EMBO Molecular Medicine

STIM1-mediated calcium influx controls antifungal immunity and the metabolic function of Th17 cells

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

12th Nov 2019

Dear Dr. Feske,

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript.

As you will see in the comments pasted below, the referees find the study to be of interest and generally well performed and written. Unfortunately, several important concerns are raised that must be addressed as described. While ref. #1 mostly requests better explanations and clarifications, ref. #2 and #3 are more critical. Indeed, ref. #2 has reservations about the assumption that one STIM1 missense mutation can give the very complex and severe clinical picture. While possible, this referee would like to see more done to support that assumption, otherwise the conclusions should be tone down. This referee also insists on using Candida stimulation on Th17 cells as well as PMA+ionomycin. Ref. #3 has two main issues, the 1st one is about the model used. Since the Vav-Cre mouse model is available in the lab, cellular assays with bone-marrow derived cells should be used to document that neutrophils from the STIM1-deficient mice have decreased antifungal capacity. The 2nd point is about the mode of action of the STIM1 mutation on ORAI1 activation at the plasma membrane.

Given these comments, we would therefore welcome the submission of a revised version within three to six months for further consideration and would like to encourage you to address all the criticisms raised as suggested to improve conclusiveness and clarity. Please note that EMBO Molecular Medicine strongly supports a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting and consult our author's guidelines for proper formatting of your revised article for EMBO Molecular Medicine.

I look forward to receiving your revised manuscript.

Yours sincerely,

Celine Carret

Celine Carret, PhD | Senior Editor EMBO Molecular Medicine

Each figure should be given in a separate file and should have the following resolution:
Graphs 800-1,200 DPI
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Colour (only CMYK) 300-400 DPI"

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***** Reviewer's comments *****

Referee #1 (Remarks for Author):

This is an important and elegant study that provides major new insight into the role of Th17 cells in orchestrating immune response to fungal infection. The authors find a novel mutation (L374P) in the second coiled coil domain of STIM1 that impairs store-operated calcium entry, T cell proliferation and cytokine production. They further demonstrate, using a combination of state-of-the-art techniques spanning the single molecule to in vivo rodent studies, a central role for STIM1 in regulating the metabolism of Th17 cells as well as antifungal immunity to systemic C. albicans infection. The study is carefully conducted, the data are convincing and the findings are novel and exciting.

I have only a few minor comments.

Fungi typically enter the body via mast cell-rich organs, such as the skin, gut, and airways. Mast cells respond to fungi both because of i) their strategic location at vascularized mucosal surfaces and ii) they express TLR2 and Dectin-1 receptors, activation of which release mediators known to be involved in antifungal responses. I wonder whether the authors looked for changes in mast cell numbers etc in the tongues of the infected mice? This is not essential for this study but the authors may already have the data.
The characterisation of the L374P STIM1 mutant is very rigorous. In Figure 2E, a clear smattering of mcherryL374P-STIM1 is seen in the TIRF images, which is not the case for the wild type mcherry STIM1. Some mcherryL374P-STIM1 clusters also appear to have formed under resting conditions. The authors explain this by drawing an analogy with their previous work on the R429C mutant, which destabilised CC3 structure and led to the exposure of the polybasic domain and subsequent accumulation in ER-PM junctions. However, looking at the data presented here and in Maus el al. I have the impression that there are more clusters/puncta of mcherryL374P-STIM1 at rest than was the case with the R429C mutant. I may of course be wrong but, if not, this might suggest that L347P promotes STIM1 clustering to some extent but prevents CAD from binding Orai1. Perhaps the authors could comment on this.
There is considerably more co-localiszation between mcherryL374P-SRTIM1 and Orai1-GFP than is the case for wild type STIM1 at rest. The distribution of Orai1 also looks a little strange to me; it seems to be in clumps and closely mirrors the distribution of mcherryL374P-STIM1. This is reflected in the Pearson coefficient graph in Figure 2E. Do the authors think this stronger co-localization under resting conditions is purely coincidental, reflecting the location of the mutant STIM1 at ER-

PM junctions or could there be some interaction with Orai1, albeit not strong enough to enable calcium entry?
In the methods, the authors state they measured the area under the curve to quantify calcium entry in P1, P2, mother and HD. This is shown in the left hand bar chart of Figure 1F. But what is analyzed in the right hand graph? The y-axis states Peak ratio but relative to what? The base line prior to TG stimulation or to the response in TG/0Ca just prior to readmission of external Ca. The way the data are presented gives the impression that there is no difference between P1 and P2 regarding SOCE, but the raw data show almost no SOCE at all for P2. It might be better to show the Peak ratio relative to the response in TG/0Ca just prior to readmission of external calcium.

Referee #2 (Remarks for Author):

The manuscript from Kahlfuss et al. describes two patients with a missense mutation in STIM1, and defects in Ca influx upon stimulation of T-cells, followed by fungal infections. Subsequently, the authors report increase in susceptibility to mucosal and systemic Candida infection in STIM1 knock-out mice.

Comments

1. The clinical picture of both patients is far more complex and severe than merely increase susceptibility to fungal infections. Indeed, extended morphological and neuro-muscular defects accompany the immunological phenotype. These defects are far more severe than it would be expected from an isolated defect in Th17 function, and most likely for a STIM1 missense mutation. The authors have focused on STIM1 sequencing, based on their earlier studies on the molecule, but the arguments that this is the only, and certainly the causative mutation, are missing. A number of missing pieces are necessary for a thorough genetic assessment of the family:
a. A comprehensive chromosomal and genetic analysis of the patients is needed and should be presented. Whole-exome/genome data would be needed, to assess the breadth of the genetic defects.
b. What is the frequency of this missense mutation in the general population? This information is crucial: a presence of the mutation in the general population of healthy individuals would invalidate the role of the mutation.
c. Ideally, a second family with this defect and the similar phenotype would be needed to validate this mutation as causative.
d. Mutation/disease segregation in the family is missing: the healthy sister and the father, who should not be homozygous of this mutation (as they are healthy). It is true that sometime it is difficult to recruit all members of a family, but that piece of information is very important to support the importance of this mutation.
2. In Figure 3 the stimulation of Th17 has been performed with PMA+ionomycin. This should be accompanied by direct stimulation with Candida albicans, which is an excellent inducer of Th17 cytokines: the release of IL-17 and IL-22 upon Candida stimulation should be shown.
3. How many mice were studied in Fig.4E, on the survival after disseminated candidiasis?
4. It is very difficult to understand the cause of death of mice with systemic Candida infection: they show very high mortality starting with day 8 post-infection (Fig.4E), but on day 6 only very few mice had any Candida in their organs (Fig.4H). Especially the majority of the control mice are almost free of fungal growth, with the exception of 1-2 mice. For mice who would succumb due to infection two days later, that is very strange. It is well known that in systemic candidiasis the target organ is the kidney in the mouse, and mice die of massive fungal infiltration and kidney insufficiency. How can

the authors explain this discrepancy?
5. The histology data in the kidney suggest hyperinflammation in the tissues, but this is a different pathophysiology than Th17 defects.

Referee #3 (Comments on Novelty/Model System for Author):

The mouse model does not adequately recapitulate the human disease

Referee #3 (Remarks for Author):

This study reports reduced antifungal CD4-mediated immunity in two patients with a point mutation in the ER Ca2+ sensor STIM1 and links this defect to a reduced metabolic function of non-pathogenic Th17 cells. A p.L347P mutation in the STIM1 channel activating domain was identified in two siblings with combined immunodeficiency suffering from recurrent bacterial and fungal infections. Store-operated Ca2+ entry was reduced s despite normal STIM1 protein expression in the patients' T cells, which failed to expand and to secrete cytokines. When expressed in HEK cells, STIM1-L374P localizes to the TIRF plane prior to store depletion and fail to form clusters and to co-localize with ORAI1 upon store depletion. In mice, conditional STIM1 deletion in T cells increased the susceptibility to systemic, but not to mucosal infection with Candida albicans, increased the expression of non-pathogenic genes in Th17 cells and reduced their glycolytic and oxidative metabolic capacity. Glycolytic function and mitochondrial respiration was also impacted in CD4 T cells from a human patient. The authors conclude that STIM1 promotes antifungal immunity by regulating differentially the metabolism of pathogenic and non-pathogenic Th17 cells.

