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BRAFV600E em células progenitoras e diferenciadas: implicações para o desenvolvimento de Câncer de Tireóide e Histiocitose das células de Langerhans

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Recife - PE
Fevereiro 2015

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MONIQUE FERRAZ DE SÁ BELTRÃO

BRAFV600E em células progenitoras e diferenciadas: implicações para o desenvolvimento de Câncer de Tireóide e Histiocitose das células de Langerhans.

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RESUMO

BRAF é conhecido como um proto-oncogene que apresenta diversas mutações, sendo a V600E a mais comumente encontrada em neoplasias humanas. A proteína BRAF quinase oncogênica tornou-se um alvo para terapia específica em oncologia por fazer parte de vias importantes como a Ras-MEK-ERK. A mutação BRAFV600E tem sido encontrada em pacientes com melanoma, câncer de tireóide, colorretal e neoplasias hematológicas. No entanto, a importância dessa molécula em carcinoma tireoidiano papilar e Histiocitose das células de Langerhans ainda não é muito clara. Para isso, modelos murinos para essas doenças foram desenvolvidos para testar a hipótese de que a mutação V600E do gene BRAF em camundongos é suficiente para induzir características fenotípicas semelhantes a observadas em pacientes com câncer tireoidiano papilifero (PTC) e Histiocitose das células de langerhnas (LCH). Nos modelos de PTC e LCH, o gene BRAF mutado é expresso nas células foliculares da tireóide (animal chamado TCB) e nas células do compartimento mielóide CX3CR1+ (animal chamado FRBRAF), respectivamente. Após 90 dias de administração de tamoxifeno para induzir a mutação, histologicamente, os animais TCB apresentam tireóide com hiperplasia folicular, protusões papilares e recrutamento leucocítico; enquanto os animais FRBRAF apresentam hepatoesplenomegalia com infiltrados inflamatórios no fígado, pulmão e baço. Análise de citometria de fluxo demonstrou aumento no número de linfócitos T predominantemente T CD4+ com presença de células Tregs Foxp3+ e Th17 na tireóide transformada em camundongos TCB. Mesma analises, em camundongos FRBRAF revelou a expansão do compartimento mielóide. Expressão de citocinas e quimiocinas são características comuns aos dois modelos animais como é o caso de CCL2, 5, 6, 8, 9/10, 17, 22 CXCL-1, 2 na tireóide de camundongos TCB e TNFα, IL-1α, M-CSF, IL-7, CCL17 no fígado de animais FRBRAF. Com destaque, observamos que no modelo de LCH, células CD11b+MHCII+Langerin+, marcador comum da lesão em humanos, também foram encontradas no fígado desses animais. Dessa forma, concluímos que a expressão de BRAFV600E em camundongos é capaz de gerar fenótipo semelhante ao de pacientes com câncer tireoidiano papilifero com infiltrado inflamatório e Histiocitose das células de langerhnas no figado. Esses resultados implicam diretamente na importância de BRAF nesses doenças.

Palavras chaves: BRAF, histiocitose, câncer de tireóide, modelo murino

ABSTRACT

BRAF is known as a proto-oncogene that has multiple mutations, V600E is commonly found in human cancers. The oncogenic protein kinase BRAF has become a target for specific therapy in oncology because of their involvemnt in the Ras-MEK-ERK pathway. The BRAFV600E mutation has been found in patients with melanoma, thyroid, colorectal cancer and hematologic malignancies. However, the importance of this molecule in papillary thryoid cancer and Langerhans cell histiocytosis is still not very clear. For this, murine models for these diseases have been developed to test the hypothesis that the BRAF V600E mutation in mice is sufficient to induce a similar phenotypic characteristics observed in patients with papillar thyroid cancer (PTC) and langerhnas cell histiocytosis (LCH). In these murine models of PTC and LCH, BRAF activacted is expressed in the thyroid follicular cells (TCB mouse), and in the myeloid cells CX3CR1+ (FRBRAF mouse), respectively. After 90 days of administration of tamoxifen to induce mutation, histologically, TCB thyroid had follicular hyperplasia, papillary protrusions and leukocyte recruitment; while FRBRAF mice had hepatosplenomegaly with inflammatory infiltrates in the liver, lung and spleen. Flow cytometric analysis demonstrated an increase in the number of T lymphocytes CD4+ predominantly Foxp3+ regulatory T cells and Th17 cells inside the TCB thyroid. Similar analyse in FRBRAF mice showed an expansion of the myeloid compartment. Expression of cytokines and chemokines are common features in two models such as CCL2, 5, 6, 8, 9/10, 17, 22 CXCL-1, 2, in TCB thyroid and TNF, IL-1α, M-CSF, IL-7, CCL17 in FRBRAF liver. Remarkably, CD11b+MHCII+ Langerin+ cells, typically found within LCH lesions in humans, were also present in the liver infiltrates. Thus, we conclude that the expression of BRAFV600E in mice is able to resemble human PTC and LCH. These results directly implicate BRAFV600E in both diseases.

Keywords: BRAF, histiocytosis, thyroid cancer, mouse model

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INTRODUÇÃO

1. INTRODUÇÃO

BRAF é um dos mais comuns oncogenes mutados implicados no desenvolvimento de cancers em humanos. Essa proteína quinase serina/ treonina é envolvida na ativação via proteína quinase mitogênica regulada por via de sinalização MAPK/ERK. Em células normais, BRAF selvagem ($BRAF^{WT}$) funciona como sinal mitogênico na via de sinalização ERK. BRAF é conhecido como um proto-oncogene que apresenta diversas mutações, sendo a V600E a corresponde a 80-90% das mutações encontrada em neoplasias humanas. A consequência dessas mutações é a desestabilização da interação hidrofóbica, bloqueio da proteína na sua conformação ativa e aumento na atividade de quinase de BRAF. A proteína BRAF quinase oncogênica tornou-se um alvo para terapia específica em oncologia por fazer parte de vias importantes como a Ras-MEK-ERK. Imunologicamente, a mutação BRAFV600E além de ativar a via de ERK, induzindo proliferação e transformação; também ativa a via de sinalização NF-kB. A mutação BRAFV600E tem sido encontrada em pacientes com melanoma, câncer de tireóide, colorretal e neoplasias hematológicas. Inibidores moleculares do BRAF como o vemurafenib vêem sendo utilizados no tratamento de melanomas e apresentando resultados promissores. Dessa forma, BRAF, torna-se uma molécula fundamental de ser avaliada em termos de mutação nos pacientes; e acompanhada com relação ao desenvolvimento de drogas inibidoras visando novas terapias moleculares. A alteração genética mais comum em carcinoma papilifero tireoidiano são as do gene BRAF as quais são observadas em 35-70% dos casos; destas, 95% corresponde a V600E. Existe uma associação direta entre mutação de BRAF com agressividade clinica do tumor tireoidiano, como invasão, metástase e recidiva do tumor. Da mesma forma, histiocitose das células de langerhans apresentam mutações BRAF em metade dos casos e etiologia pouco clara. Dessa forma, uma compreensão clara da patogênese de doenças relacionadas a expressão de BRAF ativado proporcionará melhor terapêutica, como o uso de inibidores farmacológicos dessa molécula.

REVISÃO DA LITERATURA

2. REVISÃO DA LITERATURA

2.1. Gene BRAF

BRAF é conhecido como um proto-oncogene homologo ao v-Raf de sarcoma viral em murinos e pertence a família de proteínas quinases Raf (*Rapidly accelerated fibrosarcoma*). No início da década de 80, Raf foi identificado pela primeira vez como um oncogene retroviral que codifica uma proteína quinase serina-treonina e é capaz de promover o desenvolvimento de tumores em modelos animais (Moelling, 1984; Rapp, 1983). O primeiro gene Raf a ser identificado foi v-raf (Bonner et al., 1985), homólogo do C-Raf, capaz de induz fibrossarcomas e eritroleucemias em ratos recém-nascidos (Jansen et al., 1984). Na mesma década, foi descoberto outro gene Raf em mamíferos, o B-RAF, um oncogene ativado em ensaio de fibroblastos NIH-3T3 com DNA tumoral (Ikawa et al., 1988). No total, a família Raf é composta por três proteínas: A-RAF, B-RAF e C-RAF; os quais possuem comumente três regiões conservadas: CR1, CR2 e CR3 (**Fig. 1**).

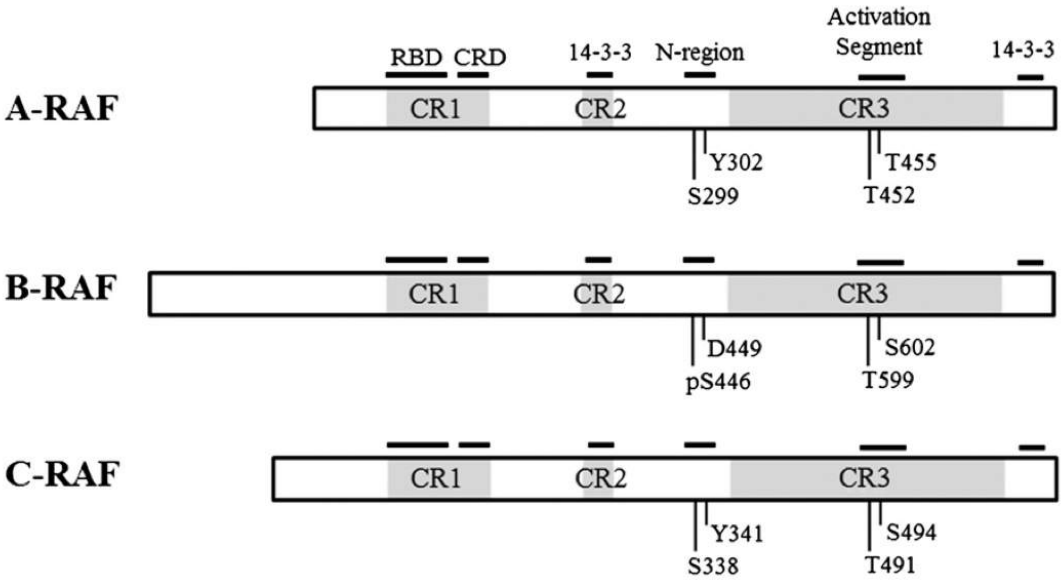

Figura 1. Estrutura linear das proteínas Raf e suas regiões conservadas. Os resíduos mais importantes em cada proteína são identificados com numerações. Em B-Raf, S446 é um resíduo constitutivamente fosforilado; em D449, o ácido aspártico substitui o resíduo de tirosina. RBD: domínio de ligação a Ras, CRD: domínio rico em cisteína, N-região: região carregada negativamente, Proteína reguladora 14-3-3 (Rahman, 2013).

CR1 e CR2 são domínios regulatórios localizados na porção N-terminal, enquanto CR3 na porção C-terminal (Wellbrock, 2004). CR1 contém o domínio de ligação a Ras (RBD, *Ras binding domain*) e o domínio rico em cisteína (CRD, *cistein rich domain*) necessário a ligação a fosfolipídeos de membrana (Roskoski, 2010). CR2 é a porção rica em serina/ treonina; sua interação com proteínas e fosforilação, implica na ativação de Raf (Guan et al., 2000; Zhang et al., 2000). A porção CR3 é o domínio quinase catalítico regulado através de fosforilacão (Roskoski, 2010).

BRAF é uma proteína quinase citoplasmática de 766 aminoácidos e 95KDa (Stephens, 1992). O gene BRAF é localizado no braço longo do cromossomo 7 (7q34) o qual é composto por 18 exons. Exons 11 e 15 que possuem regiões da porção CR3 são as mais frequentemente mutadas em pacientes com câncer (Davies, 2002).

2.1.1. Sinalização RAS/ RAF/ MEK/ ERK

BRAF é a proteína mais abundante e potente da família Raf, capaz de enviar um sinal extracelular para o complexo transmembrana receptor/Ras, ativando assim uma cascata citoplasmática. Quando fosforilada, a proteína Raf irá ativar outras quinases, a ERK (quinases reguladas por sinal extracelular, *extracellular signal-regulated kinases*), que por sua vez fosforila proteínas do citosol e nucleares, denominadas MEK (ERK quinases), desencadeando nova reação em cascata até culminar no crescimento e/ou proliferação celular (Xing M, 2005; Peyssonnaux C., 2001).

A via de sinalização MAPK (quinase mitogênica ativada, *mitogen-ativated kinase)* estimula a proliferação celular, representando uma cascata importante no desenvolvimento, uma vez que participa de inúmeras funções como crescimento, transformação e apoptose (Peyssonnaux C., 2001).

Em células normais, BRAF selvagem ($BRAF^{WT}$) funciona como sinal mitogênico na via de sinalização ERK. Essa via de sinalização é ativada após ligação com fatores mitogênicos resultando em ativação de Raf dependente de Ras (**Fig. 2**).

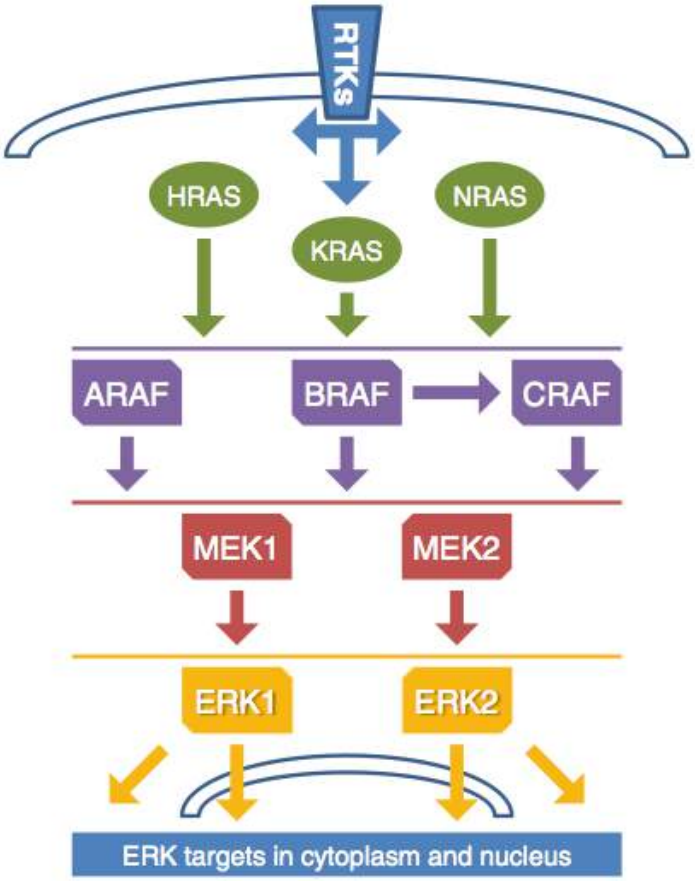

Figura 2. Via de sinalização Ras-MEK-ERK. Fatores de crescimento se ligam ao receptor tirosina-quinase (RTK) e estimulam ativação de moléculas Ras. Proteínas Ras ligadas a membrana plasmática, recrutam proteínas Raf ativando-as. Em seguida, essas, fosforiladas, ativam MEK e ERK. ERK pode fosforilar proteínas no citoplasma e no núcleo, como por exemplo fatores de transcrição, mediando assim as respostas celulares quando esta via é ativada. (Machnicki, 2014).

2.1.2. Prevalência do gene BRAF mutado e suas mutações

Mutações em BRAF são associadas com diversos tipos de neoplasias e relacionadas com malignidade, o que implica que mutações que ativam esse gene são fatores críticos para o desenvolvimento tumoral. Em termos estrutural, a maioria das mutações do gene BRAF acontecem na dobra “loop” rica em glicina e no segmento de ativação. Especificamente, estas mutações ocorrem em regiões que

estão envolvidas na interação hidrofóbica entre a dobra e o segmento de ativação (**Fig. 3**), bem como nos resíduos que contribuem para a estabilização da interação hidrófoba entre estas duas regiões (Cantwell-Dorris, 2011; Garnett and Marais, 2004). A consequência de mutações nestas regiões é a desestabilização da interação hidrofóbica, bloqueio da proteína na sua conformação ativa e aumento na atividade de quinase de B-Raf (Garnett and Marais, 2004; Davies et. al, 2002).

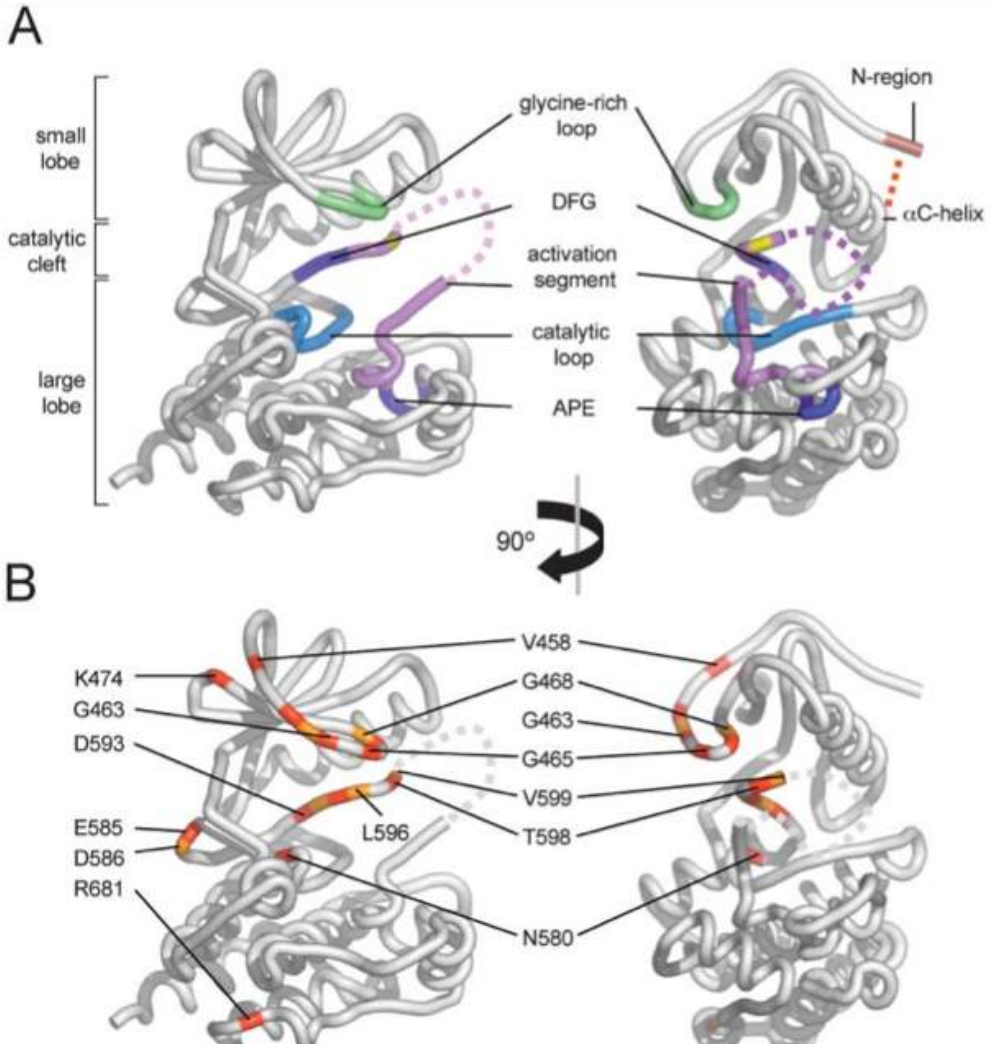

Figura 3. Estrutura da proteína quinase BRAF. Estrutura cristalizada de domínio quinase (resíduo 447-725) é apresentado. A. características catalíticas e estruturais importantes. As regiões do pequeno lobo, lobo grande e fenda catalítica são indicadas, assim como dobra rica em glicina, segmento de ativação e dobra catalítica. B. Mutações comuns do gene BRAF no câncer. Os mesmos pontos de vista apresentados em A são apresentados em B, mas com os resíduos mutantes representados na cor vermelha ou laranja. Cores alternadas são usados para maior clareza, mas não têm nenhum significado adicional (Garnett and Marais, 2004; Wan et al., 2004).

A maioria das mutações BRAF que ocorrem em neoplasias ativam essa molécula (**Fig. 4)**, mediando diretamente na sua capacidade para fosforilar MEK *in vitro* e estimular a via de ERK. No entanto, as atividades dos mutantes variam de 69 a 9200 vezes em relação ao BRAF selvagem ($BRAF^{WT}$) (Davies et al, 2002).

