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Article type : Letter to the Editor

TITLE: T-cells changes induced by desensitisation to BRAF inhibitors in two patients with DRESS.

Manuscript Acceptance Date: 23-Feb-2021

To the editor

Melanoma is an aggressive skin cancer, with low survival rates in patients with advanced disease. Melanoma patients present mutations in genes encoding kinases that participate in the Mitogen-Activated Protein Kinases (MAPK) cascade, such as RAF, encoded in BRAF gene, and MEK (1). The commonest melanoma mutation is the V600E, which produces an abnormal protein that activates the MAPK cascade inducing cellular alterations. Besides surgical excision, there are different options according to clinical stage, like the use of drug combinations as BRAF inhibitors (BRAFi) and MEK inhibitors (MEKi), which inhibit the MAPK cascade activation (2). This combination increases treatment effectiveness, decreases adverse effects, and prevents drug-resistance (3). These drugs can induce severe cutaneous allergic reactions (SCAR) (4). Generally, desensitisation in SCAR is contraindicated. Moreover, in melanoma patients, desensitisation seems to be the only procedure that allows them to continue with treatment. We have studied two melanoma patients (P1 and P2) who developed drug rash with eosinophilia and systemic symptoms (DRESS) after BRAFi administration and who tolerated this drug after desensitisation. We have assessed T-cells involvement in skin biopsies and peripheral blood samples at acute phase (T0), and immunological response after desensitisation (T1). Additionally, proliferative response was assessed at two times.

At T0, skin immunohistochemistry showed, in both patients, a perivascular cell infiltrate consisting in memory CD45RO⁺-, CD8⁺- and CD4⁺T-cells, with a CXCR3⁺ and CLA⁺ profile, CD57⁺ and CD68⁺ (Figure 1A). Peripheral blood frequencies, analysed by flow cytometry, showed higher Th2 percentage in P1 compared with control patients with melanoma and BRAFi treatment (MTCs) and with healthy controls (HCs). In P2, the Th1 percentages were higher respect to MTCs and similar to HCs. However, Th17- and

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/ALL.14807](https://doi.org/10.1111/ALL.14807)

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Treg-cells were lower in both patients, compared with MTCs and HCs. No differences in NK-cell frequencies were observed (Figure 1B). The CD4⁺Th1- and CD4⁺Th2-cell implication has been described in cell-mediated hypersensitivity reactions to other drugs (5) and DRESS.

Both patients followed successful desensitisation procedures (Table S1), P1 with dabrafenib and P2 with vemurafenib. These results agree with other desensitisation protocols similar to ours (6). Desensitisation seems to modify effector cell frequencies in both patients comparing peripheral blood at T0 and T1. For P1, Th1-, Th2-, and NK-cells decreased even below levels on MTCs, whereas Treg-cells increased, reaching levels similar to MTCs and HCs. Regarding, Th17-cell levels, although increased, they did not achieve levels observed in MTCs and HCs. Similar results were found for P2 (Figure 1C and Table S2). Changes in the proliferative response during desensitisation were analysed. P1 showed a positive proliferation of Th1- and Th17-cells at T0, becoming negative at T1, except for Th2- and NK-cells. In P2, the proliferation was higher for all cell subpopulations at T0, becoming negative for all cells except for NK-cells at T1 (Figure 2A and Table S2). No proliferations were observed in controls except for NK-cells in MTCs, indicating an unspecific cytotoxic response caused by the pharmacological effect of the drug (Figures 2A and S1A). These results suggest that the success of the desensitisation could be associated with the modulation of drug-specific T-cell proliferation. However, Treg-cells percentages in both patients showed a increase between T0 and T1 reaching the levels to MTCs at maximum concentration of the drugs. No effect was observed in HC (Figure 2B and 1SB).

We have described two patients with DRESS induced by BRAFi, and demonstrated for the first time the BRAFi-specific T-cells involvement. Moreover, desensitisation seems to induce changes in the percentage and drug-specific proliferative response of T-cells. Although further studies including more patients are needed, this study can help in the management of patients with cell-mediated hypersensitive reactions to BRAFi.

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FUNDING SOURCES

The present study has been supported by Institute of Health “Carlos III” of the Ministry of Economy and Competitiveness (grants cofunded by European Regional Development Fund (ERDF): RETIC ARADYAL RD16/0006/0001, and RD16/0006/0021; Andalusian Regional Ministry Health (grant: PI-0076-2019)).