Comments: This is a well-controlled study that presents high quality data relevant for our understanding of the cellular basis of antifungal immunity. The data are solid, well presented, and for the most part adequately interpreted. The manuscript is also very well written. My enthusiasm is somewhat limited by the use of the CD4-cre mouse model and the exclusive focus on Th17 cells, which ignores the contribution of innate immune cells and thus fail to establish a solid link between the human disease and the mouse data. I also would suggest to better document the molecular defect imparted by the L347P mutation on STIM1 conformational changes during activation. Specifically:

1. It is difficult to relate the metabolic defect of Th17 cells to the increased susceptibility of the patients to fungal infections, because the mouse model does not adequately recapitulate the human disease. Mucosal infections cannot be reproduced in Stim1fl/fl-Cd4Cre mice (Fig 4) yet can be readily generated in Stim1fl/fl-VavCre mice (Fig S4). The choice of the Cd4-Cre and the subsequent focus on Th17 cells is questionable and ignores the major contribution of innate immune cells, whose role in antifungal defence is well established. In line with this, there is an increase in GM-CSF and neutrophils in the blood of infected Stim1fl/fl-Cd4Cre mice. STIM1 was shown to regulate superoxide production by neutrophils (PMID:24493668, PMID:28724541), and one would thus expect that defective neutrophil functions contribute to the fungal infections observed in the two patients. The contribution of STIM1 in the antifungal response of neutrophils should be better documented.

2. The molecular defect induced by the STIM1-L347P mutation is not established. The authors state that STIM1-L347P fails to bind Orai1, but there is no evidence for this. Quite the contrary, Fig

2DE show that mCherry-STIM1-L347P has a high degree of co-localisation with overexpressed GFP-ORAI1 in the TIRF plane. This suggests that the two proteins co-localize at the ER-PM interface but fail to form clusters following store depletion. Lack of cluster formation cannot be used as evidence for defective STIM-ORAI binding, because cluster formation relies on the exposure of STIM1 polybasic tail rather than on binding to ORAI1. Several studies showed that STIM1 is first recruited to plasma membrane clusters via its polybasic tail and subsequently traps ORAI1 within clusters, resulting in channel activation (PMID: 25057023). This raises the possibility that STIM1-L347P is actually recruited to the PM via increased binding to ORAI1. Such a mutant would trap ORAI1 effectively, yet fail to cross-link and activate ORAI1 channels. Alternatively, STIM1-L347P might be pre-recruited to the PM via an exposed polybasic tail. To distinguish between these possibilities the authors should test whether STIM1-L347P is still recruited to the PM when expressed in the absence of ORAI1, and whether removing the polybasic tail impacts STIM1-L347P distribution. A co-IP is also required to document the binding of STIM1-L347P to endogenous and overexpressed ORAI1

Minor comments

In the text the mutation is referred to as STIM1 g.C1142T but in figure 1B and Supplementary Table 2 the mutation is T1121C. Based on p.L374P, the genetic mutation should be consistently g.T1142C.

Page 11, line 9 '...with the transcriptional identify of non-pathogenic TH-17 cells,..' should be 'identity'.

Point-by-Point response

Referee 1:

This is an important and elegant study that provides major new insight into the role of Th17 cells in orchestrating immune response to fungal infection. The authors find a novel mutation (L374P) in the second coiled coil domain of STIM1 that impairs store-operated calcium entry, T cell proliferation and cytokine production. They further demonstrate, using a combination of state-of-the-art techniques spanning the single molecule to in vivo rodent studies, a central role for STIM1 in regulating the metabolism of Th17 cells as well as antifungal immunity to systemic C. albicans infection. The study is carefully conducted, the data are convincing and the findings are novel and exciting. I have only a few minor comments.

Response: We thank the reviewer for his/her thoughtful and positive evaluation of our study and comments that have improved the manuscript.

Fungi typically enter the body via mast cell-rich organs, such as the skin, gut, and airways. Mast cells respond to fungi both because of i) their strategic location at vascularized mucosal surfaces and ii) they express TLR2 and Dectin-1 receptors, activation of which release mediators known to be involved in antifungal responses. I wonder whether the authors looked for changes in mast cell numbers etc in the tongues of the infected mice? This is not essential for this study but the authors may already have the data.

Response: We agree that mast cells are increasingly recognized to play a role in antifungal immunity (Jiao, Luo et al., 2019, Renga, Moretti et al., 2018). It is therefore tempting to speculate that overall mast cell numbers and/or function is altered in our STIM-deficient mice during systemic and/or local candida infection. In addition, it was recently demonstrated that mast cell function in house dust mite-mediated asthmatic airway inflammation depends on store-operated calcium entry (SOCE) (Lin, Nelson et al., 2018). It is therefore possible that SOCE in mast cells could be involved in antifungal immunity at mucosal surfaces. Although STIM1 and STIM2 are deleted in mast cells of *Stim1*$^{fl/fl}$*VaviCre* and *Stim1*$^{fl/fl}$*Stim2*$^{fl/fl}$*VaviCre* mice and we agree that it would be interesting to study the role of SOCE in mast cells in antifungal immunity, we had to prioritize experiments for the revision of this manuscript and focused on the role of SOCE neutrophils (as requested by reviewer 3). A detailed analysis of SOCE in mast cells will be subject of a future study.

The characterisation of the L374P STIM1 mutant is very rigorous. In Figure 2E, a clear smattering of mcherryL374P-STIM1 is seen in the TIRF images, which is not the case for the wild type mcherry STIM1. Some mcherryL374P-STIM1 clusters also appear to have formed under resting conditions. The authors explain this by drawing an analogy with their previous work on the R429C mutant, which destabilised CC3 structure and led to the exposure of the polybasic domain and subsequent accumulation in ER-PM junctions. However, looking at the data presented here and in Maus el al. I have the impression that there are more clusters/puncta of mcherryL374P-STIM1 at rest than was the case with the R429C mutant. I may of course be wrong but, if not, this might suggest that L347P promotes STIM1 clustering to some extent but prevents CAD from binding Orai1. Perhaps the authors could comment on this.

Response: Based on our analysis of the change in localization of STIM1 to the TIRF evanescent field (Figure 2G) and the colocalization of ORAI1 with STIM1 (Figure 2H) derived from the TIRF images in Figure 2D,E, we conclude that the STIM1 p.L374P mutation and the previously reported STIM1 p.R429C mutation behave very similarly (Maus, Jairaman et al., 2015). To illustrate this point, we here provide a side-by-side comparison of TIRF experiments in which HEK cells were transfected with these mutants of STIM1.