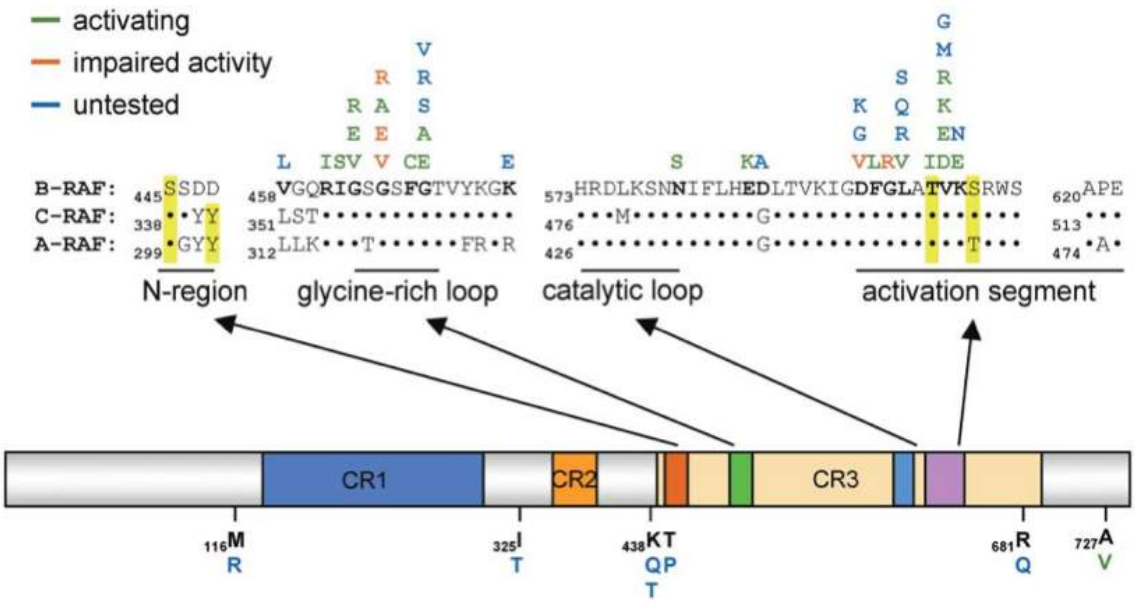

Figura 4. Associação das mutações do gene BRAF com neoplasias. A estrutura do gene BRAF é representada esquematicamente apresentando regiões conservadas (CR1, CR2, CR3), sequência de aminoácidos da região N terminal, dobra rica em glicina, dobra catalítica, e o segmento de ativação. Código de aminoácidos para B-RAF, C-RAF e A-RAF são alinhados acima do esquema. As barras amarelas destacam resíduos cuja fosforilação é necessária para a atividade da quinase e em negrito, mutações com as substituições de aminoácidos acima da sequência. Os resíduos mutantes que resultam em ativação da quinase são mostrados em verde, aqueles que prejudicam a atividade de B-RAF estão em vermelho, e os mutantes não testados estão em azul. Aminoácidos de A-RAF e C-RAF que são conservados em B-RAF são indicados por um ponto. (Garnett and Marais, 2004).

Uma característica comum a maoria das mutações BRAF é que elas interrompem a interação da dobra com o segmento de ativação rico em glicina, desestabilizando assim a conformação inativa e estimulando a atividade BRAF. No entanto, existem algumas mutações BRAF conhecidas (como mutações em resíduos E586, D587, R682, A728, K439 e T440) que não contribuem para essa desestabilização de

interação hidrofóbica entre a glicina e o segmento de ativação (Garnett and Marais, 2004). Estas mutações podem, no entanto, afetar a conformação inactiva de BRAF por mecanismos alternativos, como perturbação das interações intra-moleculares ou afetar a regulação negativa de B-Raf por outras vias e, subsequentemente, resultam na activação da via de sinalização de ERK-MEK (Garnett and Marais, 2004). Algumas mutações BRAF (incluindo G466E, G596R e G466V) diminuem a sua atividade quinase e como um resultado, essa molécula não pode ativar MEK diretamente.

Já foram identificadas mais de 40 mutações somáticas "missense", codificando aminoácido diferente, em BRAF, em exons 11 e 15. A maioria das mutações são extremamente raras, correspondendo a 0,1% a 2% de todos os casos. No entanto, é predominante a troca de timidina para adenosina no nucleotídeo 1796, convertendo valina 599 (V599) a glutamato no aminoácido 600 (Davies et. al, 2002) (**Tabela 1**).

Tabela 1. Dez mais freqüentes mutações pontuais em BRAF (Machinicki et al., 2014).

Residue	Coding sequence mutation	Amino acid mutation	Count
600	c.1799T>A	p.V600E	19,076
600	c.1798_1799GT>AA	p.V600K	432
600	c.1798_1799GT>AG	p.V600R	68
601	c.1801A>G	p.K601E	63
600	c.1799_1800TG>AA	p.V600E	51
594	c.1781A>G	p.D594G	43
469	c.1406G>C	p.G469A	30
600	c.1798G>A	p.V600M	25
466	c.1397G>T	p.G466V	19
469	c.1406G>T	p.G469V	18

A mutação V600E é a mais ativa e corresponde a 80-90% das mutações do BRAF V600 (Lovly et al 2012;. Rubinstein et al 2010). Em torno de 30% dos casos de neoplasia possuem mutações na via Ras-Raf-MEK-ERK, e destes, 8% são devido mutações em BRAF (Davies, 2002). Entretanto, a prevalência desta mutação varia significativamente entre diferentes tipos de tumor (**Tabela 2**) (Davies, 2002), como em melanoma, câncer de tireóide, câncer colorretal (Cantwell-Dorris et al., 2011; Dhomen and Marais, 2009; Pakneshan et al., 2013; Zebisch and Troppmair, 2006).

Tabela 2. Associação entre tipos de câncer a mutações distintas de BRAF (Garnett and Marais, 2004).

Amino acid[a]	Coding variant[b]	Frequency[c]	Cancer type[d] (incidence)
M116	R	0.1	Mel (1)
I325	T	0.1	Breast (1)
K438	Q, T	0.2	Lung (2)
T439	P	0.1	Lung (1)
V458	L	0.1	Lung (1)
R461	I	0.1	Colo (1)
I462	S	0.1	Colo (1)
G463	E, V, R	0.5	Colo (3), liver (1), ova (1)
G465	A, E, R, V	0.9	Mel (7), lung (2)
F467	C	0.1	Colo (1)
G468	A, E, R, S, V	1.9	Mel (4), leukemia (4), colo (3), nhl (3), liver (2), lung (2), bar (1)
K474	E	0.1	Mel (1)
N580	S	0.1	Colo (1)
E585	K	0.1	Ova (1)
D586	A	0.1	Colo (1)
D593	G, K, V	1.0	Colo (6), stom (2), mel (1), nhl (1)
F594	L	0.3	Colo (2), liver (1)
G595	R	0.2	Colo (2)
L596	Q, R, S, V	0.8	Mel (3), lung (3), liver (1), ova (1)
T598	I	0.1	Colo (1)
T598-insertion	Two amino acid insertion (T-T)	0.1	Mel (1)
V599	D, E, G, K, M, R	91[e]	Mel (442), thy (187), colo (179), ova (24), liver (11), sarcoma (7), stomach (5), glioma (4), Bar (2), breast (1), epen (1), lung (1)
V599-insertion/ deletion	Six amino acids (V599-K604) replaced by an aspartic acid	0.1	Mel (1)
K600	E, N	1.0	Thy (4), mel (3), colo (3)
R681	Q	0.1	Endo (1)
A727	V	0.1	ALL (1)

2.1.3 BRAFV600E em melanoma

Melanoma é o tipo de câncer mais frequente no Brasil e corresponde a 25% de todos os tumores malignos registrados no País (INCA, 2014). Apesar de ser um câncer facilmente tratável no inicio, ele possue alta possibilidade de metástase. Com isso, estudos pré clinicos vêem tentando colaborar no entendimento de moléculas chaves que participam de eventos chaves na patogênese do melanoma. Nesse contexto, vem chamando atenção a alta porcentagem (40-60%) de tumores de origem melanocitica que carregam BRAF mutado e ativo, onde uma única troca no códon 600 ganha destaque (Safaee 2012; Pollock, 2003).

Em melanomas, a mutação BRAFV600E é frequentemente associada com melanoma superficial (52-66%) e melanoma medular (43-55%), assim como com mutações inativadoras de p53 (Platz et al., 2008; Daniotti et al.,2004). Essa deficiência em p53 ajuda no desenvolvimento tumoral invasivo e na senescência (Michaloglou et al., 2005).

Mutações BRAF são mais relacionados a melanomas não induzido por lesão solar, ficando melanomas solares com incidência de 0-9% (Platz et al., 2008). No entanto acúmulo de agentes oxidativos tóxicos provocados pela exposição ultravioleta aumenta lesão no DNA de melanócitos contribuindo para ocorrência de mutações BRAF (Rahman, 2013; Dhomen and Marais, 2007).

Em termos terapêuticos, inibidores moleculares do BRAF como o vemurafenib vem sendo utilizado no inicio do tratamento do melanoma e apresentando resultados promissores (Sosman et al., 2012; Chapman et al, 2011). Dessa forma, BRAF, uma molécula fundamental de ser avaliada em termos de mutação nos pacientes; e acompanhada com relação ao desenvolvimento de drogas inibidoras visando novas terapias moleculares.

2.1.4. BRAFV600E em carcinoma pulmonar

BRAF ativado mutado é detectado em apenas 3% dos carcinoma de pulmão de *células não pequenas* (NSCLCs, *non-small-cell lung cancers*) (Davies et al, 2002).

Um maior número de mutações é observado para o KRAS e receptor de fator de crescimento epitelial (Shigematsu and Gazdar 2006; Brose et al 2002) nesse tipo de câncer. Além disso, essas mutações tendem a ser exclusivas e o risco de mutação BRAF é associada a pacientes fumantes (Kobayashi, et al 2011).

Em NSCLCs, os tipos de mutação para BRAF mais comuns não são do tipo Val600Glu, incluindo mais Gli468Ala e Leu596Val (Ding et al 2008).

2.1.5. BRAFV600E em câncer colorretal

Carcinoma colorretal ocorre frequentemente por acumulo de mutações da p53 e alterações na via de sinalização da ß-catenina (Cantwell-Dorris, 2011). Além desse mecanismo já bem estabelecido, o desenvolvimento de carcinoma colorretal também é associado a mutações de moléculas da via RAF-MEK-ERK, como BRAF e KRAS (Rajagopalan et al., 2002; Velho et al., 2008). Intensa correlação entre mutações de BRAF e fenótipo de metilação em ilhas CpG (CIMP) tem sido observada em pacientes com câncer colorretal (Velho et al., 2008; Weisenberger et al., 2006).

Assim como nos outros tipos de câncer, mutações em BRAF são mais prevalentes em lesões precursoras, apontando esse gene como importante nas etapas iniciais de desenvolvimento neoplásico em aproximadamente 10-15% dos casos (Cantwell-Dorris, 2011).

Em pacientes com carcinoma colorretal metastático, o inibidor oral do BRAF ativo, PLX4032 apresentou resultados menos impactantes do que comparado com pacientes com melanoma, provavelmente devido heterogenicidade de casos, resultando em respostas variadas (Kopetz et al, 2010). Sendo necessário uso de terapêutica associada a outras drogas visando aumentar eficácia.

2.1.6. BRAFV600E em neoplasma hematológico

A maioria das mutações BRAF identificadas por Davies et al (2002) foram em casos de melanomas. No entanto, essa observação tem se extendido para outros tipos de neoplasias como observado na **Tabela 3**. BRAF é uma das quinases humanas mais frequentemente mutadas em carcinomas, como também é presente em casos de não-tumores (Pollock, 2003). BRAF não parece estar diretamente envolvido na formação de leucemias e linfomas com poucas exceções como leucemia de células pilosas (LCP) em que a mutação V600E é associada a todos casos (Tiacci et al., 2011).

O inibidor de BRAF, Vemurafenib, já vem sendo usado com sucesso em tratamento de pacientes com LCP com resistência a quimioterapia (Arcaini et al., 2012). Adicionalmante, Andrulis et al. (2013) buscaram por proteína V600E em grupo de pacientes com alterações de células plasmáticas e encontraram 2.8% (7/251) dos pacientes com mieloma múltiplo expressando esse variante.

Tabela 3. Mutações de BRAF em neoplasias humanas (Machinicki et al 2014).

Alta frequência (mais de 33%)		*Moderada frequência (10- 33%)*		*Baixa frequência (menos de 10%)*	
Tricoleucemia	Mais de 100%	Adenocarcinoma endometrial	10,7%	Carcinoma adrenocortical	5,7%
Astrocitoma pilocítico	72,7%	Cancer colorretal	9,6%	Linfoma difuso de células B	4,9%
Histiocitose das células de langerhans	37,9-68,8%	Carcinoma hepatocelular	0-23,1%	Mieloma multiplo	2,8-4%
Histiocitose esclerosante poliostótica	51,4%	Carcinomas de vias biliares	2,1-21,7%	Carcinoma de células não pequenas	3,5 %
Carcinoma papilar tireoidiano	49,4%			Carcinoma escamoso de cabeça e pescoço	3,4-7,8%
Melanoma maligno	47,8%			Leucemia linfocítica crônica	0,6-2,8%
Cancer ovariano de baixo-grau	34,7%				

Seguindo os achados de leucemia de células pilosas, duas outras histiocitoses raras apresentam mutações para BRAF: histiocitose das células de Langerhans (LCH) e doença de Erdheim-Chester (ECD). LCH e ECD são doenças inflamatórias reativas ou neoplasias clonais com etiologia desconhecida (Badalian-Very et al 2013; Mazor et al 2012). Nessas patologias, mutações BRAF estão presentes em metade dos casos e apresentam cascata de ERK capaz de ser hiperativada (Badalian-Very et al 2010). No entanto, mais estudos são necessários para estabelecer a importância do gene BRAF nessas patologias.

2.1.7. Inibidores de BRAF

Diversos inibidores de BRAF vêem sendo desenvolvidos durante os últimos anos e demonstrando resultados promissores em ensaios clínicos, especialmente em melanomas metastáticos e câncer de colon (Scafani et al 2013). Dois inibidores, vemurafenib e dabrafenib foram aprovados para tratamento de melanomas. No entanto, ensaios clínicos com o sorafenib (BAY 43-9006, Nexavar) em melanomas foram desapontadores (Eisen et al., 2006; Hauschild et al., 2009).

Vemurafenib (PLX4032) inibe via da MAPK através do bloqueio de ligação ao domínio ATP da BRAF mutada (Yang et al 2012). Seu uso tem melhorado prolongado a vida de pacientes com melanoma (Chapman et al., 2011), mas menos eficiente em câncer de colon (Kopetz et al., 2010) e câncer de tireóide papilifero (Kim et al., 2013). Poucos estudos cínicos com inibidores de BRAF em outros tipos de câncer, como leucemia, câncer de pulmão e cerebral, tem sido realizado visando atividade antitumoral (Falchook et al., 2012; Falchook et al., 2013; Munoz et al., 2013; Rudin et al., 2013).

Infelizmente, muitos pacientes que respondem inicialmente bem ao tratamento adquirem resistência aos inibidores de BRAF. Dois mecanismos de resistência já foram esclarecidos: mutações secundarias NRAS e recombinação alternativa de BRAF resultando em reativação da sinalização de MAPK. Além disso, heterogenicidade intra tumoral com presença de clones tumorais com mutação

ausente para BRAF e ativação de vias alternativas podem também influenciar na resistência a inibidores de BRAF (Johansson et al 2014).

Tratamento com inibidores de BRAF sozinho ou combinando com inibidores de moléculas pequenas, imunoterapias ou quimioterapia convencional parecem a melhor maneira de tratar casos de câncer com mutação positiva para BRAF. Com isso novos testes clínicos estão sendo desenvolvidos para inibores de BRAF em diferentes tipos de câncer (**Tabela 4**).

Tabela 4. Testes clínicos envolvendo inibidores de BRAF (adaptado de Machinicki et al 2014).

Drogas testadas	**Doença**
RAF265	Melanoma metastático ou avançado
+MEK162	Tumores sólidos avançados
XL281	Tumores sólidos (câncer de pulmão, colorretal, papilar tireoidiano, melanoma)
+Cetuximab	Câncer colorretal metastático ou avançado
+Ipilimumab	Melanoma avançado
R05126766	Tumores sólidos avançados e metastático
R05212054	Tumores sólidos avançados (melanoma, neoplasma, câncer colorretal)
ARQ 736	Tumores sólidos
LGX818	Melanoma e câncer colorretal metastático
+LEE011	Melanoma
	Melanoma e câncer colorretal metastático
+MEK162+/- LEE011	Tumores sólidos avançados
+Cetuximab+/-BYL719	Câncer colorretal
+MEK162; BKM120; LEE011; BGJ398; INC280	Melanoma
+MEK162	Melanoma
CEP-32496	Tumores sólidos avançados (melanoma, câncer colorretal metastático)

2.2. Câncer de tireóide

A glândula tireóide é constituída por dois tipos celulares: células foliculares de origem epitelial responsáveis pela produção de hormônios triiodotironina (T3) e tiroxina (T4); e células C responsáveis pela produção de hormônio calcitonina. A maioria dos cânceres de tireóide é derivado das células foliculares. Os subtipos tumorais variam de carcinomas bem diferenciados, como o papilífero (PTC, *papillary*

thyroid cancer) e folicular (FTC, *follicular thyroid cancer*); carcinomas pouco diferenciados (PDTC, *poorly differentiated thyroid cancer*); e carcinomas anaplásicos (ATC, *anaplastic thyroid cancer*) (Nikiforov YE, 2011). A incidência desses subtipos de câncer tireoidianos é inversamente proporcional à sua agressividade, sendo Câncer de tireóide papilar e folicular os mais frequentes (Wreesmann e Singh, 2008).

2.2.1. Epidemiologia

Estima-se que 10% da população podem desenvolver um nódulo palpável durante a vida, devido a diversos fatores: ambientais (exposição à radiação), nutricionais (em casos de iodo-deficiência) e de etiopatogenia desconhecida (Shaktarin et al., 2003). Segundo dados do Instituto Nacional de Câncer (INCA), para o Brasil, no ano de 2014, estimam-se 1.150 casos novos de câncer de tireoide para o sexo masculino e 8.050 para o sexo feminino, com um risco estimado de 1,15 casos a cada 100 mil homens e 7,91 casos a cada 100 mil mulheres (INCA, 2014). A última estimativa mundial apontou a ocorrência de cerca de 300 mil casos novos dessa neoplasia, sendo 68 mil no sexo masculino e 230 mil no sexo feminino. Sem considerar os tumores de pele não melanoma, nas mulheres, o câncer de tireóide é o quarto mais frequente na região Sul (16,15/100 mil) e sexto nas regiões Sudeste (7,89/100 mil), Nordeste (5,68/100 mil) e Norte (3,49/100 mil) (INCA, 2014).

2.2.2. Resposta imunológica e doenças auto-imunes

A ativação de BRAF ocorre cedo na tumorigênese e pode ser um evento fundador, mas por si só não é suficiente para induzir o câncer. A mutação BRAFV600E além de ativar a via de ERK, induzindo proliferação e transformação; ela também ativa a via de sinalização NF- kB (Ikenoue et al, 2004). A ativação da via do NF-kB inibe a destruição de células importantes para a transformação, induz angiogênese, invasão e metástase através da regulação da expressão de proteínas celulares importantes, tais como ciclina D1, CDK2, VEGF, ICAM-1, VCAM-1, ELAM-1 e MMPs (Hingorani et al, 2003; Karasarides et al, 2004).

Uma das características do carcinoma tireódiano papilífero é a presença de abundante infiltrado inflamatório (**Fig. 5**).

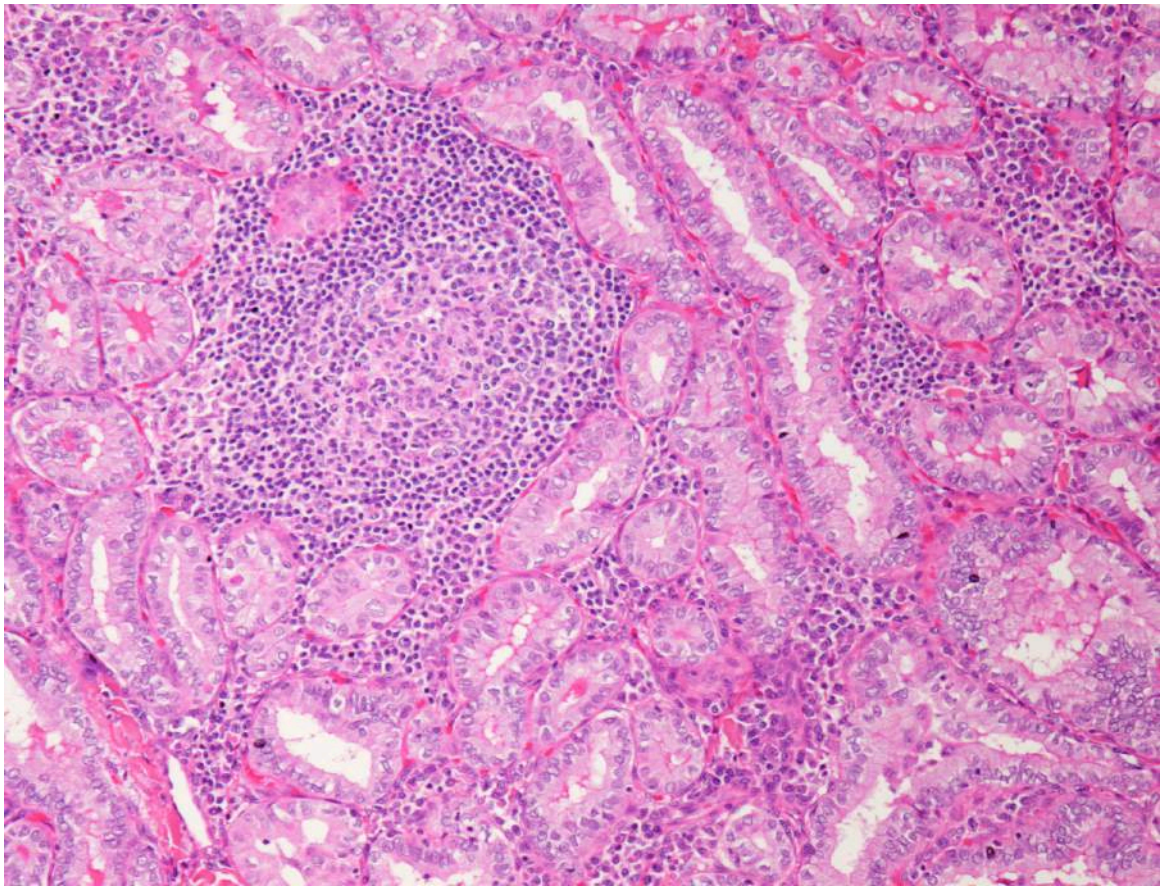

Figura 5. Infiltrado inflamatório em carcinoma papilar da tireóide. Imagem cedida como cortesia pelo Dr. David Burstein (Department of Pathology, Mount Sinai School of Medicine, NY).