CM holds a ‘Nicolas Monardes’ research contract by Andalusian Regional Ministry Health: C-0044-2012 SAS 2013. TDF hold a “Ramon y Cajal” research contract from Ministry of Economy and Competitiveness (RYC-2013-13138). FP holds a ‘Sara Borrell’ research contract by Institute of Health “Carlos III” of the Ministry of Economy and Competitiveness (CD16/00042).

CONFLICT OF INTEREST

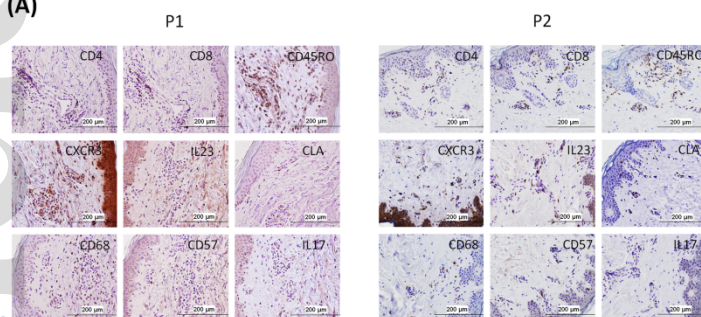
The authors declare no conflicts of interest.

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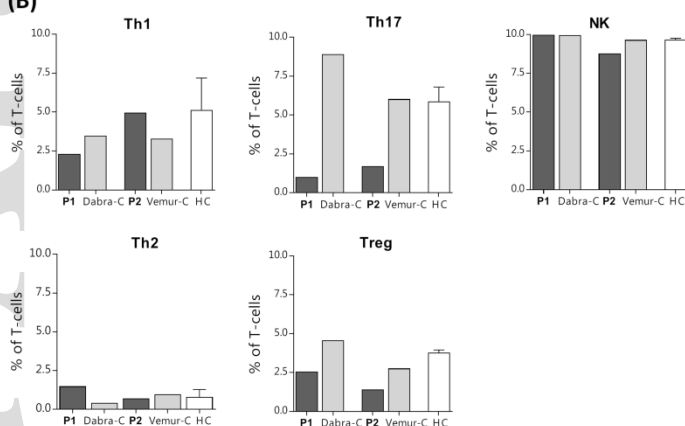
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LEGENDS TO FIGURES

Fig 1
(A)



(B)



(C)

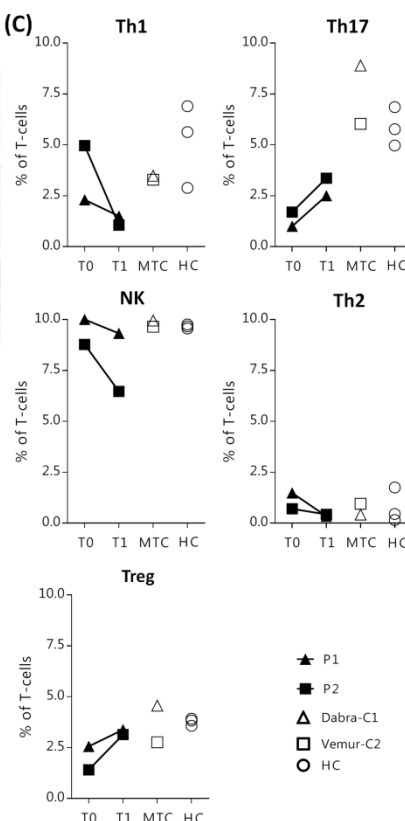


Figure 1. (A) Immunohistochemical analysis of different markers in skin biopsies obtained from the acute phase (T0) of the reaction produced by drug provocation test (DPT) in P1 and P2. Positive cells are marked in brown. **(B)** Bars represent the percentage of different CD4⁺T-cell subpopulations Th1-, Th2-, Th17-, NK-, and Treg-cells in PBMCs from P1 and P2 at T0 (dark grey bars), and a time point of controls with melanoma taking the same drugs, dabrafenib (Dabra-C) or vemurafenib (Vemur-C) (MTC, light grey bars), respectively. White bars represent mean and standard deviation of phenotypical analysis of the same CD4⁺T-cell subpopulations in healthy controls (HCs, N=3). **(C)** Sequential phenotypical analysis of differences in PBMCs from P1 and P2 at T0 and post-desensitization (T1). Frequencies of each subpopulation in MTC and HCs (N=3) are represented at in a time point.