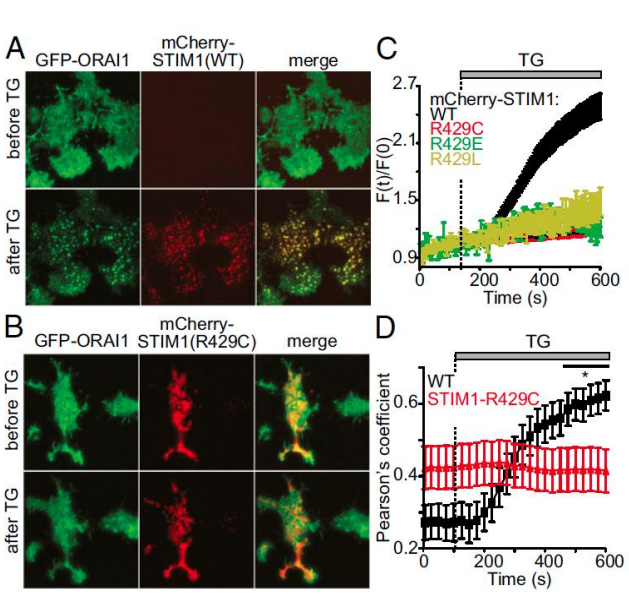

Figure 5 from Maus et al. 2015, PNAS, PMID: 25918394

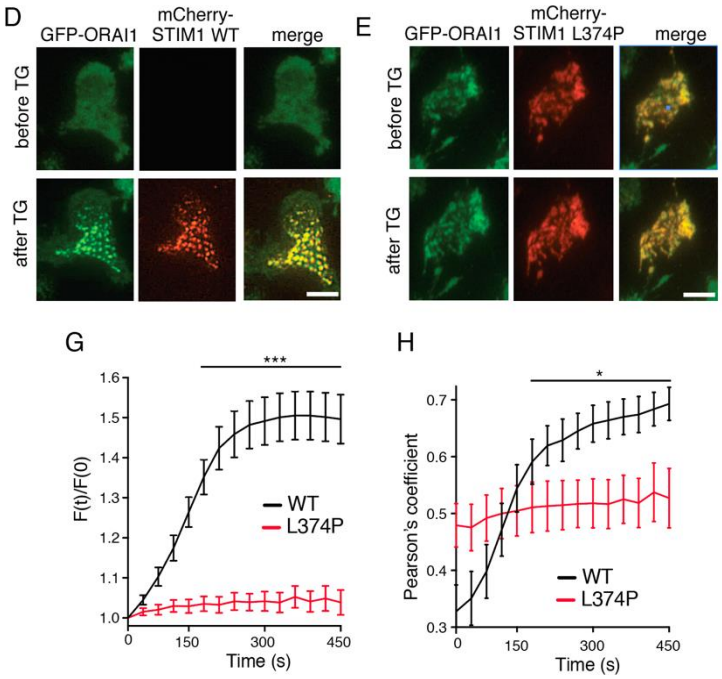

Figure 2 from the current manuscript.

There is considerably more co-localiszation between mcherryL374P-SRTIM1 and Orai1-GFP than is the case for wild type STIM1 at rest. The distribution of Orai1 also looks a little strange to me; it seems to be in clumps and closely mirrors the distribution of mcherryL374P-STIM1. This is reflected in the Pearson coefficient graph in Figure 2E. Do the authors think this stronger co-localization under resting conditions is purely coincidental, reflecting the location of the mutant STIM1 at ER-PM junctions or could there be some interaction with Orai1, albeit not strong enough to enable calcium entry?

Response: The reviewer brings up an interesting point (whether the "stronger co-localization under resting conditions reflects the location of the mutant STIM1 at ER-PM junctions or if there could be some interaction with Orai1") that was also raised by Reviewer 3. We have therefore conducted additional experiments to address the following questions: (1) is the localization of mutant STIM1 to the plasma membrane mediated by the effects of the L374P mutation on the release of the polybasic domain (PBD) of STIM1 or is it dependent on residual binding to ORAI1? To this end, we used TIRFM to analyze cells expressing a STIM1 mutant lacking the PBD and cells lacking ORAI1. (2) Does the L374P mutation impair STIM1 binding to ORAI1? To this end, we conducted co-IP experiments. A detailed description of the results addressing questions 1 and 2 is provided in our response to Reviewer 3 and **new Supplemental Figure 2**.

In the methods, the authors state they measured the area under the curve to quantify calcium entry in P1, P2, mother and HD. This is shown in the left hand bar chart of Figure 1F. But what is analyzed in the right hand graph? The y-axis states Peak ratio but relative to what? The base line prior to TG stimulation or to the response in TG/0Ca just prior to readmission of external Ca. The way the data are presented gives the impression that there is no difference between P1 and P2 regarding SOCE, but the raw data show almost no SOCE at all for P2. It might be better to show the Peak ratio relative to the response in TG/0Ca just prior to readmission of external calcium.

Response: As suggested by Reviewer 1, we have reanalyzed the calcium data and now show the peak ratio relative to the response in TG/0 calcium prior to readmission of external calcium in Figure 1F.

Referee 2:

The manuscript from Kahlfuss et al. describes two patients with a missense mutation in STIM1, and defects in Ca influx upon stimulation of T-cells, followed by fungal infections. Subsequently, the authors report increase in susceptibility to mucosal and systemic Candida infection in STIM1 knock-out mice.

Comments

1. The clinical picture of both patients is far more complex and severe than merely increase susceptibility to fungal infections. Indeed, extended morphological and neuro-muscular defects accompany the immunological phenotype. These defects are far more severe than it would be expected from an isolated defect in Th17 function, and most likely for a STIM1 missense mutation. The authors have focused on STIM1 sequencing, based on their earlier studies on the molecule, but the arguments that this is the only, and certainly the causative mutation, are missing.

Response: We thank the reviewer for his/her careful evaluation of our manuscript and comments. We agree that P1 and P2 suffer from a complex disease not only involving the immune system or Th17 cells. We do not claim that the *STIM1* mutation is only affecting Th17 cell function but it clearly has additional effects in other tissues. We and others have described mutations in *STIM1* (and in the CRAC channel encoding gene *ORAI1*), which result in a complex disease that we named CRAC channelopathy (OMIM 612782 and 612783). Hallmarks of CRAC channelopathy are (i) combined immunodeficiency syndrome with recurrent bacterial, viral and fungal infections, (ii) autoimmunity with autoantibodies against platelets and RBCs, (iii) ectodermal dysplasia with anhidrosis and amelogenesis imperfecta type III, and (iv) muscular hypotonia (Lacruz & Feske, 2015). The STIM1 p.L374P mutant patients reported in this manuscript have the same or very similar phenotype as other patients with LOF mutations in *STIM1* (or *ORAI1*). The causal relationship between the STIM1 p.L374P mutation and the observed clinical phenotype is therefore not just based on the 2 patients reported here, but also on the previous reports of STIM1 loss-of-function (LOF) mutations and the resulting clinical CRAC channelopathy phenotype. We have provided a table summarizing these studies (**Reviewer Table 1)** to illustrate the common disease phenotype resulting from STIM1 (and ORAI1) mutations.

To address the concerns of Reviewer 2, we have performed additional genetic analyses, which are summarized below and have been added to the manuscript text.

A number of missing pieces are necessary for a thorough genetic assessment of the family:

a. A comprehensive chromosomal and genetic analysis of the patients is needed and should be presented. Whole-exome/genome data would be needed, to assess the breadth of the genetic defects.

Response: We agree that in general a comprehensive chromosomal and genetic analysis is required when a mutation in a gene not previously reported in connection with a clinical phenotype is suggested to be causative for that disease. In the case of the patients reported here, we do not think that a WES/WGS analysis is necessary because of the arguments presented above, namely that the clinical phenotype of P1 and P2 (who are homozygous for the STIM1 p.L374P mutation) is practically identical to that of other patients with LOF mutations in *STIM1* (**Reviewer Table 1**). We think that this provides sufficient evidence that the STIM1 p.L374P mutation is responsible for the disease in the patients reported here.

b. What is the frequency of this missense mutation in the general population? This information is crucial: a presence of the mutation in the general population of healthy individuals would invalidate the role of the mutation.