A presença de infiltrado linfocitário é significativamente maior em pacientes com PTC do que em pacientes com outras lesões na tireóide, o que indica que essas células podem favorecer o desenvolvimento do câncer, no entanto ainda e controverso se essas células seriam capazes de contribuir para ao desenvolvimento de câncer. Além disso, não é claro como estas células inflamatórias atingem a tireóide e agem durante o desenvolvimento do tumor.

Associação epidemiológica entre tireoidite de Hashimoto e carcinoma papilífero da tireóide é outro fator que reforça a importância dessas células imunológicas na comunicação de células cancerígenas da tireóide. Estudo realizado em população no Nordeste demonstrou uma frequência de tireoidites em 31,4% de pacientes com PTC (Camandaroba et al 2009).

Alguns trabalhos sugerem que o aumento da incidência de carcinomas em pacientes com tireoidite pode ser uma condição pré-cancerosa (Zhang et al., 2014). No entanto, o presença de tireoidite linfocítica crônica em pacientes com PTC correlaciona-se com um prognóstico melhor (Marotta et al., 2013). Além disso, carcinomas da tiróide com mau prognóstico possuem celularidade reduzida de linfócitos infiltrando o tumor em comparação com PTCs, sugerindo que essas células podem desempenhar um papel protetor no câncer de tireóide (Kebebew et al 2001).

Outras células inflamatórias como macrófagos e células dendríticas, também têm sido identificados em PTCs em amostras humanas. Em geral, a presença de macrófagos em tumores tem sido associada com um mau prognóstico da doença (Fang et al, 2014).

Tomado em conjunto estes resultados sugerem que as células inflamatórias e auto-imunidade têm um papel no desenvolvimento do câncer de tiróide, mas o experimentos para comprovar essa hipótese ainda estão sendo realizados. Além disso, o mecanismo pelo qual ocorre o recrutamento e retenção das células inflamatórias para os tumores da tiróide são ainda desconhecidos.

2.2.3. Associação a mutações genéticas

Nos últimos anos, houve um grande avanço na elucidação dos eventos genéticos responsáveis pela iniciação e progressão do câncer de tireóide. Em PTC, mutações dos genes RET, NTRK, RAS e BRAF, moléculas efetoras que ativam a via MAPK são encontradas em cerca de 70% dos casos (Ouyang et al., 2014).

Na **Fig. 6** é ilustrado o mecanismo de transformação de célula tireoidiana folicular através de alterações na vias de sinalização de MAPK e PI3K-AKT. Quatro diferentes mutações têm efeito significante na progressão tumoral e diagnóstico: mutações pontuais em BRAF e RAS; e rearranjos em RET/ PTC e PAX8/PPARc (Omur, 2014).

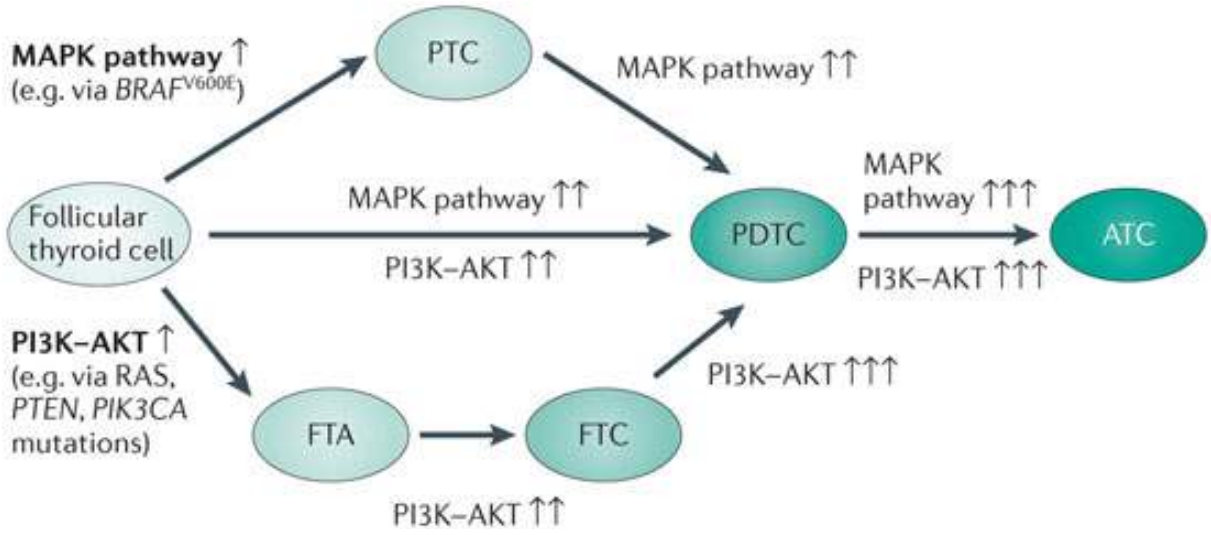

Figura 6. Tumorigênese tireoidiana via MAPK e PI3K-AKT. Ativação da cascata da MAPK por mutação BRAFV600E em câncer de tireóide papilar (PTC) a partir de células folicular da tireóide. Por outro lado, a ativação da via PI3K-AKT por mutações em RAS, PTEN e PIK3CA, leva ao desenvolvimento de adenoma folicular da tiróide (FTA) e carcinoma folicular (FTC) a partir de células folicular da tireóide. Com o acúmulo de alterações genéticas por uma das duas vias, PTC e FTC podem progredir para câncer tireoidiano pouco diferenciado (PDTC). Quando ambas vias estão totalmente ativadas através de alterações genéticas acumuladas, a conversão de PDTC para o câncer de tireóide anaplásico (ATC) é fortemente facilitada (Xing, 2013).

A alteração genética mais comum em PTCs são as do gene BRAF as quais são observadas em 35-70% dos casos (Xing, 2005); destas, 95% corresponde a V600E (Xing, 2013). Existe uma associação direta entre mutação de BRAF com agressividade clinica do tumor tireoidiano, como invasão, metástase e recidiva do tumor (Nikiforov et al, 2011).

RET (rearranjo durante a transfecção, *rearranged during transfection*), é um proto-oncogene altamente expresso nas células parafoliculares produtoras de calcitonina (células C) na glândula tiróide. A ativação do gene RET em células foliculares, como

resultado de um rearranjo cromossômico chamado RET/PTC desempenha um papel importante, especialmente na patogênese em crianças e/ou PTCs decorrentes de exposição à radiação (Fagin e tal., 2004).

Mutações do gene RAS são encontradas em 50% dos carcinomas foliculares da tireóide (Motoi et al., 2000) e são induzem tumorigenese através via clássica de MAPK ou via PI3K-AKT (Omur, 2014). Rearranjos gênicos PAX8/PPARγ compreendem 30-35% dos casos de carcinoma folicular de tireóide (Nikiforova et al., 2002), 2-3% dos adenomas foliculares e 1-5% dos carcinomas papilares com variantes foliculares (Marques et al, 2002). Informações sobre tipos de tumores tireoidianos, prevalência, sobrevida e freqüências de mutações comumente presentes são condensados na **Tabela 5**.

Tabela 5. Tumores tireoidianos, características e perfil mutacional (Omur et al., 2014).

Tumor type	Prevalence (% of thyroid cancers)	10-Year survival (%)	Mutations observed and their prevalence
Papillary carcinoma	80–85	95–98%	BRAF (V600E) 45% RET/PTC 20% RAS 10% TRK <5%
Follicular carcinoma	10–15	90–95	RAS 45% PAX8-PPARy 35% PIK3CA <10% PTEN <10% BRAF (V600E) <10%
Medullary carcinoma	3–5	60–80	Familial forms of RET >95% Sporadic RET 50%
Poorly differentiated carcinoma	<2	50	RAS35% BETA CATENIN 20% TP53 20% BRAF 15% PIK3CA 10% AKT 10%
Anaplastic carcinoma	1–2	<10	TP53 70% BETA CATENIN 65% RAS 55% BRAF 20% PIK3CA 20% PTEN <10 AKT 10%

É possível que exista também um potencial terapêutico nos casos de mutação BRAF. Como se sabe, especialmente nos pacientes mais idosos (provavelmente BRAF positivos), freqüentemente o PTC evolui com invasão local para músculo e traquéia e apresenta metástases recorrentes para linfonodos. Nesses casos, o tratamento habitual com tireoidectomia total e ablação com radioiodo não cura os pacientes, e há indicação do uso de inibidores das RAF quinases, que apresentam resultados encorajadores in vitro e em animais. Um desses inibidores, o sorafenib (Bay 43-9006), apresenta capacidade de inibir o mutante V600E em células derivadas de PTC.

Curiosamente, num ensaio clínico recente, mesmo um inibidor não-específico da via de MAPK, motesanib, o qual é um inibidor do receptor tirosina quinase do VEGF clássica, pode inibir o crescimento do tumor do tumor da tiróide, preferencialmente, em doentes que abrigava o mutação BRAF (Sherman e tal 2008). É possível que a mutação BRAF poderia aumentar o sinergismo das vias de sinalização do receptor MAPK tirosina-quinase e, por conseguinte, potenciar a sensibilidade das células para os seus inibidores.

2.2.4. Modelos animais de câncer de tireóide

Com o objetivo de elucidar a patologia e propor tratamentos pra o câncer de tireóide, alguns modelos murinos foram desenvolvidos. Basicamente, esses animais são alterados geneticamente visando modificar genes responsáveis pelo desenvolvimento tumoral, como o gene BRAF (Kim et al 2009). Por BRAFV600E ser a alteração genética mais encontrada em PTC, Dr Fagin e colaboradores, testaram a hipótese de que expressando essa mutação em células da tireóide, via promotor de tireoglobulina, eles conseguiriam induzir PTC em camundongo transgênico. Após indução de BRAF ativado por 12 ou 22 semanas, os camundongos apresentaram aumento nos níveis de TSH, redução na expressão de genes tireiodianos, tireóide aumentada e histologia característica de carcinoma de tireóide (Knauf et al 2005).

Modelos murinos também são úteis para entender papel de moléculas presente em tumores humanos como por exemplo a tirosina-fosforilada STAT3 (Couto et al 2012). Através do cruzamento de murinos transgênicos BRAFV600E com STAT3 inativado (KO, knockdown), Couto et al demonstraram que a ausência de STAT3 resultava em tumores tireoidianos papiliferos aumentados e com maior proliferação quando comparado aos camundongos que possuem STAT3. Esses achados são intrigantes, visto que STAT3 em outros tumores é apontado como mediador tumorigênico (Carpenter et al., 2014).

Recentemente, sistemas que permitem indução programada de gene em célula e tempo desejado, como o sistema Cre-ER (Hayashi et al., 2002), tem permitido construção de modelos animais que reproduzem bem as características encontradas em neoplasias humanas. O sistema semelhante induzido por doxiciclina foi utilizado pra desenvolver câncer de tireóide murino com expressão de BRAFV600E em células foliculares, e animais tratados com inibidores de MAPK possibilitaram susceptibilidade de células tumorias ao tratamento com radioiodo (Chakravarty et al 2011).

Graças aos avanços em diagnostico por imagem, é possível acompanhar metástase nesse animais e melhorar a escolha terapêutica para regressão e contenção de crescimento do tumor. Dessa forma, modelos murinos animais são ferramentas extremamente úteis para a compreensão do papel de alvos moleculares e o desenvolvimento de novas formas de diagnóstico e terapêutica.

2.3. Histiocitose das Células de Langerhans

A Histiocitose de células de Langerhans (HCL) é uma doença rara caracterizada pela proliferação de histiócitos que possuem fenótipo similar de células de Langerhans (LC) com base na expressão de CD1a. Outro marcador que confirma a identificação dessas células é a presença de grânulos de Birbeck e expressão de langerina (CD207) e proteína S100 (Badalian-Very, 2012; Cheng-Hin 2011). Causas e patologia da doença ainda não são claras. Mas sabe-se que a desregulação do sistema imunológico é um fator importante no desenvolvimento da doença, assim como sua associação com mutações do gene BRAF.

2.3.1. Sistema fagocitário mononuclear

As células dendríticas (DCs) são células apresentadoras de antígenos (APCs) capazes de induzir respostas imunes adaptativas e tolerância. Elas atuam como sentinelas nos tecidos periféricos e como APCs em órgãos linfóides secundários, filtrando e a detectando alterações ambientais que modulam o equilíbrio entre a tolerância e a resposta imune. Na pele há duas populações de DCs, as células de Langerhans (LCs) e DCs dérmicas, localizadas na epiderme e derme, respectivamente. Células dendríticas da pele e baço são oriundas de células precursoras no sangue ou a partir de precursores locais imediatos. Além da pele, LCs podem ser encontradas no pulmão, mucosa oral, esôfago, timo, etc. Elas são as mais potentes APCs do corpo (Lam, 1997).

2.3.2. Origem e sintomas da desordem histiocistica

As síndromes histiocíticas constituem um grupo de doenças com apresentações clinicas que variam de formas localizadas e benignas a disseminadas e fatais. Elas são agrupadas em três categorias: as histiocitoses das células de Langerhans ou classe I, as histiocitoses não-Langerhans ou classe II e as histiocitoses malignas ou classe III (Arceci, 1999). O primeiro caso de HCL foi descrito em 1893 onde uma criança de três anos apresentava lesões cutâneas, ósseas, exoftalmia, hepatoesplenomegalia, linfadenopatia e diabetes insipidus (Arceci, 1999). Clinicamente a doença é bem variada, evoluindo de lesões locais em pele, ossos e pulmão, ou multisistêmica, levando em casos raros a alterações neurológicas (Grois et al 2005).

A freqüência de HCL em idade pediátrica foi estimada entre 2 a 5 casos por milhão, por ano, com pico de incidência entre 1 e 4 anos e com predomínio no sexo masculino (Lichtenstein 1953). A origem da HCL não é clara. Estudos têm demonstrado que HCL é promovido por uma proliferação clonal descontrolada de células dendríticas com características de LC, o que sugere que estas células sejam oriundas de um progenitor comum. No entanto, estudo realizado em 2010, comparando células CD207+ de pacientes com HCL e LCs CD207+ isoladas da pele de pacientes saudáveis demonstrou que elas diferiam na expressão diversos genes,

com destaque para alta expressão de genes associados a células imaturas mieloides em células de pacientes com HCL (Allen et al 2010). Em termos de maturação, LCs iniciam a popular a epiderme durante o desenvolvimento do estrato córneo e permanecem negativas para CD207 e MHCII ate aproximadamente uma semana apos nascimento (Chorro et al, 2009). Merad e colaboradores identificaram os precursores monociticos dessas células CD207- (Ginhoux et al., 2006; Ginhoux et al., 2009) e as caracterizaram como CD11b+, F4/80-, CX3CR1+ e Gr1-. E colaborando com esses dados, estudo em modelos murinos demosntraram que a célula de que da origem as LCs são negativas para CD207 e CD24 (Tripp et al., 2004).

Os critérios de diagnóstico de doenças histiocíticas baseia nos aspectos clínicos, morfologia, ultra-estrutura, e nas propriedades histiocitárias imuno-histoquímica. O diagnóstico definitivo para a HCL incluem expressão de Langerina ou detecção de grânulos de Birbeck na microscopia eletrônica, assim como CD1a e S100, que são menos específicos. O pior prognóstico da doença é observado em crianças com aparecimento da doença até os dois anos de idade, com envolvimento da medula óssea, do pulmão ou do fígado e falha na resposta terapêutica sistêmica em seis semanas de tratamento (Quattrino et al 2007).

2.3.3. Resposta imunológica

A HCL não tem sido considerada doença neoplásica, mas proliferação celular ou disfunção imune onde há freqüente enriquecimento de células T regulatórias (Senechal et al 2007). Lesões de pacientes com HCL apresentam alta expressão de moléculas estimulatórias de células T como citocinas pro-inflamatórias, além de se apresentarem elevadas também sistemicamente (Allen et al 2010; Laman et al 2003). Fenotipagem de pacientes com HCL indicam que as células de Langerhans apresentam perfil mais diferenciado, principalmente quando a doença ocorre isoladamente no tecido cutâneo (Geissmann et al 2001). As lesões em geral acometem locais em que as células de Langerhans estão presentes, e a maioria das formas clínicas evoluem para processo crônico com presença de granulomas. Osteólise e fibrose são processos patológicos comuns em infiltrados inflamatórios

em tecidos de pacientes com HCL e são resultados da desregulação imunológica provocada pela tempestade de citocinas liberadas por macrófagos, linfócitos, granulocitos eosinofílicos e células gigantes multinucleadas (de Graff et al., 1996).

2.3.4. Associação a mutações genéticas

Recentemente, teste para 983 alelos para 115 genes relacionados com câncer foi usado para analisar 61 casos de LCH (Badalian-Very et al., 2010). A descoberta mais interessante foi a detecção da mutação BRAFV600E em 57% dos casos, sendo os resultados confirmados por pirosequenciamento. Ativação do gene BRAF (V600E) tem sido detectada em várias amostras de biópsia de LCH (**Tabela 6**), incluindo casos com comprometimento pulmonar (Roden et al 2014), mas não é claro se esta alteração genética é suficiente para induzir a doença, ou sua associação com o estágio da doença. Mesmo sem precisa evidência sobre o papel de BRAF em LCH, novas terapêuticas com inibidores já estão sendo aplicada (Vaiselbuh et al., 2014).

Tabela 6. Prevalência de mutações BRAF em histiocitose de Langerhnas (adaptado de Machnicki et al, 2014)

Frequência de mutação BRAF	**Artigo**
Histiocitose das células de Langerhnas	
37,9% (11/29)	Haroche et al 2012
40% (2/5)	Yousem et al 2013
41,3% (19/46)	Sahm et al 2012
57,4% (35/61)	Badalian-Very et al 2010
68,8% (11/16)	Satoh et al 2012

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OBJETIVOS

4. OBJETIVOS

Geral

Testar a hipótese de que a mutação V600E do gene BRAF em camundongos é suficiente para induzir características fenotípicas semelhantes a observadas em pacientes com câncer tireoidiano papilifero e Histiocitose das células de langerhnas.

Específicos

- Desenvolver camundongo denominado TCB expressando o gene BRAF ativado induzível em células tireoidianas
- Caracterizar alterações histológicas na tireóide de camundongos TCB
- Caracterizar por citometria de fluxo e imunofluorescência infiltrado inflamatório de camundongos TCB
- Desenvolver camundongo denominado FRBRAF expressando o gene BRAF ativado induzível em células mieloides caracterizadas por serem $CX3CR1^{+}$
- Caracterizar alterações fenotípicas nos camundongos FRBRAF
- Avaliar alterações no compartimento mieloide dos camundongos FRBRAF
- Buscar por marcadores moleculares da histiocitose das células de langerhnas em camundongos FRBRAF
- Analisar transcriptoma do fígado de camundongos FRBRAF por técnica de microarranjo

ARTIGOS PRODUZIDOS

5. ARTIGOS PRODUZIDOS

5.1.

Titulo: **Inducible thyroid specific BrafV600E activation: a mouse papillary thyroid cancer model with significant lymphocytic inflammation**

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Inducible thyroid specific BrafV600E activation: a mouse papillary thyroid cancer model with significant lymphocytic inflammation

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Abstract

Human papillary thyroid cancer (PTC) has been associated with enhanced inflammation in the thyroid. Herein, we developed a mouse PTC model with inducible conditional activation of BrafV600E (referred as TCB). 90 days after the expression of BRAFV600E in thyroid follicular epithelial cells, papillary thyroid cancer features (follicular hyperplasia, papillary protrusions, nuclear clearing and pseudo inclusions) developed together with increased recruitment of inflammatory cells, including T lymphocyte and myeloid cells and upregulation of chemokines CCL-2, 5, 6, 8, 9/10, 17, 22 CXCL-1, 2 and chemokine receptor CCR1,2,4, and CXCR1. Flow cytometry analysis demonstrated a T lymphocyte dominant infiltration in our model, especially CD4+ T lymphocyte. Further analysis revealed the coexistence of Foxp3+ regulatory T cell and IL-17 secreting Th17 cells in the microenvironment of thyroid tumor. In conclusion, we developed and characterized a mouse model of PTC with enhanced inflammation that mimics human condition. Our model provided an opportunity to further study the contribution of different inflammatory cell subsets to PTC outcome.

