCL11/CCR3 signaling. Of note, intravenous injection of CCL11₃₋₇₄ failed to induce eosinophil recruitment in DPP4i-treated mice (Fig. 9E), confirming its reduced chemotactic activity. These data support a role for DPP4 as a negative regulator of CCL11-mediated eosinophil migration *in vivo*.

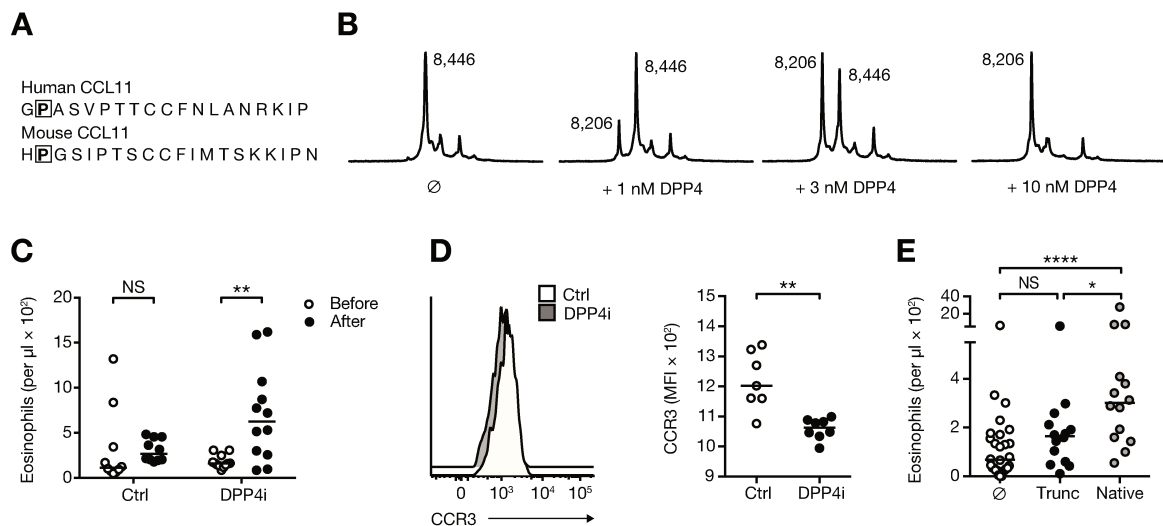


Figure 9: Fig. S2: DPP4 inhibition acts on CCL11 to enhance CCR3-activated eosinophil migration. (A) Alignment of the first 19 N-terminal amino acids of human and mouse CCL11 sequences indicated the presence of a proline (boxed) in the second position of the N terminus. (B) Recombinant mCCL11 was incubated with different concentrations of DPP4 and analyzed by SELDI-TOF-MS. Numbers indicate the molecular weight (in daltons). (C,D) Wild-type mice fed with control or DPP4i chow were injected intravenously 1 μg of CCL11 and blood was collected before and 1 h after injection. Eosinophil numbers (C) and CCR3 expression on eosinophils after injection (D) were determined by flow cytometry. (E) Wild-type mice fed with DPP4i chow were injected intravenously with PBS (\emptyset),

DPP4-truncated (trunc) or native (native) CCL11; eosinophil numbers were determined by flow cytometry. Bars show median and data are representative of two experiments (B,D) or are pooled from two independent experiments (C,E). NS, not significant; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ (Mann–Whitney test).

DPP4 inhibition delays growth of HCC independently of the adaptive immune system and ILC

Type 2 innate lymphoid cells (ILCs) are present in $Rag2^{-/-}$ mice and are major producers of IL-4, IL-5 and IL-13 during helminthic infection (203). ILCs have also been implicated in tumor immunity, having been shown to diminish the number of melanoma metastases in an IL-5 and eosinophil-dependent manner (204,205). We therefore tested the role of ILCs in the HCC model by evaluating tumor growth in $Rag2^{-/-}\gamma_c^{-/-}$ mice and $Rag2^{-/-}$ mice treated with anti-Thy1 (CD90) depleting antibodies. In both experiments, administration of DPP4i resulted in reduced tumor growth, suggesting that ILCs are not responsible for the beneficial effect observed in our model (Fig. 10A and B). Furthermore, these results suggest that ILCs are not playing a role in eosinophil-mediated delay of HCC growth.

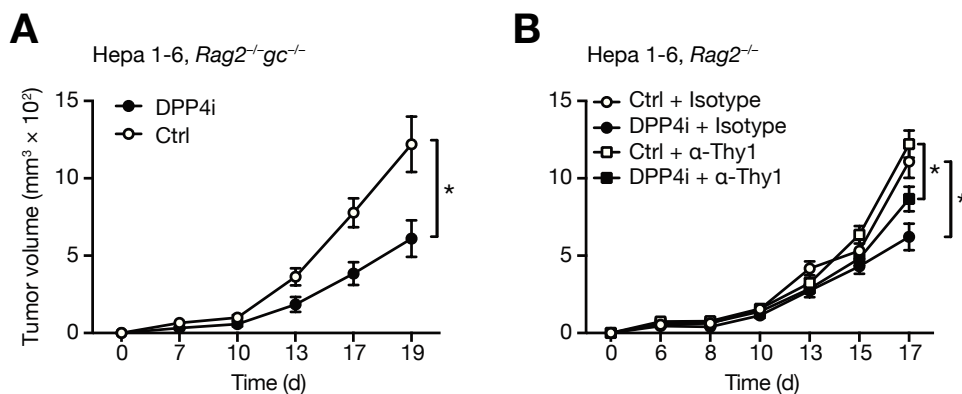


Figure 10: DPP4 inhibition delays growth of HCC independently of the adaptive immune system and ILC. (A) $Rag2^{-/-}\gamma_c^{-/-}$ mice were fed with control or DPP4i chow and Hepa 1-6 tumor cells were transferred. Tumor volumes were assessed; $n = 5$ mice per group. (B) $Rag2^{-/-}$ mice were fed with control or DPP4i chow and Hepa 1-6 tumor cells were transferred. Mice were intraperitoneally injected with anti-Thy1 depleting antibodies or with isotype control antibodies. Tumor volumes were assessed; $n = 12$ (DPP4i + isotype), 11 (Ctrl + isotype and Ctrl + α -Thy1) or 10 (DPP4i + α -Thy1) mice per group. Data show mean \pm s.e.m and are representative of two experiments. * $P < 0.05$ (two-way analysis of variance).

Tumor IL-33 expression is required and sufficient to induce eosinophil-mediated tumor immunity upon DPP4 inhibition

In an attempt to decipher the mechanisms promoting eosinophil migration into HCC tumors, we tested the hypothesis that tumor cell-derived factors may enhance production of CCL11, and that treatment with DPP4i limits its cleavage, thus supporting eosinophil recruitment. Notably, IL-33 (whose expression was enhanced in HCC tumors growing in DPP4i-treated mice (Fig. 6)) may be expressed by tumors and is known to promote eosinophil activation (206,207). Interestingly, we observed that intratumoral administration of IL-33 was capable of inducing CCL11 expression *in vivo* (Fig. 11A). When evaluating IL-33 expression in four different mouse tumor cell lines growing *in vitro*, we detected high protein expression in Hepa 1-6 cells (average 3.72 pg of IL-33 per 10^3 tumor cells (Fig. 11B)). We also observed IL-33 production by EMT6 cells (a breast carcinoma cell line syngeneic to the BALB/c strain) but not by B16-F10 or TRAMP-C2 (respectively melanoma and prostatic carcinoma cells, syngeneic to the C57BL/6 strain) (Fig. 11B).

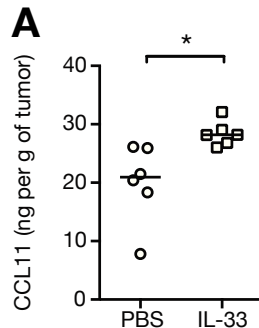


Figure 11A: IL-33 induces CCL11 expression into HCC tumor. WT C57BL/6 mice were subcutaneously injected with Hepa 1-6 cells. Seven days later, they received an intratumoral injection of PBS or 1 μ g of mIL-33. Tumors were harvested 6 h later and CCL11 expression was assessed in tumor homogenates.

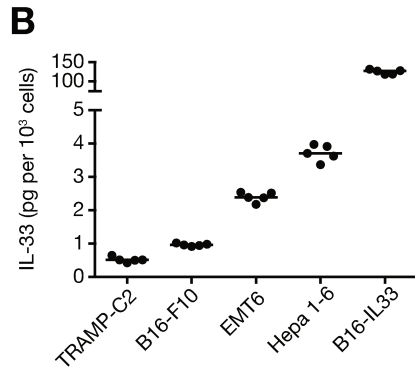
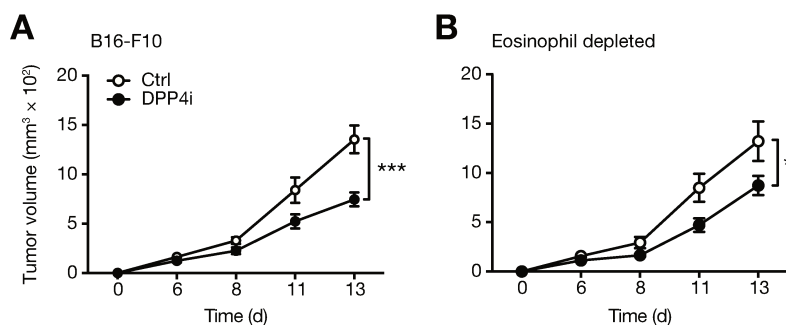


Figure 11B: IL-33 expression levels in different tumor cell lines. Quantification of mIL-33 expression in mouse tumor cell lines.

To test a possible correlation between IL-33 expression by tumor cells and eosinophil-mediated control of tumor growth, we inoculated TRAMP-C2, B16F10 and EMT6 tumor cells in WT mice treated with control or DPP4i chow. As previously described, treatment with DPP4i delayed B16-F10 growth (4) (Fig. 12A) and strikingly, also all other tumor models studied (Fig 12B and C). Next, eosinophil or T cell depletion were tested as means to assess the role of these leucocytes in DPP4i-mediated efficacy. Depletion of T cells (but not eosinophils) abolished the delay of TRAMP-C2 growth, suggesting that DPP4i enhances T-cell mediated anti-tumor immunity (Fig. 12C). Notably, in analogy with the observations made in Hepa 1-6, depletion of eosinophils was sufficient to prevent DPP4i-mediated delay of EMT6 growth (Fig. 12D), while it had no impact on the B16-F10 (Fig. 12B) and TRAMP-C2 (Fig 12C) models. These data suggest that DPP4i may enhance anti-tumor responses mediated by distinct immune-related mechanisms. Notably, expression of IL-33 by tumor cells correlated with enhanced eosinophil-dependent anti-tumor responses, suggesting that this cytokine may be assisting immune mechanisms that are enhanced by the usage of DPP4i.



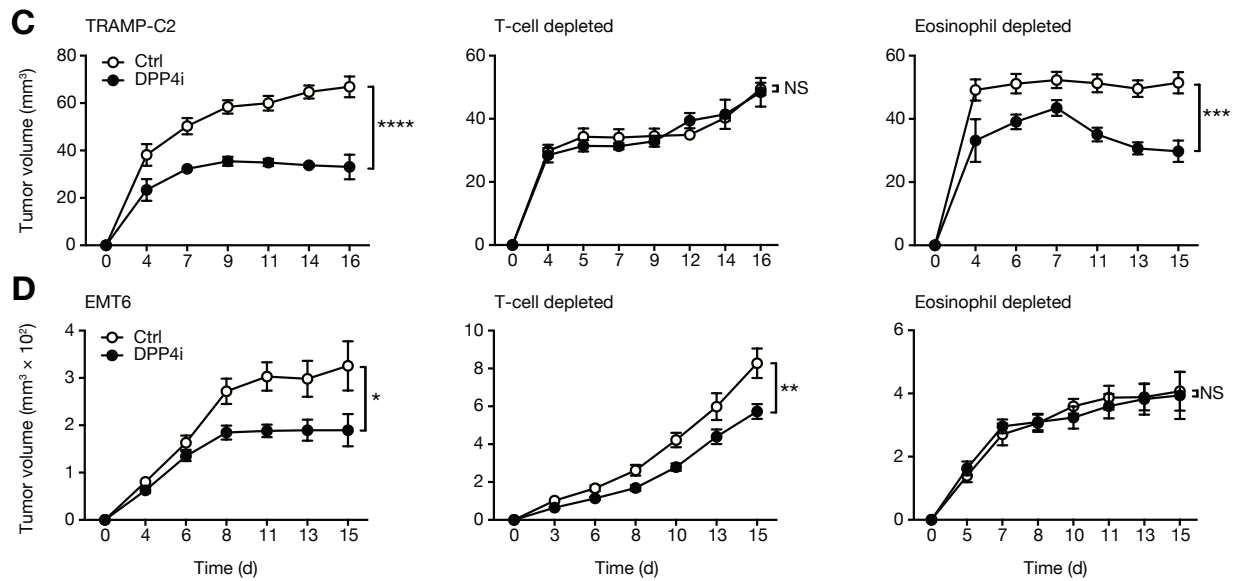


Figure 12: Tumor IL-33 expression is required to induce eosinophil-mediated tumor immunity upon DPP4 inhibition. (A) Wild-type mice were fed with control or DPP4i chow and B16F10 tumor cells were transferred. Tumor volumes were assessed; $n = 6$ mice per group. (B) Wild-type mice were fed with control or DPP4i chow and B16F10 tumor cells were transferred. Mice were T-cell depleted with anti-Siglec-F antibodies intraperitoneal injection. Tumor volumes were assessed; $n = 6$ mice per group. Data show mean \pm s.e.m and are representative of two experiments. (C and D) WT C57BL/6 or BALB/C mice were fed with Ctrl or DPP4i chow and TRAMP-C2 (C) or EMT6 (D) tumor cells were transferred. Mice were treated with anti-CD4 and anti-CD8 antibodies (T-cell depleted) or anti-Siglec-F antibody (eosinophil depleted) or with the corresponding isotype controls (left graph). Tumor volumes were assessed (mean \pm SEM, $n = 6$ mice per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significance was assessed with two-way analysis of variance.

In order to study if tumor-associated IL-33 expression is important for eosinophil-mediated tumor immunity, we utilized IL-33-expressing B16-F0 tumor cells (referred to as B16-IL33) (193) (Fig. 11B). As previously reported (193), we observed a delay in B16-IL33 growth, when compared with the parental cell line, (Fig 13A). Notably, treatment with DPP4i resulted in a significantly growth delay of B16-IL33 and administration of anti-Siglec-F antibodies abolished this phenotype (Fig. 13B and C), revealing that inhibition of DPP4 enhances eosinophil anti-tumoral functions when IL-33 is expressed.

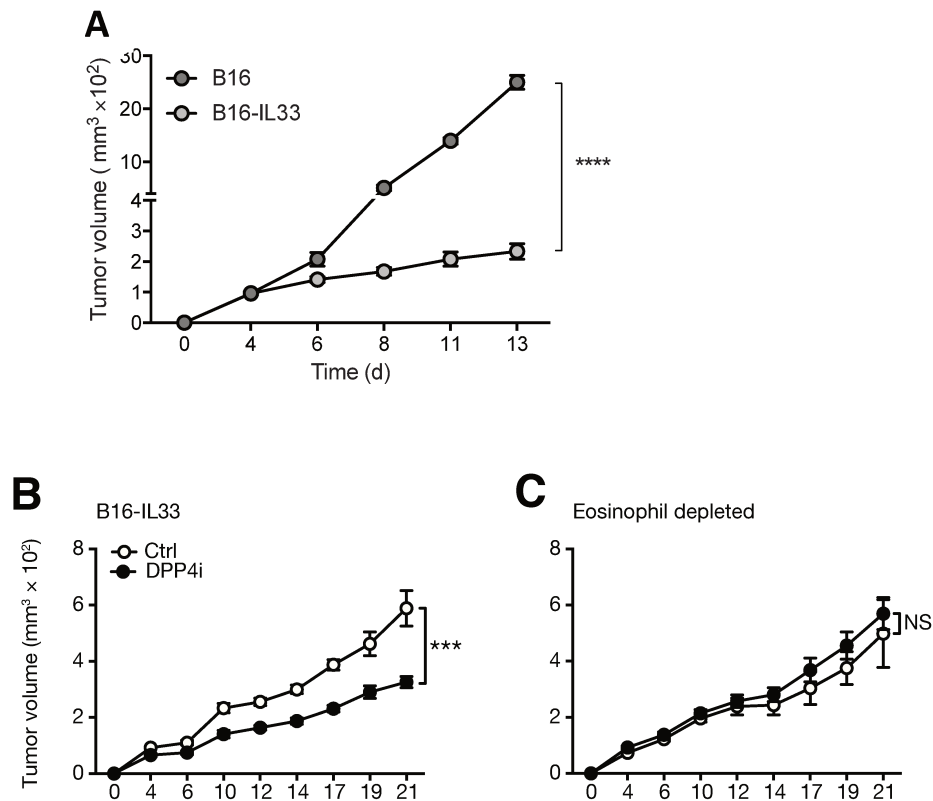


Figure 13: IL-33 is sufficient to induce eosinophil-mediated tumor immunity upon DPP4 inhibition. (A) WT mice were fed with Ctrl chow before transfer of B16-F10 or B16-IL33 tumor cells (mean \pm SEM, $n = 6$ mice per group). (B and C) WT mice were fed with Ctrl chow or DPP4i before transfer of B16-IL33 tumor cells. Eosinophil depletion was performed by intraperitoneal injection of anti-Siglec-F antibody. Tumor growth is shown (mean \pm SEM, $n = 5-6$ mice per group). NS, not significant; *** $P < 0.001$, **** $P < 0.0001$. Significance was determined using two-way analysis of variance.

To further assess the role of IL-33 as an inducer of tumor immunity, we inoculated Hepa 1-6 or EMT6 cells in WT mice treated with control or DPP4i and analyzed tumor growth upon dosing of anti-IL-33 blocking antibody (208). Strikingly, the efficacy of DPP4 inhibition was lost upon blocking of IL-33 (Fig. 14A and B).

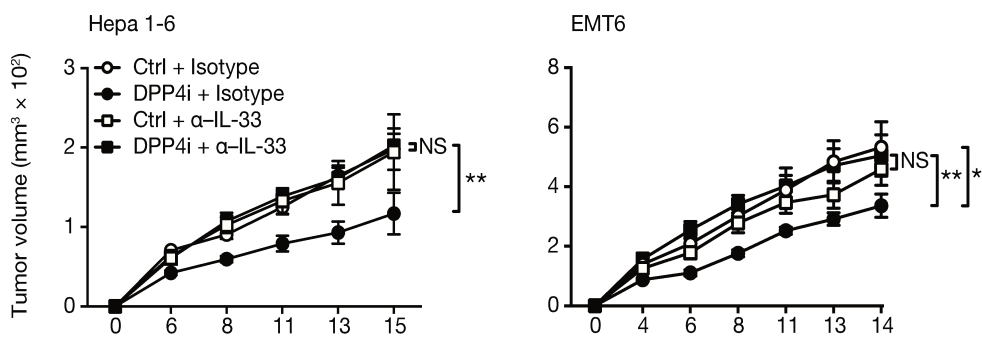


Figure 14: IL-33 is required to induce eosinophil-mediated tumor immunity upon DPP4 inhibition. WT mice were fed with Ctrl chow or DPP4i and inoculated with Hepa 1-6 (A) or EMT6 (B) tumor cells. Mice were treated with anti-IL-33 blocking antibody, or with isotype control. Tumor volumes were measured (mean \pm SEM, $n = 6$ mice per group). * $P < 0.05$, ** $P < 0.01$. Significance was determined using two-way analysis of variance.

In a similar way, treatment with DPP4i did not delay growth of Hepa 1-6 tumors when inoculated in $St2^{-/-}$ mice (deficient for expression of IL-33 receptor) (Fig. 15A). Notably, administration of DPP4i to mice inoculated with B16-F10 cells resulted in significant delay in both $St2^{+/+}$ and $St2^{-/-}$ strains (fig 15B).

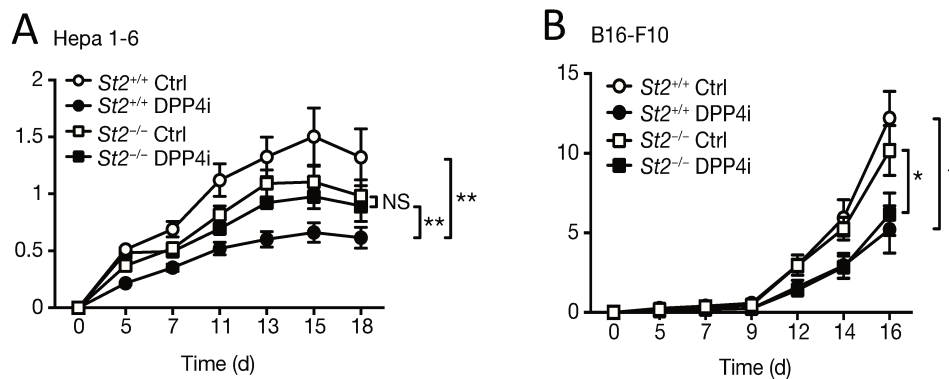


Figure 15: Eosinophil-mediated tumor immunity upon DPP4 inhibition is mediated through the IL-33/ST2 pathway in Hepa 1-6 but not in B16-F10. $St2^{+/+}$ or $St2^{-/-}$ mice were fed with Ctrl or DPP4i chow, and Hepa 1-6 (A) or B16-F10 (B) tumor cells were transferred. Tumor volumes are shown (mean \pm SEM, $n = 10$ mice per group). NS, not significant; * $P < 0.05$, ** $P < 0.01$. Significance was determined using two-way analysis of variance.

Together, these data indicate that tumor-derived IL-33 is capable of inducing eosinophil-mediated tumor immunity, and suggest that DPP4 limits this mechanism possibly through truncation and inactivation of chemokines involved in eosinophil migration.

Eosinophils contribute to checkpoint inhibitor efficacy in IL-33–producing tumors, a mechanism that is enhanced by inhibition of DPP4

Finally, we considered evaluating the role of eosinophils and DPP4i during checkpoint blockade immunotherapy. Treatment with anti-PD1 and anti-CTLA-4 was previously

reported to reduce CT26 growth and administration of DPP4i enhanced tumor rejection (4). We therefore tested if this strategy could also be applied to the models of Hepa 1-6 and EMT6. Inhibition of PD1 and CTLA-4 reduced growth of both models (Fig 16A and B); and triple combination with DPP4i resulted in a significant higher efficacy (Fig. 16).

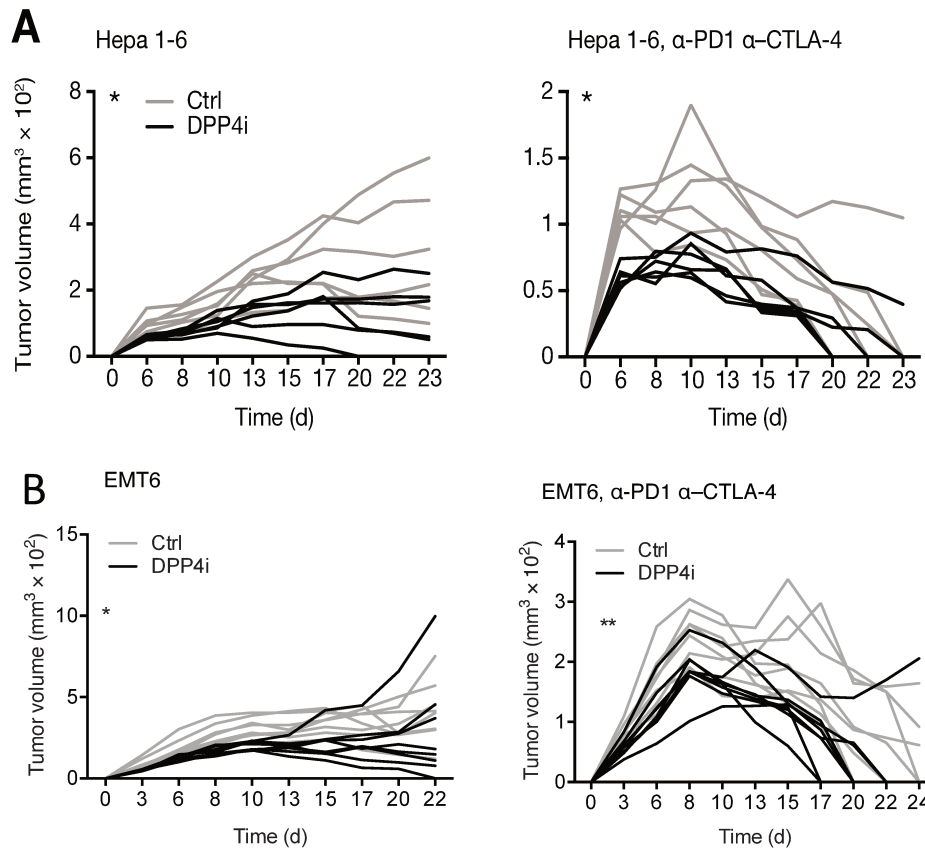


Figure 16: DPP4i improves checkpoint blockades treatment. Wild-type mice were fed with control or DPP4i chow and Hepa 1-6 (A) or EMT6 (B) tumor cells were transferred. At days 3, 6, and 9, mice were intraperitoneal injected with anti-PD1 and anti-CTLA-4 antibodies or isotype controls. Tumor volumes were assessed; lines represent single mice; $n = 6$ per group. Data are representative of two experiments. * $P < 0.05$, ** $P < 0.01$ (two-way analysis of variance).

Notably, even in the presence of a strong activator of T cell immunity (209), efficacy was significantly reduced upon depletion of eosinophils, abolishing the difference between DPP4i and control groups (Fig. 17).

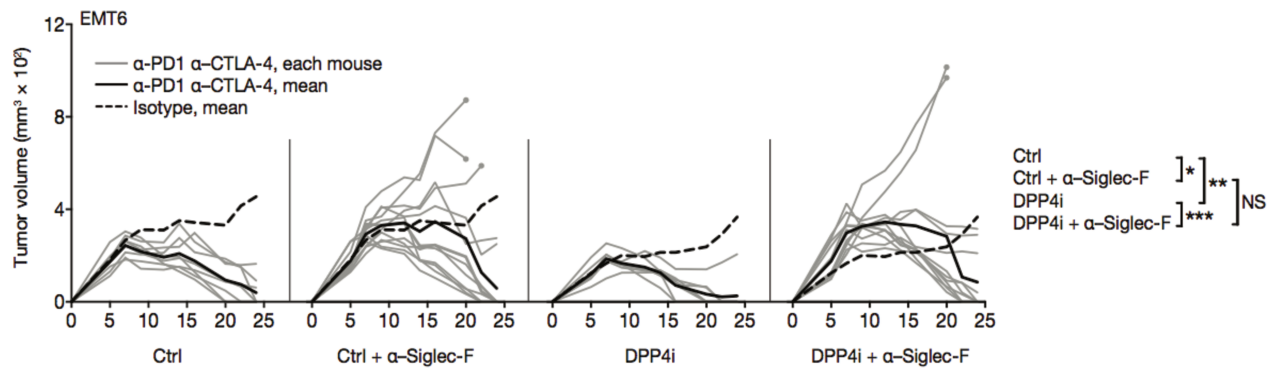


Figure 17: Eosinophils contribute to checkpoint inhibitor efficacy. WT mice fed with Ctrl or DPP4i chow were inoculated with EMT6 tumor cells. Mice were intraperitoneally injected with anti-Siglec-F or isotype control. At days 3, 6, and 9, mice were intraperitoneally injected with a combination of anti-PD1 and anti-CTLA-4 blocking antibodies, or respective isotype antibodies. Tumor volumes are shown. Anti-PD1 and anti-CTLA-4 groups: each grey line represents one mouse and solid black line represents mean. Dashed line represents mean of isotype groups. Mice that were sacrificed before the end of experiment due to oversized tumors are denoted by •. Data are representative of two experiments. NS, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-way analysis of variance).

In conclusion, we demonstrated that tumor cell derived IL-33, acting on its receptor ST2, mediated engagement of eosinophils as part of the endogenous immune response to tumors. Notably, DPP4 limited recruitment of eosinophils due to N-terminal truncation of CCL11. These findings extend the role of DPP4i, which had previously been shown to augment lymphocyte mediated tumor immunity. These results provide a new mechanism by which eosinophil degranulation may contribute to both naturally occurring and checkpoint inhibitor-induced tumor immunity. More generally, we suggest that type 1 and type 2 responses are not inherently antagonistic pathways, and in some instances, they may collaborate to control tumor growth.

Discussion

DPP4 inhibition, acting on CCL11, enhances CCR3-activated eosinophil migration and delays HCC tumor growth

We first showed that DPP4 inhibition delayed HCC tumor growth, thereby extending the initial finding that DPP4 inhibition delays melanoma tumor growth. However, in the melanoma model, DPP4-mediated post-translational modification of chemokines was shown to negatively regulate lymphocyte trafficking, and its inhibition enhanced T cell migration and tumor immunity by preserving functional CXCL10. In our HCC model, we discovered a new mechanism whereby DPP4 inhibition improves anti-tumor responses by eosinophil recruitment. Specifically, administration of DPP4i resulted in higher concentrations of CCL11 and increased CCR3-mediated eosinophil migration into mouse tumors. Enhanced tumor control was observed upon treatment with DPP4i, an effect strikingly preserved in *Rag2*^{-/-} mice, and abrogated only upon depletion of eosinophils or inhibition of their degranulation. We indeed showed that DPP4 inhibition delays growth of HCC independently of the adaptive immune system as well as innate lymphoid cells but dependently of eosinophils.

Eosinophils are cytotoxic cells able to directly act on tumors

In this work, we demonstrated that eosinophils are effector immune cells against tumor cells and can directly affect tumor growth.

In the literature, numerous of studies reported that eosinophils are mostly involved in anti-tumor responses in an indirect way. Their interactions with various innate and adaptive immune cells can indeed lead to secretion of cytokines and activation of effector cells such as CD8⁺ T cells. For example, in a T_{reg}-depleted melanoma model, eosinophils can mediate cancer rejection by enhancing infiltration of CD8⁺ T cells (130). Yet here, we showed that in T cell-depleted, *Rag2*^{-/-} or *Rag2*^{-/-} γ c^{-/-} mice, DPP4i has still a beneficial antitumor effect mediated by eosinophils. In our model, even in the absence of the adaptive immune system, eosinophils had a direct effect on tumor development.

The same study reported a second indirect way where eosinophils can participate to tumor rejection. They showed that eosinophils can change the tumor microenvironment by

normalizing tumor vasculature, illustrating by an increased expression of the adhesion molecule VCAM-1 and a decreased expression of the angiogenic factor VEGF (130). We checked in our two models, Hepa 1-6 and EMT6, if the delay of tumor growth due to the increase of eosinophil infiltration in the tumor upon DPP4i was linked to this mechanism of normalization of tumor vasculature. We didn't find any difference in terms of tumoral VCAM-1 and VEGF expressions between control mice and mice treated with DPP4i (Fig. 21 and 22), suggesting again that eosinophils have the capacity to directly affect tumor cells.

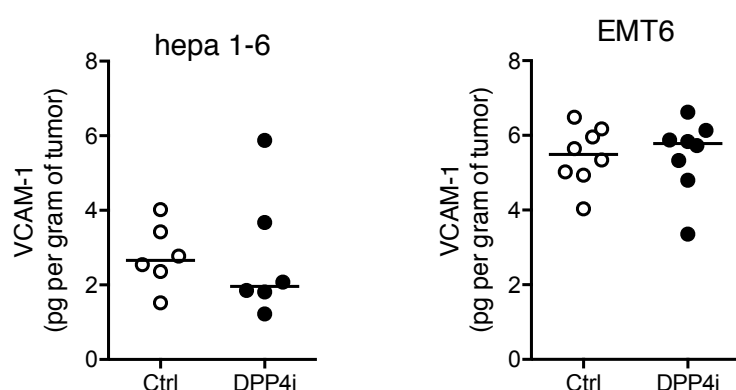


Figure 21: VCAM-1 expression in Hepa 1-6 and EMT6 tumors. WT C57BL/6 and BALBc mice were subcutaneously injected respectively with Hepa 1-6 and EMT6 cells. Seven days later tumors were harvested and VCAM-1 expression was assessed in tumor homogenates by ELISA.

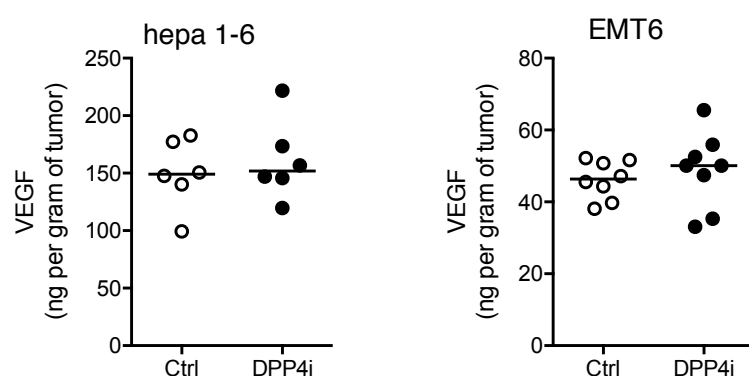


Figure 22: VEGF expression in Hepa 1-6 and EMT6 tumors. WT C57BL/6 and BALBc mice were subcutaneously injected respectively with Hepa 1-6 and EMT6 cells. Seven days later tumors were harvested and VEGF expression was assessed in tumor homogenates by ELISA.

It is known that eosinophils can display tumoricidal activities thanks to the cytotoxic activities of their granules. Several studies showed eosinophil killing tumor cells *in vitro*. Co-

culture of human eosinophils with colorectal cancer cells led to the release of eosinophil granules ECP and EDN and triggered killing of tumor cells (126). In a model of human oral squamous cell carcinoma (SCC), where human cancer cells were put in culture in presence of ECP granules, the viability of SCC cells were inversely correlated with ECP concentrations (210). We tried to reproduce this kind of experiments, putting in culture mouse Hepa 1-6 cells together with mouse eosinophils or ECP granules, but we didn't see an effect on the viability of tumor cells *in vitro*, evaluated by IncuCyte ZOOM Live-Cell Imaging system (data not shown). However, we showed that eosinophil degranulation inhibition induced the loss of the beneficial effect of DPP4i in our model, suggesting a direct tumoricidal action of eosinophils on HCC tumor cells. Probably, the tumor microenvironment provides additional factors allowing *in vivo* eosinophil degranulation in contact with tumor cells such as adhesion molecules and CCL11 expression. Notably, one study showed that granules themselves expressed CCR3 receptor and can autonomously, outside of eosinophils, respond to eotaxin (CCL11) secretion *in vivo* (211). In another *in vivo* study, the injection of IL-4 secreting tumor cell lines resulted in an antitumor activity mediated by eosinophils (212).

In summary, our study brings new evidence for a beneficial involvement of eosinophils in antitumor response and raises their direct cytotoxic effect on tumor cells.

IL-33 mediates engagement of eosinophils in antitumor response

We showed in our study that IL-33 tumor expression triggered the engagement of eosinophils against tumor development upon DPP4i. The involvement of a T_H1 or a T_H2 immune response after inoculation of different tumor cell lines was directly correlated to their IL-33 expression. Indeed, it is known from studies in the field of allergy, that eosinophils expressing ST2 receptor are activated by IL-33 which initiates—directly or indirectly through Il-5 activation—their degranulation and cytokine release (177,213). In melanoma models, recent studies described the antitumor effect of IL-33, either using IL-33-expressing tumor cells (193) or directly using systemic IL-33 treatment (214). In both cases, the restriction of tumor growth was mediated by a T_H1 response with a higher intratumoral accumulation of CD8⁺ T cells and a systemic activation of CD8⁺ T cells and NK cells. However, in the second model, authors showed that this response was eosinophil dependent and suggested an additional direct antitumor activity from eosinophils. Our work reinforces this last hypothesis as we showed the loss of beneficial effect of DPP4i in B16-IL33 tumor growth in eosinophil-

depleted condition. Our study brings the idea that tumor expression of IL-33 was required and sufficient for eosinophil-mediated anti-tumor responses when endogenous mechanisms of DPP4 immune regulation are inhibited. It also raises the idea that IL-33 might be a useful prognostic biomarker in HCC as we know that IL-33 is downregulated in HCC tumor compared to healthy or cirrhotic liver (196). Notably, we planned to retrospectively measure gene expression of *IL33* in human HCC biopsies.

Eosinophils contribute to checkpoint inhibitor efficacy upon DPP4i

We first demonstrated that DPP4i enhanced the efficacy of the combination therapy of anti-PD1 and anti-CTLA-4 in both the Hepa 1-6 and the EMT6 models. Previous studies suggested that eosinophils also participated in the efficacy of the treatment. In human, a positive correlation between increased number of eosinophils and better immunotherapy treatment outcome has been shown (134). An early increase of eosinophils during the treatment with anti-CTLA-4 was associated with better clinical response in melanoma patients (133). It is noteworthy that eosinophils can mediate part of the response induced by these treatment (124). We demonstrated this hypothesis as we showed a diminution of checkpoint inhibitor efficacy in eosinophil-depleted conditions. Finally, we suggest that type 1 and type 2 responses are not inherently antagonistic pathways, and in some instances, they may collaborate to control tumor growth. These data allowed us to consider the elaboration of a clinical trial where DPP4i would be administrated in combination with anti-PD-(L)1 in HCC patients (see perspectives section).

Perspectives

Validate our results in an orthotopic model of HCC

Our pre-clinical work was performed using a subcutaneous model of HCC. We are mindful that the potential extrapolation of our results is limited since we were not able to study the liver as site of host-tumor interaction and microenvironment. We consequently started collaboration with the Department of Cancer Immunology and the Department of in Vivo Pharmacology in Genentech Inc., South San Francisco to establish a syngeneic orthotopic model validating our results. Our collaborators directly inoculated 1 or 2 million Hepa 1-6 cells into the liver of WT or *Rag2*^{-/-} C57BL/6 mice. Fourteen days after injection, the correct implantation was checked with ultrasound imaging (Fig. 23).

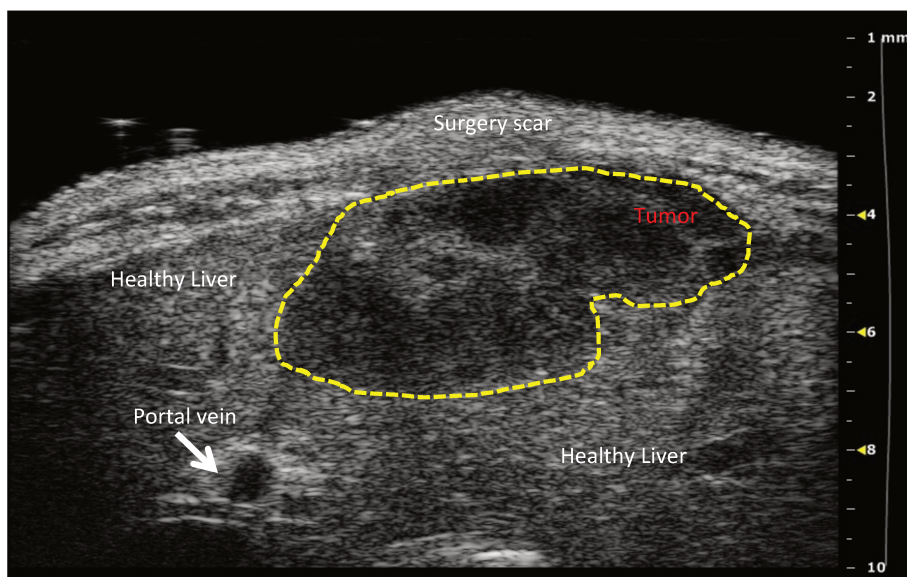


Figure 23: Picture of liver implanted HCC tumor in mouse. Ultrasound image of a liver implanted tumor, 14 days after inoculation of 2 million Hepa 1-6 cells in a *Rag2*^{-/-} mouse.

They confirmed that treatment with DPP4i induced, as observed in the subcutaneous model, an inhibition of DPP4 activity into the tumor (Fig. 24).

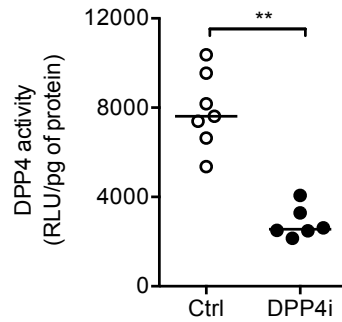


Figure 24: Sitagliptin inhibits DPP4 activity in Hepa 1-6 orthotopic tumors. *Rag2*^{-/-} C57BL/6 mice were fed with chow containing 1.1% sitagliptin (DPP4i) or with control (Ctrl) chow and orthotopically injected with Hepa 1-6 cells. Twenty days after tumor cell inoculation, tumors were collected and homogenates were prepared. DPP4 activity per tumor mass was determined and normalized to the amount of DPP4 protein (RLU, relative luminescence units). ***P* < 0.01 (Mann–Whitney test).

They also confirmed that DPP4i enhanced infiltration of immune cells into the HCC tumor. Interestingly, in the orthotopic model it seems that DPP4i promotes not only eosinophil, but also neutrophil, T cell and NK cell infiltration (Fig. 25), suggesting an even better response to DPP4i (and to a further extent to DPP4i and checkpoint inhibitor combination therapy) and again strengthening the idea of a collaboration between type 1 and type 2 responses.

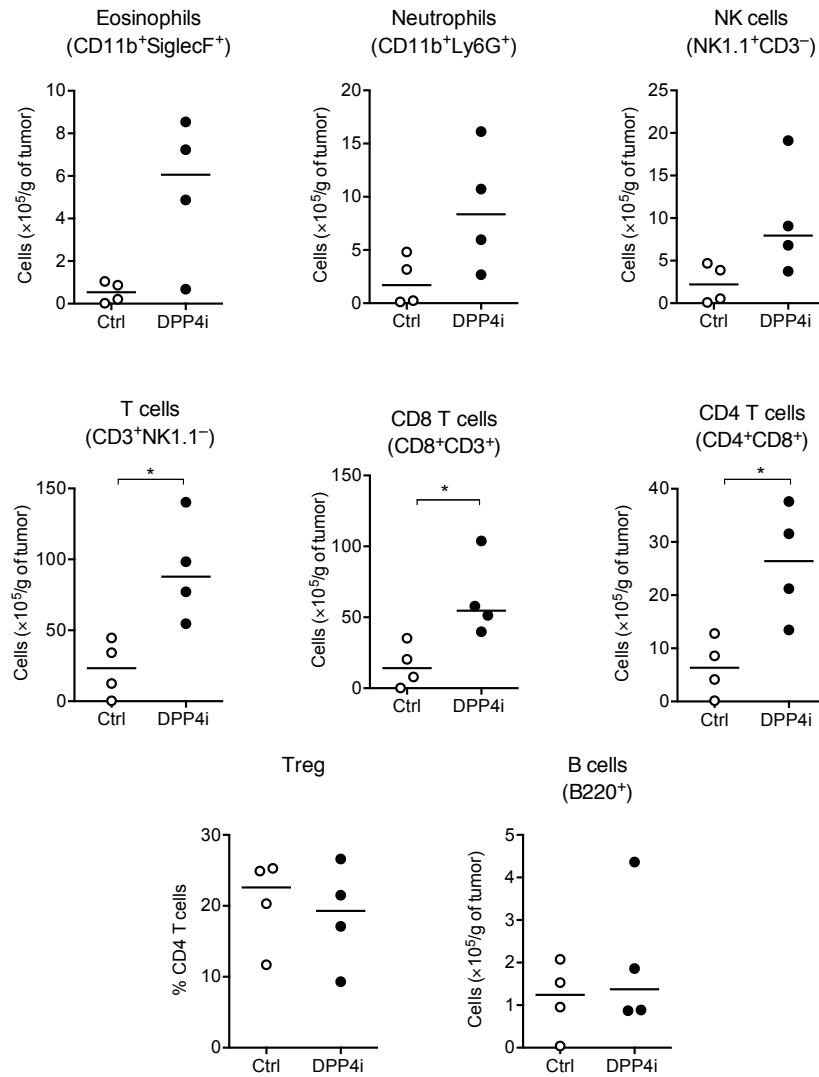


Figure 25: DPP4i enhances tumor immune cells infiltrate in orthotopic HCC. Tumors were collected 20 days after liver inoculation and tumor-associated leucocytes were analyzed by flow cytometry.

This experiment still needs to be repeated to validate these data. It will then be interesting to perform tumor growth experiments in several cell-depleted conditions to see the impact of each cell subtype on antitumor immunity.

In parallel with the repetition of these preliminary results, we are currently awaiting data regarding tumor growth (measured by luminescence) to validate that DPP4i delays tumor development in orthotopic HCC model.

III. CLINICAL PROJECT

Introduction

The results obtained with DPP4i in a preclinical model oh HCC allowed us to conduct a phase Ib pilot study to evaluate the safety of a 3-week DPP4i (sitagliptin) treatment in HCC patients undergoing liver resection.

This study was a prospective, open-labeled, monocentric pilot phase Ib study approved by the Comité de protection des personnes Ile-de-France 3 (CPP IDF 3) and by the Agence nationale de sécurité du médicament (ANSM); and sponsored by Institut national de la santé et de la recherche médicale (Inserm).

Participants were patients with hepatocellular carcinoma (stage BCLC A) for whom a curative surgery was planned in La Pitié Salpêtrière hospital, Paris, France. Enrolled patients received 100 mg ($n = 5$), 200 mg ($n = 5$) or 600 mg ($n = 5$) of sitagliptin (Januvia, Merck) daily during the time awaiting surgery after liver biopsy (28 ± 7 days) (Fig. 18). Blood samples were collected longitudinally. All participants gave written informed consent prior to inclusion in the study, conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

Here are presented results for the first group of 5 patients (dose 100 mg) recruited from February 2016 to July 2016, and partial results for the second group of 5 patients (dose 200 mg) recruited from August 2016 to June 2017.

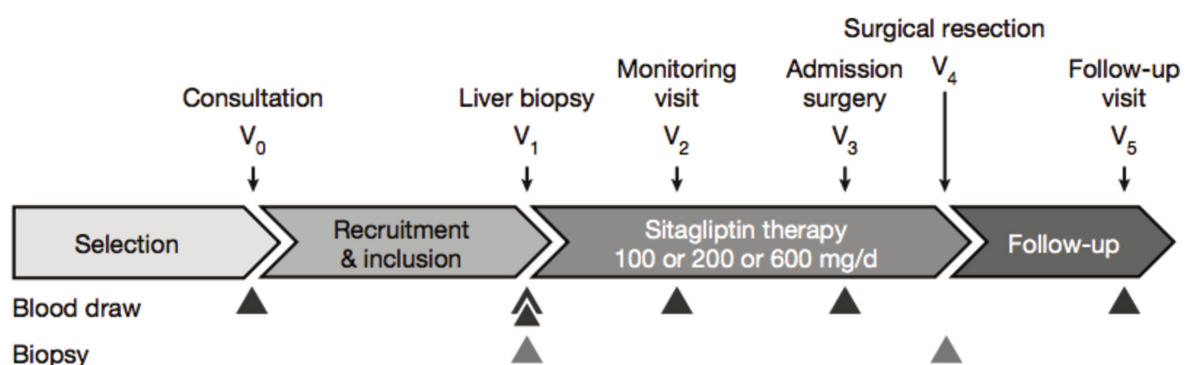


Figure 18: Study design of the clinical trial HCC-DPPiV (C15-41). Five patients with histologically proven HCC for whom curative surgery was planned were enrolled at visit 0 (V0). At V1, after liver biopsy they started treatment with the DPP4i sitagliptin (Januvia, Merck, 100 mg per day). The 5 following patients received 200 mg per day, and the last 5 patients will receive 600 mg per day. Treatment was continued until the day before surgery (21 ± 7 days of treatment). Blood samples were collected twice during treatment (V2 and V3) and 3 to 5 days after surgery (V5).

The primary outcome was the safety of sitagliptin treatment in each group of patients (100 mg, 200 mg, 600 mg).

The secondary outcomes were the evaluation of the impact of sitagliptin treatment on:

- Plasmatic DPP4 activity (DPP4 glow assay),
- Plasmatic cytokines secretion (Luminex technology),
- DPP4 substrate CXCL10 truncation (Simoa technology),
- Circulating immune cell trafficking (flow cytometry),
- Leucocyte invasion in tumor microenvironment (immunohistochemistry).

Inclusion criteria were: above 18 years of age, HCC diagnosis for whom surgery has been chosen as curative treatment and no cirrhosis or cirrhosis Child A.

Exclusion criteria were: HIV infection, portal hypertension (esophageal varices, platelet count $<100,000$, splenomegaly), diabetes and impaired liver or renal function.

The aim of this phase Ib study was to evaluate the safety of sitagliptin in this particular population and determinate the preferential dose of treatment to propose another phase Ib study evaluating combination therapy with checkpoint inhibitor molecule, or a phase II study against placebo or other therapy.

EXPERIMENTAL PLAN

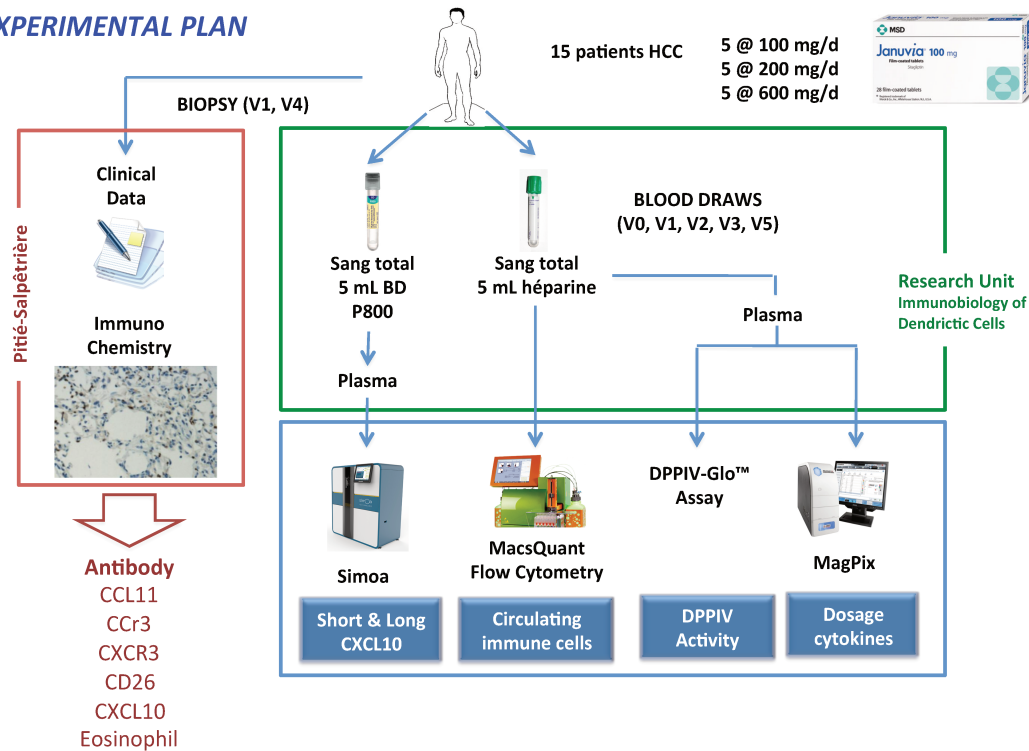


Figure 19: Experimental plan. Tissue samples stay in La Pitié Salpêtrière Hospital in the pathology unit for immunohistochemistry analyses. Blood samples are delivered in Institut Pasteur unit where analysis by flow cytometry, Simoa technology, DPP4 glow assay and Luminex technology are performed.

The inclusion of patients started in February 2016. End of inclusion of the first five patients occurred in July 2016. Then, the second group of five patients was enrolled from August 2016 to June 2017. We presented here preliminary results for these two groups of patients.

Results

Characteristics of patients

The ten patients enrolled were 8 men and 2 women, aged between 38 and 77 years. Causes of chronic liver disease were variables: HBV, HCV, OH, NASH; and six of them had a cirrhosis Child A against four with no cirrhosis (Tables 1 and 2 in appendix).

Safety

No severe adverse effect related to sitagliptin was reported. No hypoglycemia, no pancreatitis and no premature discontinuation occurred. Among severe adverse effects (grade 4) non-related to the treatment, we found one elevation of liver enzymes just after liver resection, one post-operative hypoxemia, one pre-operative bradycardia and one wrist fracture. Most common low-grade adverse effects were asthenia and fever (Tables 3–5 in appendix).

Immunological effects of DPP4 inhibition in patients with HCC

First, we showed a significant inhibition of DPP4 activity upon sitagliptin treatment, starting one hour after the first pill taken (Figure 18). However, this inhibition was more powerful and less variable in the 200 mg group, with an approximately 85% of DPP4 inhibition.

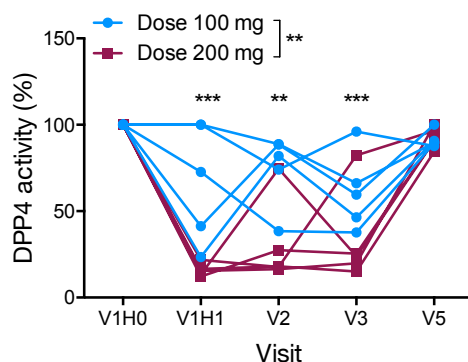


Figure 18: Plasmatic DPP4 Activity. DPP4 activity was measured in plasma and plotted as percentage of V1H0 values. Each line corresponds to 1 patient. $**P < 0.01$, $***P < 0.001$ Significance was

determined using Friedman test followed by Dunn's post test to compare visits and using two-way analysis of variance to compare the two groups of dose.

We then measured plasma levels of different cytokines and showed a significant decrease of CCL11 levels at V5 and CXCL10 at V2. No significant modification of TNF- α or IFN- γ occurred (Fig. 19).

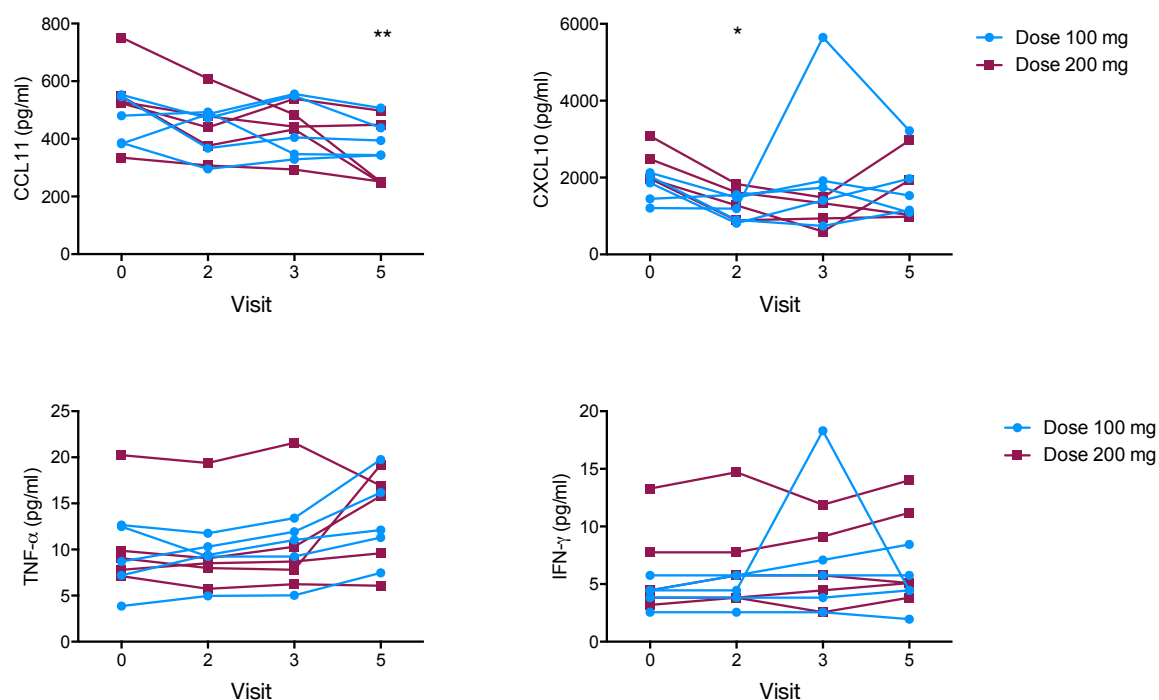


Figure 19: Plasmatic levels of cytokines. Plasmatic levels of CCL11, CXCL10, TNF- α and IFN- γ were quantified by Luminex technology. Results for IL-4, IL-5 and IL-13 were below the limit of detection.

To identify which form of CXCL10 was affected by this decrease, we measured the long, short and total forms of CXCL10 upon sitagliptin treatment for the first group. We confirmed the diminution of total CXCL10 at V2 and we showed that sitagliptin prevented the truncation of CXCL10, preserving the long agonist form at the expense of short antagonist form (Figure 20).

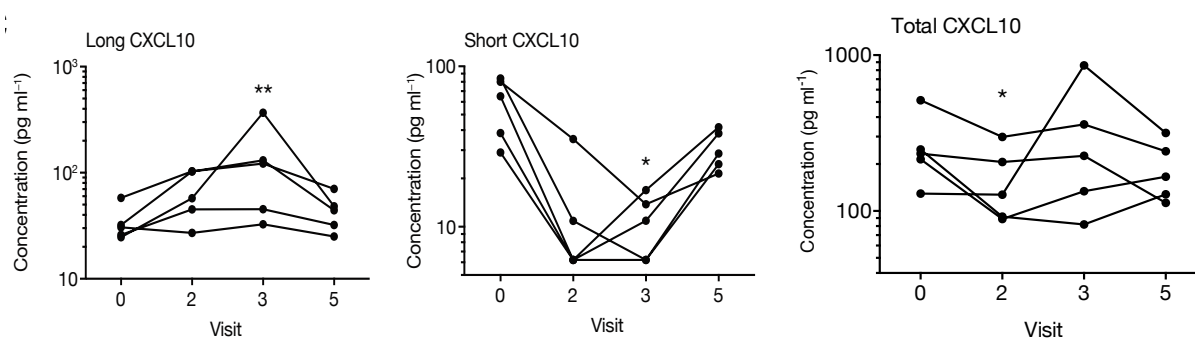


Figure 20: Quantification of long, short and total forms of CXCL10 in plasma. Plasma levels of the long form of CXCL10 (CXCL101–77) and the short form of CXCL10 (CXCL103–77) were quantified by Simoa immunoassays. Each line corresponds to 1 patient from the first group. * $P < 0.05$, ** $P < 0.01$ Significance was determined using Friedman test followed by Dunn's post test.

We then evaluated the modifications of circulating immune cells upon treatment by flow cytometry. We observed a modest reduction in the number of circulating $CD8^+$ T cells (Figure 20A and B) and we detected a statistically significant reduction in the percentage of circulating eosinophils (Figure 20A and C). Eosinophils present in the blood of patients showed a reduced expression of the chemokine receptor upon treatment with DPP4i (Figure 20D).

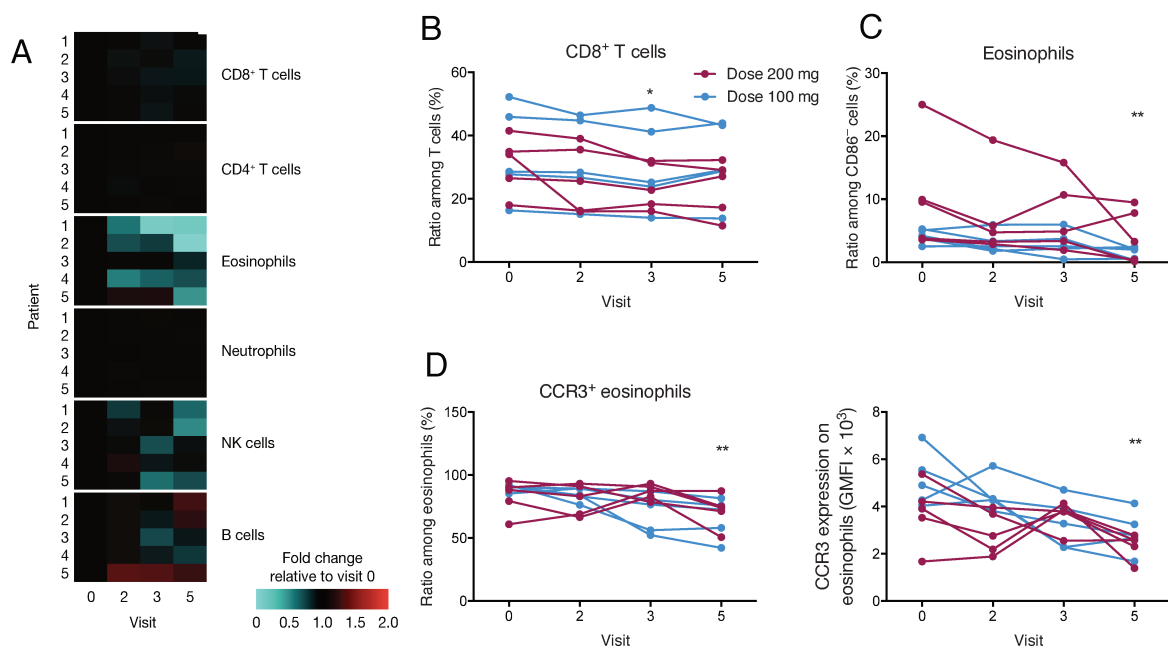


Figure 21: Circulating immune cells modifications upon DPP4i treatment. (A) Blood-associated immune cell populations (for the first group) were evaluated by flow cytometry and plotted as fold change relative to V0. (B) Percentage of $CD8^+$ T cells and (C) eosinophils in circulating blood was analyzed by flow cytometry. (D) Percentage of $CCR3^+$ eosinophils (left graph) and geometric mean of CCR3 fluorescence on eosinophils (right graph) were determined. Each line corresponds to 1 patient. * $P < 0.05$, ** $P < 0.01$ Significance was determined using Friedman test followed by Dunn's post test.

Our hypothesis is that eosinophils and $CD8^+$ T cells migrated from circulation to tumor site and we are awaiting the immunochemistry results to confirm this hypothesis.

Discussion

Sitagliptin treatment modifies chemokines and circulating immune cells expression in human

In our study, we confirmed that sitagliptin treatment modulated expression of chemokines that are substrates of DPP4, CXCL10 and CCL11. Particularly, we showed that sitagliptin, when inhibiting DPP4, preserved the long active form of CXCL10 and decreased the short antagonist form of CXCL10. This phenomenon was associated with a decrease of circulating CD8⁺ T cells. In parallel, we saw a reduction of circulating eosinophils. We hypothesize that these inflammatory immune cells went from the circulation to the inflammatory site, HCC tumor here. Histological analyses will confirm or not our hypothesis. However, it is noteworthy that this treatment is currently administrated to several millions of type 2 diabetes patients for its effect on incretin hormones. Some systematic reviews reported some correlation between antidiabetic medications and risk of hepatocellular carcinoma. Metformin showed a beneficial effect on HCC incidence, while insulin or sulphonylureas therapy was associated with higher risk of HCC (215). Metformin can impede carcinogenesis through indirect (insulin-dependent) and direct (insulin-independent) mechanisms such as the activation of the AMP-activated protein kinase (AMPK) and the inhibition of mTOR activity (216). It will be interesting to perform a similar meta-analysis to study gliptins treatment and risk of HCC. In another hand, a systematic review could be done to explore if gliptins treatment in type 2 diabetes patients exacerbates inflammatory diseases like asthma or autoimmune diseases.

Perspectives

Conduct a safety phase Ib clinical trial where DPP4i is administered in combination with anti-PD-(L)1.

In our preclinical model of HCC, we showed that DPP4i boosts the effect of anti-PD1 + anti-CTLA-4 therapy. We therefore plan to evaluate first, the safety, and second, the efficacy, of the combination therapy associating DPP4i and anti-PD-(L)1 in HCC patients. The first results of anti-PD1 treatment of cirrhotic (Child A and B7) and non-cirrhotic HCC patients are very encouraging both in terms of safety and efficacy. For the moment, this treatment is only tested in advanced HCC patients, sorafenib-untreated or sorafenib-progressor patients.

In order to improve the efficacy of both sitagliptin and anti-PD-(L)1 in treating or preventing HCC, we would aim to include patients with less advanced liver disease than those usually included in phase 3 studies of anti-PD-(L)1 for BCLC C HCC. Our choice is based on the fact that majority of patients are eligible for a loco-regional treatment (surgery, radiofrequency, chemoembolization) but numerous of them will experience HCC recurrence (around 30% at one year for early-stage HCC treated by transarterial chemoembolization (217)). We suggest to select patients for whom negative predictors of HCC recurrence are identified after liver resection and treat them with combination therapy (DPP4i + anti-PD1).

We plan to include 20 patients with HCC who underwent a curative treatment (liver resection) of HCC but with negative predictors of HCC recurrence at baseline, on the basis of a pre-defined score. From the literature about HCC recurrence after liver transplantation, some predictive factors of HCC recurrence have been identified: number of nodules >3, volume of biggest nodule >5 cm, presence of vascular micro-invasion, poor differentiation, presence of active liver disease, high level of AFP (218). We would establish a score to screen patients with a very high risk of recurrence.

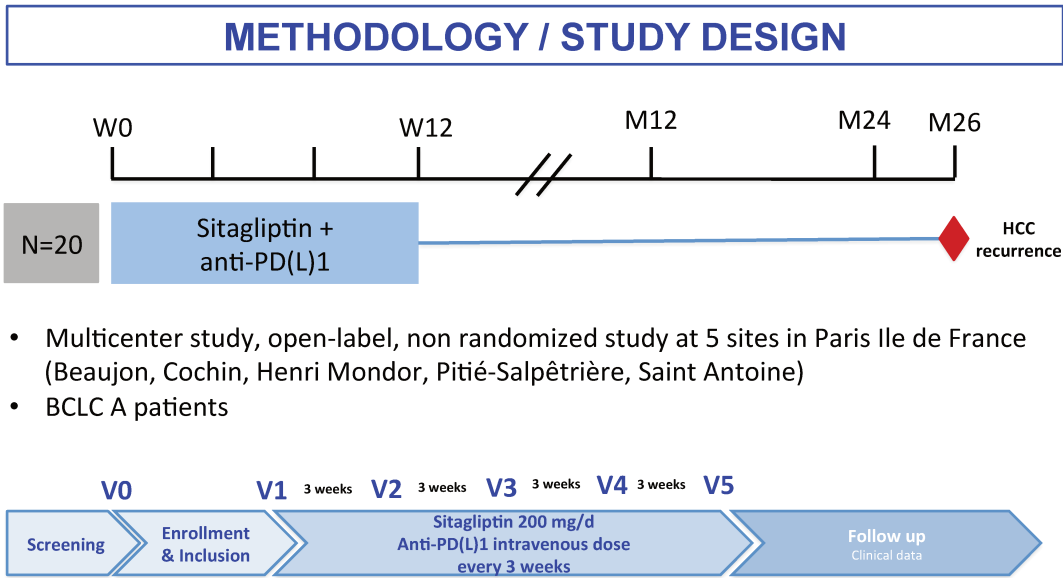
The main endpoint would be safety within a 12-month period of the combination sitagliptin (200 mg/d) + anti-PD-(L)1 for 3 months (corresponding to 3 to 4 cures).

The secondary endpoint would be the rate of recurrence at 24 months post treatment. We would also evaluate the effect of sitagliptin on the inhibition of DPP4 activity (pre- versus

under versus post-treatment), the effect of DPP4 inhibition on their substrates CXCL10 and CCL11 and the evolution of circulating immune cells levels under combined treatment.

The inclusion criteria would be: patient with HCC for which a curative surgery has been performed, with predictor factors of high risk of recurrence, with no cirrhosis or cirrhosis Child A and age >18 years.

The exclusion criteria would be presence of renal or liver function impairment and diabetes.



- Multicenter study, open-label, non randomized study at 5 sites in Paris Ile de France (Beaujon, Cochin, Henri Mondor, Pitié-Salpêtrière, Saint Antoine)
- BCLC A patients

- Blood sampling for laboratory tests : CBC-Platelets, PT-INR, creatinin, AST, ALT, GGT, ALP, AFP, Albumin, Bilirubin, Glucose, Insulinemia, Lipase
- Alpha fetoprotein every month then every 6 months
- Blood sampling for immunoassays performed at the Institut Pasteur.

3

Figure 26: Proposition of study design.

If safety is fair, we will discuss a phase 2 study aiming to compare in a randomized study a group of sitagliptin alone ($n = 15$), of sitagliptin in combination with an anti-PD(L)1 ($n = 15$) or anti-PD(L)1 alone ($n = 15$).

This clinical project, drawn from preclinical results, emphasizes the relevance of translational research and the interest of a back and forth between scientific and clinical research.

Appendix

	IK-02	MB-03	LM-04	CM-05	LJ-06
Gender	M	M	M	M	M
Age (years)	56	73	60	60	68
BMI (kg/m ²)	22,8	21,7	26,4	18,6	26,7
Liver disease	HCV under ttt	HBV under ttt	Alcohol	HCV cured	HCV cured
Cirrhosis	No (F1)	Yes (Child A)	No	No (F2-F3)	Yes (Child A)
Platelets (10 ⁹ /L) [150-400 10 ⁹ /L]	142	102	358	148	281
Hemoglobin (g/dL) [13.0 – 17.5 g/dL]	14.5	13.5	10.6	MV	13.8
TP (%) [70-120%]	97	80	84	97	98
Bilirubin (μmol/L) [2-17 μmol/L]	10	11	9	12	10
Albumin (g/dL) [35-52 g/dL]	42	39	33	42	47
Creatinin [62-106 μmol/L]	96	92	92	70	105
GFR [CKD-EPI (ml/min/1,73 m2)]	88	71	78	98	63
ASAT (UI/L) [20-32 UI/L]	26	89	28	32	33
ALAT (UI/L) [16-35 UI/L]	16	28	25	28	27
GGT (UI/L) [12-55 UI/L]	27	56	117	27	38
ALP (UI/L) [40-120 UI/L]	106	104	102	101	57
AFP (UI/L) [< 13 μg/L]	127.8	36.7	8.7	4.4	97.3
Glucose (mmol/L) [3.9 mmol/L – 5.8 mmol/L]	MV	4.6	4.6	4.4	5.6
Lipasemia (UI/L) [7-60 UI/L]	28	MV	41	22	77

Table 1: Demographics and baseline characteristics for first group of 5 patients (dose 100 mg).

	LD-07	AJ-08	TR-09	DM-10	SA-11
Gender	M	M	F	M	F
Age (years)	38	77	70	53	57
BMI (kg/m ²)	27	29,41	25,08	25,06	30.84
Liver disease	HBV under ttt	Mixte (OH/NASH)	HBV healed	HBV under ttt	HBV
Cirrhosis	Yes (Child A)	Yes (Child A)	No	Yes	Yes (Child A)
Platelets (10 ⁹ /L) [150-400 10 ⁹ /L]	122	161	198	126	149
Hemoglobin (g/dL) [13.0 – 17.5 g/dL]	14	15,1	13,1	13,3	12.7
TP (%) [70-120%]	76	84	87	101	76
Bilirubin (μmol/L) [2-17 μmol/L]	7	6	6	11	17
Albumin (g/dL) [35-52 g/dL]	38	38	39	45	28
Creatinin [62-106 μmol/L]	85	78	76	90	60
GFR [CKD-EPI (ml/min/1,73 m2)]	115	83	79	97	112
ASAT (UI/L) [20-32 UI/L]	55	26	38	32	142
ALAT (UI/L) [16-35 UI/L]	73	31	18	25	136
GGT (UI/L) [12-55 UI/L]	94	72	23	54	221
ALP (UI/L) [40-120 UI/L]	84	31	98	73	MV
AFP (UI/L) [< 13 μg/L]	3.8	5,5	38220	142	6450
Glucose (mmol/L) [3.9 mmol/L – 5.8 mmol/L]	5.3	5,4	4	5.1	5.2
Lipasemia (UI/L) [7-60 UI/L]	44	28	29	31	34

Table 2: Demographics and baseline characteristics for second group of 5 patients (dose 200 mg).

Any AEs	Dose 100mg	Dose 200mg
Serious Adverse Events (SAEs)		
Drug related	0	0
Due to other causality	2	2
Premature discontinuation	0	0
Hypoglycemia	0	0
Pancreatitis	0	0

Table 3: Serious adverse events.

Adverse Event	Dose 100 mg
Asthenia (Grade 2)	1
Cold (Grade 1)	1
Cramps (Grade 2)	1
Fatigue (Grade 1)	1
Insomnia (Grade 1)	1
Loss of appetite (Grade 1)	1
Myalgia (Grade 2)	1
RGO (Grade 1)	1
Sore Gums (Grade 1)	1
Urinary Incontinence (Grade 1)	1
Liver enzymes abnormalities (Grade 4)	1

Table 4: List of adverse events for the first dose group.

Adverse Event	Dose 200 mg
Abdominal pain (Grade 2)	1
Anxiety (Grade 1)	1
Constipation (Grade 1)	1
Dizziness (Grade 1)	1
Fatigue (Grade 1)	2
Fever (Grade 2)	1
Hypoglycemia (Grade 1)	1
Intestinal gases (Grade 1)	1
Joint aches (Grade 2)	1
Low-grade fever (Grade 2)	2
Muscles aches (Grade 2)	1
Nausea (Grade 1)	1
Sciatica (Grade 1)	1
Stomach pain (Grade 1)	1
Urinary burning (Grade 2)	1
Urinary Incontinence (Grade 1)	1
Liver enzymes abnormalities (Grade 3)	3

Table 5: List of adverse events for the second dose group.

IV. CONCLUSION

In summary, our findings first highlighted that chemokines are essential molecules for immune cell trafficking, and regulation mechanisms such as posttranscriptional modifications can considerably impede their action. Inhibiting one of their negative regulators, DPP4, led to a better efficiency of the immune response in HCC. Notably, it allowed the preservation of the CCL11 chemokine, resulting in a greater recruitment of activated eosinophils and in a better control of tumor growth. These findings extended the role of DPP4i, which was previously shown to augment T lymphocyte-mediated tumor immunity.

Second, we confirmed that eosinophils are immune cells involved and directly competent in anti-tumor responses. After its evocation in 2004, the antitumor activity of eosinophils against hepatocellular carcinoma was never more explored. Here we showed that eosinophil degranulation contributed to both naturally occurring and checkpoint inhibitor-induced tumour immunity.

Third, we demonstrated that this eosinophil-mediated antitumor response was directly linked to the expression of the IL-33 alarmin by the tumor. We thereby raised the idea that IL-33 could be a useful biomarker, predictor of a better response to DPP4i treatment.

Finally, we emphasized the idea that type 1 and type 2 responses are not inherently antagonistic pathways, and in some instances, they may collaborate to control of tumor growth.

We began to validate these findings in humans, in a clinical trial with HCC patients which will be followed by other human studies to investigate and confirm the efficacy of DPP4 inhibitors, alone or in combination therapy, in HCC pathology.

V. MATERIALS AND METHODS

Study design.

This study was a prospective, open-labeled, monocentric pilot phase Ib study approved by the Comité de protection des personnes Ile-de-France 3 (CPP IDF 3) and by the Agence nationale de sécurité du médicament (ANSM); and sponsored by Institut national de la santé et de la recherche médicale (Inserm). Participants were patients with hepatocellular carcinoma (stage BCLC A) for whom a curative surgery was planned in La Pitié Salpêtrière hospital, Paris, France. Five enrolled patients received 100 mg of sitagliptin (Januvia, Merck) daily during the time awaiting surgery after liver biopsy (28 \pm 7 days). Blood samples were collected longitudinally. All participants gave written informed consent prior to inclusion in the study, conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. Plasma was collected in BD P800 tubes, containing ethylenediamine tetraacetic acid (EDTA) and a DPP4 inhibitor to prevent extracorporeal cleavage of CXCL10. Blood collected in sodium heparin tubes was used for monitoring DPP4 activity and for performing flow cytometry. Plasma samples were stored at -80°C until analysis. Here results for the first group of 5 patients (dose 100 mg) are presented that were recruited from February 2016 to July 2016.

Mice.

Wild type C57BL/6, CD45.2, Thy1.2 and BALB/c mice were obtained from Charles River, France. *Rag2*^{-/-} and *Rag2*^{-/-} γ c^{-/-} mice were bred in the mouse facility at Institut Pasteur, or purchased from Taconic Biosciences, USA.

St2^{-/-} and *St2*^{+/+} littermates were bred in the mouse facility at Genentech. Mice used were 7–12 weeks old. For inhibition of DPP4 activity in vivo, mice were fed with chow (SAFE) formulated to contain 1.1% of the DPP4i sitagliptin (Merck). DPP4i chow was administrated to mice before treatments. Mice were maintained in a specific pathogen-free facility, and all experimental protocols were approved by the Comité d'éthique pour l'expérimentation animale (Ethics Committee for Animal Experimentation), Paris and Genentech Institutional Animal Care and Use Committee.

Tumor growth *in vivo*.

Hepa1-6, B16F10, EMT6, TRAMP-C2 and B16-IL-33 cells were cultured in DMEM (Gibco) supplemented with 10% FCS (PAA), 0.1 mM non-essential amino acids, 1mM sodium pyruvate, 10nM HEPES buffer, 70 μ M β -mercaptoethanol and 23 μ g/ml gentamycin (all from Gibco). After being thawed, cells for injections were passaged one to three times before use. WT and genetically modified mice were subcutaneously injected with 5×10^5 B16F10 or B16-IL33 cells, or 1×10^6 Hepa 1-6, EMT6, TRAMP-C2 cells in the shaved right flank. To deplete circulating immune cells, mice were intraperitoneally injected with 10 μ g of rat anti-mSiglec-F (clone E50-2440, BD Biosciences) or respective isotype antibody rat IgG2a (clone R35-95, BD Biosciences); with 100 μ g of anti-mCD8 (clone YTS 169.4, BioXCell), with 100 μ g of α -mCD4 (clone GK1.5, BioXCell), with 200 μ g of anti-mCD90 (clone T24/31, BioXCell) or with respective isotype antibody rat IgG2b (clone LTF-2, BioXCell). Intraperitoneal injections were performed one day before and every six days after tumor cell inoculation. To neutralize CCR3⁺ circulating cells, mice were intraperitoneally injected with 100 μ g of rat anti-mouse CD193 (CCR3) (clone J073E5, BioLegend) or respective isotype antibody rat IgG2a (clone RTK2758, BioLegend). To block IL-33, mice were intraperitoneally injected with 3.6 μ g of anti-mIL-33 (R&D systems) or with the respective isotype control goat IgG (R&D systems). Intraperitoneal injections were done one day before and every two days after tumor cell injections. For immunotherapy protocols, mice were intraperitoneally injected with a combination of 100 μ g anti-CTLA-4 (clone 9D9, Bio X Cell) and 200 μ g anti-PD1 (clone RMP1-14, Bio X Cell), or control isotypes (100 μ g rat IgG2b (clone MPC-11) and 200 μ g rat IgG2a (clone 2A3), both from Bio X Cell) on days 3, 6 and 9 after tumor cell implantation. For inhibition of eosinophil degranulation, cetirizine dihydrochloride from Sigma Aldrich (25 mg/kg/mouse) was directly incorporated in the drinking water. Tumor largest and smallest diameters were measured with a caliper, and tumor volume was calculated (elliptical volume = smallest diameter² \times largest diameter \times 0.523).

Flow cytometry.

Human

Fluorochrome-conjugated antibodies used were anti-hCD56 (clone TULY56, eBioscience), -hCD3 (clone UCHT1, BD Biosciences), -hCD8b (clone SIDI8BEE, eBioscience), -hCD19 (clone HIB19, BD Bioscience), -hCD4 (clone OKT4, eBioscience), -hCD14 (clone 61D3, eBioscience), -hSiglec-8 (clone 7C9, BioLegend), -hCD16 (clone

B73.1, BD Bioscience), -hCD45 (clone HI30, BD Bioscience), -hCD193 (CCR3) (clone eBio5E8-G9-B4, eBioscience), -hCD86 (clone 2331, BD Bioscience), and -hCD279 (clone MIH4, BD Bioscience). Flow cytometry was done with a MACSQuant Analyzer 10 (Miltenyi Biotec) and computer analysis was performed with FlowJo (Treestar).

Mouse

Fluorochrome-conjugated antibodies used were anti-mCD3 (clone 145-2C11, BD Biosciences), -mNK1.1 (clone PK136, BD Biosciences), -mCD49b (clone DX5, BD Biosciences), -mLy-6G (clone 1A8, BD Biosciences), -mCD11b (clone M1/70, BD Biosciences), -mSiglec-F (clone E50-2440, BD Biosciences). For exclusion of dead cells, Live/Dead fixable Aqua reagent (Invitrogen) was used. Cell suspensions were incubated with mCD16/CD32 Fc-blocking antibody (BD Biosciences) before incubation with fluorochrome-conjugated antibodies. For analysis of tumor infiltrates, Hepa1-6 and EMT6 tumors were collected and digested in phosphate buffered saline (PBS) supplemented with 2.7 mg/ml Collagenase (Roche) and 23 U/ml DNase I (Invitrogen) for 30 min at 37 °C. Digestion was terminated by PBS supplemented with 2% fetal bovine serum (FCS) and 5 mM EDTA (Gibco). Tumor cell suspensions were obtained after filtration over a 70-µm cell strainer. For evaluation of circulating eosinophil migration, 1 µg/mouse of full length mCCL11(1–74) (Peprotech), DPP4-truncated mCCL11(3–74) or PBS was intravenously injected. Truncated mCCL11 was obtained after 37°C overnight incubation of 35 µg of CCL11 with 10 µg of mDPP4 (R&D). One hour after IV injection, blood was collected and flow cytometry analyses were performed. Blood was collected with a 50 µl heparin-coated capillary and blood was transferred in Eppendorf tubes containing 100 µl of ethylenediaminetetraacetic acid (EDTA) 100mM. Blood was resuspended with 1 ml of red blood cell lysis buffer (1.66 % NH₄Cl in water) for an incubation of 6 min at 37°C. After spin, blood was resuspended in FACS Buffer (PBS) + 2 %) before antibody staining. For the determination of cell numbers, AccuCheck Counting Beads reagent (Invitrogen) was used. Flow cytometry was done with a BD LSRFortessa, with DIVA software. Computer analysis was done with FlowJo (Treestar).

Histology

Hepa 1-6 cells were subcutaneously injected in mice treated with Ctrl or DPP4i. After 10 days of growth, tumors were collected and froze. Eosinophil infiltration was evaluated on Hematein & Eosin (H&E) staining of frozen sections (5 µm).

ELISA/Luminex assays.

For the detection of mCCL11, mDPP4, mIL-33, m-VEGF, m-ICAM-1, DuoSet ELISA kits (R&D Systems) were used. Plates were read at 450 nm in a Lab-systems Multiskan MS (Thermo) reader.

For the detection of mouse and human IL-4, IL-5, IL-13, CXCL10, CCL11, IFN- γ , TNF- α , MILLIPLEX MAP mouse/human cytokine/chemokine magnetic bead panel (MCYTOMAG-70K, Merck) was used. Plates were read in a MAGPIX System (Merck). Computer analysis was done with MILLIPLEX Analyst 5.1 software.

Mass spectrometry.

Recombinant chemokines were purchased from Peprotech. For surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry, mCCL11 was incubated in the presence or absence of 1, 3 or 10 nM mDPP4 (R&D) for 30 min at 37 °C. The digested product was applied to an H4 protein chip according to the manufacturer's instructions and analyzed with the ProteinChip Systems Series 4000 (Ciphergen). Data were analyzed using CiphergenExpress software. Human chemokines were incubated in the presence or absence of 100 nM hDPP4 (R&D) overnight at 37 °C, and analyzed by liquid chromatography–mass spectrometry (LC-MS). Samples were injected onto a Q Exactive Plus EMR mass spectrometer (Thermo Fisher) using a Dionex UltiMate 3000 UHPLC (Thermo Scientific). Prior to injection, samples were separated at 0.3 μ l/min on a 80°C heated, 2.1 x 50 mm MAbPac Reverse-Phase column (Thermo Fisher). A 20% to 65% Buffer B gradient over 4.5 minutes was performed with 0.1% formic acid, 0.02% trifluoroacetic acid in water (Buffer A) and 0.1% formic acid, 0.02% trifluoroacetic acid, 9.88% water in acetonitrile (Buffer B), followed by a 90% Buffer B wash for 6.4 minutes. Acquired mass spectrometric data was analyzed using Intact Mass software (Protein Metrics)

CXCL10 quantification.

Human plasma concentration of total (R&D clone 33036), long (CXCL10₁₋₇₇) (AbD Serotec clone 12010), and short (CXCL10₃₋₇₇) (AbD Serotec clone 09852) CXCL10 was measured using Simoa technology (Quanterix). Simoa assays were carried out as previously described (219).

Statistical analysis.

No statistical methods were used to predetermine sample size. For animal studies, mice were randomly allocated to different cages before experiments. *P* values ≥ 0.05 were considered non-significant. For two-way ANOVA, significance of the effect of treatment was reported. In all instances, statistical tests were done using Prism GraphPad.

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MANUSCRIPT

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DPP4 inhibition reveals IL-33-dependent eosinophil-mediated control of tumour growth

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Dipeptidyl peptidase 4 (DPP4 or CD26)–mediated post-translational modification of chemokines has been shown to negatively regulate lymphocyte trafficking, and its inhibition enhances T cell migration and tumour immunity by preserving functional CXCL10. In extending these initial findings to humans and pre-clinical hepatocellular carcinoma model, we discovered a new mechanism whereby DPP4 inhibition improves anti-tumour responses by eosinophil recruitment. Specifically, administration of DPP4 inhibitors (DPP4i) resulted in higher concentrations of CCL11 and increased CCR3-mediated eosinophil migration into mouse tumours. Enhanced tumour control was observed upon treatment with DPP4i, an effect strikingly preserved in *Rag2*^{−/−} mice, and abrogated only upon depletion of eosinophils or inhibition of their degranulation. We further demonstrated that tumour expression of interleukin-33 (IL-33) was necessary and sufficient for eosinophil-mediated anti-tumour responses, and that this mechanism contributed to checkpoint inhibitor efficacy. These findings provide new insight into IL-33- and eosinophil-mediated tumour control, revealed when endogenous mechanisms of DPP4 immune regulation are inhibited.

Tumour growth and immune evasion have been associated with poor cytotoxic T cell responses. This may occur as a result of the failure to prime tumour antigen–specific T cells, or due to the presence of tumour-associated immunosuppressive cells¹. Additionally, it has been described that lymphocyte migration may be perturbed by the post-translational modification (PTM) of CXCR3 ligands². Our prior work established that DPP4-mediated N-terminal cleavage of CXCL10 limits T and NK cell migration in pre-clinical models of melanoma and colorectal carcinoma (CRC)³. DPP4 inhibitor (DPP4i)–treated mice showed both improved naturally occurring tumour immunity and enhanced responses to T cell-mediated immunotherapy. Moreover, clinical data suggest that elevated DPP4 expression

within tumours correlated with worse overall survival in CRC⁴, bladder cancer⁵, and gastrointestinal stromal tumour patients⁶.

We initiated a phase Ib interventional clinical trial (Fig. 1a) using DPP4i in hepatocellular carcinoma (HCC) patients to evaluate safety and to test the prediction that DPP4 inhibition stabilizes the long agonist form of plasma CXCL10₁₋₇₇. Additional blood-based pharmacodynamic biomarkers included the monitoring of immune cell subsets with the expectation that enhanced chemokine activity would result in lower numbers of lymphocytes in circulation. As part of a pilot study, five patients were recruited following histological diagnosis of early stage HCC (Barcelona Clinic Liver Cancer A (BCLC A)). Upon informed consent, patients were treated for 3–4 weeks with 100 mg per day of the DPP4i sitagliptin, while awaiting surgical resection of their tumours (Fig. 1a). No reported adverse events were associated with DPP4i treatment and all patients showed normal glycaemia levels (Extended Data Table 1). Blood biomarkers were tested pre-treatment, on-treatment and post-DPP4i treatment. Administration of DPP4i resulted in decreased plasma dipeptidyl peptidase activity (Fig. 1b), increased levels of the full-length agonist long isoform of CXCL10₁₋₇₇ (Fig. 1c) and a striking reduction in the N-terminally antagonist cleaved form, CXCL10₃₋₇₇ (Fig. 1d). Consistent with these results, we observed a modest reduction in the number of circulating CD8⁺ T cells (Fig. 1e, f and Extended Data Table 2). These data provided additional *in vivo* validation that DPP4 mediates PTM of a T cell chemoattractant⁷, and support our previous observations that DPP4 modulates lymphocyte trafficking.

In evaluating other potential effects of DPP4i, we detected a statistically significant reduction in the percentage of circulating eosinophils (Fig. 1e, g, Extended Data Table 2).

While several chemokines were previously described as potential DPP4 substrates⁸, no systematic biochemical evaluation has been performed. We therefore tested 36 human chemokines as potential substrates for DPP4 and identified 16 that are sensitive to N-terminal truncation (Extended Data Table 3). Among these, the CCR3 ligand CCL11, but not CCL24 or CCL26 (also known as Eotaxin-1, -2 and -3, respectively) was efficiently truncated by DPP4 (Extended Data Fig. 1). We also detected DPP4-mediated loss of NH₂ dipeptide in other CCR3 ligands including CCL5, CCL7 and CCL13⁹. Of note, CCL7 and CCL13 revealed incomplete DPP4-mediated truncation (table S3), due to the presence of a pyroglutamic N-terminus residue, as previously reported¹⁰. Chemokine binding to CCR3 was shown to induce receptor internalization and loss of N-terminal dipeptides from CCL11 prevents this process¹¹. We therefore monitored CCR3 expression as a surrogate biomarker for active chemokines. Eosinophils present in the blood of patients showed a reduced expression of the chemokine receptor upon treatment with DPP4i (Fig. 1h). These data led us to hypothesize that DPP4i enhances stability of CCR3 ligands, leading to receptor internalization and possible mobilization of eosinophils into tissues such as the tumour.

To explore the role of DPP4 in the context of HCC, we used an experimental mouse model of Hepa 1-6 inoculated in syngeneic C57BL/6 mice. Animals were fed with control chow or with chow containing DPP4i (1.1% sitagliptin)³. The level of tumour-associated DPP4 enzymatic activity was diminished upon DPP4i administration in a subcutaneous model of Hepa 1-6, confirming the expected pharmacodynamic effects of this treatment (Fig. 2a). Next, we studied the development of the tumour, and observed significantly reduced growth (Fig. 2b) and tumour mass (Fig. 2c) in DPP4i-treated mice. Of note, DPP4i did not alter the growth of the tumour cells *in vitro*, thus excluding a direct effect on tumour cell proliferation or metabolic activity (Fig. 2d). When examining tumour-associated leucocytes, we observed

no differential accumulation of T lymphocytes, natural killer (NK) cells or neutrophils between the control and treatment groups (Fig. 2e, f), but there was a significantly greater infiltration of eosinophils in tumours from mice receiving DPP4i (Fig. 2e, g). Histological analysis confirmed higher number of eosinophils infiltrating the Hepa 1-6 tumours of DPP4i-treated mice (Fig. 2h). Furthermore, we detected elevated levels of cytotoxic granule-associated eosinophil peroxidase (EPX) and eosinophil cationic protein (ECP) in tumours isolated from DPP4i-treated mice (Fig. 2i). Together, these data suggest that DPP4i administration is beneficial for the control of Hepa 1-6 tumour growth and indicate eosinophil accumulation and degranulation as a possible tumouricidal mechanism.

Despite the unchanged number of lymphocytes, we tested the role of the adaptive immune system as mediator of anti-tumour responses in DPP4i-treated animals. Tumours were implanted in wild-type (WT) mice that received a combination of anti-CD4 and anti-CD8 depleting antibodies or alternatively, inoculation was done in *Rag2*^{-/-} mice (lacking T and B lymphocytes). While the beneficial effect of DPP4i treatment on B16-F10 melanoma cells was shown to be dependent on T cells³, the positive effect of DPP4i treatment on tumour growth remained evident in the Hepa 1-6 model (Fig. 3a, b). These findings supported DPP4 as a negative regulator of anti-tumour responses, but challenged enhanced T cell migration as the suggested mechanism of action in this model.

To decipher the mechanisms behind DPP4i-mediated control of HCC, we performed protein analysis on extracts of tumours grown in WT mice fed with control or DPP4i chow. The amount of the T cell chemoattractant CXCL10 was similar between the different experimental groups (Fig. 3c), providing an explanation for the unchanged lymphocyte

accumulation. Furthermore, the levels of interferon- γ (IFN- γ) were lower in tumour samples from DPP4i-treated mice (Fig. 3c). By contrast, we observed higher level of CCL11 and increased concentration of interleukin-4 (IL-4), IL-5 and IL-33 upon DPP4i treatment (Fig. 3D). While CCL11 was previously shown to regulate eosinophil migration, IL-4, IL-5 and IL-33 play an important role in eosinophil homeostasis and activation^{12,13}. Therefore, we directly tested the role of eosinophils in our HCC model by treating mice with anti-Siglec-F antibodies, capable of selectively depleting eosinophils¹⁴, and studied tumour growth in the presence and absence of DPP4i. Strikingly, depletion of eosinophils in WT and *Rag2*^{-/-} mice abrogated the anti-tumour responses initiated by DPP4i treatment (Fig. 3e, f). In order to study if CCR3-mediated migration and eosinophil-degranulation were critical responses, we treated mice with anti-CCR3 blocking antibodies or with cetirizine, a second generation histamine H1 receptor antagonist¹⁵, and observed a loss of the beneficial effect initiated by DPP4i (Fig. 3g, h). Together, these data demonstrate that inhibition of DPP4 enhances anti-tumour responses in a manner that is dependent on CCR3 signalling and eosinophil degranulation.

To gain insight into DPP4 regulation of eosinophil migration, we examined its role in modifying the main eosinophil chemoattractant in mouse, CCL11. As observed with human CCL11 (Extended Data Fig. 1), we detected N-terminal truncation of two amino acids from recombinant mouse CCL11 upon incubation with recombinant DPP4 (Extended Data Fig. 2a, b). The cleaved form of CCL11 (referred to as CCL11₃₋₇₄) has been previously reported to have reduced chemoattractive properties¹⁶. In order to access the in vivo role of DPP4 enzymatic activity on CCL11-mediated eosinophil recruitment, we fed WT mice with control chow or DPP4i and studied eosinophil mobilization after intravenous CCL11₁₋₇₄ administration. Notably, eosinophil recruitment was only evident upon treatment with DPP4i

(Extended Data Fig. 2c). Furthermore, we observed downregulation of CCR3 expression on eosinophils (Extended Data Fig. 2d), suggesting that inhibition of DPP4 enhances CCL11/CCR3 signalling. Of note, intravenous injection of CCL11₃₋₇₄ failed to induce eosinophil recruitment in DPP4i-treated mice (Extended Data Fig. 2e), confirming its reduced chemotactic activity. These data support a role for DPP4 as a negative regulator of CCL11-mediated eosinophil migration *in vivo*.

Type 2 innate lymphoid cells (ILCs) are present in *Rag2*^{-/-} mice and are major producers of IL-4, IL-5 and IL-13 during helminthic infection¹⁷. ILCs have also been implicated in tumour immunity, having been shown to diminish the number of melanoma metastases in an IL-5 and eosinophil-dependent manner^{18,19}. We therefore tested the role of ILCs in the HCC model by evaluating tumour growth in *Rag2*^{-/-}*γc*^{-/-} mice and *Rag2*^{-/-} mice treated with anti-Thy1 (CD90) depleting antibodies. In both experiments, administration of DPP4i resulted in reduced tumour growth, suggesting that ILCs are not responsible for the beneficial effect observed in our model (Extended Data Fig. 3a, b). Furthermore, these results suggest that ILCs do not play a role in eosinophil-mediated delay of HCC growth.

In an attempt to decipher the mechanisms promoting eosinophil migration into HCC tumours, we tested the hypothesis that tumour cell-derived factors may enhance production of CCL11, and that treatment with DPP4i limits its cleavage supporting eosinophil recruitment. Notably, IL-33, whose expression was enhanced in HCC tumours grown in DPP4i-treated mice (Fig. 3d), may be expressed by tumours and is known to promote eosinophil activation^{20,21}. Interestingly, we observed that intratumoural administration of IL-33 was capable of inducing CCL11 expression *in vivo* (Fig. 4a). When evaluating IL-33 expression in

four different mouse tumour cell lines grown *in vitro*, we detected high protein expression in Hepa 1-6 cells (average 3.72 ng of IL-33 per 10⁶ tumour cells, Fig. 4b). We also observed IL-33 production by EMT6 cells (a breast carcinoma cell line syngeneic to the BALB/c strain) while B16-F10 and TRAMP-C2 (respectively, melanoma and prostatic carcinoma cells, syngeneic to the C57BL/6 strain) presented low IL-33 levels (Fig. 4b). To test for a correlation between IL-33 expression by tumour cells and eosinophil-mediated control of tumour growth, we inoculated TRAMP-C2, EMT6 and B16-F10 tumour cells in WT mice treated with control or DPP4i chow. As shown, treatment with DPP4i delayed growth in all tumour models studied (Fig. 4c, d and Extended Data Fig. 4a). Next, eosinophil or T cell depletion were tested as a means to assess the role of these leucocytes in DPP4i-mediated efficacy. Depletion of T cells – but not eosinophils – abrogated the effect of DPP4i in the TRAMP-C2 model, suggesting a critical role for T cell-mediated anti-tumour immunity (Fig. 4c). Similar findings were evident when using B16-F10 (Extended Data Fig. 4b)³. By contrast, depletion of eosinophils led to the loss of DPP4i-mediated control of EMT6 tumour growth (Fig. 4d). These data suggest that DPP4i may enhance anti-tumour responses by two different immune mechanisms. Notably, expression of IL-33 by tumour cells correlated with enhanced eosinophil-dependent anti-tumour responses, suggesting that this cytokine may assist immune mechanisms that are enhanced by the usage of DPP4i.

In order to assess whether tumour-associated IL-33 expression is critical for eosinophil-mediated tumour immunity, we utilized IL-33-expressing B16-F0 tumour cells (referred to as B16-IL33) (Fig. 4b)²². As previously reported²², we observed a delay in B16-IL33 growth, as compared to the parental cell line (data not shown). Notably, treatment with DPP4i resulted in a significant growth delay of B16-IL33 and administration of anti-Siglec-F antibodies abolished this phenotype (Fig. 4e, f), revealing that DPP4 inhibition enhances

eosinophil anti-tumoural activity when IL-33 is expressed. To further assess the role of IL-33 as an inducer of tumour immunity, we inoculated Hepa 1-6 or EMT6 cells in WT mice and analysed the impact of anti-IL-33 blocking antibody²³. Strikingly, the efficacy of DPP4 inhibition in these models was lost upon IL-33 blockade (Fig. 4g, h). Similarly, treatment with DPP4i did not delay growth of Hepa 1-6 tumours when *St2*^{-/-} recipient mice were used (deficient for expression of IL-33 receptor) (Fig. 4i). Notably, administration of DPP4i to mice inoculated with B16-F10 cells resulted in significant delay in both *St2*^{+/+} and *St2*^{-/-} strains (Extended Data Fig. 4c). These results indicate that tumour-derived IL-33 is capable of inducing eosinophil-mediated tumour immunity, a mechanism of tumour control that is limited by DPP4 truncation and inactivation of CCR3 ligands.

Finally, we considered the role of eosinophils during checkpoint blockade or combination immunotherapy that includes the use of DPP4i. Treatment with anti-PD1 and anti-CTLA-4 was previously reported to reduce CT26 growth and administration of DPP4i enhanced tumour rejection³. We therefore tested if this strategy could also be applied to the models of Hepa 1-6 and EMT6. Inhibition of PD1 and CTLA-4 reduced growth in both models and triple therapy (with DPP4i) resulted in a significantly lower tumour burden (Fig. 4j and Extended Data Fig. 5). Notably, even in the presence of a strong activator of T cell immunity²⁴, treatment efficacy was reduced upon depletion of eosinophils, rendering treatment with DPP4i similar to the control group (Fig. 4j). These data suggest that type 1 and type 2 immune responses can collaborate in achieving improved tumour control and highlight a novel mechanism by which tumour growth may be constrained, revealed upon DPP4 inhibition.

Eosinophils are traditionally regarded as important for the clearance of parasites such as helminths, and are mediators of allergic asthma¹². They have also been shown to participate in the control of tumour growth²⁵⁻³⁰. Specifically, tumour eosinophilia and evidence of eosinophil degranulation were associated with better prognosis in several human solid tumours, including oral squamous cell carcinoma³¹, pulmonary adenocarcinoma, bladder cancer, prostate cancer, and colon carcinoma^{32,33}. Additionally, eosinophils can be activated by danger signals released from necrotic epithelial cells³⁴, such as the alarmin IL-33³⁵. While IL-33 is expressed in many tumour tissues, its expression is down-regulated as compared to healthy tissue²⁰. In sum, we demonstrated that tumour cell derived IL-33, acting on its receptor ST2, mediated engagement of eosinophils as part of the endogenous immune response to tumours. Notably, DPP4 limited recruitment of eosinophils due to N-terminal truncation of CCL11. These findings extend the role of DPP4i, which had previously been shown to augment T lymphocyte mediated tumour immunity³. These results provide a new mechanism by which eosinophil degranulation may contribute to both naturally occurring and checkpoint inhibitor-induced tumour immunity. More generally, we suggest that type 1 and type 2 responses are not inherently antagonistic pathways, and in some instances, they may collaborate to control tumour growth.

MATERIALS

Study design. This study was a prospective, open-labeled, monocentric pilot phase Ib study approved by the Comité de protection des personnes Ile-de-France 3 (CPP IDF 3) and by the Agence nationale de sécurité du médicament (ANSM); and sponsored by Institut national de la santé et de la recherche médicale (INSERM). Participants were patients with hepatocellular carcinoma (stage BCLC A) for whom a curative surgery was planned in La Pitié Salpêtrière hospital, Paris, France. Five enrolled patients received 100 mg of sitagliptin (Januvia, Merck) daily during the time awaiting surgery after liver biopsy (28 \pm 7 days). Blood samples were collected longitudinally. All participants gave written informed consent prior to inclusion in the study, conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. Plasma was collected in BD P800 tubes, containing ethylenediamine tetraacetic acid (EDTA) and a DPP4 inhibitor to prevent extracorporeal cleavage of CXCL10. Blood collected in sodium heparin tubes was used for monitoring DPP4 activity and for performing flow cytometry. Plasma samples were stored at -80°C until analysis. Here results for the first group of 5 patients (dose 100 mg) are presented that were recruited from February 2016 to July 2016.

Mice. Wild type C57BL/6, CD45.2, Thy1.2 and BALB/c mice were obtained from Charles River, France. *Rag2*^{-/-} and *Rag2*^{-/-}*γc*^{-/-} mice were bred in the mouse facility at Institut Pasteur, or purchased from Taconic Biosciences, USA. *St2*^{-/-} and *St2*^{+/+} littermates were bred in the mouse facility at Genentech. Mice used were 7–12 weeks old. For inhibition of DPP4 activity *in vivo*, mice were fed with chow (SAFE) formulated to contain 1.1% of the DPP4i sitagliptin (Merck). DPP4i chow was administrated to mice before treatments. Mice were maintained in a specific pathogen-free facility, and all experimental protocols were approved by the Comité d'éthique pour l'expérimentation animale (Ethics Committee for Animal Experimentation), Paris and Genentech Institutional Animal Care and Use Committee.

Tumour growth *in vivo*. Hepa1-6, B16F10, EMT6, TRAMP-C2 and B16-IL-33 cells were cultured in DMEM (Gibco) supplemented with 10% FCS (PAA), 0.1 mM non-essential amino acids, 1mM sodium pyruvate, 10nM HEPES buffer, 70 μ M β -mercaptoethanol and 23 μ g/ml gentamycin (all from Gibco). After being thawed, cells for injections were passaged one to three times before use. WT and genetically modified mice were subcutaneously injected with 5×10^5 B16F10 or B16-IL33 cells, or 1×10^6 Hepa 1-6, EMT6, TRAMP-C2 cells in the shaved right flank. To deplete circulating immune cells, mice were intraperitoneally injected with 10 μ g of rat anti-mSiglec-F (clone E50-2440, BD Biosciences) or respective isotype antibody rat IgG2a (clone R35-95, BD Biosciences); with 100 μ g of anti-mCD8 (clone YTS 169.4, BioXCell), with 100 μ g of anti-mCD4 (clone GK1.5, BioXCell), with 200 μ g of anti-mCD90 (clone T24/31, BioXCell) or with respective isotype antibody rat IgG2b (clone LTF-2, BioXCell). Intraperitoneal injections were performed one day before and every six days after tumour cell inoculation. To block CCR3 cell receptor, mice were intraperitoneally injected with 100 μ g of rat anti-mouse CD193 (CCR3) (clone J073E5, BioLegend) or respective isotype antibody rat IgG2a (clone RTK2758, BioLegend). To block IL-33, mice were intraperitoneally injected with 3.6 μ g of anti-mIL-33 (R&D systems) or with the respective isotype control goat IgG (R&D systems). Intraperitoneal injections were done one day before and every two days after tumour cell injections. For immunotherapy protocols, mice were intraperitoneally injected with a combination of 100 μ g anti-CTLA-4 (clone 9D9, BioXCell) and 200 μ g anti-PD1 (clone RMP1-14, BioXCell), or control isotypes (100 μ g rat IgG2b (clone MPC-11) and 200 μ g rat IgG2a (clone 2A3), both from BioXCell) on days 3, 6 and 9 after tumour cell implantation. For inhibition of eosinophil degranulation, cetirizine dihydrochloride from Sigma Aldrich (25 mg/kg/mouse) was directly incorporated in the drinking water. Tumour largest and smallest diameters were measured

with a calliper, and tumour volume was calculated ($\text{elliptical volume} = \text{smallest diameter}^2 \times \text{largest diameter} \times 0.523$).

Flow cytometry. *Human.* Fluorochrome-conjugated antibodies used were anti-hCD56 (clone TULY56, eBioscience), -hCD3 (clone UCHT1, BD Biosciences), -hCD8b (clone SIDI8BEE, eBioscience), -hCD19 (clone HIB19, BD Bioscience), -hCD4 (clone OKT4, eBioscience), -hCD14 (clone 61D3, eBioscience), -hSiglec-8 (clone 7C9, BioLegend), -hCD16 (clone B73.1, BD Bioscience), -hCD45 (clone HI30, BD Bioscience), -hCD193 (CCR3) (clone eBio5E8-G9-B4, eBioscience), -hCD86 (clone 2331, BD Bioscience), and -hCD279 (clone MIH4, BD Bioscience). Flow cytometry was done with a MACSQuant Analyzer 10 (Miltenyi Biotec) and computer analysis was performed with FlowJo (Treestar).

Mouse. Fluorochrome-conjugated antibodies used were anti-mCD3 (clone 145-2C11, BD Biosciences), -mNK1.1 (clone PK136, BD Biosciences), -mCD49b (clone DX5, BD Biosciences), -mLy-6G (clone 1A8, BD Biosciences), -mCD11b (clone M1/70, BD Biosciences), -mSiglec-F (clone E50-2440, BD Biosciences). For exclusion of dead cells, Live/Dead fixable Aqua reagent (Invitrogen) was used. Cell suspensions were incubated with mCD16/CD32 Fc-blocking antibody (BD Biosciences) before incubation with fluorochrome-conjugated antibodies. For analysis of tumour infiltrates, Hepa1-6 and EMT6 tumours were collected and digested in phosphate buffered saline (PBS) supplemented with 2.7 mg/ml Collagenase (Roche) and 23 U/ml DNase I (Invitrogen) for 30 min at 37 °C. Digestion was terminated by PBS supplemented with 2% fetal bovine serum (FCS) and 5 mM EDTA (Gibco). Tumour cell suspensions were obtained after filtration over a 70- μm cell strainer. For evaluation of circulating eosinophil migration, 1 μg /mouse of full-length mCCL11(1–74) (Peprotech), DPP4-truncated mCCL11(3–74) or PBS was intravenously injected. Truncated mCCL11 was obtained after 37°C overnight incubation of 35 μg of CCL11 with 10 μg of mDPP4 (R&D). One hour after intravenous (IV) injection, blood was

collected and flow cytometry analyses were performed. Blood was collected with a 50 µl heparin-coated capillary and blood was transferred in Eppendorf tubes containing 100 µl of ethylenediaminetetraacetic acid (EDTA) 100 mM. Blood was resuspended with 1 ml of red blood cell lysis buffer (1.66 % NH₄Cl in water) for an incubation of 6 min at 37°C. After spin, blood was resuspended in FACS Buffer (PBS) + 2% fetal bovine serum) before antibody staining. For the determination of cell numbers, AccuCheck Counting Beads reagent (Invitrogen) was used. Flow cytometry was done with a BD LSRFortessa, with DIVA software. Computer analysis was done with FlowJo (Treestar).

Histology. Hepa 1-6 cells were subcutaneously injected in mice fed with Ctrl or DPP4i chow. After 10 days of growth, tumours were collected and froze. Eosinophil infiltration was evaluated on hematein & eosin (H&E) staining of frozen sections (5 µm).

ELISA/Luminex assays. For the detection of mCCL11, mDPP4, mIL-33, DuoSet ELISA kits (R&D Systems) were used. Plates were read at 450 nm in a Lab-systems Multiskan MS (Thermo) reader. For the detection of mIL-4, mIL-5, mCXCL10, mCCL11, mIFN γ , MILLIPLEX MAP mouse cytokine/chemokine magnetic bead panel (MCYTOMAG-70K, Merck) was used. Plates were read in a MAGPIX System (Merck). Computer analysis was done with MILLIPLEX Analyst 5.1 software.

Mass spectrometry. Recombinant chemokines were purchased from Peprotech. For surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry, mCCL11 was incubated in the presence or absence of 1, 3 or 10 nM mDPP4 (R&D) for 30 min at 37 °C. The digested product was applied to an H4 protein chip according to the manufacturer's instructions and analysed with the ProteinChip Systems Series 4000

(Ciphergen). Data were analysed using CiphergenExpress software. Human chemokines were incubated in the presence or absence of 100 nM hDPP4 (R&D) overnight at 37 °C, and analysed by liquid chromatography–mass spectrometry (LC-MS). Samples were injected onto a Q Exactive Plus EMR mass spectrometer (Thermo Fisher) using a Dionex UltiMate 3000 UHPLC (Thermo Scientific). Prior to injection, samples were separated at 0.3 µl/min on a 80°C heated, 2.1 x 50 mm MAbPac Reverse-Phase column (Thermo Fisher). A 20% to 65% Buffer B gradient over 4.5 minutes was performed with 0.1% formic acid, 0.02% trifluoroacetic acid in water (Buffer A) and 0.1% formic acid, 0.02% trifluoroacetic acid, 9.88% water in acetonitrile (Buffer B), followed by a 90% Buffer B wash for 6.4 minutes. Acquired mass spectrometric data was analysed using Intact Mass software (Protein Metrics).

CXCL10 quantification. Human plasma concentration of total (R&D clone 33036), long (CXCL10_{1–77}) (AbD Serotec clone 12010), and short (CXCL10_{3–77}) (AbD Serotec clone 09852) CXCL10 was measured using Simoa technology (Quanterix). Simoa assays were carried out as previously described³⁶.

Statistical analysis. No statistical methods were used to predetermine sample size. For animal studies, mice were randomly allocated to different cages before experiments. *P* values ≥ 0.05 were considered non-significant. For two-way analysis of variance, significance of the effect of treatment was reported. In all instances, statistical tests were done using Prism GraphPad.

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AUTHOR CONTRIBUTIONS

C.H., S.P., R.B.d.S. and M.L.A. designed the study. C.H. and R.B.d.S. designed, carried out and analysed experiments. J.B. performed statistical analyses and provided technical and intellectual assistance. V.B. conducted Simoa and mass spectrometry experiments. L.B. provided tumor cell lines. V.P. conducted histological experiments. O.S., E.S., E.M., T.B., A.L., D.D. and V.M. provided technical and intellectual assistance for the clinical trial. G.E. provided intellectual assistance. C.H., R.B.d.S. and M.L.A. wrote the manuscript. M.L.A., R.B.d.S. and S.P. supervised the study.

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FIGURES

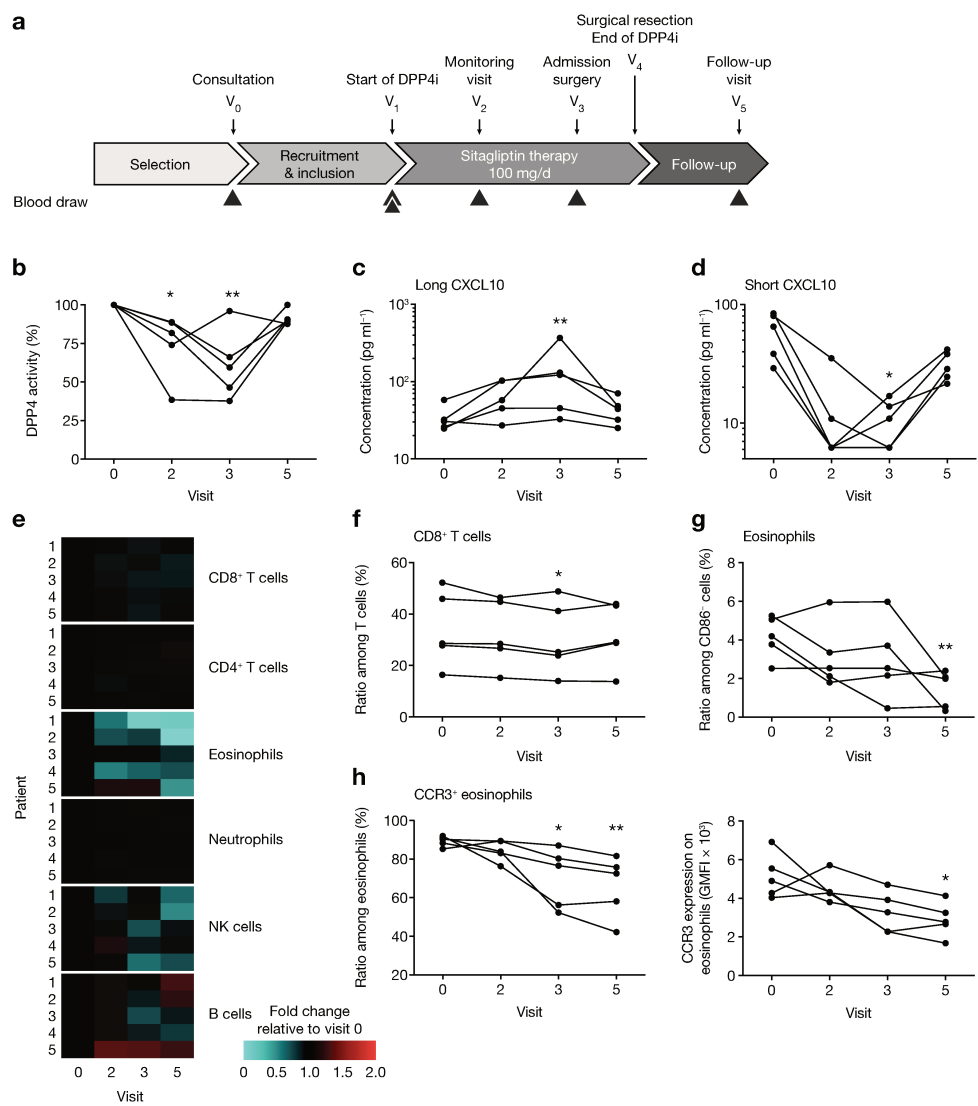


Figure 1 | Immunological effects of DPP4 inhibition in patients with HCC. **a**, Study design of the clinical trial HCC-DPPIV (C15-41). Five patients with histologically proven HCC for whom curative surgery was planned were enrolled at visit 0 (V0). At V1, they started treatment with the DPP4i sitagliptin (Januvia, Merck, 100 mg per day). Treatment was continued until the day before surgery (21 ± 7 days of treatment). Blood samples were collected twice during treatment (V2 and V3) and 3 to 5 days after surgery (V5). **b**, DPP4 activity was measured in plasma and plotted as percentage of V0 values. **c,d**, Plasma levels of the long form of CXCL10 (CXCL10₁₋₇₇, c) and the short form of CXCL10 (CXCL10₃₋₇₇, d) were quantified by Simoa immunoassays. **e**, Blood-associated immune cell populations were evaluated by flow cytometry and plotted as fold change relative to V0. **f,g**, Percentage of CD8⁺ T cells (f) and eosinophils (g) in circulating blood was analysed by flow cytometry. **h**, Percentage of CCR3⁺ eosinophils (left graph) and geometric mean of CCR3 fluorescence on eosinophils (right graph) were determined. In all graphs, each line corresponds to 1 patient. * $P < 0.05$, ** $P < 0.01$ Significance was determined using Friedman test followed by Dunn's post test.

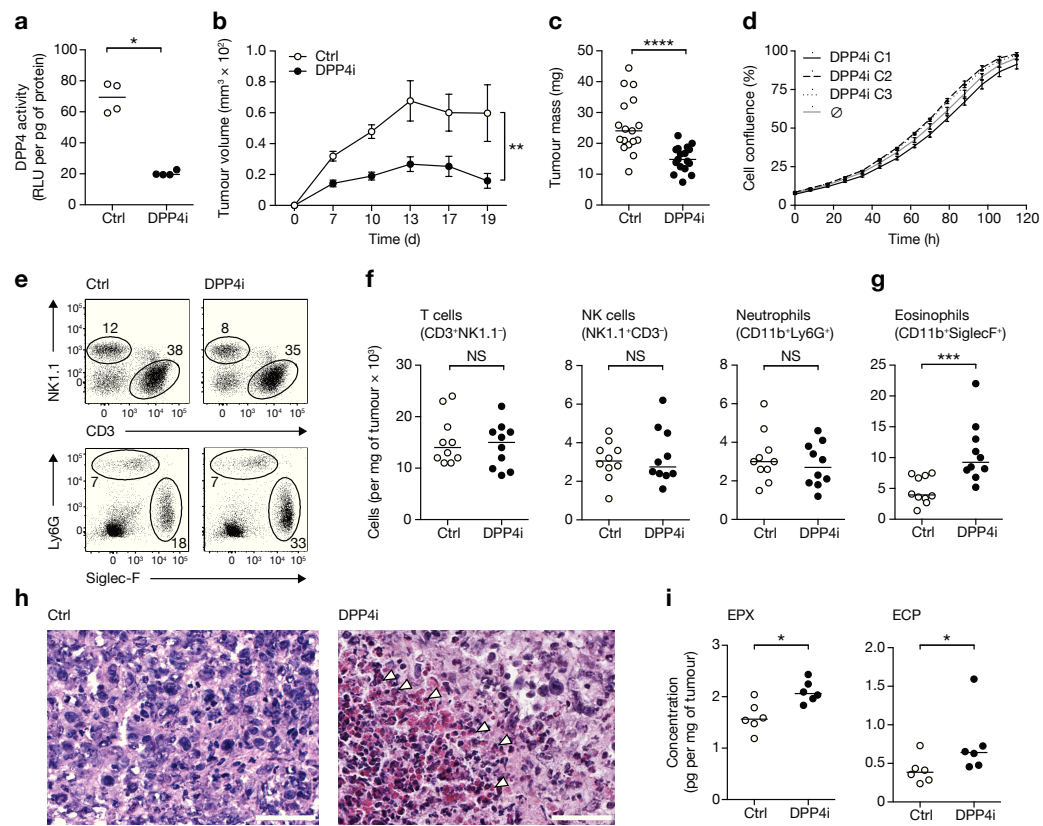


Figure 2 | DPP4 inhibition delays growth of HCC and enhances eosinophil migration.

a, Wild-type (WT) C57BL/6 mice were fed with chow containing 1.1% sitagliptin (DPP4i) or with control (Ctrl) chow and subcutaneously injected with Hepa 1-6 cells. Eight days after tumour cell inoculation, tumours were collected and homogenates were prepared. DPP4 activity per tumour mass was determined and normalized to the amount of DPP4 protein (RLU, relative luminescence units). **b**, Mice were treated as described in (a) and tumour volumes were measured over time (mean \pm SEM, $n \geq 16$ mice per group). **c**, Tumours were collected at day 10 post-inoculation and tumour mass was determined. **d**, Hepa 1-6 cells were cultured in the presence of 5 (C1), 0.05 (C2) or 0.005 (C3) $\mu\text{g/ml}$ of DPP4i, or left untreated (\odot). Cell confluence was measured over time using intra-incubator microscopy (mean \pm SEM, $n = 3$ technical replicates). **e**, Mice were treated as in a. Tumours were collected 10 days after inoculation and tumour-associated leucocytes were analysed by flow cytometry. Representative flow cytometry plots for the analysis of T cells ($\text{CD3}^+\text{NK1.1}^-$), NK cells ($\text{CD3}^-\text{NK1.1}^+$), neutrophils ($\text{CD11b}^+\text{Ly6G}^+$) and eosinophils ($\text{CD11b}^+\text{Siglec-F}^+$) are shown. **f,g**, Number of tumour-associated leucocytes is shown. **h**, Representative illustration of eosinophils infiltration in Hepa 1-6 tumours in mice receiving Ctrl (left) or DPP4i (right) after hematein and eosin staining. Arrows indicate eosinophils. Scale, 100 μm . **i**, Eosinophil peroxidase (EPX) and eosinophil cationic protein (ECP) expression was quantified by ELISA in extracts from Hepa 1-6 tumours collected 8 d after inoculation and normalized to tumour mass. Each dot corresponds to one mouse. Data are representative of two experiments (a, d and i) or pooled from two to three (b, c, f and g) independent experiments. NS, not significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. Significance was determined using Mann–Whitney test (a, c, f, g, i) or two-way analysis of variance (b).

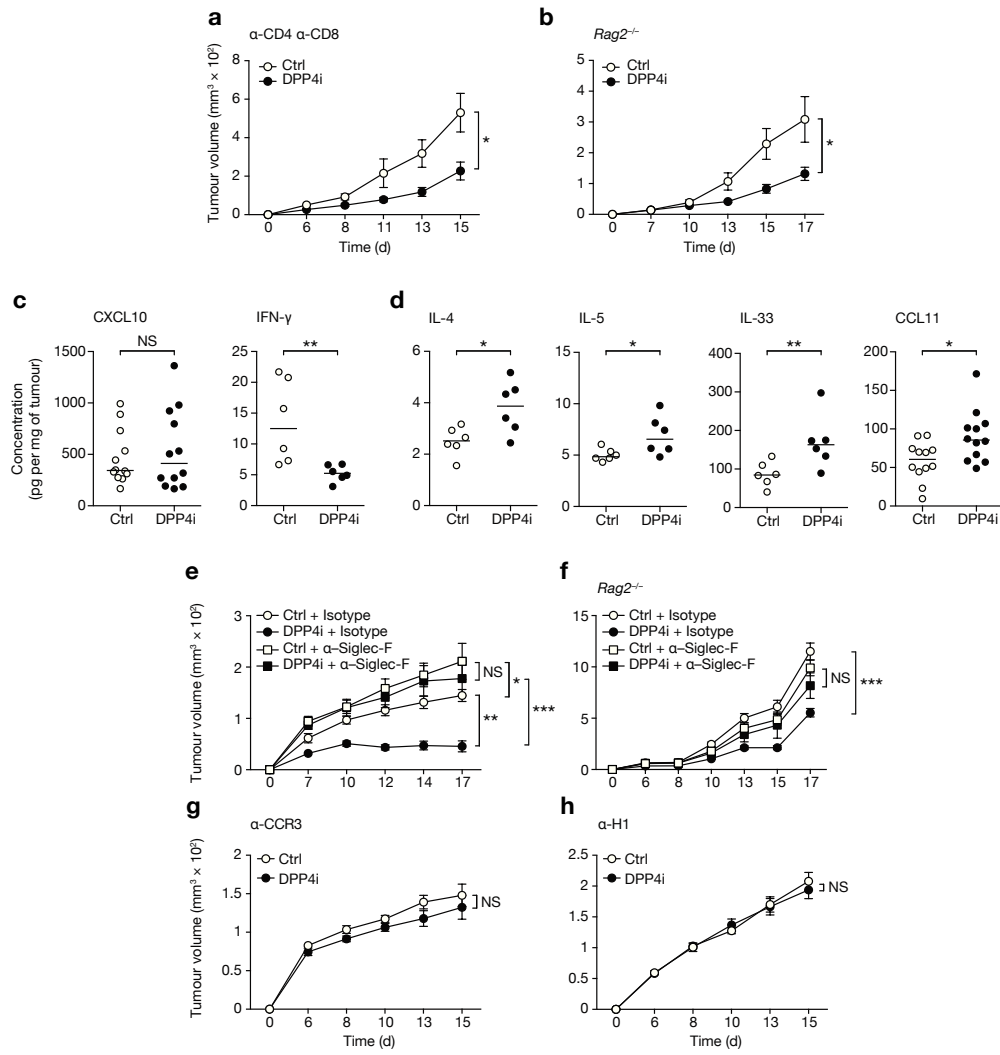
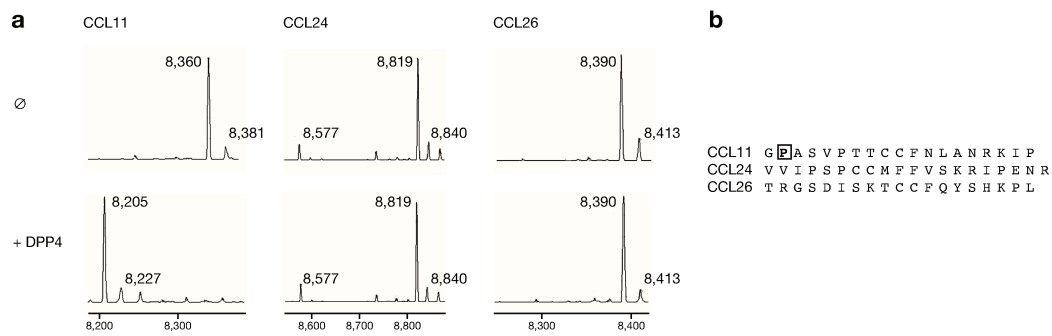


Figure 3 | DPP4 inhibition delays HCC growth in an eosinophil-dependent manner. a, WT C57BL/6 mice were fed with chow containing 1.1% sitagliptin (DPP4i) or with control (Ctrl) chow and subcutaneously injected with Hepa 1-6 cells. Mice were treated with anti-CD4 and anti-CD8 depleting antibodies. Tumour volumes were assessed (mean \pm SEM, $n = 11$ mice per group). **b,** Hepa 1-6 cells were inoculated in *Rag2*^{-/-} mice treated with Ctrl or DPP4i chow. Tumour volumes are shown (mean \pm SEM, $n = 5$ mice per group). **c, d,** Quantification of cytokine expression in extracts from Hepa 1-6 tumours collected 8 d after inoculation. **e,** WT mice were treated as in a. Depletion of eosinophils was performed by intraperitoneal injection of anti-Siglec-F antibody. Parallel groups were injected with isotype control. Tumour volumes were measured (mean \pm SEM, $n \geq 9$ mice per group). **f,** *Rag2*^{-/-} mice were treated as in (E). Tumour volumes were measured over time (mean \pm SEM, $n \geq 10$ mice per group). **g,** WT mice fed with Ctrl or DPP4i chow were inoculated with Hepa 1-6 tumour cells and treated with anti-CCR3 blocking antibody. Tumour volumes are shown (mean \pm SEM, $n \geq 11$ mice per group). **h,** WT mice fed with Ctrl or DPP4i chow were treated with cetirizine (anti-H1) incorporated in drinking water. Hepa 1-6 tumour cells were inoculated and tumour growth was assessed; (mean \pm SEM, $n \geq 11$ mice per group). Each dot corresponds to one mouse. Data are pooled from two (a, c CXCL10, D CCL11, g, h) or are representative of two independent experiments (b, c IFN- γ , d IL-4, IL-5 and IL-33, e, f). NS, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significance was determined using Mann–Whitney test (c, d) or two-way analysis of variance (a, b, e–g).

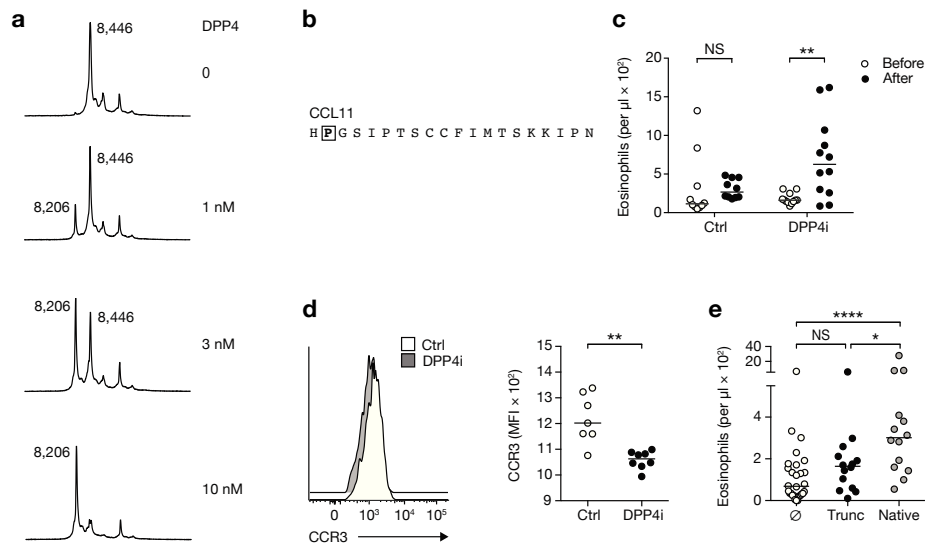
Figure 4 | Tumour IL-33 expression is required and sufficient to induce eosinophil-mediated tumour immunity upon DPP4 inhibition. **a**, WT C57BL/6 mice were subcutaneously injected with Hepa 1-6 cells. Seven days later, they received an intratumoural injection of PBS or 1 µg of mIL-33. Tumours were harvested 6 h later and CCL11 expression was assessed in tumour homogenates. **b**, Quantification of mIL-33 expression in murine tumour cell lines. **c, d**, WT C57BL/6 or BALB/c mice were fed with Ctrl or DPP4i chow and TRAMP-C2 (C) or EMT6 (D) tumour cells were transferred. Mice were treated with anti-CD4 and anti-CD8 antibodies (T cell depleted) or anti-Siglec-F antibody (eosinophil depleted) or with the corresponding isotype controls (left graph). Tumour volumes were assessed (mean ± SEM, $n = 6$ mice per group). **e, f**, WT mice were fed with Ctrl chow or DPP4i before transfer of B16-IL33 tumour cells. Eosinophil depletion was performed by intraperitoneal injection of anti-Siglec-F antibody. Tumour growth (e) and tumour volumes at day 21 (f) are shown (mean ± SEM, $n = 5-6$ mice per group). **g, h**, WT mice were fed with Ctrl chow or DPP4i and inoculated with Hepa 1-6 (g) or EMT6 (h) tumour cells. Mice were treated with anti-IL-33 blocking antibody, or with isotype control. Tumour volumes were measured (mean ± SEM, $n = 6$ mice per group). **i**, WT or *St2^{-/-}* mice were fed with Ctrl or DPP4i chow, and Hepa 1-6 tumour cells were transferred. Tumour volumes are shown (mean ± SEM, $n = 10$ mice per group). **j**, WT mice fed with Ctrl or DPP4i chow were inoculated with EMT6 tumour cells. Mice were intraperitoneally injected with anti-Siglec-F or isotype control. At days 3, 6, and 9, mice were intraperitoneally injected with a combination of anti-PD1 and anti-CTLA-4 blocking antibodies, or respective isotype antibodies. Tumour volumes are shown. Anti-PD1 and anti-CTLA-4 groups: each grey line represents one mouse and solid black line represents mean. Dashed line represents mean of isotype groups. Mice that were sacrificed before the end of experiment due to oversized tumours are denoted by ×. Data are representative of two experiments. NS, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$,

**** $P < 0.0001$. Significance was determined using two-way analysis of variance (c–e, g–j) or Mann–Whitney test (a, f).

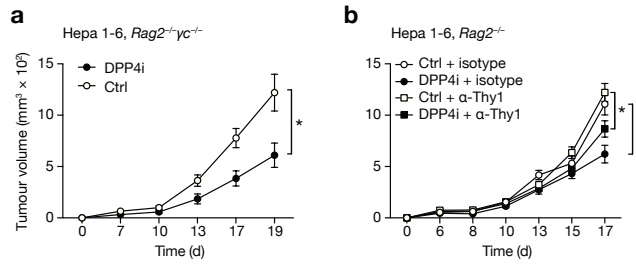
EXTENDED DATA



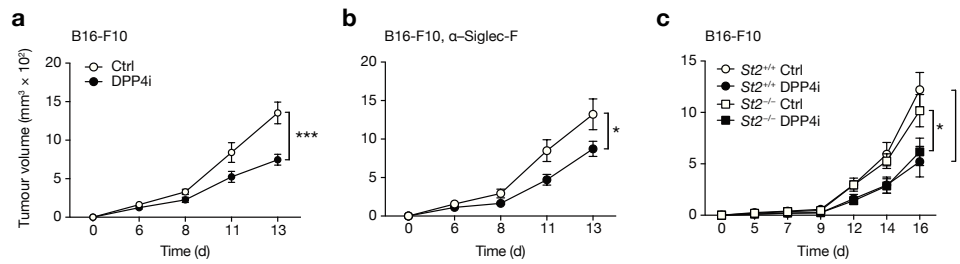
Extended Data Figure 1 | DPP4 truncates human CCL11. **a**, Recombinant hCCL11, hCCL24 and hCCL26 were incubated alone, or in the presence of 100nM of recombinant hDPP4 and intact protein mass measurement was obtained by liquid chromatography–mass spectrometry (LC-MS) analysis. Numbers indicate the molecular weight (in Daltons). **b**, Alignment of the N-terminal amino acids of hCCL11, hCCL24 and hCCL26 sequences indicated the presence of a proline (boxed) in the second position of the N terminus of hCCL11.



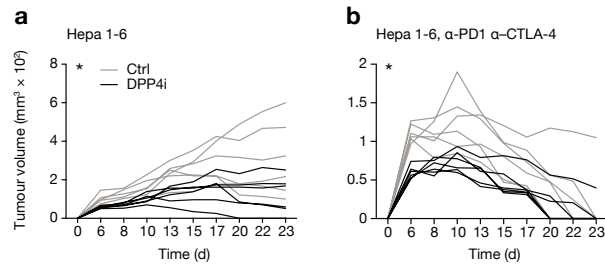
Extended Data Figure 2 | DPP4 inhibition acts on CCL11 to enhance CCR3-activated eosinophil migration. **a**, Recombinant mCCL11 was incubated with different concentrations of DPP4 and analysed by SELDI-TOF-MS. Numbers indicate the molecular weight (in Daltons). **b**, Alignment of the first 19 N-terminal amino acids of mouse CCL11 sequences indicated the presence of a proline (boxed) in the second position of the N terminus. **c**, **d**, Wild-type mice fed with control or DPP4i chow were injected intravenously 1 μg of CCL11 and blood was collected before and 1 h after injection. Eosinophil numbers (**c**) and CCR3 expression on eosinophils after injection (**d**) were determined by flow cytometry. **e**, Wild-type mice fed with DPP4i chow were injected intravenously with PBS (\square), DPP4-truncated (trunc) or native (native) CCL11; eosinophil numbers were determined by flow cytometry. Data show median and are representative of two experiments (**b**, **d**) or are pooled from two independent experiments (**c**, **e**). NS, not significant; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ (Mann–Whitney test).



Extended Data Figure 3 | DPP4 inhibition delays growth of HCC independently of the adaptive immune system and ILC. a, *Rag2*^{-/-} γ *c*^{-/-} mice were fed with control or DPP4i chow and Hepa1–6 tumour cells were transferred. Tumour volumes were assessed; *n* = 5 mice per group. **b,** *Rag2*^{-/-} mice were fed with control or DPP4i chow and Hepa1–6 tumour cells were transferred. From the day before tumour injection and every 3 days throughout the experiment, mice were Thy1 cells-depleted by intraperitoneal injection of anti-Thy1 antibodies or injected with isotype control antibodies. Tumour volumes were assessed; *n* = 12 (DPP4i + isotype), 11 (Ctrl + isotype and Ctrl + α -Thy1) or 10 (DPP4i + α -Thy1) mice per group. Data show mean \pm s.e.m and are representative of two experiments. **P* < 0.05 (two-way analysis of variance).



Extended Data Figure 4 | DPP4 inhibition delays tumour growth of B16 melanoma independently of eosinophils. **a**, WT mice were fed with Ctrl or DPP4i chow and B16-F10 tumour cells were transferred. Tumour volumes were assessed; $n = 6$ mice per group. **b**, Wild-type mice were fed with control or DPP4i chow and B16F10 tumour cells were transferred. From the day before tumour injection throughout the experiment, mice were eosinophil depleted with anti-Siglec-F antibodies intraperitoneal injection every 6 days. Tumour volumes were assessed; $n = 6$ mice per group. **c**, St2^{+/+} and St2^{-/-} mice were fed with Ctrl or DPP4i chow and B16-F10 tumour cells were transferred. Tumour volumes were assessed; $n = 8$ to 11 mice per group. Data show mean \pm s.e.m and are representative of two experiments. * $P < 0.05$, *** $P < 0.001$ (Mann–Whitney test).



Extended Data Figure 5 | DPP4i improves checkpoint blockade treatments in Hepa 1-6 tumour. Wild-type mice were fed with control or DPP4i chow and Hepa 1-6 tumour cells were transferred. At days 3, 6, and 9, mice were intraperitoneal injected with isotype control (a) or anti-PD1 and anti-CTLA-4 (b) antibodies. Tumour volumes were assessed; lines represent single mice; $n = 6$ per group. Data show mean \pm s.e.m and are representative of two experiments. $*P < 0.05$ (two-way analysis of variance).

Extended Data Table 1 | Reported adverse events during phase Ib clinical trial.

Adverse events	Number of cases
Serious adverse events (SAEs)	
Drug related	0
Due to other aetiologies	2
Premature discontinuation	0
Hypoglycaemia	0
Pancreatitis	0

Extended Data Table 2 | Median fold change of leucocyte ratios for the 5 patients during DPP4i treatment.

Visit	CD8 T cells	CD4 T cells	Eosinophils	Neutrophils	NK cells	B cells
0	1	1	1	1	1	1
2	0.96 (0.89–0.99)	1.01 (0.92–1.05)	0.64 (0.48–1.18)	1.00 (0.98–1.02)	0.95 (0.71–1.18)	1.09 (1.09–1.43)
3	0.88 (0.85–0.93)	1.01 (0.96–1.07)	0.70 (0.11–1.18)	1.01 (0.96–1.03)	0.85 (0.53–1.08)	0.84 (0.66–1.42)
5	0.96 (0.83–1.04)	1.00 (0.97–1.08)	0.41 (0.06–0.79)	1.01 (0.96–1.06)	0.65 (0.44–0.93)	1.28 (0.72–1.36)

Extended Data Table 3 | Susceptibility of human chemokines to DPP4-mediated NH₂ truncation.

Chemokine	NH ₂ sequence	Truncation (%)
CCL2	QP	11.1
CCL3	AS	21
CCL4	AP	62
CCL5	SP	96.1
CCL7	QP	76
CCL11	GP	96.5
CCL13	QP	55
CCL14	GP	95.5
CCL15	QF	0
CCL16	QP	71.7
CCL17	AR	0
CCL18	QV	0
CCL19	GT	0
CCL20	AS	0
CCL21	SD	0
CCL22	GP	100
CCL23	RV	0
CCL24	VV	0
CCL25	QG	0
CCL26	TR	0
CCL27	FL	74
CXCL1	AS	0
CXCL2	AP	100
CXCL3	AS	0
CXCL4	EA	0
CXCL5	AA	0
CXCL6	VL	0
CXCL7	AE	0
CXCL8	AV	0
CXCL9	TP	80.1
CXCL10	VP	100
CXCL11	FP	100
CXCL12	KP	99
CXCL13	VL	0
CXCL16	NE	0
CX3CL1	QH	0

ABSTRACT

Dipeptidyl peptidase-4 (DPP4 or CD26)–mediated post-translational modification of chemokines has been shown to negatively regulate lymphocyte trafficking, and its inhibition enhances T cell migration and tumor immunity by preserving functional CXCL10. In extending these initial findings to humans and pre-clinical hepatocellular carcinoma models, we discovered a new mechanism whereby DPP4 inhibition improves anti-tumor responses by eosinophil recruitment. Specifically, administration of DPP4 inhibitors (DPP4i) resulted in higher concentrations of CCL11 (or eotaxin) and increased CCR3-mediated eosinophil migration into mouse tumors. Enhanced tumor control was observed upon treatment with DPP4i, an effect strikingly preserved in *Rag2*^{−/−} mice, and abrogated only upon depletion of eosinophils or inhibition of their degranulation. We further demonstrated that tumor expression of IL-33 was necessary and sufficient for eosinophil-mediated anti-tumor responses, and that this mechanism contributed to checkpoint inhibitor efficacy. These findings provide new insight into IL-33- and eosinophil-mediated tumor control, revealed when endogenous mechanisms of DPP4 immune regulation are inhibited.

Keywords: Dipeptidyl peptidase 4, eotaxin, hepatocellular carcinoma, eosinophils, IL-33, immune checkpoint inhibitor.

Résumé

La modification post-traductionnelle des chimiokines par la dipeptidyl peptidase-4 (DPP4 ou CD26) régule négativement le trafic des lymphocytes, et son inhibition améliore la migration des lymphocytes T et l'immunité anti-tumorale en préservant la forme fonctionnelle de CXCL10. En étendant ces résultats initiaux aux humains et à un modèle préclinique de carcinome hépatocellulaire, nous avons découvert un nouveau mécanisme par lequel l'inhibition de DPP4 améliore les réponses anti-tumorales par le recrutement des éosinophiles. Plus précisément, l'administration d'inhibiteurs de DPP4 (DPP4i) conduit à des concentrations tumorales plus élevées de CCL11 (ou eotaxine) et à une augmentation de la migration des éosinophiles exprimant CCR3 dans les tumeurs. Un meilleur contrôle de la croissance tumorale a été observé lors du traitement par DPP4i, un effet conservé chez les souris *Rag2*^{−/−} mais abrogé uniquement lors de la déplétion des éosinophiles ou de l'inhibition de leur dégranulation. Nous avons également démontré que l'expression tumorale d'IL-33 était nécessaire et suffisante pour une réponse anti-tumorale médiée par les éosinophiles et que ce mécanisme contribuait à l'efficacité des inhibiteurs de points de contrôle immunitaires. Ces résultats révèlent un nouveau mécanisme par lequel le contrôle tumoral est médiée par IL-33 et les éosinophiles, mécanisme ici révélé lorsque les mécanismes endogènes de régulation immunitaire par DPP4 sont inhibés.

Mots clés : Dipeptidyl peptidase 4, eotaxine, carcinome hépatocellulaire, éosinophiles, IL-33, inhibiteur de point de contrôle immunitaire.