Response: We thank the reviewer for his/her comment as we had not adequately addressed this in the previous version of the manuscript. We performed additional genetic analyses: (1) gnomAD Analysis (https://gnomad.broadinstitute.org/) using the gnomAD v3 data set (GRCh38 / hg38) from unrelated individuals sequenced as part of various disease-specific and population genetic studies. The input term (gene name) “STIM1“ and a search for the variants “p.Leu374Pro” and “c.1121T>C” yielded that the

p.Leu374Pro STIM1 mutation is extremely rare with an allele frequency of 6.98e-6 (1 allele out of 143330).

Gene	Mutation	Immuno-defi-ciency	Auto-immu-nity	Muscular hypotonia	Ectodermal dysplasia		References
					Anhidrosis	Amelogenesis imperfecta	
ORAI1	p.R91W	Y	n.r.	Y	n.r.	n.r.*	Feske et al. (1996); Feske et al. (2006)
	p.R91W	Y	Y	Y	Y	Y	Feske et al. (2000); Feske et al. (2006)
	p.V181SfsX8	Y	n.r.	Y	Y	Y	Lian et al. (2018)
	p.L194P	Y	Y	Y	Y	*	Lian et al. (2018)
	p.G98R	Y	Y	Y	Y	Y	Lian et al. (2018)
	p.G98R	Y	Y	Y	Y	Y	Lian et al. (2018)
	p.A88SfsX25	Y	Y	Y	Y	n.r.*	Partiseti et al. (1994); McCarl et al. (2009)
	p.A103E/p.L194P	Y	n.r.	Y	Y	Y	Le Deist et al. (1995); McCarl et al. (2009)
	p.H165PfsX1	Y	n.r.	Y	n.r.	n.r.*	Chou et al. (2015)
	p.R270X	Y	n.r.	Y	n.r.	n.r.*	Badran et al. (2016)
STIM1	p.E128RfsX9	Y	Y	Y	n.r.	Y	Picard et al. (2009)
	p.E128RfsX9	Y	Y	Y	n.r.	Y	Picard et al. (2009)
	C1538-1 G>A	Y	n.r.	Y	n.r.	n.r.	Byun et al. (2010)
	p.R429C	Y	Y	Y	Y	Y	Fuchs et al. (2012), Maus et al. (2015)
	p.R429C	Y	Y	Y	Y	Y	Fuchs et al. (2012), Maus et al. (2015)
	p.R426C	n.r.	n.r.	n.r.	Y	Y	Wang et al. (2014)
	p.P165Q	Y	Y	Y	Y	Y	Schaballie et al. (2015)
	p.P165Q	Y	n.r.	Y	Y	Y	Schaballie et al. (2015)
	p.L74P	Y	Y	n.r.	Y	Y	Parry et al. (2016)
	p.L74P	Y	n.r.	n.r	Y	Y	Parry et al. (2016)
	p.L374P (P1)	**Y**	**n.r.**	**Y**	**Y**	**Y**	**This study**
	p.L374P (P2)	**Y**	**Y**	**Y**	**Y**	**Y**	**This study**

Reviewer Table 1: CRAC channelopathy due to mutations in the genes *ORAI1* and *STIM1*. *STIM1* mutations at the bottom of the table are those reported in this manuscript. Abbreviations: n.r., not reported or not tested; N, No; Y, Yes. * Patients died before complete dentition.

As "CRAC channelopathy" due to mutations in the genes *ORAI1* or *STIM1* follows an autosomal recessive inheritance, the homozygous p.Leu374Pro mutation in *STIM1* detected in both patients in the current study
is compatible with their disease ('CRAC channelopathy'). (2) We also calculated the scaled Combined Annotation-Dependent Depletion (CADD) score for the STIM1 c.1121T>C, p.Leu374Pro mutation by using the Single Nucleotide Variant (SNV) lookup tool https://cadd.gs.washington.edu/snv The (maximum) CADD score at this position is 28.5. (Request: Chromosome 11, Position 4103565, CADD GRCh37-v1.4), which indicates that the mutation is within the top 0.1% of the most deleterious variants in the human genome. We now report these findings in the manuscript.

c. Ideally, a second family with this defect and the similar phenotype would be needed to validate this mutation as causative.

Response: We agree that studying a second family with the same mutation and phenotype would be ideal. However, mutations in STIM1 (and ORAI1) are extremely rare and no other family with the exact same STIM1 p.L374P mutation is known. However there are several other patients and families with LOF mutations in STIM1 that present with the same clinical phenotype (**Reviewer Table 1)**, which in our opinion validates the STIM1 p.L374P mutation as causative of the disease in our patients. (We would like

to point out that the novelty of our study is not the disease phenotype itself but the in-depth analysis of the role STIM1 and SOCE in antifungal immunity, which has never been reported before.)

d. Mutation/disease segregation in the family is missing: the healthy sister and the father, who should not be homozygous of this mutation (as they are healthy). It is true that sometime it is difficult to recruit all members of a family, but that piece of information is very important to support the importance of this mutation.

Response: We agree that the father and sister II.3 should not be homozygous for the mutation. They are clinical healthy (as far as we could ascertain) and do not show any hallmark symptoms of CRAC channelopathy. Unfortunately, neither the sister nor the father (who is estranged from the rest of the family and incarcerated) are available for genetic testing.

2. In Figure 3 the stimulation of Th17 has been performed with PMA+ionomycin. This should be accompanied by direct stimulation with Candida albicans, which is an excellent inducer of Th17 cytokines: the release of IL-17 and IL-22 upon Candida stimulation should be shown.

Response: We thank the reviewer for this comment and have conducted the suggested experiments. First, we have cultured expanded human T cells from patient 1, his mother and two healthy donors (HD) in the presence of *C. albicans* for 2 and 4 weeks in vitro and restimulated T cells with PMA and ionomycin (P+I) to induce production of Th17 cytokines. In addition, we treated HD T cells with the selective CRAC channel inhibitor GSK-7975 (Rice, Bax et al., 2013, Wen, Voronina et al., 2015) for 6 hours during the restimulation of T cells with P+I. We found that CD4+ and CD8+ T cells from patient 1 and T cells from HDs treated with GSK-7975 have an almost complete defect in the production of IL-17A (and IL-2) compared to T cells from the mother and a HD. We were not able to detect IL-22 production in either HD or patient cells. Second, we cultured PBMC from a HD for 6 days in the presence of *C. albicans* and then restimulated cells for 6 hours with P+I in the presence of GSK-7975 or DMSO as control. CD4+ and CD8+ T cells showed a near complete defect in the production of IL-17A. We now show the experiments proposed by the reviewer within a new **Figure 4** as we think they strengthen our findings. Third, it is noteworthy that T cells from P1 and P2 that were stimulated with *C. albicans* and analyzed for T cell proliferation as part of their clinical evaluation for immunodeficiency showed a severe defect in C. albicans-specific proliferation (**Supplemental Table 2).**

3. How many mice were studied in Fig.4E, on the survival after disseminated candidiasis?

Response: Five *Stim1$^{fl/fl}$Cd4Cre* and 12 WT mice were used for these experiments. We have added this information to the manuscript. We thank the reviewer for drawing our attention on this omission.

4. It is very difficult to understand the cause of death of mice with systemic Candida infection: they show very high mortality starting with day 8 post-infection (Fig.4E), but on day 6 only very few mice had any Candida in their organs (Fig.4H). Especially the majority of the control mice are almost free of fungal growth, with the exception of 1-2 mice. For mice who would succumb due to infection two days later, that is very strange. It is well known that in systemic candidiasis the target organ is the kidney in the mouse, and mice die of massive fungal infiltration and kidney insufficiency. How can the authors explain this discrepancy?