Introduction

Thyroid cancer is the most common malignancy of endocrine organs (Nikiforov and Nikiforova 2011). Its incidence significantly increased over the past three decades with an annual increase of 6.4% from 1997 to 2010. The age-adjusted incidence rate now is 12.2 per 100,000 men and women per year. (Li, Lee et al. 2012). The most common form of thyroid cancer is papillary thyroid carcinoma (PTC), comprising 65-88% of all differentiated thyroid cancers (Enewold, Zhu et al. 2009). An inflammatory component is present in the microenvironment of most neoplastic tissues. It has been proposed that cancer related inflammation might be considered as the “seventh hallmark” of cancer (Colotta, Allavena et al. 2009). Thyroid cancer often has an inflammatory cell infiltrate, which includes lymphocytes, macrophages, dendritic cells and mast cells (Guarino, Castellone et al. 2010). However, it is unclear how these inflammatory cells reach the thyroid during cancer development and whether they indeed contribute to PTC development.

The T1799A nucleotide transversion in the BRAF gene is a prominent oncogenic mutation in PTC and occurs in around 45% of cases. This mutation causes a valine-to-glutamic acid change in codon 600 of the BRAF protein, resulting in BRAFV600E, which possesses elevated serine/threonine protein kinase activities and constitutively activates the mitogen-activated protein kinase signaling pathway in human cancer. In a recent published retrospective multicenter study, the presence of BRAFV600E mutation was significantly associated with increased cancer-related mortality among patients with PTC (5.3% vs 1.1% in BRAFV600E-positive vs mutation-negative patients) (Xing, Alzahrani et al. 2013).

Here, we described the development of transgenic mice with tamoxifen inducible expression of BrafV600E in thyroid epithelial cell and generated a mouse model recapitulating human PTC and characterizing the features of inflammatory infiltrate during cancer development.

Materials and Method

Mice

To generate a mouse expressing an inducible form of Cre in the thyroid, the cDNA encoding ERT2-Cre-ERT2 was cloned into a pBS-TG vector that contains a 2 kb fragment of the rat thyroglobulin promoter. After sequence verification, the transgene was isolated from the plasmid by restriction enzyme digestion and gel purification and microinjected into C57BL6/J mouse eggs. The resulting litters were screened with primers detecting ERT2-Cre-ERT2: 5'-TGGAGATCTTCGACATGCTG-3' and 5'-GATGTGGGAGAGGATGAGGA-3'. We refered these animals as TGCRET2 mice. To generate thyroid-specific conditional expression of BRAFV600E we intercrossed the mice containing a floxed BRAFV600E allele (a gift from Dr. Martin McMahon, UCSF) with the TGCRET2 mice described above. We refer to these animals as TCB mice. Adult TCB mice were treated with repeated injections of Tamoxifen dissolved in peanut oil (2mg i.p. x 6) and were monitored for the development of phenotypes. All mice were housed under specific-pathogen-free conditions in individually ventilated cages at the animal facility of Icahn School of Medicine at Mount Sinai. All experiments were performed following institutional guidelines.

Preparation of the samples

For haematoxylin-eosin (HE) and immunofluorescence study, the removed thyroid tissues were fixed by immersion in 10% phosphate-buffered formalin over night and then processed for paraffin sections. Routinely, 4-μm sections were cut and stained.

Immunofluorescence staining.

For immunohistochemical staining, deparaffinized slides were treated with citrate-based antigen retrieval solution (DAKO, Carpinteria, CA) for 15 min. Sections were blocked, and incubated with primary Abs in a humidified atmosphere for 1 h at room temperature followed by incubation with the appropriate labeled secondary Abs for 30 min. Primary Abs used were Cre (MAB 3120) from Millipore; pan-keratin (ab6401), Ki67 (ab15580), CD3 (ab5690) from Abcam; CD45 (14-0451), F4/80 (14-4801), Foxp3 (14-5773) from eBioscience and TTF-1 (sc-13040) from Santa Cruz; rabbit anti-mouse CCL6 and CCL17 polyclonal antibodies (kind gift of Dr. Steven L. Kunkel, University of Michigan). Secondary Abs used were Alexa Fluor 488 and 594 goat anti-rat IgG (nos. A-11006 and A-11007), Alexa Fluor 488 goat anti-mouse IgG1 (A-21121) and Alexa Fluor 488 and 594 goat anti-rabbit IgG (nos. A11008 and A11037) (all from Invitrogen). The slides were washed and mounted with Fluoromount-G (Southern Biotech). Images were captured using a Nikon fluorescence microscope and processed using Adobe Photoshop CS3.

Flow cytometry

To prepare single cell suspensions, individual thyroids were minced in RPMI 1640 media with 10% FBS and 2.5 mg/ml Collagenase D and incubated for 1h at 37°C. Thyroid cells were incubated for 30 min at RT with 5 μg/ml Fc block (BD Pharmingen,

San Diego, CA) and then stained for 30 min with directly conjugated primary mAbs at 4°C. Directly conjugated Abs against CD45 (30F-11), CD3 (145-2C11), B220 (RA3-6B2), CD8 (53-6.7), CD4 (RM4-5), CD11b (M1/70), CTLA4 CD152 (UC10-4B9), Foxp3 (FJK-16S) and IL-17 (eBio17B7) were obtained from eBioscience. For intracellular staining, cells were stimulated for 3h with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml), and ionomycin (1 μg/ml, both from Sigma-Aldrich) in the presence of monensin (2μM) (eBioscience) at 37°C. For detection of IL-17, cells were fixed and permeabilized according to manaufacturer's protocol (eBioscience). For detection of Foxp3, Foxp3 Staining Buffer Set (eBioscience) was used for fixation and permeabilization. FACS Canto cytometer (BD Biosciences, Franklin Lakes, NJ) was used to acquire events. All flow cytometry studies were analyzed with FloJo software (TreeStar).

Reverse transcription polymerase chain reaction and real-time polymerase chain reaction

Total RNA was extracted from the thyroid using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed with 1 μg of total RNA. PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and of 60°C for 1 min. Relative expression levels were calculated as $2^{(Ct\ ubiquitin\ -\ Ct\ gene)}$ (Ct indicates cycle threshold. For details, see User Bulletin No. 2, ABI PRISM 7700; Applied Biosystems) Two-tailed Student t test was used to evaluate statistically significant differences in expression levels of specific genes. Primers were designed by using Primer Express 2.0 software (Applied Biosystems). Primer sequences are described in Table 1.

Results

Inducible BRAFV600E activation in thyrocytes results in papillary thyroid cancer model with classical clinical features.

We generated a transgene in which the rat thyroglobulin promoter drives expression of tamoxifen-inducible form of cre recombinase (Figure 1A). Q-PCR analysis showed Cre expression in one of these lines (Figure 1B). Immunostaining of the thyroid of these animals, but not of controls, showed expression of Cre in the nuclei of thyrocytes 24h after tamoxifen administration. (Figure 1C). We then intercrossed these mice with mice containing a floxed BRAFV600E allele. The obtained strain was referred as TCB mice (Figure 1D). This method enabled us to induce an oncogene (BRAFV600E) activation in the thyroid epithelial cells with temporal control. As shown in Figure 1E and F, 90d after tamoxifen injection, we obtained a phenotype of enlarged and lobulated thyroid glands with increased thyroid weight. Histological analysis of the thyroid showed features similar to human PTC: enlarged, multilayer follicles devoid of colloid, papillary protrusions and nuclear changes including nuclear clearing, overlapping and nuclear inclusions (Figure 1G). Marked elevation of proliferating thyroid epithelial cells are present, as shown by pan-keratin and Ki-67 co staining and quantified by absolute number of double positive cells per thyroid lobe (Figure 1H). Furthermore, we assessed the thyroid endocrine function in our model. A decrease in thyroglobulin and concomitant increase in pituitary TSH mRNA are shown in Figure 1I, suggesting hypothyroidism associated with BrafV600E

expression in thyrocyte. Thus, we are able to generate an inducible mouse PTC model that recapitulates human condition.

BrafV600E activation in the thyroid leads to enhanced inflammation in situ.

Since inflammation is a feature frequently observed in human PTC patients, we assessed the inflammatory infiltrate in our mouse model. Similarly, a dense inflammation demonstrated by intra-thyroid CD45+ cell flow cytometry and immunofluorescence staining (Figure 2A) was observed 90d after TAM injection. Further analysis of different populations of thyroid infiltrating CD45+ cells showed a significant increase of CD3+T cells (Figure 2B) and both CD4+ and CD8+ T cell subsets (Figure 2C). A less striking elevation in myeloid population, as shown by flow cytometry of CD11b+ cell and F4/80 immunofluorescence staining (Figure 2D) were noticed as well. A consistent upregulation of proinflammatory cytokines IL-1α, IL-6, IFN-γ and TNF-α in the thyroid (Figure 1G) was also observed. Thus, PTC induced in our model was accompanied by an enhanced intrathyroidal inflammation.

Expression of BrafV600E affects expression of chemokines in the thyroid.

Chemokines and their receptors are key factors controlling leukocyte recruitment into tissue (Lira and Furtado 2012). To test whether the expression of chemokines was altered in our PTC model, we performed Q-PCR for chemokine/chemokine receptor genes in the thyroid after tamoxifen administration. As shown in Figure 3A, we observed marked increase in the expression of several chemokine genes, including CCL5, CCL17, CCL22 and their receptor CCR1 and CCR4 that are mainly responsible for T lymphocyte recruitment (Scarpino, Stoppacciaro et al. 2000). We also observed a significant increase of CCL2, 6, 8, 9/10 CXCL1 and CXCL2 and their

receptors CCR2 and CXCR1 that are primarily involved in myeloid cell recruitment (Figure 3B) (Tanaka, Kurebayashi et al. 2009). Immunofluorescence staining in Figure 3C and 3D indicated F4/80+ cells served as the major source of CCL6 and CCL17, suggesting the production of these chemokines by macrophages.

Both regulatory T cell and Th17 cells are present among thyroid infiltrating T cells in BrafV600E induced PTC model.

We noticed a 10 fold increase in the number of CD3+T cells after tamoxifen administration (Figure 2C). Since the increase of T lymphocytes, especially the CD4+T cell (Figure 2e) was more striking than the myeloid population (Figure 2f-g), we focused on characterizing CD4+ T cells by flow cytometry. Further analysis demonstrated the emergence of Foxp3+CTLA-4+ regulatory T cells (Treg) in the thyroid 90 days after Tamoxifen injection (Figure 4A and 4C). The presence of Treg was confirmed by immunofluorescence staining of Foxp3 (Figure 4B) and was consistent with the upregulation of Treg related cytokines including IL-10, TGF-β1 and TGF-β2 (Figure 4D) in the thyroid. Moreover, intracellular cytokine flow cytometry showed an appearance of the IL-17+CD4+T cells (Figure 5A). Elevated IL-17A mRNA is also observed (Figure 5B). Thus, the inflammation in our PTC model was mainly composed of a dense infiltrate of T lymphocytes, especially the CD4+T cells. Treg and Th17 cells co-existed in the microenvironment of thyroid tumor.

Discussion

In our study, inducible BrafV600E expression in thyroid epithelial cells results in mouse PTC with features similar to human disease. We characterized the cancer

features and inflammatory infiltration in our TCB mouse and establish it as a useful tool to study the contribution of different immune compartments in PTC progression.

There are several genetically engineered mouse models of PTC being reported. Oncogenes or oncoproteins such as BrafV600E (Charles, Iezza et al. 2011; Franco, Malaguarnera et al. 2011), RET/PTC1 (Jhiang, Sagartz et al. 1996; Santoro, Chiappetta et al. 1996; Cho, Sagartz et al. 1999), RET/PTC3 (Powell, Russell et al. 1998) and TRK-T1(Russell, Powell et al. 2000) driven by thyroid specific promoter can initiate mouse PTC which harbors some human PTC features, such as: increased follicle size and loss of colloid, papillary protrusion, follicular cell hyperplasia, and nuclear invaginations. However, although lymphocytes were frequently found within and surrounding primary thyroid tumors in human studies (Hirabayashi and Lindsay 1965; Okayasu, Fujiwara et al. 1995; Okayasu 1997; Buyukasik, Hasdemir et al. 2011), none of these published mouse PTC models showed lymphocytic infiltrates in the thyroid (Russell, Powell et al. 2000). Thus, we report for the first time a mouse PTC model with both cancer features and a significant lymphocytic infiltration that mimic human conditions.

In addition, we use flow cytometry to characterize the different lymphocytic subsets and cytokine production. Similar to what was reported in human papillary thyroid cancer (French, Weber et al. 2010), a significantly increased thyroid CD4+T cell infiltration is also indicated in our model. Furthermore, we found a co-existence of both Foxp3+CTLA4+ regulatory T cell (Treg) and CD4+IL17+ Th17 cell in the cancerous thyroid as well. The accumulation of both Treg and Th17 cells in tumor microenvironment have been shown in different types of tumors, including prostate

cancer (Sfanos, Bruno et al. 2008), bladder cancer (Chi, Lu et al. 2010) and invasive breast cancer(Benevides, Cardoso et al. 2013). Tregs that express the transcription factor Foxp3 are indispensible for the maintenance of immune tolerance (Sakaguchi, Yamaguchi et al. 2008). Increased Treg in the thyroid of our mouse PTC model is consistent with what were reported in human PTC (French, Weber et al. 2010; Gogali, Paterakis et al. 2012). Treg accumulation in cancer has been generally linked to unfavorable disease outcomes as reported for many tumors (Curiel, Coukos et al. 2004) (Petersen, Campa et al. 2006). Treg is considered as a key player in tumor immune escape and angiogenesis (Facciabene, Peng et al. 2011) (Vignali, Collison et al. 2008). In thyroid cancer, the increased Treg frequency in the thyroid also correlates with more aggressive disease, including increased incidence of lymph node metastasis (French, Weber et al. 2010), intrathyroidal metastasis and more advanced TNM (tumor-lymph nodes-metastasis) stages (Gogali, Paterakis et al. 2012). A distinct lineage of IL-17 producing CD4+T cells (Th17 cells) were identified in 2005 (Park, Li et al. 2005). These cells are important in host defense and pathogenesis of a broad array of inflammatory and autoimmune diseases (Tesmer, Lundy et al. 2008). Th17 cells have been found in many different types of human tumors. However, their function in tumor immunity remains largely controversial. Its pro-tumor effects in several models such as bladder carcinoma, skin cancer and hepatocellular carcinoma were believed to be mediated by promoting tumor cell survival, motility and invasiveness (Wang, Yi et al. 2009) (Li et al. 2012), inducing tumor promoting microenvironments at tumor site (He, Li et al. 2010) and increasing angiogenesis (Numasaki, Fukushi et al. 2003) (Luo et al. 2009). While in other models, mounting evidence suggests that Th17 cells may also have potent antitumor immune effects. In mice melanoma models, the anti-tumor roles of Th17 cells have

been associated with increased production of IFN-γ (Muranski, Boni et al. 2008) and activation of tumor specific CD8+T cells (Martin-Orozco, Muranski et al. 2009). The exact impacts of regulatory T cells and Th17 cells in PTC growth are still unknown and merit further investigation.

Chemokines are crucial for cancer-related inflammation (Mukaida and Baba 2012). We demonstrate several chemokines/chemokine receptors upregulation in tumor microenvironment. For example, increased CCL5 and CCL2 can mediate the recruitment of Th17 cells (Su, Ye et al. 2010). Also, in human PTC, expression levels of CCL-2 were positively correlated with tumor size and lymph node involvement, serving as an independent predictive factor for recurrence (Tanaka, Kurebayashi et al. 2009). We showed for the first time elevated CCL17, CCL22 and their receptor CCR4 in mouse PTC model, which was consistent with previous results that CCL17 and CCL22 were responsible for intratumoral Treg infiltration (Mizukami, Kono et al. 2008).

In summary, we established a mouse PTC model with inducible thyroid specific expression of BrafV600E. Our model was featured by intense CD4+ T cell infiltration containing both Th17 cell and Treg, which mimic the human disease. Our model provided basis for further study of the contribution of different T cell subsets in thyroid cancer. Although current therapy is quite effective for most PTC patients, a considerable number (~15%) of PTC patients develop distant metastasis and recurrent disease (Prescott, Sadow et al. 2012). In these patients, novel tumor immunotherapy targeting Tregs can be considered.

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Figure legend

Figure 1: Development of an inducible thyroid specific BrafV600E activation induced mouse papillary thyroid cancer model. (a) Diagram of the TGCRET2 transgene. Transgenic founders carry the TGCRET2 transgene(*). ZP3 was used as internal control amplification. **(b)** Q-PCR analysis of thyroid mRNA shows express of Cre in the thyroid. **(c)** Cre is present in the nuclei of TGCRET2 thyrocytes after tamoxifen treatment,but not in controls as indicated by Cre immunofluorescence staining (40x). **(d)** Schematic representation of the generation of TCB mice and experimental design. **(e)** Representative images of the thyroid gland from TCB mice without (left) or with (right) TAM administration. **(f)** Thyroid weight of mice from wild type group, TCB mice without or with TAM administration. **(g)** Histological analysis of thyroid tissue from either treated or non-treated mice (top:4x; bottom:60x). **(h)** Immunofluorescence staining and quantification of proliferating thyrocytes by Ki67 (red) and pan-karatin (green) (20x). **(i)** Decreased thyroglobulin mRNA and increased pituitary TSH after TAM treatment.

Figure 2: Thyroid specific BrafV600E activation induced an enhanced inflammation in situ.

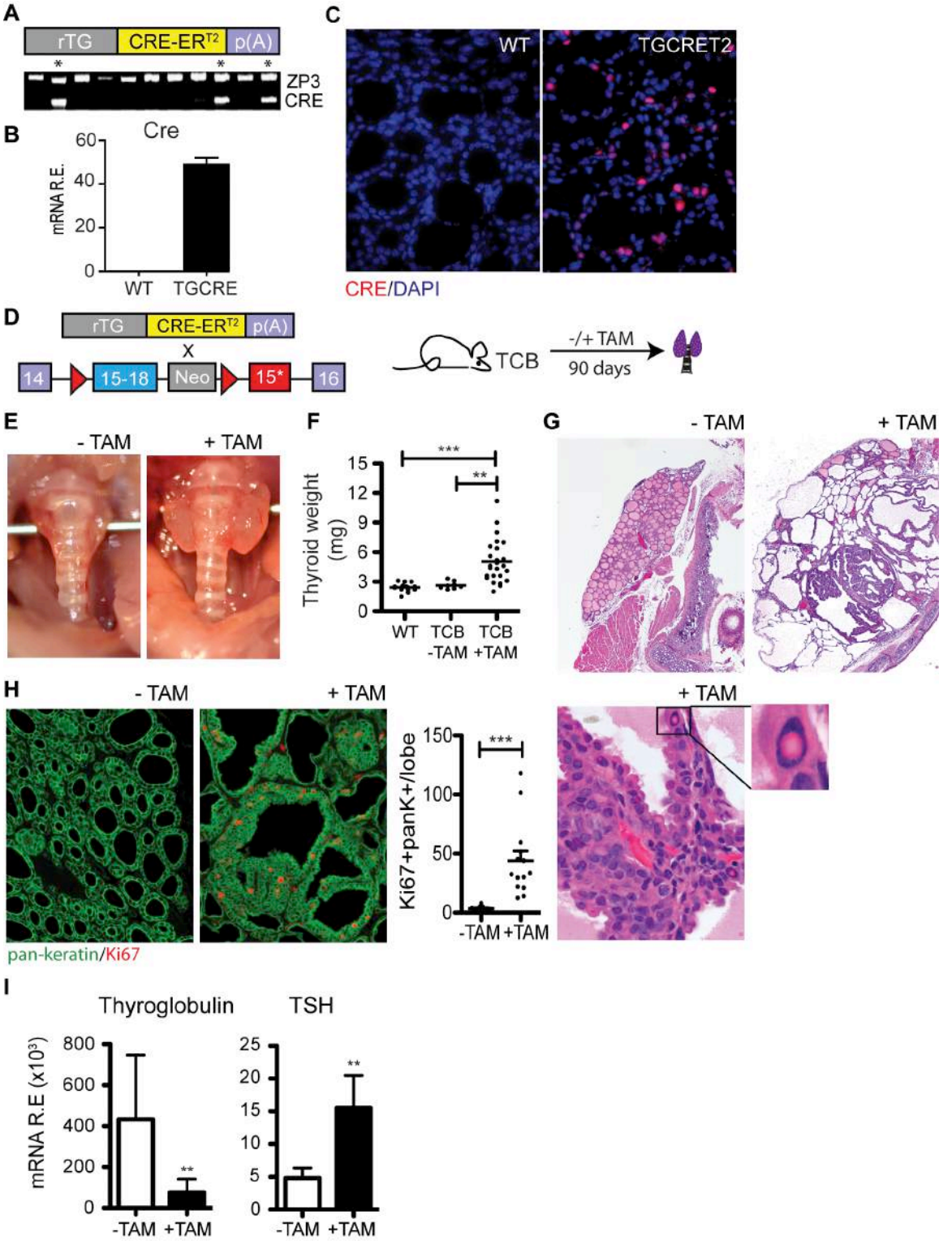
(a-b) Increased CD45+ cell in the thyroid of TCB mice after TAM administration as indicated by flow cytometry **(a)** and CD45 immunofluorescence staining (10x) **(b). (c-d)** Increased CD45+ cells were composed of CD3+ T cell as shown by flow cytometry **(c)** and CD3 immunofluorescence staining (20x) **(d). (e)** Both CD4 and CD8+ T cell subsets were increased. **(f-g)** Increased myeloid population as shown by CD11b+ flow cytometry **(f)** and F4/80 immunofluorescence staining (20x) **(g). (h)**

Upregulation of proinflammatory cytokines IL-1α, IL-6, TNF-α and IFN-γ indicated by Q-PCR.