Fig 2

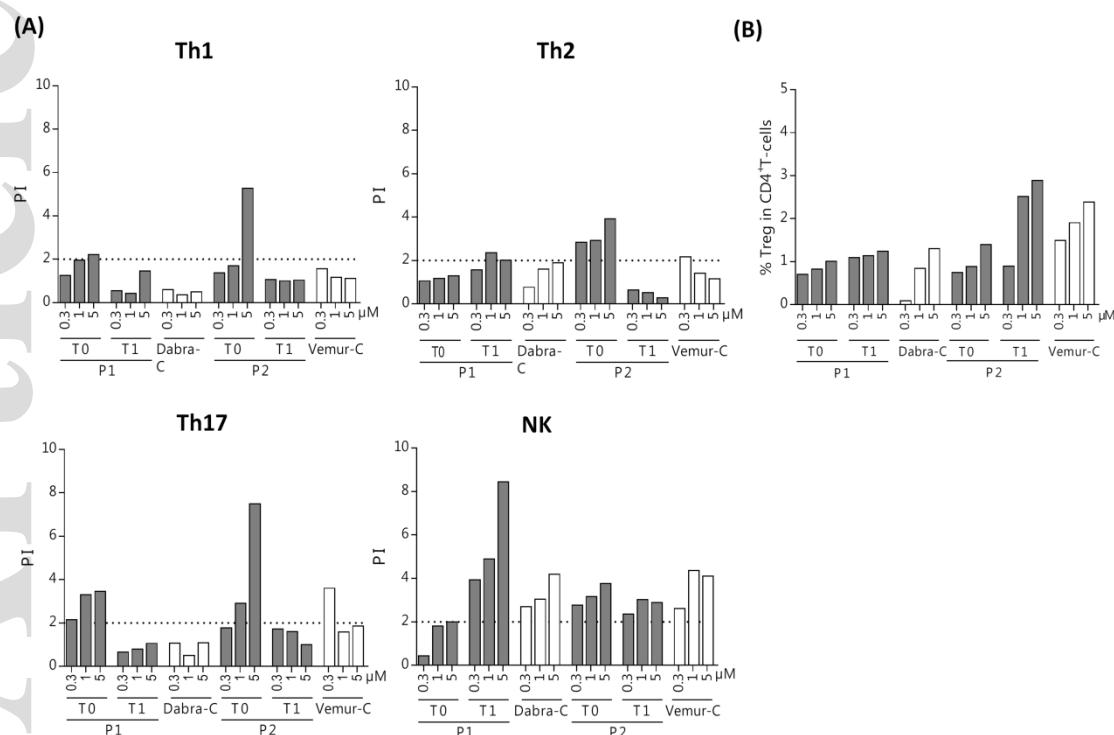
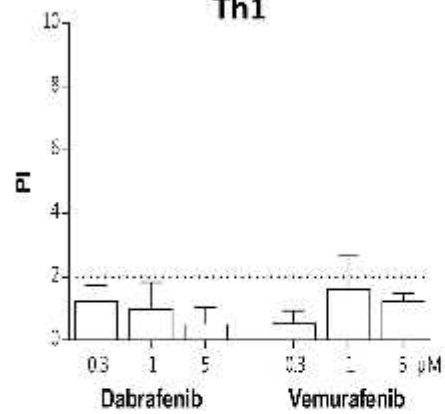


Figure 2. (A) Proliferative response after stimulating with different concentrations of the drug used in the desensitisation procedure, dabrafenib for P1 and vemurafenib for P2, and the corresponding controls with melanoma and similar treatment, dabrafenib (Dabra-C) or vemurafenib (Vemur-C). Dotted line represents the cut-off to consider positive or negative the proliferation index (PI). The IP > 2 is considered positive. (B) Treg-cell percentages at different concentrations of the drug used in the desensitisation procedure and two times. The bars represent the proliferation index (PI) of the different cell subpopulations and Treg-cell percentages at the acute phase (T0) and post-desensitisation (T1) for patients (grey bars) and for MTC at a time point (white bars).

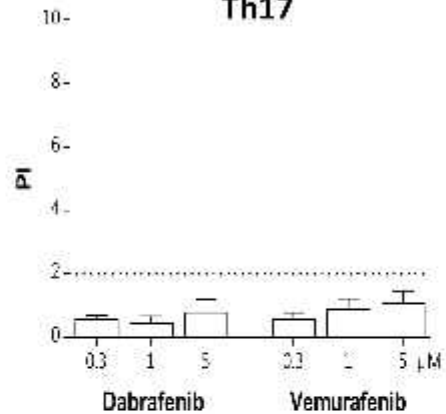
Figure S1

(A)

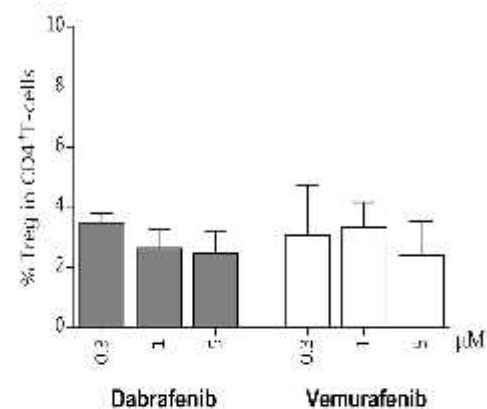
Th1



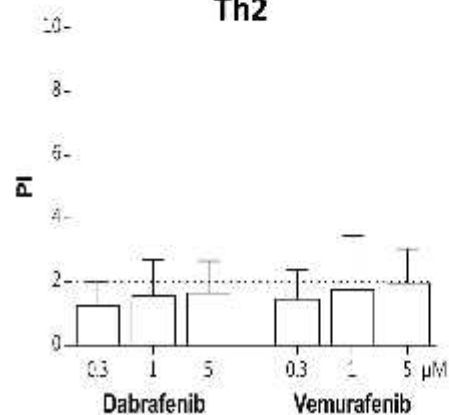
Th17



(B)



Th2



NK

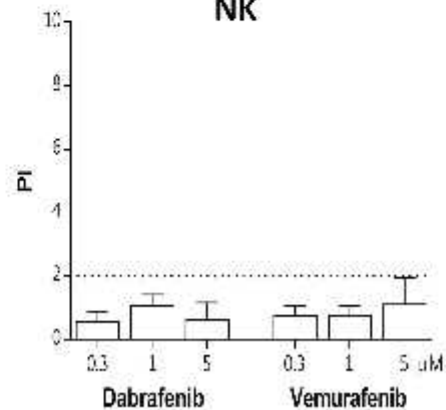
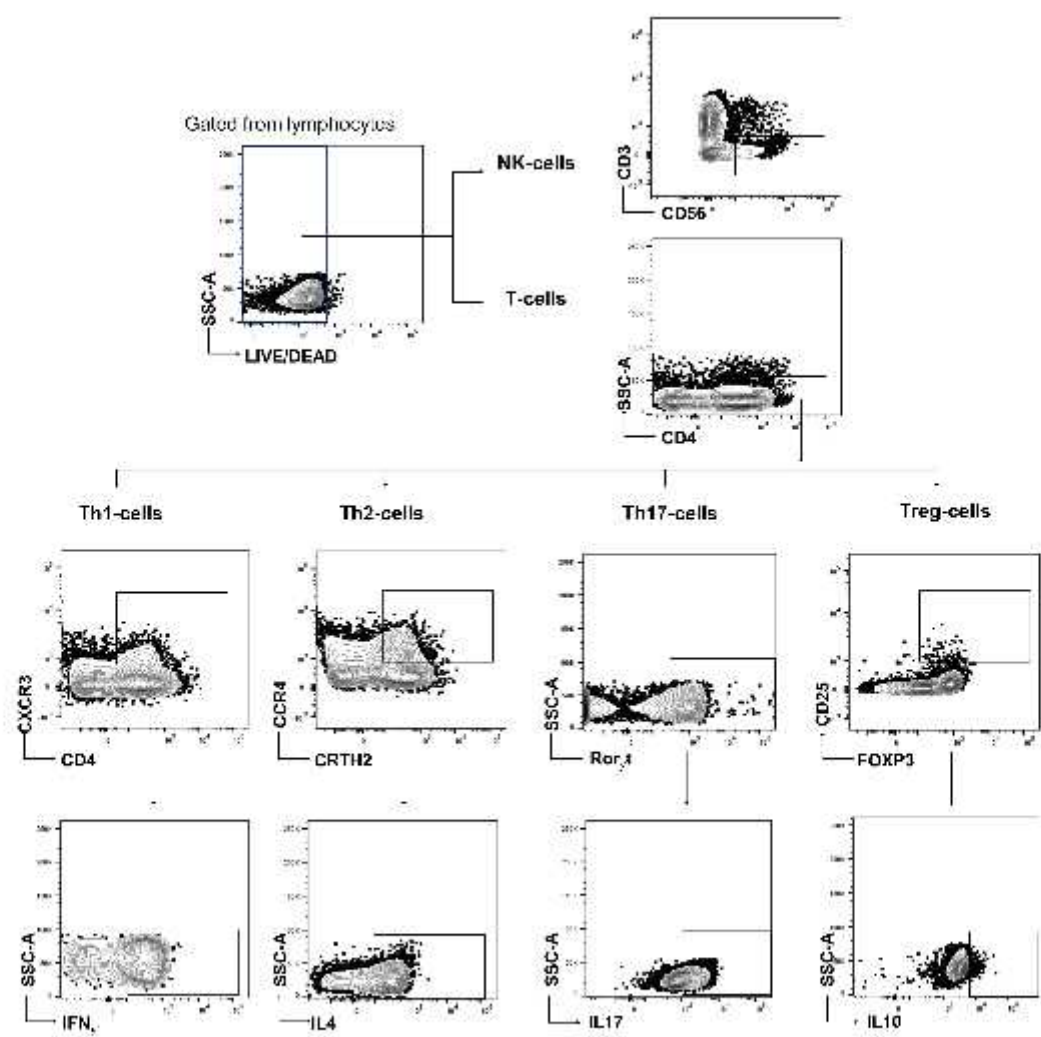


Figure S2



ONLINE SUPPLEMENTARY

TITLE: T-cells changes induced by desensitisation to BRAF inhibitors (BRAFi) in two patients with DRESS

DETAILED EXPLANATIONS OF METHODS

Patients

Two patients (P1 and P2) who developed non-immediate reactions to the BRAFi-MEKi combination were included in the study.

P1 was a 47-year-old woman with cutaneous melanoma diagnosed in 2007. In 2016, the disease progressed to a IIIB cutaneous melanoma, so she initiated treatment with nivolumab. A new progression was observed and a daily treatment with full doses of vemurafenib (960 mg/12 hours) and cobimetinib (60 mg/24 hours) was initiated (both from Roche Pharma, Grenzach-Wyhlen, Germany). She developed fever, eosinophilia ($0.75 \times 10^9/L$), rash with >50 % of skin involvement, and kidney injury 10 days after. She was admitted to hospital at day 11 and initiated a 15-day corticosteroid and antihistamine treatment with good evolution, but a 10-day hospitalisation was necessary. Other potential causes like autoimmune disorders, bacterial and viral infections were ruled out, so she was diagnosed with a probable DRESS according to RegiSCAR (1). Twenty days after the reaction onset, and with complete resolution of clinical symptoms, treatment was re-started with only vemurafenib at half dose (480 mg/12 hours) and, 4 hours after the first dose, she developed generalised erythroderma and sickness. Treatment was stopped and the patient was sent for evaluation to the Allergy Unit, where diagnosis of vemurafenib-induced DRESS was confirmed. We recommended alternative treatment with drugs of different chemical structure from vemurafenib, but this was not possible due to the lack of response to checkpoint inhibitors and the low life expectancy with all other drugs. Due to the severity of the reaction, drug provocation test (DPT) was not performed with the culprit but with dabrafenib (Novartis Pharma, Nuremberg, Germany), being positive as the patient developed generalised erythroderma 4 hours after the first dose. She was treated with metilprednisolone and dexchlorfeniramine, and symptoms resolved after 12 hours. Cross-

reactivity between vemurafenib and dabrafenib was confirmed. A multidisciplinary tumour board proposed desensitisation to dabrafenib.