Response: *Stim1$^{fl/fl}$Cd4Cre* mice started to succumb to systemic *C.albicans* infection at day 8 p.i. and at day 17 p.i. all mice had died (**revised Figure 6E**). The fungal burden (CFU per gram of infected organs) at day 6 p.i. show high CFU counts in 3 *Stim1$^{fl/fl}$Cd4Cre* mice, which correspond to the mice that succumbed to *C.albicans* early post-infection (**revised Figure 6H**). *Stim1$^{fl/fl}$Cd4Cre* mice with lower fungal burdens at day 6 p.i. died at later time points. The same correlation between fungal burdens and time of death was observed for WT mice. At day 6 p.i. only 2 WT mice showed high fungal burdens and

these mice died at day 10 or 11 p.i. The remaining WT mice that had no detectable *C. albicans* in their kidneys at day 6 p.i. (**Figure 6H**) died between days 11 and 20 or survived the systemic *C. albicans* infection. To further illustrate the increased fungal burdens in the kidneys of *Stim1$^{fl/fl}$Cd4Cre* mice compared to WT littermate controls, we added new Periodic acid–Schiff (PAS) stains to detect polysaccharides in the cell wall of *C. albicans* in the kidneys of *Stim1$^{fl/fl}$Cd4Cre* and WT mice at day 6 post-infection (**new Figure 6I**, right panels). Taken together, we think that the increased fungal burdens of *Stim1$^{fl/fl}$Cd4Cre* mice compared to WT controls correlates well with the their faster and more complete (100%) death from systemic *C. albicans* infection.

While answering reviewer #2's comment, we did however notice a mistake in the x-axis labeling of the original **Figure 4F (**now **revised Figure 6F)**, which we corrected. Fungal burdens were measured at day 6p.i.

5. The histology data in the kidney suggest hyperinflammation in the tissues, but this is a different pathophysiology than Th17 defects.

Response: This is an interesting point and we welcome the opportunity to comment on it. The kidney histologies of *C. albicans* infected *Stim1$^{fl/fl}$Cd4Cre* mice indeed show increased leukocyte infiltration (inflammation) compared to WT mice (revised **Figure 6I**).This finding correlates well with the higher numbers of Cd11b^{+} Gr-1^{+} neutrophils in the blood of infected *Stim1$^{fl/fl}$Cd4Cre* mice (revised **Figure 6K**) and the increased frequencies of GM-CSF-producing CD4^{+} T cells in the spleen of these mice (revised **Figure 6J**) compared to infected WT littermates. These results are also consistent with our RNA sequencing data of *in vitro* differentiated non-pathogenic Th17 cells. *Stim1$^{fl/fl}$Cd4Cre* non-pathogenic Th17 cells show significantly elevated expression of *Csf2* compared to WT Th17 cells (revised **Figure 7C**). We think that this enhanced GM-CSF production by CD4^{+} T cells of *Stim1$^{fl/fl}$Cd4Cre* mice is responsible for neutrophil mobilization from the bone marrow, which are subsequently recruited to the *C. albicans* infected kidneys of these mice. However, the lack of many other important Th17 (and Th1) effector cytokines in *Stim1$^{fl/fl}$Cd4Cre* mice (**Figure 6J**) may result in an overall impaired innate and adaptive immune response to systemic *C. albicans* infection compared to WT mice.

A similar kidney inflammation was recently described in *Il17ra$^{\Delta RTEC}$* mice with conditional deletion of the IL-17 receptor A (IL-17RA) in renal tubular epithelial cells (RTEC). 7 days after systemic infection with *C. albicans* the authors of this study noted "increased tissue pathology in the cortex and inner and outer medullary regions" consistent on H&E staining of serial kidney sections with leukocyte infiltration; see Figure 5A of (Ramani, Jawale et al., 2018). Systemic *C. albicans* infection in these mice resulted in impaired kidney function and death of mice by 10 days p.i. similar to findings in *Stim1$^{fl/fl}$Cd4Cre* mice after systemic *C. albicans* infection. We have added a sentence to the discussion of our manuscript mentioning the kidney inflammation in *Stim1$^{fl/fl}$Cd4Cre* and *Il17ra$^{\Delta RTEC}$* mice after systemic *C. albicans* infection.

Referee 3:

This study reports reduced antifungal CD4-mediated immunity in two patients with a point mutation in the ER Ca2+ sensor STIM1 and links this defect to a reduced metabolic function of non-pathogenic Th17 cells. A p.L347P mutation in the STIM1 channel activating domain was identified in two siblings with combined immunodeficiency suffering from recurrent bacterial and fungal infections. Store-operated Ca2+ entry was reduced s despite normal STIM1 protein expression in the patients' T cells, which failed to expand and to secrete cytokines. When expressed in HEK cells, STIM1-L374P localizes to the TIRF plane prior to store depletion and fail to form clusters and to co-localize with ORAI1 upon store depletion. In mice, conditional STIM1 deletion in T cells increased the susceptibility to systemic, but not to mucosal infection with Candida albicans, increased the expression of non-pathogenic genes in Th17 cells and reduced their glycolytic and oxidative metabolic capacity. Glycolytic function and mitochondrial respiration was also impacted in CD4 T cells from a human patient. The authors conclude that STIM1 promotes antifungal immunity by regulating differentially the metabolism of pathogenic and non-pathogenic Th17 cells.

Comments: This is a well-controlled study that presents high quality data relevant for our understanding of the cellular basis of antifungal immunity. The data are solid, well presented, and for the most part adequately interpreted. The manuscript is also very well written.

Response: We thank the reviewer for his/her careful evaluation of our manuscript and positive assessment.

My enthusiasm is somewhat limited by the use of the CD4-cre mouse model and the exclusive focus on Th17 cells, which ignores the contribution of innate immune cells and thus fail to establish a solid link between the human disease and the mouse data. I also would suggest to better document the molecular defect imparted by the L347P mutation on STIM1 conformational changes during activation.

Response: To address the reviewers two main points we performed additional experiments and provide additional discussion within the manuscript. **(1)** Regarding the contribution of innate immune cells to antifungal immunity, we completely agree with the reviewer and have included additional data and restructured the manuscript (see below). **(2)** Regarding the molecular defect caused by the STIM1 p.L374P mutation, we have conducted additional experiments, which were added **new Supplemental Figure 2** and are discussed in detail below.

Specifically: 1. It is difficult to relate the metabolic defect of Th17 cells to the increased susceptibility of the patients to fungal infections, because the mouse model does not adequately recapitulate the human disease. Mucosal infections cannot be reproduced in Stim1fl/fl-Cd4Cre mice (Fig 4) yet can be readily generated in Stim1fl/fl-VavCre mice (Fig S4). The choice of the Cd4-Cre and the subsequent focus on Th17 cells is questionable and ignores the major contribution of innate immune cells, whose role in antifungal defence is well established. In line with this, there is an increase in GM-CSF and neutrophils in the blood of infected Stim1fl/fl-Cd4Cre mice. STIM1 was shown to regulate superoxide production by neutrophils (PMID:24493668, PMID:28724541), and one would thus expect that defective neutrophil functions contribute to the fungal infections observed in the two patients. The contribution of STIM1 in the antifungal response of neutrophils should be better documented.

Response: We completely agree with the reviewer regarding the important role of innate immune cells for antifungal immunity. We therefore decided to include our data showing impaired immunity to mucosal infection with *C. albicans* in *Stim1*$^{fl/fl}$ and *Stim1/2*$^{fl/fl}$*-Vav-iCre* mice to the main figures **(new Figure 5**). Since *Stim1*$^{fl/fl}$ and *Stim1/2*$^{fl/fl}$*-Vav-iCre* mice lack SOCE in all immune cells, they better recapitulate the immunodeficiency in human patients, who also lack SOCE in all immune cells. Our data show that *Stim1*$^{fl/fl}$ and *Stim1/2*$^{fl/fl}$*-Vav-iCre* mice show strongly increased susceptibility to mucosal infection with *C. albicans*, which is in line with the mucocutaneous *Candida* infections observed in several patients with CRAC channelopathy (**Table 1**).