Figure 3: Increased expression of intrathyroidal chemokines and chemokine receptors CCL5, CCL17, CCL22, CCR1 and CCR4 **(a);** CCL2, CCL6, CCL8, CCL9/10, CXCL1, CXCL2, CCR2 and CXCR1 **(b). (c-d)** Costaining of CCL6 and CCL17 with F4/80. All the images are shown at 40x.

Figure 4: Emergence of regulatory T cell (Treg) in the TCB thyroid after TAM administration. (a-c) Increase number of Treg (CD3+Foxp3+) as indicated by flow cytometry and quantification **(a)** and immunofluorescence staining (40x) **(b).** Tregs are confirmed by the expression of CTLA-4**(c). (d)** Increase expression of TGF-b1 TGF-b2, IL-10 and Foxp3 by Q-PCR.

Figure 5: Presence of Th17 cell in the TCB thyroid after TAM administration. **(a)** Flow cytometry shows increased number of CD4+IL-17+Th17 cells (plots gated on CD3+cells). **(b)** Increased expression of IL-17A mRNA in the thyroid after TAM injection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rTG
CRE-ER^{T2}
p(A)
ZP3
CRE
B
Cre
mRNA R.E.
WT
TGCRE
C
WT
TGCRET2
CRE/DAPI
D
rTG
CRE-ER^{T2}
p(A)
X
14
15-18
Neo
15*
16
TCB
-/+ TAM
90 days
E
- TAM
+ TAM
F
Thyroid weight (mg)
WT
TCB -TAM
TCB +TAM
G
- TAM
+ TAM
H
- TAM
+ TAM
pan-keratin/Ki67
Ki67+panK+/lobe
-TAM
+TAM
+ TAM
I
Thyroglobulin
TSH
mRNA R.E (x10^3)
-TAM
+TAM
-TAM
+TAM

Figure 1.

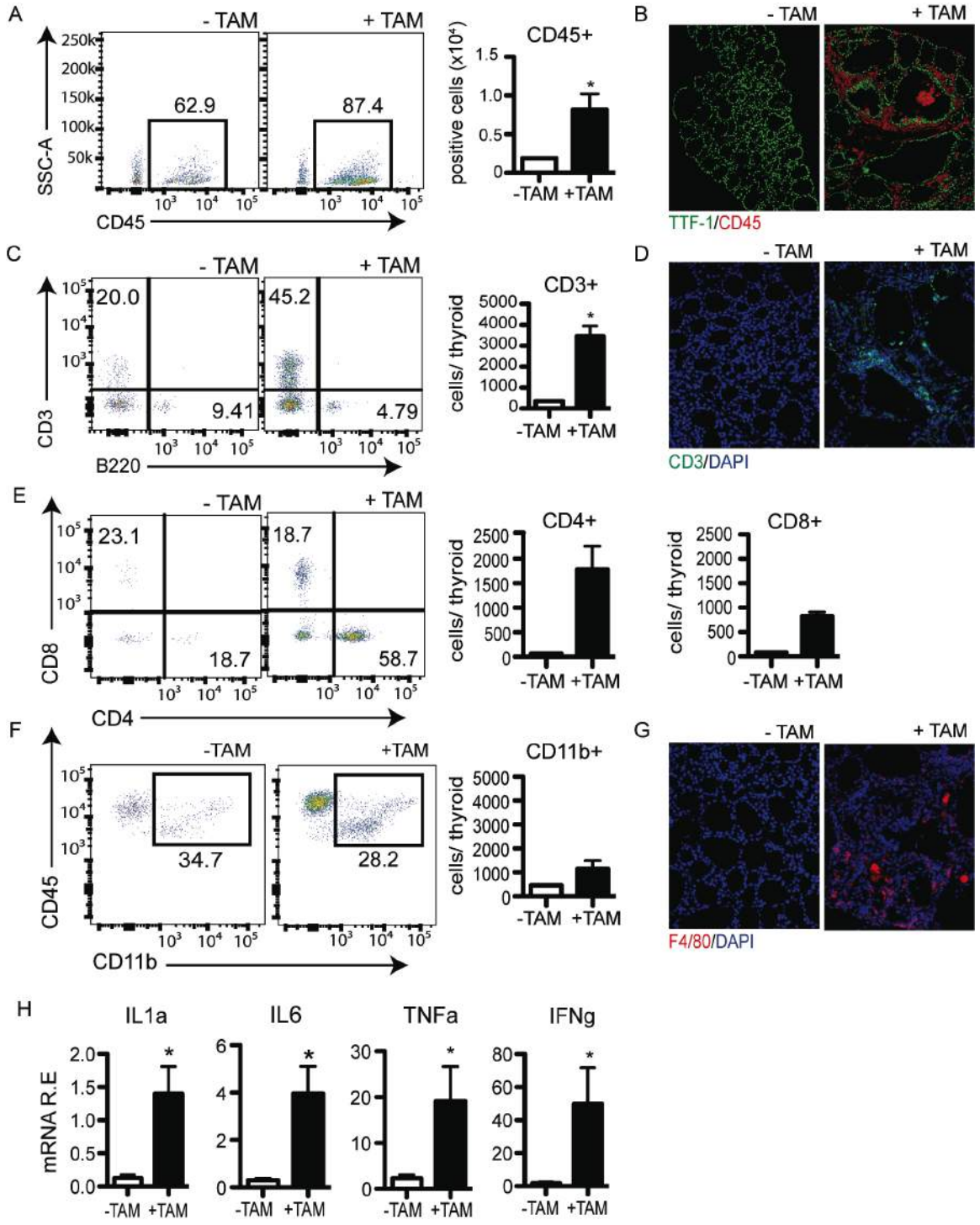
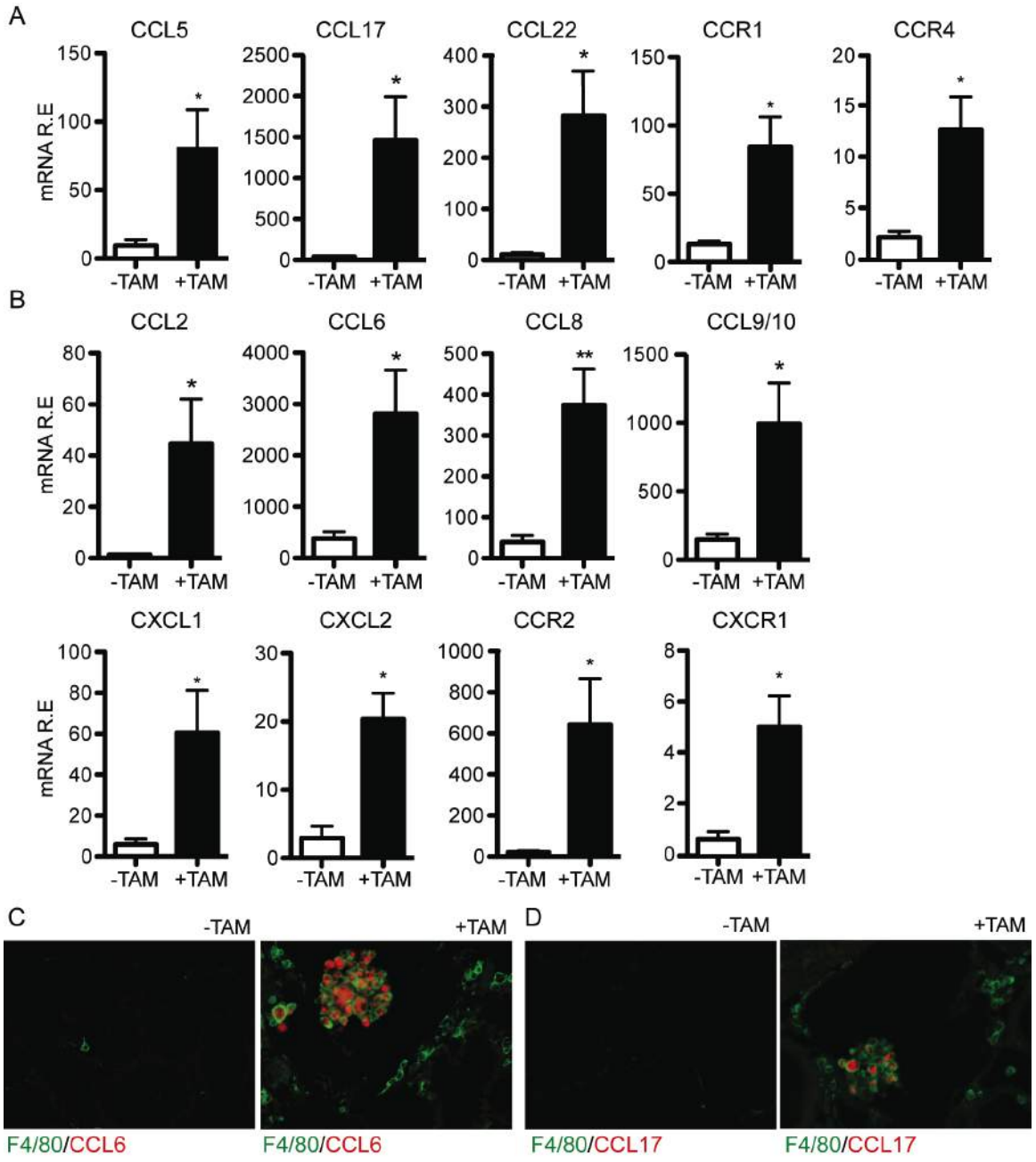

A
- TAM
+ TAM
SSC-A
CD45
62.9
87.4
CD45+
positive cells (x10⁴)
-TAM +TAM
B
- TAM
+ TAM
TTF-1/CD45
C
CD3
B220
20.0
45.2
9.41
4.79
CD3+
cells/ thyroid
D
CD3/DAPI
E
CD8
CD4
23.1
18.7
18.7
58.7
CD4+
CD8+
F
-TAM
+TAM
CD45
CD11b
34.7
28.2
CD11b+
G
F4/80/DAPI
H
IL1a
IL6
TNFa
IFNg
mRNA R.E

Figure 2.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CCL22
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CCR4
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+TAM
B
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CCL6
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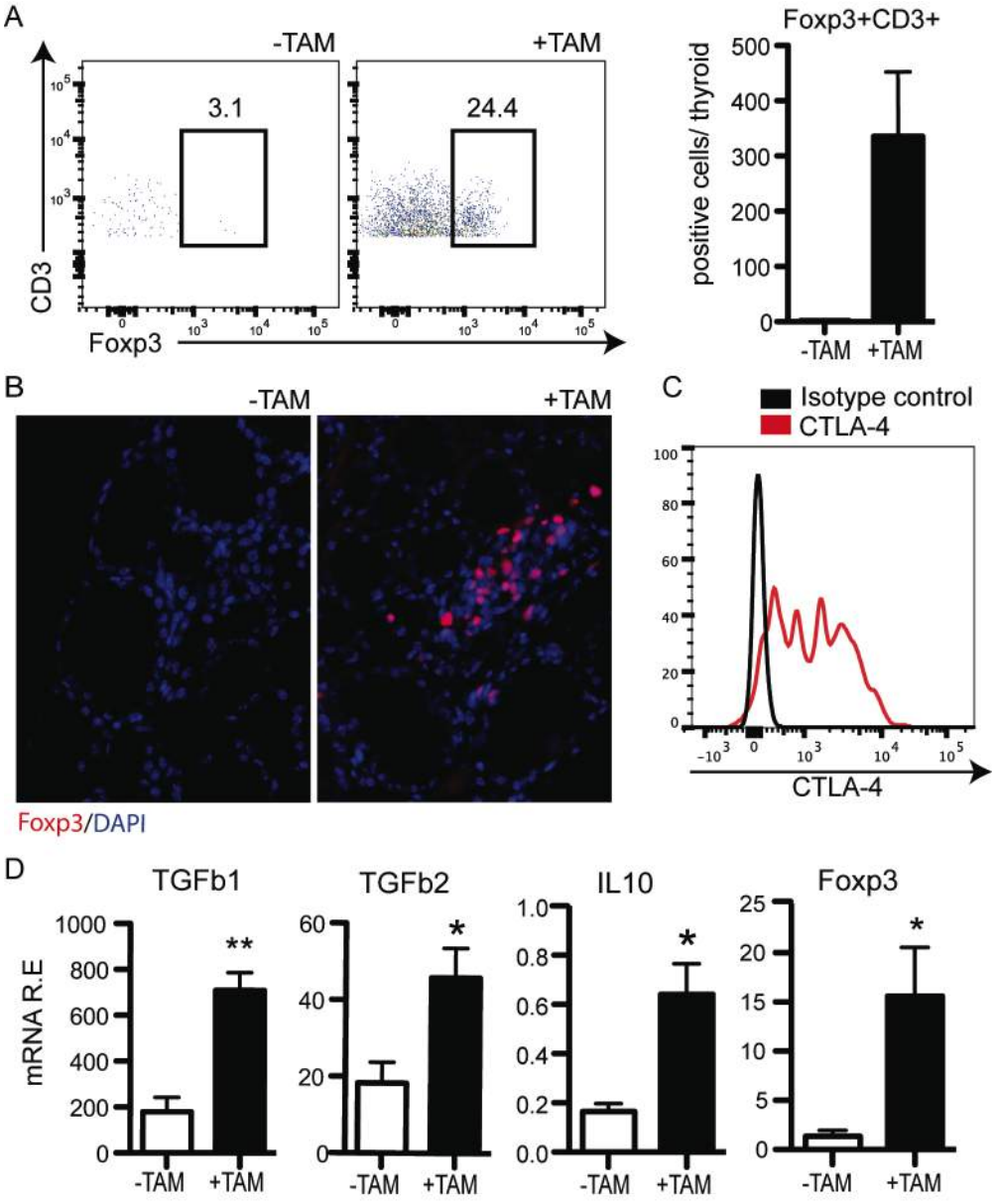
CCL9/10
CXCL1
CXCL2
CCR2
CXCR1
C
D
F4/80/CCL6
F4/80/CCL6
F4/80/CCL17
F4/80/CCL17

Figure 3.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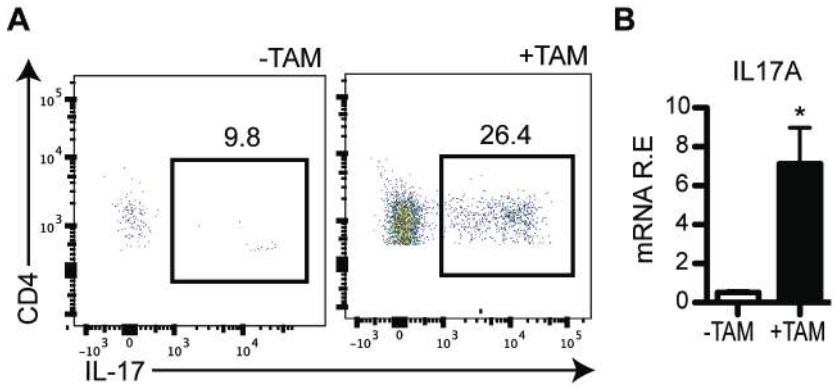
+TAM
3.1
24.4
CD3
Foxp3
Foxp3+CD3+
positive cells/ thyroid
500
400
300
200
100
0
-TAM +TAM
B
-TAM
+TAM
Foxp3/DAPI
C
Isotype control
CTLA-4
CTLA-4
D
TGFb1
TGFb2
IL10
Foxp3
mRNA R.E
1000
800
600
400
200
0
60
40
20
1.0
0.8
0.6
0.4
0.2
0.0
25
20
15
10
5
**
*
-TAM +TAM

Figure 4.



Figue 5.

5. ARTIGOS PRODUZIDOS

5.2.

Titulo: **Inducible expression of BRAFV600E in CX3CR1+ cells triggers myeloid expansion and development of a Langerhans cell histiocytosis-like disease**

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Inducible expression of BRAFV600E in CX3CR1+ cells triggers myeloid expansion and development of a Langerhans cell histiocytosis-like disease

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Abstract

Langerhans cell histiocytosis (LCH) is a rare disease characterized by an accumulation of histiocytes and Langerhans cells. LCH can involve single or multiple organs, and has a worse prognosis when it affects risk-organs such as, liver, spleen and lung. The origin of LCH is unclear. Studies have shown that LCH is promoted by an uncontrolled clonal proliferation of dendritic cells with LC characteristics, suggesting that these cells share a common myeloid progenitor. An activating mutation of the BRAF gene (V600E) has been detected in many LCH biopsy samples, but it is unclear if it is sufficient to induce disease. To test the hypothesis that BRAFV600E is sufficient to cause LCH, we generated *FRBRAF* mice. These mice express BRAFV600E in the mononuclear phagocyte system (CX3CR1+ cells) upon administration of tamoxifen (TAM). After 3 months of TAM treatment, mice appeared hunched, had an enlarged abdomen and were neurologically affected (hind limb weakness and spasticity). Upon necropsy, the mice had marked hepatosplenomegaly. Flow cytometric analysis of the blood showed an expansion of the myeloid compartment. Histological analyses revealed the presence of marked inflammatory infiltrates in the liver, lung, and spleen and occasional mild inflammatory infiltrates in the spinal cord and the brain. In some instances, granuloma-like aggregates, rich in giant cells, myeloid cells, eosinophils and lymphocytes were detected in liver and the lungs. Remarkably, CD11b+MHCII+ Langerin+ cells, typically found within LCH lesions in humans, were also present in these infiltrates. Analysis of the liver transcriptome of the FRBRAF mice showed an increase in the expression of several cytokines, chemokines, and chemokine receptors known to be expressed within LCH lesions (TNFα, IL-1α, M-CSF, IL-7, CCL17 and CCR6). We show that expression of BRAFV600E in CX3CR1+ cells in mice promotes expansion of the myeloid compartment and development of a disease that resembles human LCH. These results directly implicate BRAFV600E in the pathogenesis of LCH.

Introduction

Langerhans cell histiocytosis (LCH) is a rare disease caused by uncontrolled clonal proliferation in which accumulation of cells with langerhans cells characteristics. LCH affects mainly young children and features accumulation of CD1a+ Birbeck granules+ cells within the skin, the bones (Geissman et al 2001), and occasionally lymphoid organs, lungs (Suri H et al 2012), kidney (Segerer et al 2008), liver (Hatemi et al 2010), CNS (Grois N et al 2005; Baumann M et al 2012). Cases in adults are less frequent and reported resulting in remaining several questions about (Arico M 2004, Cantu M et al 2012).

The origin for LCH is still unclear. However, studies have been showed that LCH is caused by an uncontrolled clonal proliferation of dendritic cells with LC characteristics (Girolomoni et al 2002). Because of this, it is most likely that the cells that give origin to the LCH cells comes from the same myeloid compartment that give origin to the LC (Guillaume Hoeffel et al 2012).

LCH can involve single or multiple organ/ tissue systems. Single system LCH can be restricted to one organ affected (bone unifocal or multifocal), skin, not draining lymph node or lungs. When cranial facial lesions happen, central nervous system can be affected. However CNS involvement in LCH is a rare disorder (Grois et al 1998). In contrast, multi system LCH is usually involving risk-organs as hematopoietic, liver, spleen and lung. LCH has been proposed to be a clonal disorder more than simple reactive disease. The idea of neoplastic process is supported by incidence of genetic mutations, as such BRAFV600E. The presence of BRAF V600E in 57% of archived LCH bone lesions from two different institutions suggest BRAF role in LCH (Badalian-Very et al 2010). The potential consequences, to be tested, of BRAFV600E in LCH is the dysregulation signaling through MEK-ERK pathways and thereby increase survival, enhance proliferation and impaired differentiation (Nichols et al 2010).

An activating mutation of the BRAF gene (V600E) has been detected in many LCH biopsy samples, but it is unclear if it is sufficient to induce disease. To test the hypothesis that BRAFV600E is sufficient to cause LCH, we generated *a* mice that express BRAFV600E in the mononuclear phagocyte system (CX3CR1+ cells) upon administration of tamoxifen. In this study, we demonstrate that BRAF-V600E expression in CX3CR1 cells is sufficient to induce an LCH-like phenotype in mice with multi system involvement.