P2 was a 60-year-old woman with IIB cutaneous melanoma diagnosed in 2013. At the beginning, she started treatment with Interferon-alfa, but it was stopped due to adverse gastrointestinal toxicity with weight loss. She started treatment with vemurafenib and cobimetinib at full dose, developing 8 days after, fever, eosinophilia ($0.9 \times 10^9/L$), rash with >50 % of skin involvement, and liver and kidney injury. As in P1, other potential causes were ruled out, so definitive DRESS diagnosis was reached according to RegiSCAR.(1) Treatment with prednisone, ebastine, and ranitidine was initiated, with complete resolution of symptoms in 10 days. The patient was sent to the Allergy Unit for evaluation. Considering the clinical history, she was diagnosed with definitive vemurafenib-induced DRESS and desensitisation to vemurafenib was proposed by the multidisciplinary tumour board.

In addition, none of the patients (P1 and P2) presented other allergies or skin diseases.

Desensitisation Procedure

Desensitisation was schemed in a weekly-stepped way to increase doses of the drug until reaching complete dose using prednisone and ranitidine as premedication (see table S1). P1 reached dabrafenib 150 mg/12 hours and trametinib 2 mg/24 hours after 5 weeks, and P2 vemurafenib 480 mg/12 hours and cobimetinib 60 mg/24 hours after 4 weeks. P2 could not reach the recommended 960 mg vemurafenib dose due to musculoskeletal toxicity.

Cellular studies

Sample collection and storing

Skin biopsies were obtained at acute phase (T0): during positive DPT with dabrafenib in P1, and during reaction to vemurafenib in P2, for both patients these samples at T0 were obtained in absence of glucocorticoid therapy. Sequential peripheral blood samples were obtained at T0 (absence of glucocorticoid therapy) and 2 weeks after the patient had reached the whole dose of BRAFi (T1). Moreover, blood samples from melanoma treated controls (MTC), one patient

treated for minimum 5 months with vemurafenib/cobimetinib and one treated with dabrafenib/trametinib; and from three healthy controls (HC) were obtained for cellular studies. Results from patients with melanoma treated with each drug were compared with the corresponding P1 or P2 patient. The media of the results obtained from HC was compared with both patients.

Blood was obtained from all subjects by venopunction. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Paque density gradient (Rafer S.L, Zaragoza, Spain), frozen in liquid nitrogen, and biobanked in Málaga Regional University Hospital-IBIMA Biobank, Andalusian Public Health System Biobank. All sequential samples from the same patient were assayed at the same time.

Cellular phenotype

The frequency of different lymphocyte subpopulations at T0 and T1 was analysed by flow cytometry (2) using fluorescent monoclonal antibodies (BD (BD™ Pharmigen, San Diego, CA and Biolegend, San Diego, CA), and fixed with Fixation/Permeabilisation solution with Golgi stop kit (BD Bioscience, San Diego, CA). Cell subpopulation phenotypes were defined as: CD4⁺CXCR3⁺IFN γ ⁺ (Th1); CD4⁺CCR4⁺CRTH2⁺IL-4⁺ (Th2); CD4⁺IL-17⁺ROR γ t⁺ (Th17); CD4⁺CD25^{High}FOXP3⁺IL-10⁺ (Treg); and CD3⁺CD56⁺ (NK cells). For cell staining of the last population, in addition to FOXP3 MoAb, the human Foxp3 buffer set was used (BD™ PharMingen, San Diego, CA). Results were expressed as percentage of cells in the T-cells population and analysed following the gate strategy shown in the Figure S2.

T-cell proliferation assay

PBMCs (1.5x10⁶/mL) were incubated with different BRAFi concentrations (0.3, 1 and 5 μ M) as described (3) for 7 days at 37°C and 5% CO₂ and with complete medium (RPMI 1640 (Lonza, Basel, Switzerland) plus 10% foetal bovine serum (Gibco; Thermo Fisher Scientific, Waltham, MA). T-cell subpopulation proliferation was determined assessing cells with low expression of 5,6-carboxyfluorescein diacetate N-succinimidyl ester (CFSE, Thermo Fisher Scientific) by flow cytometry. Phytohemagglutinin (10 μ g/mL, Sigma, St Louis, MI, USA) and culture media were used as positive and negative controls, respectively. After incubation, cells were stained

with Live/Dead NearIR (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) for 15–20 min to determine cell viability. None of the drug concentrations used showed significant cell toxicity. Results were expressed as proliferation index (PI) calculated as % of stimulated cells expressing CFSE^{low} versus % of unstimulated cells CFSE^{low}. A PI greater than 2 was considered positive as described (4, 5).

Immunohistochemistry in skin biopsies

Microtome sections (8 mm) of skin biopsies were processed as described (4) for immunohistochemical staining using anti-CD4, -CD8 (Ventana Medical Systems, Roche Diagnostics, Barcelona, Spain), -CD57, -CD45RO, (Dako, Ely, Cambridgeshire, UK), and -CLA (BD Pharmingen, San Diego, CA), -CXCR3, -IL23, -IL17 (Abcam, Cambridge, UK). Furthermore, the skin samples came from the same biopsy, but they were not obtained consecutively. They were stained with different markers and for each marker several sections were made. The binding of these primary antibodies (rabbit and goat IgG) was detected using a broad-spectrum super picture HRP polymer conjugate (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). The visualisation of the signal was carried out using 3-3'-diaminobenzidine substrate kit (Sigma, St Louis, MI, USA) in an optical microscope and the optical microscope areas were randomly chosen.

The study was conducted following the standard operational procedure for desensitisations of the Allergy Unit and according to the Declaration of Helsinki. All participants (patients and controls) signed their informed consent. Protocols were approved by the Ethical Committee of Málaga.

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Table S1. Desensitisation protocol of P1 with dabrafenib and of P2 with vemurafenib.

P1	Week	Prednisone (MD)	Ranitidine (MD)	Dabrafenib (MD-ED)	Trametinib (ED)
	1	20 mg/24h	150 mg/24h	50 mg - 0	0,5 mg/24h
	2	20 mg/24h	150 mg/24h	50 mg – 50 mg	0,5 mg/24h
	3	20 mg/24h	150 mg/24h	75 mg – 50 mg	0,5 mg/24h
	3*	20 mg/24h	150 mg/24h	75 mg – 75 mg	0,5 mg/24h
	4	10 mg/24h	150 mg/24h	150 mg – 75 mg	0,5 mg/24h
	5	10 mg/24h	150 mg/24h	150 mg – 150 mg	0,5 mg/24h
	5*	10 mg/24h	150 mg/24h	150 mg – 150 mg	2 mg/24h
	7	No	No	150 mg – 150 mg	2 mg/24h

P2	Week	Prednisone (MD)	Ranitidine (MD)	Vemurafenib (MD-ED)	Cobimetinib (ED)
	1	40 mg/24h	150 mg/24h	240 mg - 0	20 mg/24h
	2	25 mg/24h	150 mg/24h	240 mg – 240 mg	20 mg/24h
	3	25 mg/24h	150 mg/24h	480 mg – 240 mg	20 mg/24h
	3*	25 mg/24h	150 mg/24h	480 mg – 480 mg	40 mg/24h
	4	25 mg/24h	150 mg/24h	480 mg – 480 mg	60 mg/24h
	7	No	No	480 mg – 480 mg	60 mg/24h

* The second increasing dose in the same week. MD: morning dose. ED: evening dose. Prednisone and ranitidine were used as premedication.

Table S2. Summary of changes in the different effector T-cells and the proliferative capacity for the two patients.

Group	Phenotype		Response*	
	T0	T1	T0	T1
P1	↑ Th1	↓ Th1	+	-
	↑ Th2	↓ Th2	-	+
	↓ Th17	↑ Th17	+	-
	↓ Treg	↑ Treg	+(**)	+(**)
	↑ NK	↓ NK	-	+
P2	↑ Th1	↓ Th1	+	-
	↑ Th2	↓ Th2	+	-
	↓ Th17	↑ Th17	+	-
	↓ Treg	↑ Treg	+(**)	+(**)
	↑ NK	↓ NK	+	+

(*) Proliferative response and (**) Treg-cell percentages obtained with the concentration of the drugs at 5 µM; + (positive proliferative response) and - (negative proliferative response).

FIGURES AND FIGURE LEGENDS

Figure S1. (A) Bars represent mean and SEM of the proliferation index (PI) of Th1-cells, Th17-cells, NK-cells and Th2-cells. (B) Treg-cell percentages after stimulating with different concentrations of dabrafenib and vemurafenib in healthy controls (N=3). Results are expressed as PI of the different cell subpopulations and as percentages for Treg-cells. Dotted line represents the cut-off to consider positive or negative the proliferation index (PI). The IP > 2 is considered positive.

Figure S2. Representative example of the gate strategy followed to determinate the phenotype of T-cells and NK cells using flow cytometry.