We also agree with the reviewer's comment that "*STIM1 was shown to regulate superoxide production by neutrophils [..] and one would thus expect that defective neutrophil functions contributes to the fungal infections observed in the two patients".* We have therefore conducted new experiments to test the role of SOCE in ROS production and candicidal function of neutrophils. Using poly-I:C injected *Stim1*$^{fl/fl}$ and *Stim1/2*$^{fl/fl}$*-Mx1-Cre* mice, which lack SOCE in all immune cells (similar to *Stim1*$^{fl/fl}$ and *Stim1/2*$^{fl/fl}$*-Vav-iCre* mice), we found impaired ROS production in the absence of STIM1 or both STIM1/STIM2 and decreased fungal killing after coincubation of *C. albicans* with STIM1 or STIM1/STIM2 deficient neutrophils. We added these results to **new Figure 5H,I** and have updated the results and discussion sections of the manuscript accordingly.

We would like to briefly comment on two additional points raised by the reviewer: (1) "*Mucosal infections cannot be reproduced in Stim1fl/fl-Cd4Cre mice (Fig 4) yet can be readily generated in Stim1fl/fl-VavCre mice (Fig S4).*" The finding that *Stim1*$^{fl/fl}$*Cd4Cre* mice are not more susceptible to mucosal *Candida* infection than WT mice (revised **Figure 6A-D**) is consistent with studies showing that mice lacking T cells are not significantly more susceptible to mucosal *Candida* infection than WT mice either (Conti, Peterson et al., 2014, Gladiator, Wangler et al., 2013). (2) "*It is difficult to relate the metabolic defect of Th17 cells to the increased susceptibility of the patients to fungal infections.*" We here show that Th17 cell function of

Stim1$^{fl/fl}$*Cd4Cre* mice is impaired, which makes these mice more susceptible to systemic *Candida* infection.This is in line with the recently reported role of Th17 cells in mediating immunity to systemic *Candida* infection (Shao, Ang et al., 2019). Our data show that STIM1 is important for Th17 cell function in antifungal immunity, and that STIM1 is critical for regulating Th17 cell metabolism as metabolic pathways were among the most deregulated pathways in STIM1-deficient Th17 cells as determined by RNA-Seq analysis.

2. The molecular defect induced by the STIM1-L347P mutation is not established. The authors state that STIM1-L347P fails to bind Orai1, but there is no evidence for this. Quite the contrary, Fig 2DE show that mCherry-STIM1-L347P has a high degree of co-localisation with overexpressed GFP-ORAI1 in the TIRF plane. This suggests that the two proteins co-localize at the ER-PM interface but fail to form clusters following store depletion. Lack of cluster formation cannot be used as evidence for defective STIM-ORAI binding, because cluster formation relies on the exposure of STIM1 polybasic tail rather than on binding to ORAI1. Several studies showed that STIM1 is first recruited to plasma membrane clusters via its polybasic tail and subsequently traps ORAI1 within clusters, resulting in channel activation (PMID: 25057023). This raises the possibility that STIM1-L347P is actually recruited to the PM via increased binding to ORAI1. Such a mutant would trap ORAI1 effectively, yet fail to cross-link and activate ORAI1 channels. Alternatively, STIM1-L347P might be pre-recruited to the PM via an exposed polybasic tail. To distinguish between these possibilities the authors should test whether STIM1-L347P is still recruited to the PM when expressed in the absence of ORAI1, and whether removing the polybasic tail impacts STIM1-L347P distribution. A co-IP is also required to document the binding of STIM1-L347P to endogenous and overexpressed ORAI1

Response: We agree with the reviewer that it is interesting to study how the L374P mutation abolishes SOCE despite being localized at the PM and partially colocalizing with ORAI1. We had initially not planned a more comprehensive analysis to dissect the effects of the L374P mutation because (i) the paper is mostly focused on the role of SOCE in immunity to C. albicans and (ii) because the effects of the STIM1 p.L374P mutation are similar to those of a STIM1 p.R429C mutation we had reported earlier (Maus et al., 2015). To repond to the reviewer's request we have, however, conducted several additional experiments to address the concerns raised. The results are summarized in **new Supplemental Figure 2**.

(1) We tested the hypothesis that mutant STIM1 is localized at the PM because the L374P mutation results in an "exposed polybasic tail" which traps STIM1 at ER-PM junctions. To this end we used HEK293 cells stably expressing GFP-ORAI1 and transfected them with mCherry-tagged wildtype STIM1, STIM1 lacking the polybasic domain (ΔK), STIM1 p.L374P or STIM1 p.L374P-ΔK. We analyzed the localization the behaviour of these STIM1 proteins with regard to localization at the PM, cluster (puncta) formation and colocalization with ORAI1 by total internal reflection fluorescence microscopy (TIRFM). As shown in **new Supplemental Figure 2A-C**, deletion of the polybasic domain does not affect the TG-induced translocation of STIM1-ΔK to the PM, puncta formation or colocalization with ORAI1 (**panel B**), which is consistent with (Park, Hoover et al., 2009), who also showed that STIM1-ΔK forms puncta in cells overexpressing ORAI1 as in our experiments. As shown in our original data (Figure 2E), we again found here that STIM1 p.L374P is prelocalized at the PM and partially colocalizes with ORAI1 (**panel B**). Deletion of the polybasic domain had no significant effect on the behaviour of STIM1 p.L374P-ΔK, which was still localized at the PM and partially colocalized with ORAI1, suggesting that the localization of the STIM1 mutant is not dependent on its polybasic domain.

(2) We next tested the hypothesis that mutant STIM1 is localized at the PM because of binding to ORAI1. To this end we transfected HEK293 cells in which ORAI1 was deleted by CRISPR/Cas9 gene editing with either WT STIM1 or the three mutant STIM1 constructs described above and analyzed the localization of STIM1 at the PM by TIRFM. As shown in the **Figure added for the reviewers below**, deletion of ORAI1 abolished the translocation of WT STIM1 to the PM after thapsigargin stimulation. Importantly, deletion of ORAI1 does not prevent the prelocalization of STIM1 p.L374P at the PM. Even combined deletion of ORAI1 and the polybasic domain of STIM1 p.L374P-ΔK had no significant effect on

the localization of mutant STIM1 at the PM. This finding is unexpected but indicates that the localization of mutant STIM1 p.L374P at ER-PM junctions is independent of its polybasic domain and ORAI1. At present, we can only speculate about the mechanism. It is possible that STIM1 p.L374P has a partially active configuration which allows it to oligomerize with endogenous STIM1 or STIM2 in HEK293 cells and piggybacks on them to translocate to ER-PM junctions. This is plausible, as coexpression of STIM2 with STIM1ΔK in HEK293 cells was shown to result in coclustering of both proteins suggesting that STIM2 recruits STIM1 to ER-PM junctions (Ong, de Souza et al., 2015). We planned to test this idea by deleting STIM2 in HEK293 cells and overexpressing STIM1 p.L374P-ΔK, but these experiments were stopped when our lab was shut down due to the COVID19 outbreak.