Results

Activation of BRAF^{V600E} driven through the CX3CR1 locus in adult mice result in multi-organ disease

In the an attempt to target activated BRAF on myeloid cells utilizing the CX3CR1 locus, we created the mice expressing BRAFV600E under the CRE^{ERT2} tamoxifen inducible system located in the CX3CR1 locus. These mice express BRAFV600E in the mononuclear phagocyte system (CX3CR1+ cells) upon administration of tamoxifen (TAM) (**Fig 1a**). After 15 weeks of the first TAM treatment, mice appeared hunched, had an enlarged abdomen and were neurologically affected (hind limb weakness and spasticity). Upon necropsy, the mice had marked hepatosplenomegaly **(Fig 1b-c).** PCR analysis of liver and lung of tamoxifen treated mice displayed floxed BRAF. The recombinant allele (334pb) was detected in tamoxifen-administered mice, and was absent in tissues of untreated mice that displayed the targeted and wild type allele, 307pb and 187pb, respectively **(Fig 1d)**.

FRBRAF mice display an LCH-like disease with an influx of inflammatory cells into the liver and the lung

Adult untreated *FRBRAF* mice greater than three months of age did not display any significant abnormalities. To further explore the enlargement of livers observed in the *FRBRAF* mice treated with tamoxifen, histological analysis was done after four months of the initial tamoxifen treatment. Several foci of inflammatory infiltrate cells were observed dispersed throughout the liver, especially around the lobule areas showing granuloma-like structure (**Fig 2a-b**). Presence of giants cells were frequentlly observed in the FRBRAF spleen (**Fig 2c**).

BRAFV600E mutation, in adenovirus-infected system, has been shown to result in benign lung tumors that sometimes progressed to adenocarcinoma (Dankort et al 2007). However, increased inflammation in the lung driven by BRAFV600E has not been reported. Additionally, pulmonary Langerhans cell histiocytosis (PLCH) is a relatively common find in adults, especially in cigarette smokers (Suri et al 2012). *FRBRAF* mice display an accumulation of inflammatory cells resulting in inflammatory cellular lesions distributed mainly in the bronchiolar region (**Fig 2d**).

Upon a deeper analysis of areas where an inflammatory infiltrate is present, multinucleated giant cells are also detected (**Fig 2e**). Additionally it is also observed that the inflammatory infiltrate in the lung is occasionally adhered to walls of blood vessels. As described in humans with PLCH, *FRBRAF* mice display diffused infiltration of Langerhans cells with vesicular nuclear chromatin in the lungs (**Fig 2f**).

CX3CR1-BRAFV600E mice show myeloid expansion

The differentiation of myeloid progenitors to mature, terminally differentiated cells is a highly regulated process. Conditional BRAF mutation in myeloid progenitors CX3CR1+ induce myeloid expansion in the FRBRAF blood and liver. FRBRAF mice treated with tamoxifen showed 1.6 x more CD11b+ cells in the blood compared with littermates control (**Fig 3a**). Similar expansion of CD11b+ cells was observed in the FRBRAF liver associated to expansion of cells presenting myeloid/ monocytic markers such (**Fig 3b**). Special increase was observed in the macrophages/ monocytes in the FRBRAF liver compared to dendrit cells and granulocytes population (**Fig 3c**).

Because multiple APCs, including Langerhans cells and macrophages, are involved in the lesion formation, we hypothesize that LCH is a disease of myeloid blood precursors. To test this hypothese in the LCH mouse model, we performed flow cytometry analysis in the bone marrow of these animals. Changes in myeloid progenitors in the bone marrow of untreated and tamoxifen treated mice were assessed using flow cytometry (CMP (common myeloid progenitor): Lin^- c-Kit^+ Sca-1^- FcgRII $^-$ $CD34^+$, GMP (granulocytic/monocytic progenitor),: Lin^- c-Kit^+ Sca-1^- FcgRII $^+$ $CD34^+$; MEP (megakaryocytic/erythroid progenitor): Lin^- c-Kit^+ Sca-1^- FcgRII $^-$ $CD34^-$). Expansion of GMP population (control 27 and Tamoxifen-treated mice 64 followed by reduction of MEP (control 38 and Tamoxifen-treated mice 11) was observed in the bone marrow of BRAF activated mice (**Fig 3d**).

$BRAF^{V600E}$ expression in CX3CR1 cells result in increased expression of inflammatory molecules in the liver associated with LCH halmarks

The increased presence of myeloid and lymphocytic cells in the livers showing an LCH-like disease in the tamoxifen treated *FRBRAF* mice advocates the potential role of cytokines in the pathogenesis of the disease. To explore this possibility, we

compared the differences in expression of inflammatory molecules in the livers between the treated and untreated *FRBRAF* mice. To better characterize the inflammatory changes observed in these mice, FRBRAF liver was processed to perform a mouse microarray assay (Illumina®). Analysis of the liver transcriptome of the FRBRAF mice showed the presence of chemokines, cytokines, cell adhesion molecules, Matrix metalloproteinases and a hallmark of the disease, CD207 or langerin, top-ranked differentially regulated genes (**Fig 4**).

Validation by qPCR confirmed the expression of several cytokines, chemokines, and chemokine receptors known to be expressed within LCH lesions (TNFα, IL-1α, M-CSF, IL-7, CCL17 and CCR6) (**Fig 4**).

An increase in the mRNA expression of Tumor necrosis factor alpha (TNFa) was observed in the treated *FRBRAF* mice (**Fig 4**). However others pro-inflammatory cytokines such as IL1α and IL-6 were not upregulated (data not shown). Additionally, monocytic chemotactic protein (CCL2) was upregulated in the livers and lungs of the treated mice (**Fig 4**). Interestingly, TNFα expression has been reported in LCH bones lesions in human (Egeler et al 1999; Graaf et al 1996), and TNFα is known to be able to stimulate the migration of normal langerhans cells in vitro (Wang et al 1999), promote local accumulation of leukocytes and induce injury in tissues. Our results suggest that the presence of TNFα associated with increased recruitment of myeloid cells in the livers and lungs of mice with an LCH-like disease mirror human findings and supports the possibility of this cytokine as a target to therapy.

Increased expression of CCR6 was also observed in the tissues of the *FRBRAF* treated mice (**Fig 4**). CCR6 is reported to be found in LCH cells and epidermal LCs but not in activated dendritic cells (Laman et al 2003). Furthermore, resting LCs express CCR6, but when mature LCs are recruited for another environment they lose the chemokine receptor expression (Flemming et al 2003). It has been shown that LCH cells do not express CD83 and CD-Lamp (markers of mature dendritic cells), but that the majority of cells express CD14 (marker of immature dendritic cells) (Geissmann et al 2001). Based on the aforementioned, our data suggests that the LCH cells accumulated in these tissues are most likely that of an immature state.

TGFβ, CXCL14 and CX3CL1 were up-regulated in the livers of treated *FRBRAF* mice (**Fig 4**). The presence of TGFβ in LCH lesions is an important cytokine that play

a role in lung fibrosis (Fukuda et al, 1990). CXCL14 and CX3CL1 are important chemotactic for monocytes and monocytes/ lymphocytes, respectively.

Hematopoietic growth factors have been related with the disease (Rolland et al, 2005; Soler et al, 2003) and were investigated. GM-CSF and G-CSF showed difference, however M-CSF was not different by RT-PCR in the CX3CR1 BRAF liver (data not show). Also, no difference in the transcripts for CCR2, CCR7 and CX3CR1 was detected by RT-PCR in the CX3CR1 BRAF liver with LCH like phenotype (data not showed). To correlate the mouse model with LCH human finds, we checked the expression of inflammatory chemokine reported to be express by CD1a+ cells, such as CCL5, CXCL11 and CCL20, that are responsible for own recruitment and the last one for the lymphocyte recruitment. CCL5 and CXCL11 upregulation were observed in the mouse tissue, however CCL20 did not change (data not show). A combination of inflammatory molecules found to be expressed differencially expressed between untreated and tamoxifen treated mice were presented with LCH clinical human report of this molecule (**Table 1**).

Taken together, our data indicate that, LCH like disease lesions in CX3CR1 BRAF treated mice are characterized by a complex cytokine, chemokine and growth factors network, which may coordinate LCH cells initiation and accumulation through the tissues and play role in the pathogenesis of the disease.

Characterization of the langerin+ cells in the liver

The presence of an LCH-like phenotype in the *FRBRAF* mice was done through examining the presence of a known hallmark of the disease, langerin or CD207. Langerin mRNA expression was ten times increased in the livers of mice treated with tamoxifen when compared to untreated littermates (**Fig 4**). An increase of langerin+ cells was further corroborated through immunofluorescent staining, where several clusters of langerin+ cells were visualized in the livers of treated *FRBRAF* mice (**Fig 5A**) and not detected in the untreated littermates.

To better characterize the langerin+ cells in the FRBRAF liver, double immunofluorescent staining was utilized to co-localize the expression of langerin with myeloid markers. Co-expresssion of langerin positive cells with F4/80 (**Fig 5b**) or CD11b (**Fig 5c**) or CD11c (**Fig 5d**) or MHCII (**Fig 5e**) positive cells were observed. The majority of Langerin+ clustered cells in the liver co-localized with F4/80 and CD11b+. Little co-localization was observed with CD11c and MHCII markers. In

summation, our results suggest that BRAFV600E in CX3CR1+ cells in adult mice result in an LCH-like disease, predominately affecting the liver, where a influx of myeloid cells with LCH-like phenotype and hallmarks of LCH such as langerin+ cells are present.

Discussion

Langerhans cell histiocytosis is a rare disease with unknown etiology. Formerly, it was recognized as different pathologies such as histiocytosis X, eosinophilic granuloma, Abt-Letterer-Siwe disease and Hand-Schüller-Christian disease because of its variable clinicopathological spectrum (Baladian-Very et al, 2013). The feature that defines LCH is the abnormal infiltration of immune cells and presence of specific LC markers within lesions located mainly in the bones, skin, liver, spleen and lung (Weitzman et al., 2008; Satter et al., 2008).

In this study, we demonstrate that driving the expression of the proto-oncogene BRAF-V600E in the control locus of the CX3CR1 receptor in adult mice is sufficient to promote an LCH-like disease. Outstandingly, our *in vivo* model resembles multisystem LCH hallmarks with: (1) affected organs (lacking phenotype on bones and skin where the transgene is down modulated), (2) langerin-positive cells expansion and accumulation within the lesions (lacking presence of Birbeck granules) and (3) marked up existence of inflammatory cytokines and chemokines profile comparable to the human records.

Clinical reports from adult human patients describe cases of multisystem disease with splenomegaly and hepatomegaly as life threatening diagnostics (Weitzman et al., 2008). Affected human livers contain aggregates of langerin+ cells that can be clustered with macrophages, eosinophils and T cells. In our model, the most striking altered organ was the liver where langerin+ cells could be found grouped around portal veins and sinuses together with inflammatory cells and cytokines.

CX3CR1 is known to be expressed on the mononuclear phagocyte system (macrophages, monocytes, DCs, NK and microglia cells) as well as in the bone marrow myeloid precursors (Combadiere et al., 1998; Yona et al., 2013). Recent findings have shown that tissue-resident macrophages, such as peritoneal, splenic and alveolar macrophages, as well as Kupffer cells and Langerhans cells, down-regulate CX3CR1 expression after they mature *in situ*, yet these populations arise

from CX3CR1 positive cells within the myeloid precursors of the bone marrow, fetal liver or yolk sac (Merad et al., 2002; Hoeffel et al, 2012). Hence, in our model, we used this receptor to drive BRAF mutation expression in myeloid progenitor compartiment via CRE-ER system.

LCs are originated embryonically during the development of the stratum corneum, where monocyte lineage progenitors populate the epidermis (Tripp CH et al., 2004; Chorro L et al., 2009). These cells are langerin and MHC class II negatives up to one week after birth and its precursor features the expression of monocyte markers such as CD11b, F4/80 and CX3CR1. In our model, the expression of BRAF induced by tamoxifen can only be achieved on cells that harbor the expression of the CX3CR1 and we promote this mutation after LC settlement in the skin and its down-regulation of the oncogene-promoter gene. This may explain why no LCH-like alterations on the bone and skin were found on *FRBRAF* mice and speculate why LCH is predominantly found on newborns up to young adolescents (Arico et al., 2004).

We used the YFP reporter signal to track BRAF floxed mutation. *FRBRAF* mice treated with tamoxifen expanded YFP+/CD11b+ population in the blood and liver as seen by FACs. This findings correlate with data from the lesions appreciated by immunofluorescence and indicate that the mononuclear phagocyte system is the major cell population targeted in our model.

Whether the disease comes from inflammatory reaction or is triggered by a neoplastic process is matter of debate. Studies showed that all types of LCH are monoclonal, except the adult pulmonary LCH (William CL et al, 1994, Yu RU et al, 1994). And the involvement of neoplastic mutation in LCH lesions supports the notion of a malignant transformation as the main cause of this cellular expansion (Baladian-Very et al, 2010). Because pathological LCH cells share specific marks with Langerhan cells, LCH was initially thought to arise from aberrant epidermal LC (Yousem SA et al., 2001; da Costa CE et al., 2009). After an inflammatory stimulus, LCs are capable to leave the epidermis and migrate to the draining lymph nodes, so this cell could expand and go to other organs guided by chemokine gradients (Romani et al., 2010). However, genetic analyses comparing langerin positive cells from normal skin and from LCH lesion revealed that these cells do not form a unique expression pattern and LCH cells had increased expression of early myeloid precursor markers (Allen et al, 2010). Together with this concept, analysis of LCH cells showed expression of immature markers (Geissmann et al., 2001). And it have

been demonstrated that blood from LCH patients had elevated levels of myeloid DC precursors as well as hemopoietic cytokines such as FLT3-L and M-CSF (Rolland et al., 2005). These data indicate that a common progenitor of DCs, LC and/or macrophages could be the source of LCH.

Material Methods

Mice.

FRCRE-YFP and *BRAF* mice have been described (Parkhurst et al, 2013; Dankort et al, 2009; 2007). All mice were housed under specific-pathogen-free conditions in ventilated cages. All experiments involving mice were performed in accordance with the guidelines of the Animal Care and Use Committee of Mount Sinai School of Medicine.

Histology and immunohistochemistry.

For immunofluorescent staining on frozen sections, organs were dissected, and subsequently frozen in O.C.T compound (Sakura) and snap frozen in 2-methylbutane (Merck) chilled in dry. 8 µm sections were sectioned, and fixed in -20°C acetone for 15 minutes. Sections were incubated with purified anti- langerin (Santa Cruz), anti-CD11c (BD biosciences), or anti-CD11b (BD Biosciences) for 1 hour at room temperature and then incubated with Alexa Fluor 488 or Alexa Fluor 594 labeled antibodies for 30 min

For immunofluorescent staining on paraffin embedded embedded sections, organs were dissected, fixed in 10% formalin and processed for paraffin sectioning. Four-micron sections were de-waxed by immersion in xylene and hydrated by serial immersion in ethanol and PBS. Antigen retrieval was performed by incubating sections in a pressure cooker for 15 min in Target Retrieval Solution (DAKO). Sections were washed with PBS (2 x 10 min) and blocking buffer (TBS containing 10% BSA and 0.3% Triton X-100) was added for 1 hour. Sections were incubated with primary antibody in blocking-buffer over-night at 4°C and then incubated with Alexa Fluor 488 or 594-labeled secondary antibodies for 30 min.

Sections were mounted with Fluoromount-G (Beckman Coulter). Antibodies were obtained from Santa Cruz Biotech (langerin), Abcam (Pan-Keratin), BD Biosciences (CD11c, CD11b), and e-Bioscience (CD45, F4/80).

Fluorescent imaging

Immunofluorescent imaging was performed using Cy3 HYQ, FITC HYQ and a fluorescence microscope (E600; Nikon) with Plan Apo objective lenses. Images were acquired using a digital camera (DXM1200F; Nikon) and Nikon Act-1 software version 2.63. Images were composed in Adobe Photoshop CS3.

RNA extraction and Quantitative PCR

Total RNA was extracted using the RNeasy mini Kit (Qiagen) according to the manufacturers protocol. Reverse transcription was performed using 3 μg of total RNA. Quantitative PCR was conducted in triplicates using 10 - 25 ng of reverse-transcribed cDNA and 0.4 μM of each primer in a 10 μL final reaction volume containing 1x SYBR Green PCR Master Mix (Applied biosystems). PCR cycling conditions were: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec, 60°C for 1 min. Relative expression levels were calculated as $2^{(Ct\ Ubiquitin\ -\ Ct\ gene)}$. Primers were designed using primer express 2.0 software (Applied biosystems).

Microarray Analysis

Total RNA from three *FRBRAF* transgenic mice was isolated with the RNeasy kit (QIAGEN) after 90d of the injection of Tamoxifen (n = 3/ group). After cDNA synthesis and overnight biotin-labeled cRNA amplification (MessageAmp II aRNA Amplification; Ambion), 20 μg of biotin-labeled cRNA was hybridized to Affymetrix Mouse Genome 430 2.0 arrays and processed according to Affymetrix procedures. Fold changes and statistical significance were determined using contrasts in Linear Models for MicroArrays (limma v3.14.4, Bioconductor/R)[50]. Quantile-normalized expression values were corrected for multiple testing (Benjamini-Hochberg), filtered for FDR < 0.01 and fold-change > 3. The expression values were plotted with heatmap.2 (gplots v2.11.0, CRAN/R). For enrichment analysis of biological process ontology and KEGG pathway, probe lists were analyzed in DAVID[18]. GO and KEGG terms were selected based on $p < 0.05$ and sorted for the number of involved genes.

Cell isolation and flow cytometry

The liver was microdissected following a perfusion through the portal vein with PBS from FRBRAF animals at indicated ages and incubated in 0.1mg/ml collagenase (sigma-aldrich) to obtain single-cell suspensions. Bone marrow cells were flushed thoroughly from mouse femurs with PBS. Peripheral blood leukocytes were obtained through retro-orbital bleeding by heparinized capillary tubes. Red blood cells from the bone marrow and peripheral blood were removed by RBC lysis buffer (eBioscience). Cells were stained with selected antibodies and analyzed in a FACSCanto cytometer (Becton Dickinson). Data were analyzed using the FlowJo software (Tree Star, Inc). Cells were stained with selected antibodies and analyzed in a FACSCanto cytometer (Becton Dickinson). Directly conjugated antibodies against CD45, CD11b, CD11c, MHCII, Ly6G, Gr-1, Lin, c-KIT, Sca-1, FcrRII and CD34 were obtained from BD Biosciences.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 4.0b for Macintosh (GraphPad Software). Differences among means were evaluated by either a two-tailed T-test or ANOVA followed by Dunnett's post-test. All results shown represent mean ± s.e.m. For all analyses, the null hypothesis was rejected at the 0.05 level.

Acknowledgements

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Authorship contributions

MFB, MEP, GCF, JJ, and LC performed the experiments. CP, JJL and MM contributed reagents and materials. SAL designed the study and wrote the manuscript.

Conflict of Interest Disclosures

The authors declare no financial interests

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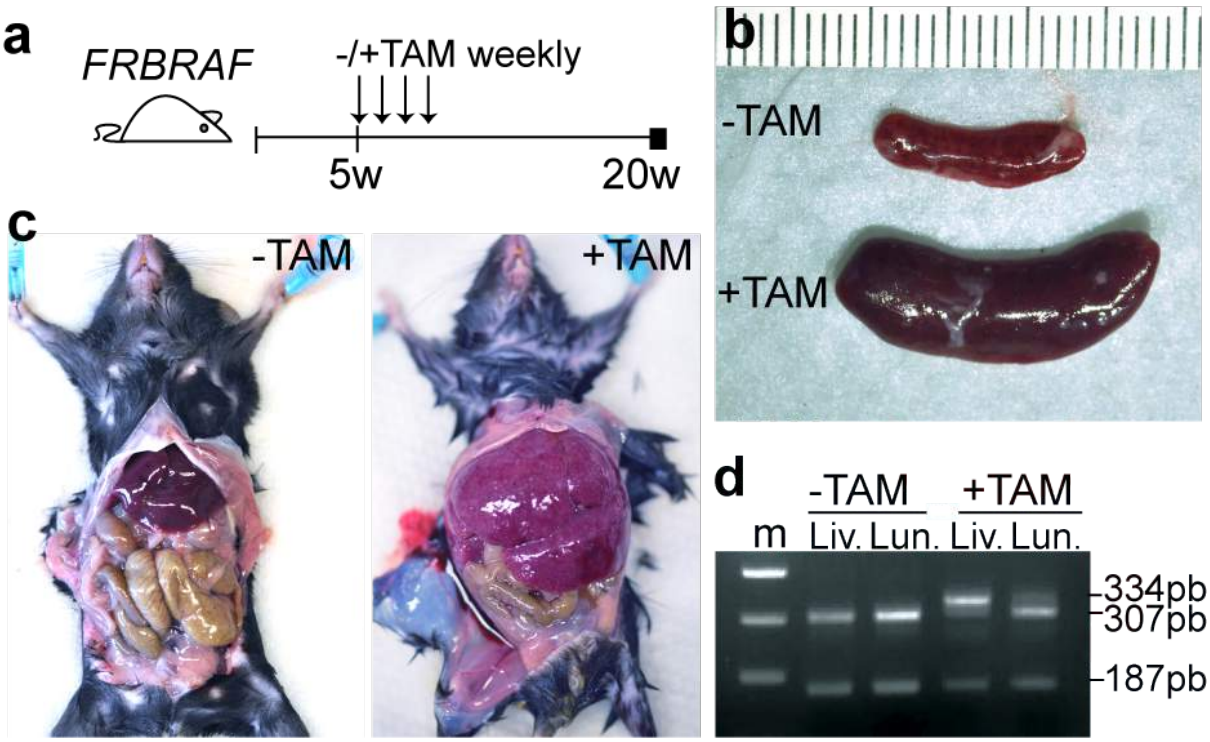


Figure 1. Mouse model of inducible activation of BRAF^{V600E} in CX3CR1 cells leads to hepatosplenomegaly (a) Schematic of FRBRAF mice treatment. **(b)** Activation of BRAF result in **(b, c)** Hepatosplenomegaly in FRBRAF mice and **(d)** Floxing of BRAF in liver and lung of treated mice.

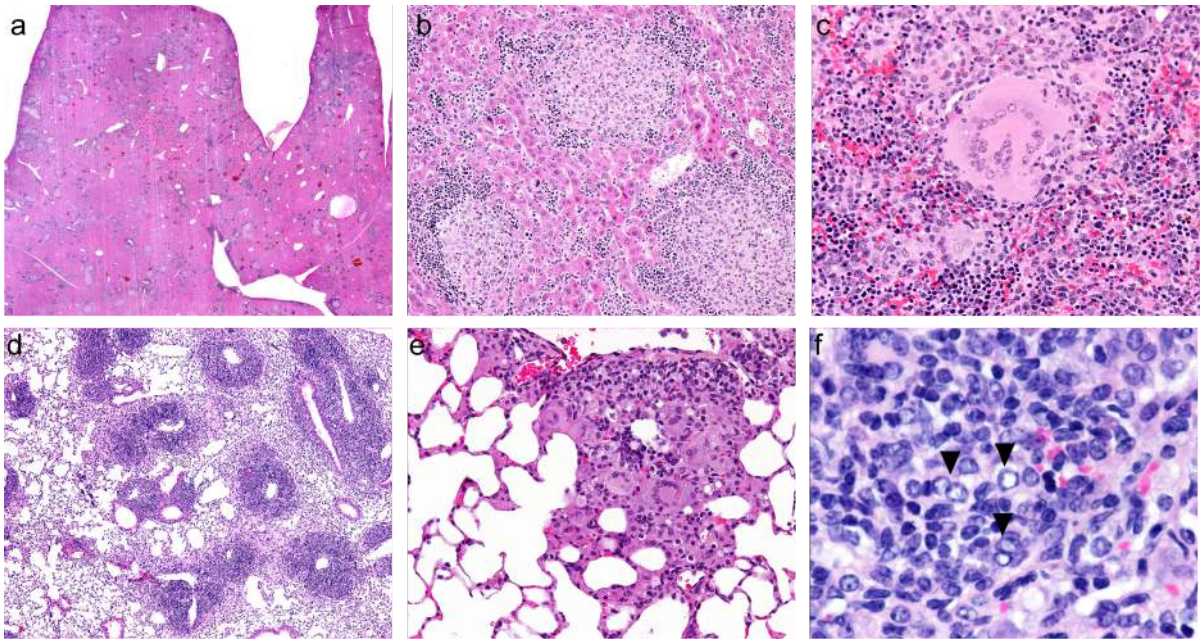


Figure 2. Histiocytic infiltrate into the liver, spleen and lung. Liver tissue (**a-b**) sections from FRBRAF mice after tamoxifen stained with hematoxylin and eosin show **(a)** several inflammatory infiltrates **(b)** higher magnification of granuloma-like structure **(c)** Multinucleated giant cells in inflammatory aggregates in the spleen (**d)** Low-power of the lung tissue shows a densely cellular infiltrate of histiocytes with presence of (**e**) giant cells and (**f**) vesicular nuclear chromatin.

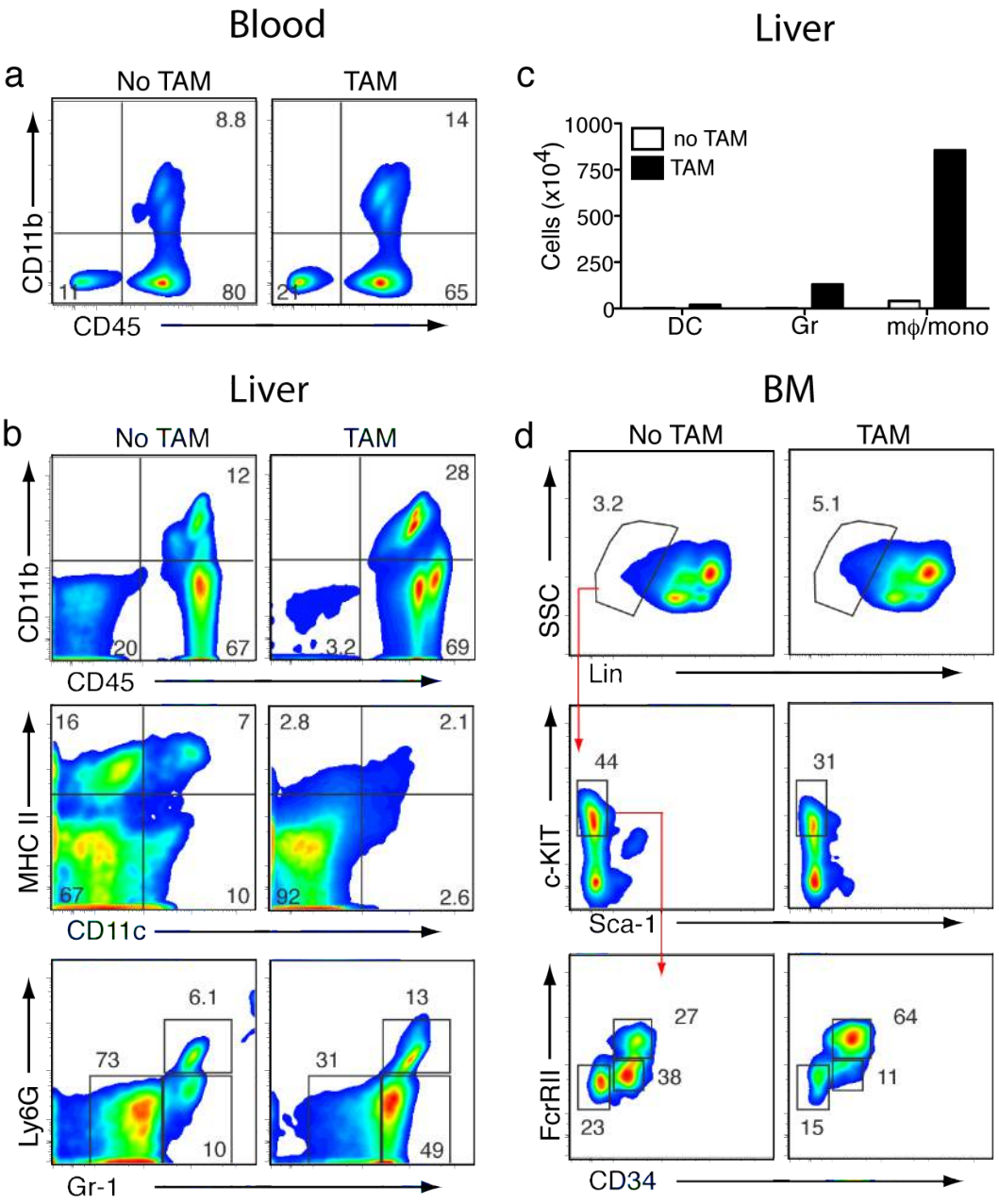


Figure 3. BRAF^{V600E} in CX3Cr1 expressing cells induces myeloid and GMP progenitors expansion. FACS analysis of blood (**a**) and single-cell suspension of the liver (**b-c**) of FRBRAF mice indicated expansion of cells presenting myeloid/ monocytic markers. (**d**) Bone marrow cells were stained for GMP (granulocytic/monocytic progenitor), CMP (common myeloid progenitor) and MEP (megakaryocytic/erythroid progenitor). Note the increase of the GMP population in FRBRAF TAM (n=3).

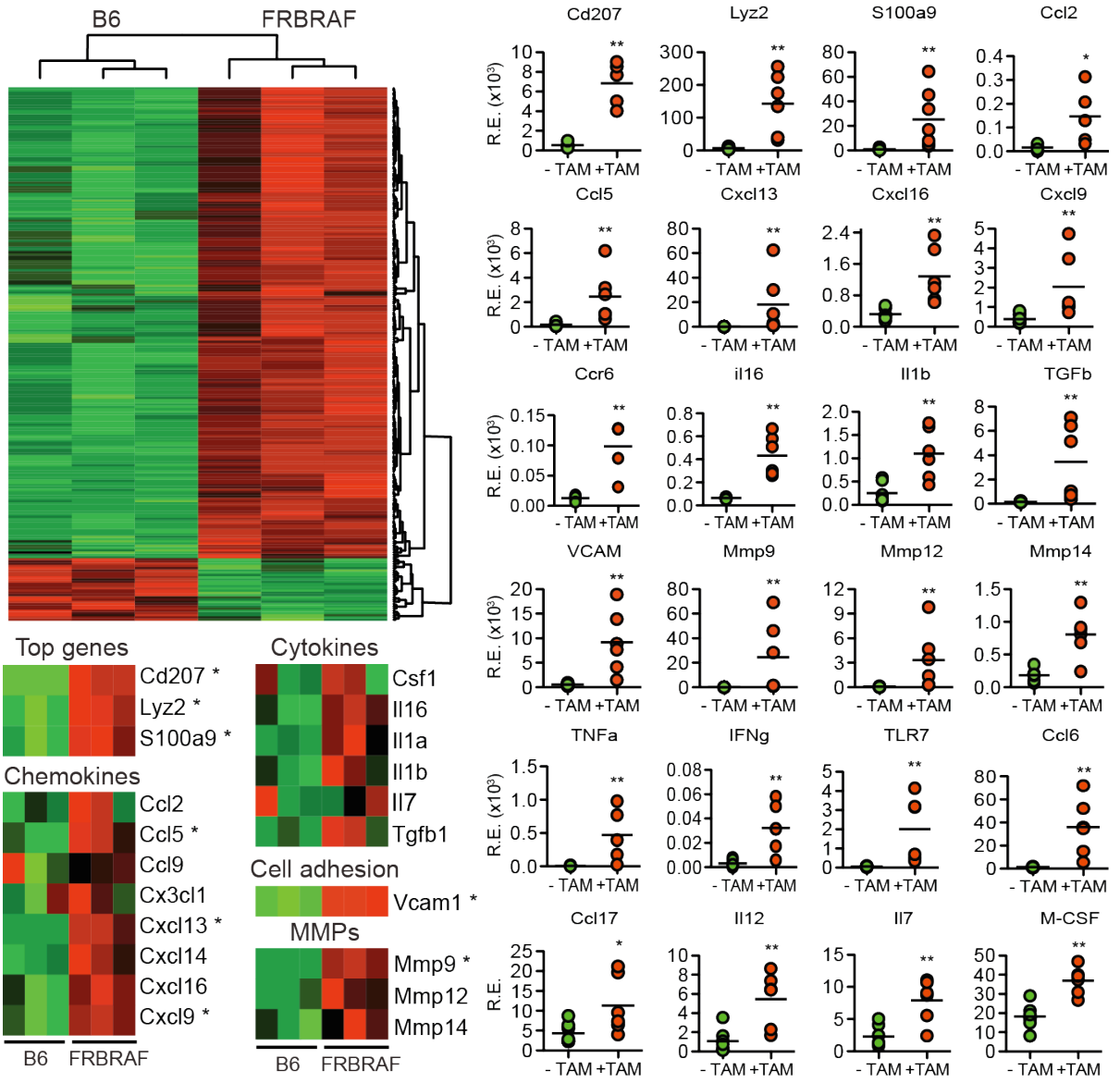


Figure 4. Gene expression in FRBRAF liver reveals alteration in molecules relevant for immune cell chemotaxis, remodeling and alteration of key genes expressed by Langerhans cells (**a, b**) Microarray analysis of liver genes expression (mean) from wild-type mice (B6) and treated FRBRAF (n=3) (one mouse per 'lane'). Red indicates genes with intensity greater than the mean intensity of the genes presented here; green indicates genes with intensity lower than that mean intensity. (*) Indicated statistic significance. (**c**) Validation of expression of genes involved in Langerhans cell pathway, chemokines, cytokines, cell adhesion and MMPs. ***P < 0.0001, **P = 0.001 and *P > 0.01

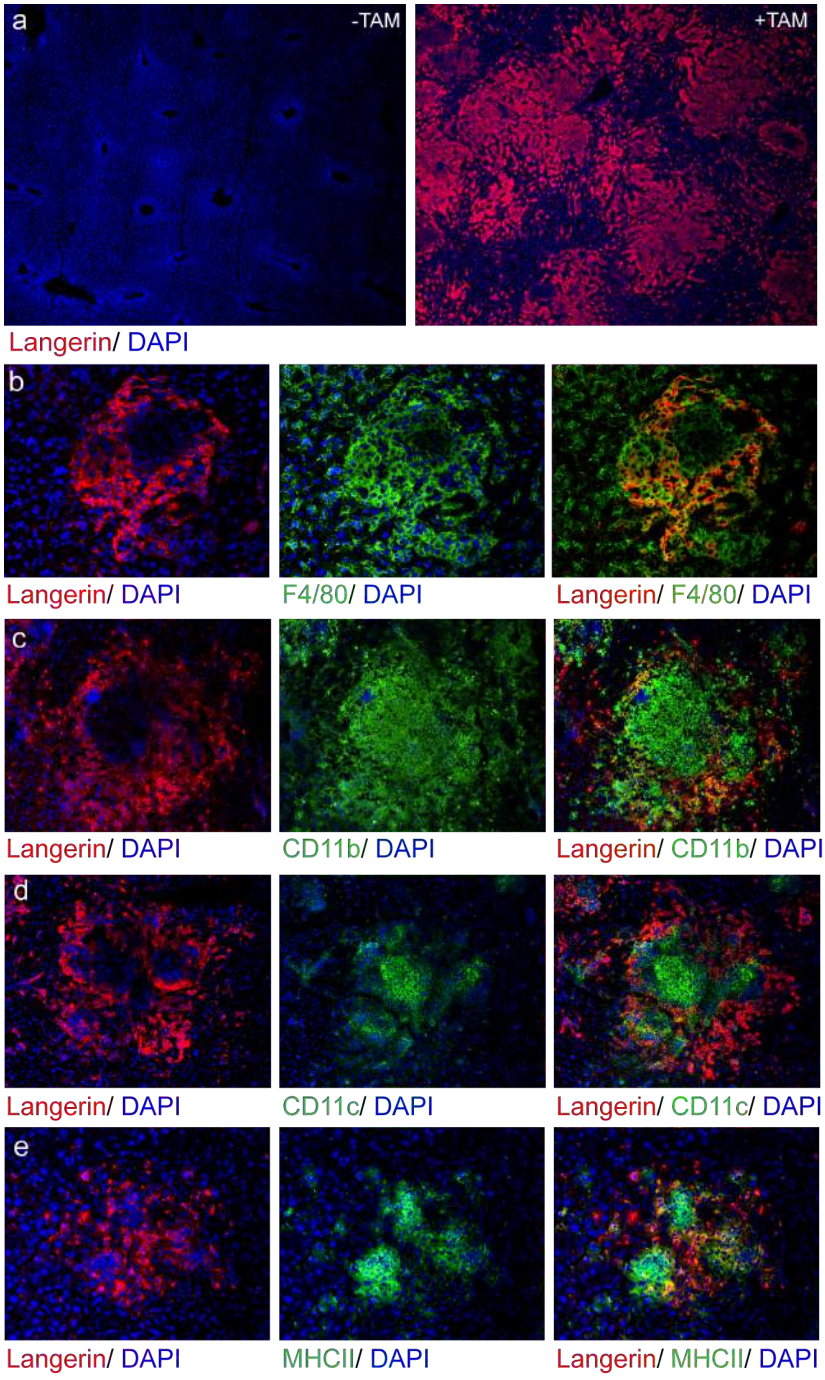


Figure 5. Immunohistochemical analysis of langerin positive cells in the liver **(a, b)** presence of clusters of langerin + cells in the liver of FRBRAF mice on TAM. Langerin + cells clusters in the liver, for the most part, co localize with **(b)** F4/80 positive cells and **(c)** CD11b positive cells and few with **(d)** CD11c positive cells and (**e**) MHCII positive cells.

Table 1. Inflammatory molecules and genes associated with Langerhans cell histiocytosis upregulated in the FRBRAF liver. P values were calculated comparing FRBRAF mice vs. wild-type. [a] p<0.001,***; p<0.01,**;p<0.05,*; p>0.05,NS.

Gene	P value	[a]	No of mice analysed/ group	References
Cytokines				
IL1a	0.0314	*	6	de Graaf et al. 1996; Egeler et al. 1999; Kannourakis et al. 1994
IL1b	0.0017	**	7	de Graaf et al. 1996; Kannourakis et al. 1994
IL2	0.4784	NS	6	Egeler et al. 1999
IL3	0.0828	NS	6	Egeler et al. 1999; Kannourakis et al. 1994
IL7	0.0023	**	6	Egeler et al. 1999
IL10	0.1397	NS	5	Egeler et al. 1999
IL12	0.0032	**	6	No reports
IL16	0.0001	***	7	No reports
M-CSF	0.0004	***	6	Rolland et al. 2005
GM-CSF	0.4844	NS	6	de Graaf et al. 1996; Egeler et al. 1999; Kannourakis et al. 1994
G-CSF	0.2589	NS	6	
TNFa	0.0273	*	7	de Graaf et al. 1996; Egeler et al. 1999; Kannourakis et al. 1994
IFNg	0.0050	**	7	de Graaf et al. 1996; Egeler et al. 1999;
TGFB1	0.0162	*	7	de Graaf et al. 1996; Kannourakis et al. 1994
Chemokines				
CCL2	0.0378	*	5	Abla et al. 2010
CCL5	0.0085	**	6	Annels et al. 2003; Abla et al. 2010
CCL17	0.0346	*	6	Rust et al. 2006
CCL20	0.6514	NS	7	Annels et al. 2003; Abla et al. 2010
CCL22	0.1534	NS	6	Rust et al. 2006
CXCL9	0.0288	*	7	No reports
CXCL14	0.0029	**	5	Schaerli et al. 2005
CXCL16	0.0042	**	7	No reports
CX3CL1	0.0103	*	5	No reports
Chemokine-receptors				
ccr2	0.0933	NS	5	Abla et al. 2010
ccr6	0.0022	**	5	Annels et al. 2003; Abla et al. 2010
ccr7	0.5248	NS	5	Fleming et al 2003
cx3cr1	0.1241	NS	5	No reports
Matrix metalloproteinases				

mmp12	0.0497	*	7	Rust et al. 2006; Kis-Toth et al. 2013
mmp14	0.0003	***	7	No reports
Adhesion molecules				
vcam	0.0065	**	7	Ruco et al. 1993; de Graaf et al. 1995
Langerhans' cell phenotype				
CD207	0.0002	***	5	Rust et al. 2006
Lyz2	0.0029	**	7	Rust et al. 2006
s100a9	0.0171	*	7	Rust et al. 2006

CONSIDERAÇÕES FINAIS E PERSPECTIVAS

6. CONSIDERAÇÕES FINAIS E PERSPECTIVAS

No decorrer desta tese foi testada a hipótese de que a mutação V600E do gene BRAF em camundongos seria suficiente para induzir características fenotípicas semelhantes as observadas em pacientes com câncer tireoidiano papilifero e Histiocitose das células de langerhnas.

No primeiro artigo, nós caracterizamos um modelo murino de câncer tireoidiano papilifero provocado pela indução da mutação BRAFV600E em células da foliculares da tireóide. Esses animais apresentaram alterações histológicas e inflamação semelhante as observadas em humanos com a doença. Nesse trabalho demonstramos que a presença da mutação é capaz de causar neoplasia tireoidiana e caracterizamos populações imunológicas presentes no microambiente tumoral.

No segundo artigo, nós demonstramos que a expressão da mutação BRAFV600E em células expressando o receptor de quimiocina CX3CR1, encontrado principalmente no compartimento celular do tipo mieloide, é capaz de promover proliferação dessas células e desenvolvimento de patologia que se assemelha a histiocitose das células de langerhans em humanos. O modelo murino desenvolvido apresenta infiltrados inflamatórios hepáticos com presença de células expressando marcador padrão da doença, o CD207 ou langerin.

Esses resultados implicam diretamante na etiopatologia de ambas doenças e na relevância da mutação BRAFV600E em pacientes com câncer tireoidiano papilifero e Histiocitose das células de langerhnas.

Além disso, os modelos murinos criados a partir do gene mutado BRAF podem ser importantes ferramentas para avaliar a história da doença frente diversos regimes experimentais como tratamento com silenciadores ou ativadores de gene-alvo, cruzamento com linhagens murinas mutantes, tratamento com anticorpos para depleção de célula-alvo, indução com citocinas/ quimiocinas, dentre tantas outras opções.

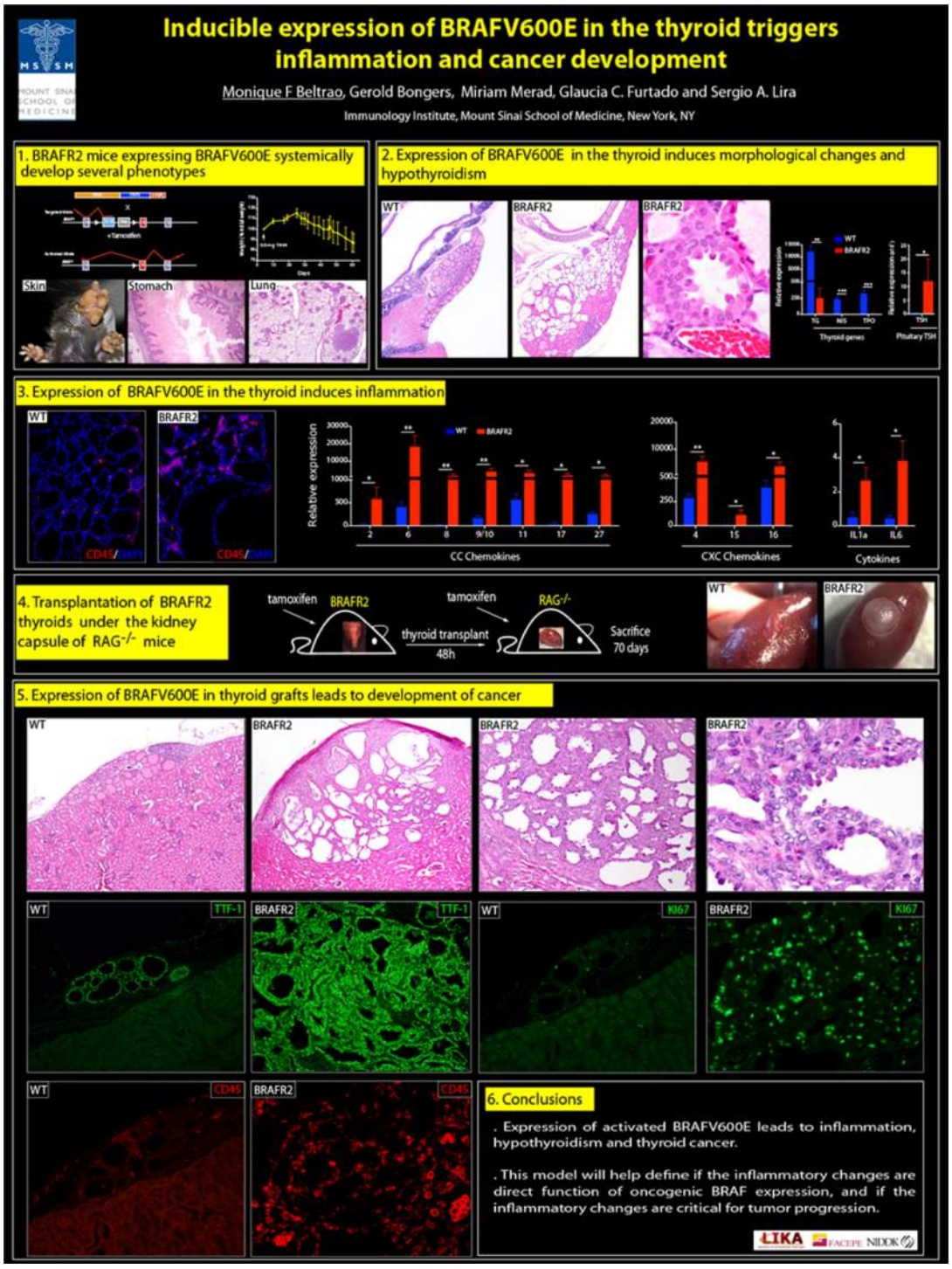
Especificamente, os camundongos TCB e FRBRAF podem ser úteis para definir se modificações inflamatórias podem impactar diretamente na patologia provocada pelo gene BRAF ativado, assim como se essas células imunológicas são necessárias para a progressão tumoral.

ANEXOS

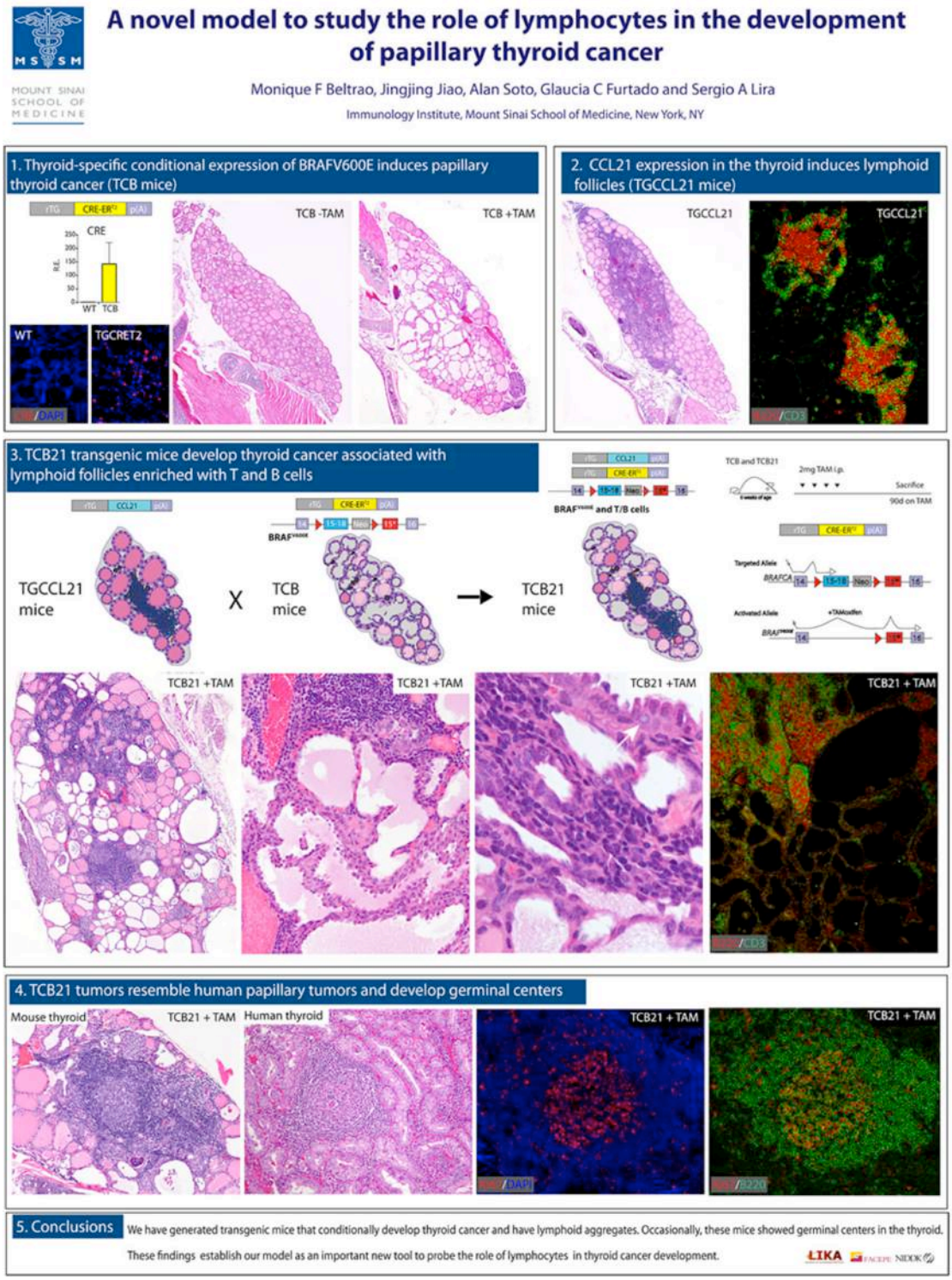
7. ANEXOS

7.1. Apresentação de trabalhos em congressos/ Institutional meetings

7.1.1. **Retreat 2011**, Mount Sinai School of Medicine, New York, USA



7.1.2. **Retreat 2012**, Mount Sinai School of Medicine, New York, USA



7.1.3. **11th World Congress on Inflammation (2013)**, SBI, Natal, Brasil

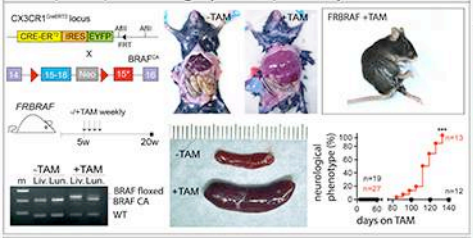
Inducible expression of BRAFV600E in CX3CR1+ cells triggers myeloid expansion and development of a LCH-like disease

Monique Ferraz de Sa Beltrao[1,2], Michelle E Pacer[1], Rafael S Czepielewski[1], Jingjing Jiao[1], Lili Chen[1], Alan Soto[1], Gerold Bongers[1], Glaucia C. Furtado[1] and Sergio A. Lira[1]

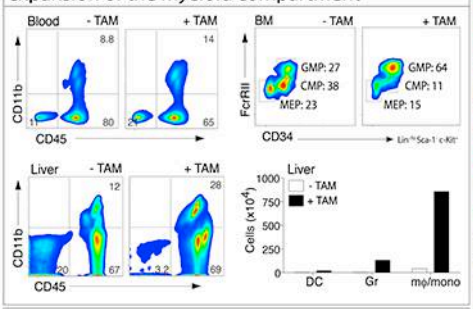
[1] Immunology Institute, Mount Sinai School of Medicine, New York, NY; [2] LIKA/ UFPE, PE, Brazil

Introduction. Langerhans cell histiocytosis (LCH) is a rare disease characterized by an accumulation of histiocytes and Langerhans cells. LCH can involve single or multiple organs, and has a worse prognosis when it affects risk-organs such as, liver, spleen and lung. The origin of LCH is unclear. Studies have shown that LCH is promoted by an uncontrolled clonal proliferation of dendritic cells with LC characteristics, suggesting that these cells share a common myeloid progenitor. An activating mutation of the BRAF gene (V600E) has been detected in many LCH biopsy samples, but it is unclear if it is sufficient to induce disease. To test the hypothesis that BRAFV600E is sufficient to cause LCH, we generated FRBRAF mice. These mice express BRAFV600E in the mononuclear phagocyte system (CX3CR1+ cells) upon administration of tamoxifen (TAM).

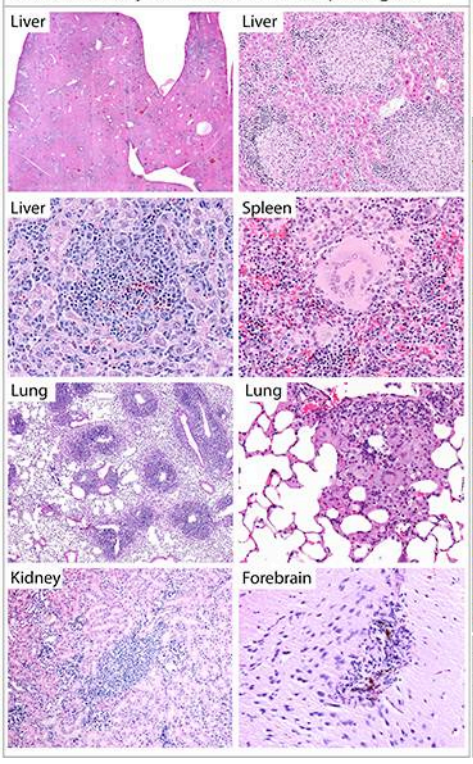
1. Expression of BRAFV^{600E} in CX3CR1^{+} cells leads to hepatosplenomegaly and spasticity



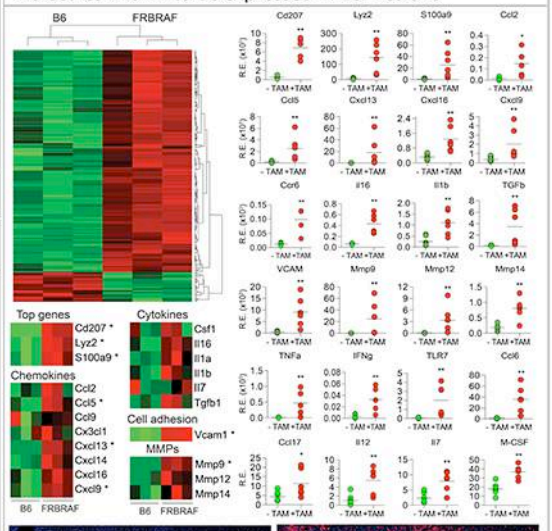
2. Expression of BRAFV^{600E} in CX3CR1^{+} cells induces expansion of the myeloid compartment



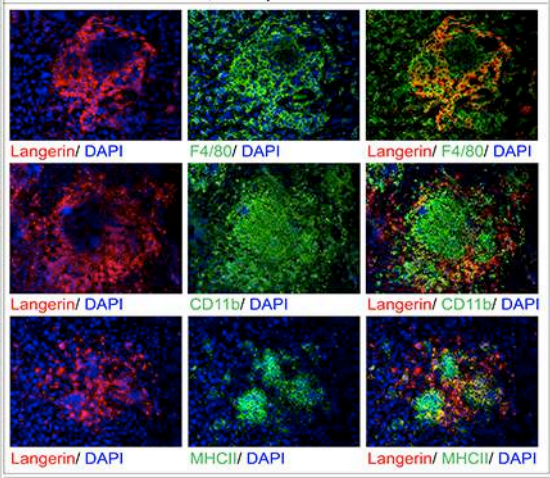
3. Expression of BRAF^{V600E} in the myeloid cells induce histiocytic infiltrates in multiple organs



4. Microarray and qPCR analysis of the FRBRAF livers reveal upregulation of cytokines, chemokine receptors and others molecules known to be expressed in LCH lesions



5. F4/80^{+}CD11b^{+}MHCII^{+}Langerin^{+} cells, typically found in LCH lesions in humans, were present in the FRBRAF liver



Conclusion. We show that expression of BRAFV600E in CX3CR1+ cells promotes expansion of the myeloid compartment, histiocytic infiltrates in multiple organs and development of a disease that resembles human LCH. These results directly implicate BRAFV600E in the pathogenesis of LCH.

7.1.4. **XXXIX Congresso of the Brazilian Society of Immunology (2014)**, SBI, Búzios, Brasil

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IMMUNO BÚZIOS 2014
XXXIX Congress of the Brazilian Society of Immunology
VII ESCI - Extra Section of Clinical Immunology

BEST POSTER AWARD
PhD STUDENT

"EXPRESSION OF BRAFV600E IN CX3CR1+ CELLS DRIVES DEVELOPMENT OF A DISEASE RESEMBLING LCH IN THE LIVER"

RAFAEL SANGUINETTI CZEPIELEWSKI[1]; MONIQUE FERRAZ DE SÁ BELTRÃO[2]; MICHELLE E PACER[3]; JINGJING JIAO[4]; LILI CHEN[5]; ALAN SOTO[6]; GLAUCIA C FURTADO[7]; SERGIO A LIRA[8].

1.LABORATORY OF CELLULAR AND MOLECULAR IMMUNOLOGY, PUCRS, PORTO ALEGRE - RS - BRAZIL; 2,3,4,5,6,7,8.IMMUNOLOGY INSTITUTE, ICAHN SCHOOL OF MEDICINE AT MOUNT SINAI, NEW YORK - UNITEDSTATES .

7.2. Manuscritos publicados durante o doutorado

7.2.1. J Exp Med. 2014 Apr 7;211(4):669-83. doi: 10.1084/jem.20130977. Epub 2014 Mar 17.

BRAF-V600E expression in precursor versus differentiated dendritic cells defines clinically distinct LCH risk groups

Marie-Luise Berres, Karen Phaik Har Lim, Tricia Peters, Jeremy Price, Hitoshi Takizawa, Helene Salmon, Juliana Idoyaga, Albert Ruzo, Philip J. Lupo, M. John Hicks, Albert Shih, Stephen J. Simko, Harshal Abhyankar, Rikhia Chakraborty, Marylene Leboeuf, **Monique Beltrão**, Sérgio A. Lira, Kenneth M. Heym, Venetia Bigley, Matthew Collin, Markus G. Manz, Kenneth McClain, Miriam Merad, and Carl E. Allen

Abstract

Langerhans cell histiocytosis (LCH) is a clonal disorder with elusive etiology, characterized by the accumulation of CD207+ dendritic cells (DCs) in inflammatory lesions. Recurrent BRAF- V600E mutations have been reported in LCH. In this study, lesions from 100 patients were genotyped, and 64% carried the BRAF-V600E mutation within infiltrating CD207+ DCs. BRAF-V600E expression in tissue DCs did not define specific clinical risk groups but was associated with increased risk of recurrence. Strikingly, we found that patients with active, high-risk LCH also carried BRAF-V600E in circulating CD11c+ and CD14+ fractions and in bone marrow (BM) CD34+ hematopoietic cell progenitors, whereas the mutation was restricted to lesional CD207+ DC in low-risk LCH patients. Importantly, BRAF-V600E expression in DCs was sufficient to drive LCH-like disease in mice. Consistent with our findings in humans, expression of BRAF-V600E in BM DC progenitors recapitulated many features of the human high-risk LCH, whereas BRAF-V600E expression in differentiated DCs more closely resembled low-risk LCH. We therefore propose classification of LCH as a myeloid neoplasia and hypothesize that high-risk LCH arises from somatic mutation of a hematopoietic progenitor, whereas low-risk disease arises from somatic mutation of tissue-restricted precursor DCs.

7.2.2. Arabian Journal of Chemistry (2014), DOI: 10.1016/j.arabjc.2014.05.023

Electrochemical DNA biosensor for the detection of Human Papillomavirus E6 gene inserted in recombinant plasmid

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Abstract

In the current study, we describe a novel, simple, inexpensive, sensitive, specific, stable and label-free electrochemical DNA biosensor used to identify a target gene cloned into a plasmid. The biosensor was designed with a 23-mer oligonucleotide of guanine-free, which was immobilized on the pencil graphite electrode (PGE) for E6 gene detection from human papillomavirus 16 type (HPV16). The E6 gene was used due to its clinical importance. The optimal probe concentration was obtained in 500 nM. The hybridization detection showed a good linearity in the range of 40-5,000 pg/μL with a detection limit of 16 pg/μL. The electrochemical method showed higher sensitivity and specificity when compared with the agarose gel electrophoresis assay. This technology could be postulated as a new and attractive alternative for cloning analysis in plasmids.

7.2.3. Mucosal Immunol. 2014 Aug 27. doi: 10.1038/mi.2014.77.

IL-23 activates innate lymphoid cells to promote neonatal intestinal pathology

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Abstract

Interleukin-23 (IL-23) responsive group 3 innate lymphoid cells (ILC3s) have been implicated in immune homeostasis and pathogenesis in the adult, but little is known about their roles in the newborn. Here we show that IL-23 promotes conversion of embryonic intestinal $Lin^{-}IL\text{-}23R^{+}Thy1^{+}$ cells into IL-22-producing $Thy1^{+}Sca\text{-}1^{hi}$ ILC3s in vitro. Gut-specific expression of IL-23 also activated and expanded $Thy1^{+}Sca\text{-}1^{hi}$ ILC3s, which produced IL-22, IL-17, IFN-γ, and GM-CSF and were distinct from canonical $CD4^{+}$ lymphoid tissue inducer (LTi) cells. These ILC3s accumulated under the epithelium in intercellular adhesion molecule (ICAM)-1 positive cell aggregates together with neutrophils that disrupted the epithelium, leading to the formation of discrete intestinal erosions, bleeding, and neonatal death. Genetic and antibody depletion of ILC3s rescued the mice from neonatal death. Antibiotic treatment of pregnant mothers and offspring prolonged survival of IL-23 transgenic mice, suggesting a role for the commensal flora on ILC3-induced pathogenesis. Our results reveal a novel role for the IL-23-ILC3s axis in the pathogenesis of neonatal intestinal inflammation.

7.2.4. Arch Gynecol Obstet. 2015 Mar;291(3):481-91. doi: 10.1007/s00404-014-3480-5.

Site of infections associated with human papillomavirus.

Beltrão M, Wanderley MS, de Santana NA, Bruneska D, de Lima Filho JL.

Abstract

INTRODUCTION: Human papillomavirus (HPV) is the most clinically common sexually transmitted infection due to its carcinogenic power and the high number of lesions that it causes at different sites of the human body.

MATERIAL AND METHODS: Genital tract organs are the most common sites where the virus can be found, but by increasing the sensitivity of diagnostic technique, it is possible to identify viral presence in different regions of the body such as the stomach, the lung, and the urinary tract. These findings break with the traditional HPV skin/genital tropic profile and demonstrate that the virus is capable of infecting a wide variety of cells, tissues, and organs or can, at least, survive in these areas. The widespread presence of the HPV in the human body, often in latent form, led us to consider the hypothesis that HPV latency may be associated with no disease.

CONCLUSION: This observation raises further questions about the possibility of the virus not causing disease in specific sites of the human body, but rather, behaving like a commensal/opportunistic microorganism.