(3) Finally, we tested whether mutant STIM1 is able to bind to ORAI1 to respond to the reviewer's comments "*a co-IP is also required to document the binding of STIM1-L347P to endogenous and overexpressed ORAI1".* To this end, we cotransfected HEK293 cells with Flag-ORAI1 and either WT or mutant STIM1 p.L374P. Cells were left unstimulated or stimulated with thapsigargin in the absence of extracellular Ca2+ to maximally deplete ER stores and induce STIM1 activation. ORAI1 was immunoprecipitated with anti-Flag beads and STIM1 binding detected by SDS-PAGE using an anti-STIM1 antibody. Our results from three repeat experiments show that even in unstimulated cells WT STIM1 can be co-IP'ed with ORAI1, and as expected this co-IP increased when cells were stimulated with thapsigargin **(new Supplemental Figure 2D,E).** The STIM1 p.L374P mutant showed stronger co-IP with ORAI1 in unstimulated cells. This increased co-IP is consistent with the constitutive localization of STIM1 p.L374P at the PM we show by TIRFM in Figure 2 and S2. Since this constitutive PM localization of STIM1 p.L374P does not depend on ORAI1 (as shown in the Figure for reviewers) we speculate that the constitutive co-IP of STIM1 p.L374P and ORAI1 may be facilitated by the recruitment of STIM1 p.L374P to the PM by endogenous STIM1 or STIM2 as discussed above. In other words, the recruitment of mutant STIM1 to the PM does not depend on ORAI1, but once it is at the PM it is able to bind to ORAI1. After stimulation with thapsigargin to deplete ER stores, the co-IP between STIM1 p.L374P and ORAI1 was strongly reduced to levels similar to those found for WT STIM1 in unstimulated cells **(new Supplemental Figure 2D,E)**. This result was unexpected because TG stimulation has no effect on the PM localization of STIM1 p.L374P (as shown in the Figure for reviewers). However, according to our model that STIM1 p.L374P may be recruited to the PM by endogenous STIM1 or STIM2, we speculate that TG-induced store depletion results in increased binding of endogenous STIM1 and STIM2 to ORAI1, which compete with and replace mutant STIM1 p.L374P. As stated above, we planned to test this idea by deleting STIM2 in HEK293 cells and repeating the co-IP experiments, but this was no longer possible when our lab was shut down due to the COVID19 outbreak.

[Figure for reviewers removed upon authors request]

Taken together, we propose the following **model** how the p.L374P mutant affects STIM1 function: L374P results in a conformational change in the C-terminus of STIM1 that partially activates it and allows STIM1 to translocate to the PM. This recruitment is not dependent on the polybasic domain of STIM1 or the presence of ORAI1 in the PM, at least not under conditions of ectopic overexpression of STIM1 p.L374P. We speculate that overexpressed STIM1 p.L374P binds to endogenous STIM1 or STIM2, which bring it to the PM and facilitate its binding to ORAI1. This binding, observed by partial colocalization in TIRFM and co-IP experiments, is weak and not sufficient to activate CRAC channel opening as apparent from (a) impaired SOCE and T cell function, and (b) reduced co-IP of ORAI1-STIM1 p.L374P after thapsigargin stimulation, likely because mutant STIM1 is outcompeted by ORAI1 binding of endogenous STIM1 or STIM2. What we cannot fully explain is why the mutation retains its ability to bind to ORAI1 but fails to activate it. One explanation is that CRAC channel activation requires the formation of STIM1 clusters (Luik, Wang et al., 2008). Whereas STIM1 p.L374P is located near the PM and binds ORAI1, it fails to form puncta after store depletion with thapsigargin, suggesting that the mutation interferes with proper oligomerization of STIM1 molecules, which is required for the formation of macromolecular complexes that have the correct STIM1:ORAI1 stoichiometry needed to active the CRAC channel (Yen & Lewis, 2019). An additional explanation for impaired SOCE could be that the L374P mutation results in conformational changes that do not abrogate STIM1 binding to ORAI1 but specifically interfere with its ability to gate the CRAC channel. This interpretation is supported by the finding that the binding of mutant STIM1 to ORAI1 observed in co-IP experiments is lost after cell stimulation, likely because it is replaced by active endogenous WT STIM1 that has a higher affinity for ORAI1. This changed or weaker binding implies that the interaction of mutant STIM1 to ORAI1 may be different in a fundamental way and prevents proper ORAI1 gating.

Minor comments

In the text the mutation is referred to as STIM1 g.C1142T but in figure 1B and Supplementary Table 2 the mutation is T1121C. Based on p.L374P, the genetic mutation should be consistently g.T1142C.

Response: We thank the reviewer for poining out this mistake which we have corrected in the text. The correct annotation of the mutation in P1 and P2 is c.1121T>C and p.Leu374Pro.

Page 11, line 9 '...with the transcriptional identify of non-pathogenic TH-17 cells,..' should be 'identity'.

Response: We have corrected this mistake.

Note that the RNA-Seq data shown in Figure 7 and 8 were reanalyzed to account for an updated version of R (v. 3.6.2) and the KEGG database (2019; the previous analysis was done using the 2016 version of KEGG accessed through DAVID). The reanalysis confirmed the results we had presented in the original mansuscript, but resulted in slightly more accurate lists of differentially expressed genes (DEG) and KEGG pathways.

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13th May 2020

Dear Prof. Feske,

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final editorial amendments:

1) Please provide a point-by-point letter including my comments and your detailed responses to their comments (as Word file).

2) Please carefully check the authors guidelines for formatting your supplemental information: Expanded view and Appendix (see: https://www.embopress.org/page/journal/17574684/authorguide#expandedview)
Please label your supplemental information document "Appendix" and change the nomenclature as "Appendix figure S1", "Appendix Table S1" and so on. Remove the graphical abstract from this file. Include a Table of content on the 1st page. Have all text black. This document must be provided as pdf, it won't be edited nor typeset.

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6) Authors' contribution: the contributions of Dimitrius Raphael, Mate Maus, and Zhengxi Sun are missing

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- their clinical impact = Impact
This may be edited to ensure that readers understand the significance and context of the research. Please refer to any of our published articles for an example.

9) Every published paper now includes a 'Synopsis' to further enhance discoverability. Synopses are displayed on the journal webpage and are freely accessible to all readers. They include a short stand first (maximum of 300 characters, including space) as well as 2-5 one sentence bullet points that summarise the paper. Please write the bullet points to summarise the key NEW findings. They should be designed to be complementary to the abstract - i.e. not repeat the same text. We encourage inclusion of key acronyms and quantitative information (maximum of 30 words / bullet point). Please use the passive voice. Please attach these in a separate file or send them by email, we will incorporate them accordingly.

10) As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts.

In the event of acceptance, this file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. Let us know whether you agree with the publication of the RPF and as here, if you want to remove or not any figures from it prior to publication.

Please note that the Authors checklist will be published at the end of the RPF.

11) Data availability: To list the primary data generated in your study, we would kindly ask you to include a formal "Data availability" section (after Materials & Methods) that follows the example below:

The datasets produced in this study are available in the following databases:

- [data type]: [full name of the resource] [accession number/identifier] ([doi or URL or identifiers.org/DATABASE:ACCESSION])

example
* RNA-Seq data: Gene Expression Omnibus GSExxxxx (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSExxxxx)
Please add the accession number now and make sure that the data is available as soon as the paper is accepted.

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Celine Carret

Celine Carret, PhD
Senior Editor
EMBO Molecular Medicine

*** Instructions to submit your revised manuscript ***

To submit your manuscript, please follow this link:

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Please do not share this URL as it will give anyone who clicks it access to your account.

When submitting your revised manuscript, please include:

1) a .doc formatted version of the manuscript text (including Figure legends and tables)

2) Separate figure files*

3) supplemental information as Expanded View and/or Appendix. Please carefully check the authors guidelines for formatting Expanded view and Appendix figures and tables at https://www.embopress.org/page/journal/17574684/authorguide#expandedview

4) a letter INCLUDING the reviewer's reports and your detailed responses to their comments (as Word
file).

***** Reviewer's comments *****

Referee #2 (Remarks for Author):

[The authors have responded adequately and the article is suitable for publication].

Referee #3 (Remarks for Author):

The authors have addressed all the points raised in the first round of review. The new data clarify the role of innate immune cells in the pathogenesis of the fungal infection. They also provide new insights into the mechanisms of activation of the mutant protein. The authors have to be congratulated for their fine work in addressing the comments. I have no further suggestion for change.

Point-by-Point response

Editor: *Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final editorial amendments:*

Referee #2 (Remarks for Author):
[The authors have responded adequately and the article is suitable for publication].

Referee #3 (Remarks for Author):
The authors have addressed all the points raised in the first round of review. The new data clarify the role of innate immune cells in the pathogenesis of the fungal infection. They also provide new insights into the mechanisms of activation of the mutant protein. The authors have to be congratulated for their fine work in addressing the comments. I have no further suggestion for change.

Response: *We thank all 3 reviewers for their time and expertise used to review this manuscript, which has helped to improve the quality of the published work.*

2nd Revision - Editorial Decision 25th May 2020

25th May 2020

Dear Prof. Feske,

Thank you for revising your article as suggested. We are pleased to inform you that your manuscript is accepted for publication and is now being sent to our publisher to be included in the next available issue of EMBO Molecular Medicine.

Please read below for additional IMPORTANT information regarding your article, its publication and the production process.

Congratulations on your interesting work,

Celine Carret

Celine Carret, PhD
Senior Editor
EMBO Molecular Medicine

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dr. Stefan Feske
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2019-11592

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor	ARRIVE Guidelines
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http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Animal group sample size were determined using power analysis (power=90% and alpha=0.05) based on the mean and standard deviation from our previous studies and/or pilot studies.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The numbers of mice used in most of the experiments were based upon results of pilot experiments to achieve reproducible results statistical significance (P<0.05), this was done to have sufficient statistical power to detect difference of 1.6, 1.8 and 2.0 standard deviations with 72%, 80% and 87% power using a two-tailed t-test. We aimed to detect effect sizes of 2.0 standard deviations, and thus expected to have sufficient power. Statistical significance between different groups was determined by using the two-tailed unpaired Student's t test when a normal distribution was assumed or otherwise by Mann Whitney U test. Where indicated, data was analyzed by one-way ANOVA followed by Tukey's honestly significant difference (HSD) post hoc test for multiple comparisons. p values < 0.05 were considered significant; different levels of significance are indicated as follows: p<0.05*; p<0.01**; p <0.001***.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	The criteria for animal experiments were pre-established. Mice with signs of severe pain, weight loss more than 20%, apathy, or insufficient eating and drinking were sacrificed.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	For some of the readouts, e.g. determing Candida CFUs, the investigator was blinded.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Animal experiments were conducted unblinded. For certain readouts (e.g. determing Candida CFUs within different organs) the investigator was blinded.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistical significance between different groups was determined by using the two-tailed unpaired Student's t test when a normal distribution was assumed or otherwise by Mann Whitney U test. Where indicated, data was analyzed by one-way ANOVA followed by Tukey's honestly significant difference (HSD) post hoc test for multiple comparisons. p values < 0.05 were considered significant; different levels of significance are indicated as follows: p<0.05*; p<0.01**; p <0.001***.
Is there an estimate of variation within each group of data?	Yes, there is an estimate of variation shown as shown by error bar indicating the standard error mean for each group data. Whereever possible, we show individual data points in figures (Figure 4 B,C,D; Figure 5 B,E,G; Figure 6 B,H,J; Figure EV4 C,D.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All commercial antibodies used in this study are listed in Supplemental Table 3. Antigen Manufacturer Clone Conjugation (Anti-human antibodies) CD3 Biolegend SK7 APC-Cy7 CD3 Biolegend UCHT1 Alexa700 CD4 Biolegend OKT4 BV510 CD4 Biolegend RPA-T4 PE, PE-Cy7 CD4 BioLegend OKT4 Alexa700 CD8 Biolegend HIT8a Alexa700 CD8 Biolegend RPA-T8 PE-Cy7 CD8 Biolegend SK1 Pacific-Blue CD45RO BD Pharmingen UCHL1 PE-Cy7 CD45RO Biolegend UCHL1 APC-Cy7 CD44 Biolegend IM7 PerCP CD57 Biolegend HCD57 Pacific-Blue CD16 Biolegend 3G8 Alexa488 CD56 Biolegend HCD56 PerCP-Cy5.5 CD27 Biolegend O323 PE-Cy7 CCR7 R&D 150503 FITC HLA-DR Biolegend L243 APC-Cy7

	Va24Ja18 TCR Biolegend 6B11 APC Foxp3 BioLegend 259D PE IL-2 Biolegend MQ1-17H12 Alexa700, APC IL-22 eBioscience 22URTI eF710 TNF-alpha Biolegend MAb11 A488 IL-17A eBioscience eBio64CAP17 PE IFN-gamma eBioscience 4S.B3 Alexa488 γδ TCR BD B1 biotinylated +streptavidin Biolegend - BV605 ViabDye eBioscience - eF506 Phopsho mTor Ser2448 eBioscience MRRBY eFluor660 Phospho S6 Ser235/236 eBioscience cupk43k APC Anti-mouse antibodies Antigen Manufacturer Clone Conjugation (Anti-mouse antibodies) CD4 eBioscience GK1.5 PE-Cy7, eFluor450 CD44 eBioscience IM7 Pacific-Blue, FITC IL-2 eBioscience JES6-5H4 FITC IFN-gamma eBioscience XMG1.2 APC, PE TNF-alpha eBioscience MP6-XT22 APC IL-17A eBioscience eBio17B7 APC IL-17A Biolegend TC11-18H10.1 FITC IL-17F eBioscience eBio18F10 PerCP eFluor710 GM-CSF Biolegend MP1-22E9 PerCP-Cy5.5 CD45 Biolegend 30-F11 PE-Cy7 MHC-II eBioscience M5/114.15.2 eFluor450 Cd11b eBioscience M1/70 APC Gr-1 (Ly6G) eBioscience RB6-8C5 PE Gr-1 (Ly6G) Biolegend 1A8 AlexaFluor647
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines used in the lab are regularly tested for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All mice used in this study and their origins are described in the Materials and Methods section. Stim1fl/fl and Stim1fl/flStim2fl/fl has been described before (Oh-Hora et al., 2008). Stim1fl/fl and Stim1fl/flStim2fl/fl mice were further crossed to Cd4-Cre mice (B6.Cg-Tg(Cd4-cre)1Cwi/BfluJ, JAX strain 22071), Vav-iCre mice (B6.Cg-Commd10Tg(Vav1-icre)A2Kio/J, JAX strain 008610) and Mx1Cre mice (B6.Cg-Tg(Mx1-cre)1Cgn/J, JAX strain 003556) as described (Saint Fleur-Lominy, Maus et al., 2018, Vaeth, Zee et al., 2015). All animals are on the C57BL/6 genetic background and were maintained under SPF conditions in accordance with institutional guidelines for animal welfare approved by the IACUC at NYU School of Medicine.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal work was performed in accordance with institutional guidelines for animal welfare approved by the IACUC at NYU School of Medicine.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Written informed consent was obtained from both patients, in accordance with research ethics board policies at the University of British Columbia and Oregon Health & Science University. Experiments using deidentified cell samples of patients were conducted with Institutional Review Board approval at the New York University School of Medicine.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Written informed consent was obtained from both patients, in accordance with research ethics board policies at the University of British Columbia and Oregon Health & Science University. Experiments using deidentified cell samples of patients were conducted with Institutional Review Board approval at the New York University School of Medicine.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Consent from the patients was obtained. Within the manuscript no pictures are shown that allow identification of the patients.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	RNA Sequencing raw files will be uploaded to the GEO database at the time of paper acceptance.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA