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Lionel Le Bourhis, Emmanuel Martin, Emmanuel Martin, Isabelle Peguillet ...+25 more authors

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Anti-microbial activity of mucosal associated invariant T cells

Lionel Le Bourhis^{1,2}, Emmanuel Martin^{1,2}, Isabelle Péguillet^{1,3*}, Amélie Guihot^{4*}, Nathalie Froux⁵, Maxime Coré^{1,2}, Eva Lévy^{1,2}, Mathilde Dusseaux^{1,2}, Vanina Meyssonier⁴, Virginie Premel^{1,2}, Charlotte Ngo⁶, Béatrice Riteau⁷, Livine Duban^{1,2}, Delphine Robert^{1,3}, Martin Rottman⁸, Claire Soudais^{1,2}, Olivier Lantz^{1,2,3}

¹ *Institut curie, Département de Biologie des Tumeurs, Paris, 75005, France.*

² *INSERM U932, Paris, 75005, France.*

³ *Center of Clinical Investigations CICBT507 IGR/Curie*

⁴ *UPMC Univ Paris 06, INSERM, UMR945, AP-HP, Hôpital Pitié-Salpêtrière, Laboratoire d'Immunologie Cellulaire et Tissulaire, Service des Maladies Infectieuses et Tropicales, F-75013, Paris, France*

⁵ *CNRS, UPS44, TAAM, Institut de Transgenèse, 45071, Orléans cedex 2, France*

⁶ *Institut curie, Département de Chirurgie, Paris, 75005, France.*

⁷ *Unité de Virologie et Immunologie Moléculaires, UR 892, INRA, Domaine de Vilvert, 78352 France.*

⁸ *Laboratoire de Microbiologie, Hôpital R. Poincaré (AP-HP) and EA 3647, University Versailles Saint-Quentin. 92380, Garches, France.*

**IP and AG contributed equally*

Running title: MAIT cell activation by microbial infection

Correspondence to: O. Lantz,

Laboratoire d'Immunologie and Inserm U932, Institut Curie, Paris, France, 26 rue d'Ulm, 75005 Paris.

Phone: 33144324218

Fax: 33144324444

Email: olivier.lantz@curie.net

Abstract

Mucosal associated invariant T (MAIT) lymphocytes are characterized by two evolutionarily conserved features: an invariant TCR α chain and restriction by the MHC-related protein, MR1. Here we show that MAIT cells are activated by cells infected with different strains of bacteria and yeasts, but not viruses, both in human and mouse. This activation requires cognate interaction between the invariant T cell receptor (TCR) and MR1, which can present a bacteria-derived ligand. In humans, we observe a striking diminution of MAIT cell blood-numbers in patients with bacterial infections such as tuberculosis. In mouse, MAIT cells protect against infections by *Mycobacterium* and *Escherichia coli*. Thus, MAIT cells are evolutionarily conserved innate-like lymphocytes that sense and help fight off microbial infections.

‘Innate-like lymphocytes’ display features of both the innate and adaptive immune systems. In particular, B1 B cells^{1, 2}, certain types of $\gamma\delta$ T cells³ and Natural Killer T (NKT) cells⁴ express antigen receptors with limited diversity. These invariant lymphocyte subsets follow specific ontogenic pathways, home to particular tissues and have elevated clonal sizes compared to conventional lymphocytes⁵. These innate cells display immediate effector functions after stimulation, such as antibody production or cytokine secretion^{2, 5}.

Mucosal associated invariant T (MAIT) cells are another lymphocyte subset that expresses an evolutionarily conserved invariant TCR α chain, composed of the *iV α 7.2* segment in humans and *iV α 19* in mice associated with the *J α 33* segment. They are selected on the highly phylogenetically conserved major histocompatibility complex (MHC) class I related molecule, MR1 (90% homologous α 1 and α 2 domains between mouse and human, vs. 60-70% for other MHC class I)^{6, 7}. MAIT cells are abundant in human blood (1-8% of T cells vs. 0.01-1% for NKT cells), the intestinal mucosa and mesenteric lymph nodes (MLN)⁸. Human MAIT cells display a memory phenotype early in life, but are small in numbers and naïve in cord blood, suggesting MAIT cells expand after birth and acquire their memory phenotype in the presence of commensal flora⁸. Supporting this hypothesis, MAIT cells are not detectable in germ-free mice⁷. However, their number is increased in transporter associated with antigen processing and invariant chain double-deficient mice (*Tap^{-/-}Ii^{-/-}*), in which conventional T cell selection is impaired^{7, 9}. Despite the availability of transgenic animals expressing MAIT cell-specific V α 19 and V β 6 chains^{8, 10, 11}, little is known about the function of these cells. However, their location in the gut and their remarkably conserved features in mice and humans, suggest an important role in response to microbes.

Here we show that MAIT cells respond to antigen presenting cells (APC) cocultured with bacteria in an MR1-dependent manner both in humans and mice. This activation is induced by a wide variety of bacteria and yeasts, but not by viruses. APCs acquire their stimulatory capacity very quickly after infection and a cognate interaction between the *i*TCR and MR1 is required. Moreover, this interaction has antibacterial potential *in vivo*, as MR1-deficient animals are more susceptible to *Mycobacterium abscessus* and *Escherichia coli* infection. Finally, in humans, the number of MAIT cells is reduced in peripheral blood from patients with infectious diseases such as tuberculosis, while they are detected at the site of infection. We conclude that MAIT cells are evolutionarily conserved innate-like T cells with anti-microbial properties.

Results

MR1- and bacteria-dependent activation of human MAIT cells

The expansion and acquisition of a memory phenotype by human MAIT cells shortly after birth⁸ suggest that these cells could respond to bacteria. We isolated monocytes from peripheral blood mononuclear cells (PBMCs) and cocultured them with *E. coli*. Autologous sorted-V α 7.2 cells were seeded on these APCs at a 1:1 ratio and cultured overnight. Most MAIT cells, which are V α 7.2⁺CD161⁺ and either CD8 β ^{int} or CD4⁻CD8 β ⁻ double negative cells (DN)⁸, were activated as indicated by CD69 up-regulation (**Fig. 1a**). This activation was specific, as neither V α 7.2⁺CD161⁻ conventional T cells from the same culture (**Supplementary Fig. 1a**), nor V α 7.2⁻ CD4 and CD8 T cells cultured in parallel were activated (**Supplementary Fig. 1b**). MAIT cell activation was modulated by the bacteria:monocytes ratio, the multiplicity of infection (MOI) (**Fig. 1b**), and was completely abrogated by an anti-MR1 antibody (**Fig. 1c**). In contrast to mainstream memory T cells, MAIT cells secreted interferon (IFN)- γ (**Fig. 1d**) after stimulation with *E. coli*-infected monocytes, but not large amounts of interleukin (IL)-2, -4, -5, -13 or -17 (data not shown). Blockade of both CD69 upregulation (**Fig. 1c**) and IFN γ secretion (**Fig. 1e**) by the anti-MR1 antibody indicates that cognate interaction between the TCR and the MHC-Ib molecules is required for MAIT activation.

Decreased MAIT cell blood numbers in bacteria infected patients

The bacterial reactivity of MAIT cells suggests their involvement in anti-bacterial defenses. We observed a significant decrease in MAIT cell proportions and absolute numbers in the blood of patients with pulmonary bacterial pathologies, including tuberculosis, as compared to healthy donors or patients with cancers (**Fig. 2a** and **Supplementary Fig. 2a**). The frequency of $\gamma\delta$ T cells was unchanged while their absolute numbers were slightly decreased compared to healthy controls due to the lymphopenia found in some of these patients (**Fig. 2b** and **Supplementary Fig. 2b**). Taken together, these results indicate that the reduced MAIT cell frequency represents a true decrease in numbers and not a dilution by infection-reactive mainstream T cells. One possibility was that upon infection MAIT cells infiltrate tissues, decreasing their number in blood. To test this hypothesis, we visualized MAIT cells by tissue-immunofluorescence using anti-CD3, anti-V α 7.2 and anti-IL-18R α , the latter of which correlates with CD161 expression (**Fig. 2c**). We observed MAIT cells in the lung lesions from two patients with *Mycobacterium tuberculosis* infection (**Fig 2d**). Similarly,

we obtained ascites fluid from patients with suspected ovarian malignancy. One patient analyzed had a higher MAIT cell number compared to blood and the other ascites (32% vs 0.1-18.6%) (**Fig. 2e**). Strikingly, this patient was subsequently diagnosed with disseminated tuberculosis. These results indicate that human MAIT cells seem to migrate into infected tissues, suggesting that MAIT cells might have anti-microbial functions.

Infected APCs rapidly stimulate mouse MAIT cells

To investigate mouse MAIT cell activation by infected APCs, we cultured bone marrow-derived dendritic cells (BMDCs) from MR1-sufficient (*Mr1*⁺) or -deficient (*Mr1*^{-/-}) animals and cocultured them with *E. coli*. As a source of MAIT cells, we used *iV*_{α19} and *iV*_{α19}-V_{β6} transgenic (Tg) C_α mice, which have elevated frequencies of these cells⁸. T cells from *iV*_{α19}-V_{β6} (**Fig. 3a**) and *iV*_{α19} (**Fig. 3b**) Tg mice strongly up-regulated CD69 expression when co-cultured with infected *Mr1*⁺, but not *Mr1*^{-/-} BMDCs. Similarly to human MAIT cells, only CD8 and DN T cells were activated (**Fig. 3a**). We also observed MR1-dependent CD25 up-regulation (**Fig. 3c**) and low amounts of IL-2 secretion by both *iV*_{α19} single and *iV*_{α19}-V_{β6} double Tg MAIT cells (**Fig. 3d**), consistent with their low proliferation capacities (**Fig. 3e**). Peritoneal macrophages cocultured with *E. coli* were also able to induce MAIT cell activation (data not shown), indicating that mouse MAIT cells respond to various types of infected-APCs in an MR1-dependent manner.

To examine the role of soluble factors in MAIT cell activation, we fixed *Mr1*⁺ or *Mr1*^{-/-} BMDCs with glutaraldehyde at different time points after infection, before adding *iV*_{α19}-V_{β6} Tg T cells. Three hours after infection, *Mr1*⁺ BMDCs were already able to stimulate MAIT cells (**Fig. 4a**), indicating that BMDC very rapidly acquire the MAIT cell stimulatory capacity. This also demonstrates that soluble factors such as cytokines are not necessary for MAIT cell activation.

To further examine the cellular events required for MAIT cell activation, we treated BMDCs with several drugs modifying antigen processing before infection. Treatment of the BMDCs with dynasore, cytochalasin D and chloroquine (inhibitors of endocytosis and/or phagocytosis and endosomal acidification, respectively) strongly inhibited MAIT cell activation (**Fig. 4b**), indicating that bacteria or bacterially-derived compounds need to be internalized and processed. The proteasome inhibitor, lactacystin, did not show any effect (**Fig. 4b**) although it prevented ovalbumin-reactive OT-I TCR-Tg cell activation (data not

shown). Additionally, infected BMDC derived from *Tap^{-/-}Ii^{-/-}* animals activated MAIT cells equally well compared to wild-type *Tap^{+/+}Ii^{+/+}* cells (**Fig. 4c**). Taken together, these results indicate that MAIT cell activation by BMDCs is independent of conventional MHC class I and class II antigen presentation pathways.

Conserved interactions between invariant-TCR and MR1

To study the evolutionary conservation of the TCR-MR1 interactions, we tested if human MAIT cells are activated by infected mouse APCs and *vice versa*. Sorted human MAIT cells were activated by *E. coli* infected APCs from *Mr1⁺* but not *Mr1^{-/-}* mice (**Fig. 4d**). Similarly, mouse hybridomas (6C2 and 8D12) expressing the *iV_α19-V_β8* chains were stimulated by infected human monocytes as indicated by IL-2 secretion, which was completely abrogated by the addition of anti-MR1 (**Fig. 4e**). These results indicate that mouse and human MAIT cell TCRs interact similarly with MR1 on infected APCs.

Looking at the TCR repertoire involved in MR1 reactivity, we observed that in *iV_α19* single Tg mice, most CD8⁺ and DN T cells expressing the V_β6 or V_β8 segments (known to be preferentially found in MAIT cells⁸) responded to *Mr1⁺*, but not to *Mr1^{-/-}* infected BMDCs (**Fig. 3b**). In contrast, 80% of the CD8⁺ and DN T cells expressing neither of these V_β segments did not respond (**Fig. 3b**), excluding the possibility of an as yet unknown *iV_α19*-binding super-antigen. Additionally, MAIT cells did not require selection by MR1, but only expression of permissive V_β segments in association with the *iV_α19* TCR α chain. Indeed, T cells from *iV_α19-V_β6* double or *iV_α19* single Tg MR1-deficient mice were activated by *E. coli* infected BMDCs (**Supplementary Fig. 3a & 3b**). Finally, 10% of DN T cells from *Tap^{-/-}Ii^{-/-}* V_β6 single Tg mice were activated by infected BMDC in an MR1-dependent manner (**Supplementary Fig. 3c**) in line with our previous *iV_α19* quantification by RT-QPCR⁸. Thus, bacteria-infected APCs activate all mouse MAIT cells through MR1, regardless of the fine specificity imparted by the TCR β chain indicating that expression of the *iV_α19* TCR α chain together with a permissive V_β chain is sufficient to recognize MR1 on infected APCs.

MAIT cells sense a wide variety of microbes

These results suggest an extreme conservation of the bacterial component(s) involved in MAIT cell activation. To begin this characterization, we used heat-killed or paraformaldehyde (PFA)-fixed bacteria. Live bacteria were more efficient than dead microbes at inducing a MAIT cell response (**Fig. 5a**), but PFA-treated bacteria were able to trigger better activation

than heat-killed organisms (**Fig. 5a**), indicating that the compound responsible for MAIT activation is partially heat-labile but preserved by fixation.

We then tested if one specific class of bacteria induces MAIT cell activation. Gram-negative bacilli *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, Gram-positive *Lactobacillus acidophilus* and Gram-positive cocci *Staphylococcus aureus* and *Staphylococcus epidermidis*, all induced a strong response by both $iV_{\alpha}19$ - $V_{\beta}6$ and $iV_{\alpha}19$ Tg MAIT cells (**Fig. 5b** and data not shown, respectively). Surprisingly, *Enterococcus faecalis* and *Streptococcus* Group A, two bacteria closely related to *Staphylococci*, did not induce MAIT cell activation, even though all bacteria induced effective BMDC activation (data not shown). We obtained similar activation patterns for human MAIT cells by monocytes for the same panel of bacteria (data not shown). As *Streptococci* and *Staphylococci* share many microbial-associated molecular patterns (MAMPs)^{12, 13}, these results suggest that some bacteria provide the necessary trigger for MAIT cell response while others do not. Alternatively, *E. faecalis* and *Streptococci* could actively inhibit the presentation of MR1 and its ligand. However, this hypothesis is refuted by the fact that PFA-fixed *E. faecalis* still do not induce MAIT cell activation (**Fig. 5c**) and infections with a mixed suspension of PFA-killed *E. coli* and *E. faecalis* in a 1 to 10 ratio still do (**Fig. 5c**). These data strongly suggest that *E. faecalis* lacks the necessary compound for MR1-dependent MAIT activation.

To address the specificity of MAIT cell responses to bacteria, as compared to other microbes, we tested viruses and yeasts in our *in vitro* model. *Saccharomyces cerevisiae*, *Candida glabrata* and *Candida albicans* infected BMDCs were able to induce a strong MAIT response in an MR1-dependent manner (**Fig. 5d**). On the other hand, several unrelated viruses (including Encephalomyocarditis, Sendai, Newcastle disease, Herpes simplex and Parainfluenza3 viruses), despite an ability to activate BMDC, failed to activate MAIT cells (**Supplementary Fig. 4**). These results suggest that MAIT cells respond preferentially to bacteria and yeasts.

MAIT cells respond to a conserved exogenous antigen

All these microbes can stimulate APCs by innate receptors such as Toll-like receptors (TLR) and Nod-like receptors (NLR) through recognition of common MAMPs. We tested if one of the TLR ligands could recapitulate bacteria-driven MAIT cell activation. Despite efficient activation of the BMDCs by Pam3Cys, polyIC, lipopolysaccharide (LPS) or CpG, (ligands of TLR2, 3, 4 and 9, respectively¹⁴) (**Supplementary Fig. 5a**), no MR1-dependent activation was detected on $iV_{\alpha}19$ - $V_{\beta}6$ Tg MAIT cells (**Supplementary Fig. 5b**). We obtained

similar results using human monocytes and autologous MAIT cells (data not shown). We then used *Myd88*^{-/-}, *Trif*^{-/-} or *Myd88*^{-/-}-*Trif*^{-/-} BMDCs, which are impaired in their response to many TLR ligands and IL-1 β ^{15, 16, 17}, to study the response towards whole bacteria. *iV α 19-V β 6* Tg MAIT cells were still receptive to stimulation by infected BMDCs from all knockout genotypes, although less efficiently compared to wild-type cells for MyD88-deficient BMDCs (**Supplementary Fig. 6a**). This reduction could be explained by impaired cellular activation of the BMDCs as indicated by lack of CD40 up-regulation (**Supplementary Fig. 6b**). Several other innate pathways were also not involved because MAIT cell activation was unaffected when using BMDCs from mice deficient for *Nod1*- and *Nod2*^{-18, 19}, *Nlrp3*- and *Asc*⁻²⁰⁻²², or *Ips1*⁻²³⁻²⁵ (**Supplementary Fig. 6a and 6b**). These results indicate that DC-activation through the main microbial recognition pathways is not sufficient for MAIT cell activation.

Both human (**Fig. 1a**) and mouse *iV α 19* Tg (**Fig. 2b**) MAIT cells were activated by autologous *E. coli*-infected APCs. However, previous reports show these cells do not react to transduced cell-lines expressing high amounts of human or mouse MR1 at their cell surface^{26, 27}. This discrepancy strongly suggests that a ligand, presented by MR1, is provided during the APC-bacteria coculture and is necessary for MAIT activation. Therefore, we tested the capacity of cell lines to activate MAIT cells after bacterial coculture. WT3 mouse fibroblasts co-cultured with *E. coli*, which does not invade this cell type, could activate purified *iV α 19* and *iV α 19-V β 6* Tg T cells (**Fig. 6a** and **Supplementary Fig. 7a**, respectively). This activation was significantly increased when using cells over-expressing mMR1 (WT3-mMR1) and was completely blocked by anti-MR1 antibody, indicating that this response depends on MR1 expression by the WT3 cells. We obtained similar results using human MAIT cells stimulated by HeLa cells expressing human MR1 (**Supplementary Fig. 7c**). To test for the presence of an exogenous ligand, we fixed WT3 and WT3-mMR1 cells with glutaraldehyde prior to bacterial co-culture so that no processing and/or upregulation of an endogenous ligand could occur. We observed a significant proportion of *iV α 19* and *iV α 19-V β 6* Tg MAIT cells activated by WT3-mMR1 but not WT3 cells (**Fig. 6b** and **Supplementary Fig. 7b**). The activation of *iV α 19* Tg cells by WT3-mMR1, even fixed, in the presence of bacteria, indicates that MAIT cells recognize an exogenous ligand presented by MR1.

MAIT cells have an antibacterial function *in vivo*

The reactivity of MAIT cells towards cells co-cultured with bacteria, the acquisition of a memory phenotype after birth in human and their absence in germ-free mice⁷ suggest that

colonization by the commensal flora induces expansion of these T cells *in vivo*. To test this hypothesis, we reconstituted germ-free animals with mono-microbial flora. Consistent with our previous findings⁷, *iV α 19-J α 33* mRNA levels in the mesenteric lymph nodes (MLN) of *Tap^{-/-}Ii^{-/-}MrI⁺* germ free and *Tap^{-/-}Ii^{-/-}MrI^{-/-}* mice were comparable (**Fig. 7a**). Feeding germ-free *Tap^{-/-}Ii^{-/-}* animals with a single strain of bacteria, including either *Enterobacter cloacae*, *Lactobacillus casei*, *Bacteroides thetaiotaomicron* or *Bifidobacterium animalis*, increased *iV α 19-J α 33* mRNA levels in the MLN back to those found in conventional *Tap^{-/-}Ii^{-/-}MrI⁺* mice (**Fig. 7a** and data not shown), demonstrating that single microbial flora are sufficient to induce MAIT cell expansion. In contrast, feeding *E. faecalis* failed to generate a detectable MAIT population (**Fig. 7a**), further suggesting that this bacterium does not provide the ligand necessary for MAIT cell activation.

We then examined whether MAIT cells display anti-bacterial properties *in vivo*. We followed MAIT cell activation and *E. coli* clearance after intraperitoneal injection. Both *iV α 19* and *V β 6 Tg MrI^{-/-}* mice harbored elevated colony forming units (CFUs) in the spleen 3 days after infection, as compared to their *MrI⁺* counterparts (**Fig. 7b**). *iV α 19 Tg* mice exhibited better protection compared to *V β 6 Tg* mice, correlating with higher MAIT numbers⁸. T cells in *iV α 19 Tg MrI⁺* animals accumulated in the peritoneal cavity (**Fig. 7c**) and up-regulated CD25 surface expression (**Fig. 7d**). Moreover, in an *MrI⁺* background, T cells expressing the *V β 6* or *V β 8* segments displayed higher CD25 expression compared to cells expressing neither of these *V β* segments (**Fig. 7d**), further suggesting activation is MAIT cell-specific. Furthermore, consistent with our *in vitro* data (**Supplementary Fig. 5**), MR1-deficient mice were not more sensitive to influenza viral infection than wild-type (WT) mice (data not shown). Taken together, these results indicate that MAIT cells are recruited and activated at the site of infection and have anti-bacterial properties.

We next examined the role of MAIT cells in a mycobacterium infection model using *Mycobacterium abscessus*²⁸. *M. abscessus*-infected BMDCs strongly stimulated mouse MAIT cells in a dose- and MR1-dependent manner (**Fig. 8a**), while *MrI⁺* and *MrI^{-/-}* BMDCs were activated similarly by the bacteria (data not shown). We injected intravenously 10⁶ bacteria into non-transgenic, *iV α 19* or *V β 6 Tg* mice, as well as *Tap^{-/-}Ii^{-/-}* and *Cd3^{-/-}* mice on either MR1-sufficient or -deficient backgrounds. *iV α 19-MrI^{-/-}* and *V β 6-MrI^{-/-}* Tg mice harbored higher bacterial load in the spleen 15 days after infection than their *MrI⁺* littermates (**Fig. 8b**). No significant difference was observed between *MrI⁺* and *MrI^{-/-}* non-transgenic animals. *Tap^{-/-}Ii^{-/-}* animals (that have impaired conventional T cell and increased MAIT cell numbers),

exhibited increased bacterial burdens compared to wild-type mice but even more so in the absence of MR1 suggesting that the increased proportion of MAIT cells found in these mice leads to some protection. Similarly, *Cd3^{-/-}* animals had an impaired response to *M. abscessus* compared to WT, however no difference could be seen between *Mr1⁺* and *Mr1^{-/-}* mice in this genotype confirming that the protection observed is T cell mediated. Altogether, these data indicate an important role for MAIT-mediated immune responses against mycobacterium infections.

Discussion

MAIT cells are highly conserved between species and very abundant in humans, but no function has yet been described. We now show that human and mouse MAIT cells respond to bacteria- or yeasts-infected APCs in an MR1-dependent manner. MAIT cells react to APCs cocultured with various bacteria including *Enterobacteriaceae*, *Staphylococci* and *Mycobacterium*, but not *Streptococci* or *E. faecalis*. Activation is independent of the classical MHC class I and II pathways and from the main innate immune receptors. In mice, MAIT cell activation mediates antibacterial functions in two different infection models. Finally, MAIT cell frequencies are significantly decreased in the blood of bacterially-infected patients. This observation can be explained by the migration of these cells into infected tissues suggesting that, like in mice, human MAIT cells play a role in the antibacterial response.

The acquisition of memory phenotype by human MAIT cells after birth suggested that the commensal flora induces MAIT cell maturation and expansion. In mice, MAIT cells are naïve and in small numbers⁸, which could result from a missing genetic element arising from the genetic bottleneck of laboratory mice or from the cleanliness of mouse facilities. We show that MAIT cells are absent in germ-free mice but can be recovered after colonization with bacteria, clearly indicating an interaction between MAIT cells and the commensal flora. Hence, the gut flora could drive the expansion of MAIT cells directly by MR1-dependent interactions or indirectly through the constitution of trophic niches. The fact that human and mouse MAIT cells are activated by ubiquitous bacteria does not exclude that, *in vivo*, the expansion of MAIT cells could be driven by one particular undefined microbe with special properties, as observed in the case of *Bacteroides fragilis*²⁹ or segmented filamentous bacteria^{30,31}.

The wide specificity of MAIT cells for phylogenetically distant microbial entities, and the observed cross-reactivity between human and mice, suggest that MAIT cells recognize a conserved antigen. Human MAIT cells did not react against cells over-expressing human MR1²⁶ suggesting that a ligand might have been missing in the MR1 groove. We now find that human MAIT cells can recognize MR1 expressed on APC or hMR1 over-expressing fibroblasts cocultured with bacteria. Similarly, V β 6⁺ and V β 8⁺ T cells from *iV α 19* single Tg mouse recognize mMR1 on infected mouse APC but not on uninfected MR1-transduced cell lines. Altogether, these results suggest that human and mouse MR1 present a ligand, which is either provided or induced by the bacteria.

The characterization of the ligand presented by MR1 is the next main challenge in MAIT cell research. Previous reports have proposed that MAIT cells were activated by a

lipidic compound, α -Mannosyl-ceramide, presented by MR1³². We could not reproduce this data using this compound in our experimental settings (data not shown). We also failed to see any effect of N-butyldexynojirimycin, a drug that inhibits glycolipid biosynthesis³³ (data not shown) arguing against the possibility of a lipidic ligand loaded on MR1. Even though the human MAIT cell population is oligoclonal⁸, most of these cells are activated by various types of bacteria and yeasts, suggesting the absence of fine TCR specificity towards various unrelated microbes. One possible explanation would be that all these microbes induce the upregulation of an endogenous ligand, which might be also necessary for thymic selection. However, our observations that fixed cells expressing MR1 can activate MAIT cells, in the presence of bacteria, indicates the existence of an exogenous antigen. This ligand could be either of multiple composition, but the MR1-*i*TCR interaction would be non-discriminative, or an extremely conserved compound among microbes.

The existence of a highly conserved microbial-derived product inducing immunological responses is reminiscent of the ligands of the innate immune system^{34, 35}. It is therefore surprising that none of the tested cells deficient for major innate immune pathways (MyD88, Trif, Nod1 and/or Nod2, Nlrp3 or Asc, Ips1) impacts strongly on MAIT cell activation. Also, pure TLR ligands could not recapitulate the activation triggered by whole bacteria. Thus, cellular activation alone cannot trigger the APC to become competent to activate MAIT cells. These results contrast with NKT cell activation where TLR-9 and -4 ligands induce the upregulation of an endogenous ligand loaded on CD1d^{36, 37}.

B cells have been implicated in MAIT cell expansion in the periphery^{7, 8}. However, to date, there are no satisfactory results showing a direct activation of MAIT cells by B cells. Further experiments are necessary to formally address this question. Nonetheless, we show that MAIT cells can be activated by various types of cells (DCs, macrophages, epithelial and fibroblastic cells), suggesting that the pathway necessary for MR1 processing and loading are broadly expressed. TAP and the Ii chain, essential players in the MHC class I and class II pathways, are not required for MAIT cell ontogeny^{7, 9}. In this study, we establish that they are also not necessary for MR1-dependent activation. Studies have reported that some exogenous antigen processing could occur in the absence of TAP, a process named vesicular processing^{38, 39}. MR1 could utilize such a pathway to stimulate MAIT cells after bacterial triggering. Our results showing that fixed cells over-expressing MR1 can activate MAIT cells suggest that a bacterial product can be directly loaded on the MR1 molecule without processing. However, such circumstances are non-physiological because MR1 expression at the cell surface has not been detected in non-transduced settings²⁶. Accordingly, MR1 loading

with the putative ligand could occur in a specific compartment of the endosomal pathway. The microbial trigger could induce the production and trafficking of MR1 through this compartment en route to the cell membrane similarly to what has been described for the MHC class Ib molecules H2-M3^{40, 41}.

MAIT cells have a clear antibacterial function that could be attributed to the production of IFN- γ ⁴². The concentrations are moderate compared to conventional memory T cells stimulated with CD3 and CD28 antibodies. However, the absence of conventional CD4 and CD8 T cell activation with infected APCs is striking. In mice, increased MAIT numbers correlates with increased protection against infection of *E. coli*. In the *M. abscessus* infection model, we observe no significant difference between *Mr1*⁺ and *Mr1*^{-/-} non-transgenic mice that have low MAIT frequency, while we do in V α 19 and V β 6 transgenic mice that have increased MAIT numbers, closer to what is observed in humans. We can speculate that MAIT cell function resides in the prompt response of these cells at the site of infection. MAIT cell ontogeny and their ability to respond to a wide variety of bacteria including commensals in the gut might suggest a dual role; in defense against infections, depicted in the present study, and possibly in mucosal homeostatic mechanisms.

The results presented here show that MAIT cells respond to microbes and participate in anti-bacterial immune responses in both human and mouse. MAIT cells represent an evolutionarily conserved innate-like lymphocyte population that senses and participates to immune responses against microbes. MAIT cells represent the first T cell population that distinguishes between microbial and viral challenges. Considering the abundance of this cell type in humans, their wide microbial specificity and their protective capacity, MAIT cell manipulation could have significant impact on vaccine and therapeutic drug development against infectious diseases.

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Author Contributions

LLB, EM, MC, EL and CS performed most experiments. MR provided *M. abscessus* strain and performed *M. abscessus* experiments with CS. AG, VM and CN provided patient samples; IP, DR and VP analyzed them. NF managed germ free mice and performed the reconstitution experiments with LD and EM. BR performed *in vivo* flu experiments. LLB, EM, CS and OL designed experiments. LLB, CS and OL wrote the manuscript.

Figure legends

Figure 1: Human MAIT cells respond to bacteria-infected monocytes in an MR1-dependent manner and are decreased in the blood of bacterial infected patients.

(a) $V_{\alpha}7.2^{+}$ sorted cells activation by *E. coli* infected APCs. Gray: non-infected. Black: infected. Representative of more than 5 experiments.

(b) Activation of human MAIT cells by monocytes cocultured with increasing MOI: 0 (gray), 0.1 (thin black), 1 (heavy gray) and 10 (heavy black). Representative of 3 experiments.

(c) Blockade of sorted $V_{\alpha}7.2^{+}$ cell activation by 26.5 anti-MR1 antibody (red) or isotype control (black). Gray: non-infected. Representative of 3 experiments.

(d) Production of IFN- γ by flow-sorted memory ($CD45RO^{+}$) CD4 and CD8 conventional and MAIT ($V_{\alpha}7.2^{+}CD161^{+}$) T ($CD3^{+}$) cells after 48 hour co-culture with autologous monocytes infected with *E. coli* at various MOI (0: white, 1: light gray, 10: gray and 100: dark gray) or monocytes alone (-). Anti-CD3/CD28 stimulated T cells (black) are shown as controls. Mean and SEM of 2 different donors. Representative of 3 experiments.

(e) Blockade of IFN- γ production by FACS sorted MAIT cells as in (d) with or without blocking anti-MR1 antibody in control condition (0) and with *E. coli* infection (MOI of 10). Mean and SEM of 3 independent experiments (* $p < 0.01$, paired t-test).

Figure 2: Human MAIT are decreased in the blood of bacterially infected patients.

(a) Proportion of MAIT ($V_{\alpha}7.2^{+}CD161^{+}CD3^{+}TCR\gamma\delta^{-}$ cells) and (b) $\gamma\delta$ T cells in the blood of healthy donors or patients with the indicated pathology. (* $p < 0.001$, NS: non-significant. Mann-Whitney test)

(c) Human T cells were gated on $CD3^{+}V_{\alpha}7.2^{+}CD4^{-}$ cells. $CD161^{+}$ cells (defined previously as MAIT cells) correlated well with $IL-18R\alpha^{+}$ cells.

(d) *M. tuberculosis* infected lung H&E and immunostaining. Arrows indicate triple-stained MAIT cells.

(e) Representative plot of indicated tissues. Selected plot representative of the geometric median indicated above the gate and numbers below indicate range of percent cells in gate for each group for the indicated number of patients.

Figure 3: T cells from mouse transgenic (Tg) for the specific MAIT TCR V_{α} and/or V_{β} chains respond to *E. coli* infected APCs through MR1.

(a) Activation of mouse $iV_{\alpha}19-V_{\beta}6$ Tg MAIT cells by bacteria-infected $Mr1^{+}$ or $Mr1^{-/-}$

BMDCs. Gray: non-infected; Black: infected. Representative of more than 5 experiments.

(b) Activation of *iV α 19* transgenic T cells according to V β 6 or V β 8 segment expression. Gray: non-infected; Black: infected. Representative of more than 5 experiments.

(c) T cells as in **(a)** co-stained for CD69 and CD25 after overnight co-culture with non-infected (0) or infected (10) *MrI*⁺ or *MrI*^{-/-} BMDCs. Representative of more than 5 experiments.

(d) IL-2 production by MAIT cells after stimulation by infected *MrI*⁺ (closed triangle) or *MrI*^{-/-} (open triangle) BMDCs. Representative of 2 experiments. Mean and SEM are plotted. (*p<0.05, ANOVA)

(e) *iV α 19-V β 6* bead-sorted MAIT cells were CFSE-stained and stimulated as in **(a)**. Activation assayed by CD25 up-regulation and proliferation by CFSE dilution was assessed at day 2 and 5. Representative of 2 experiments.

Figure 4: MAIT cell activation requires a highly conserved cognate interaction between MR1 and the TCR, as well as internalization of exogenous compounds but is independent of classical MHC class I and class II presentation pathways.

(a) *MrI*⁺ and *MrI*^{-/-} BMDC were fixed at the indicated time points after infection before MAIT cell (*iV α 19-V β 6 Tg*) overnight co-culture. Gray: non-infected. Black: infected. Representative of 2 experiments.

(b) *E. coli* (*E.c.*) infected (black) or non-infected (white) BMDCs were treated with indicated drugs or left untreated before infection and stimulation of MAIT cells. Representative of 3 experiments. (*p<0.05, Mann-Whitney test)

(c) BMDC from WT (upper panels) or *Tap*^{-/-}*Ii*^{-/-} mice (lower panels) in a *MrI*⁺ (left) or *MrI*^{-/-} (right) background were infected (black) or left uninfected (gray) before overnight co-culture with responding T cells. Representative of 2 experiments.

(d) Human monocytes (hMono) or mouse *MrI*⁺ (*mMrI*⁺) or *MrI*^{-/-} (*mMrI*^{-/-}) macrophages were infected with *E. coli* at the indicated MOI. Sorted human MAIT cells were seeded on these three populations and activation assessed after overnight co-culture by CD69 staining. Representative of 2 experiments.

(e) Human monocytes were infected as indicated in **(a)** and hybridomas expressing mouse MAIT specific TCR used as responding cells. Activation was assessed by IL-2 secretion. Representative of 2 experiments.

Figure 5: MAIT cells respond to phylogenetically diverse microbes.

(a) Activation of $iV_{\alpha}19-V_{\beta}6$ Tg T cells by BMDC cultured with live- (closed triangle) or paraformaldehyde (PFA)-treated- (closed circle) or heat-killed-(HK) (closed square) *E. coli* or non-infected control (open diamond). Representative of 2 experiments.

(b) *E. coli* (closed triangle, Ec), *P. aeruginosa* (closed inverted triangle, Pa), *K. pneumoniae* (closed square, Kp), *L. acidophilus* (closed circle, La), *S. aureus* (open square, Sa), *S. epidermidis* (open circle, Se), *Streptococcus* group A (open triangle, SA), and *E. faecalis* (inverted open triangle, Ef) were tested for their ability to activate $iV_{\alpha}19-V_{\beta}6$ Tg T cells. Non-infected controls (open diamond). Representative of 3 experiments.

(c) BMDCs from wild type or MR1-deficient mice were infected with mixed suspensions of PFA-fixed bacteria at different ratios of *E. coli* : *E. faecalis*, while the total MOI remained constant at 1 or 10. Bacteria were fixed with PFA to avoid differential growth rates by the two different strains. $iV_{\alpha}19-V_{\beta}6$ Tg T cell activation by this APCs was assessed by CD69 and CD25 up-regulation. Representative of 3 experiments.

(d) *C. albicans* (closed circle, Ca), *C. glabrata* (closed diamond, Cg), and *S. cerevisiae* (closed square, Sc) infected-BMDC were fixed 4 hours after infection and $iV_{\alpha}19-V_{\beta}6$ Tg T cells added for overnight stimulation. Non-infected controls (open diamond). Representative of 3 experiments.

Figure 6: Fibroblastic cells can induce MAIT cell activation through MR1, which can present an exogenous bacteria-derived ligand.

(a) The mouse fibroblastic cell line WT3, and the stably-transfected cell line over-expressing mouse MR1 (WT3-mMR1) were co-cultured with various MOI of *E. coli* to serve as APC to $iV_{\alpha}19$ Tg T cells. MAIT activation was assessed by CD69 and CD25 up-regulation in the presence or absence of blocking anti-MR1 antibody ($\tilde{\alpha}$ MR1). Pool of 4 independent experiments. Bars represent mean and SEM; * $p < 0.05$, paired t-test.

(b) WT3 and WT3-mMR1 cells were fixed by glutaraldehyde prior to co-culture with *E. coli* at the indicated MOI and extensively washed after 3 hours of stimulation. Then, $iV_{\alpha}19$ Tg T cells were seeded on the cell layer and activation assessed by CD69 and CD25 up-regulation. Pool of 4 independent experiments. Bars represent mean and SEM; * $p < 0.05$, paired t-test.

Figure 7: MAIT cell bacteria-dependent expansion, activation at the site of infection and anti-bacterial function.

(a) Mono-microbial flora infection allows reconstitution of MAIT cell numbers in germ-free mice. $iV_{\alpha}19$ mRNA levels in the MLN of MrI^+ or $MrI^{-/-}$ mice in a $Tap^{-/-}Ii^{-/-}$ background. Germ-free $MrI^+Tap^{-/-}Ii^{-/-}$ mice were reconstituted with the indicated bacteria: *E. cloacae* (Ec), *L. acidophilus*, (La) and *E. faecalis* (Ef). Individual mice were analyzed in different batches and pooled data are presented. (* $p < 0.05$; Student's t-test) $n = 4$ to 6 per group.

(b) Lower bacterial counts in mice over-expressing MAIT cells after 10^7 CFU *E. coli* intra-peritoneal injection. CFU counts in the spleen of $iV_{\alpha}19$ or $V_{\beta}6$ transgenic mice in an MrI^+ (closed triangle) or $MrI^{-/-}$ (open triangle) background. Bars represent Median. Pooled data of 2 independent experiments. (* $p < 0.05$; ** $p < 0.01$; Mann Whitney test). $n = 8$ to 16 per group.

(c) MAIT cells accumulate at the site of infection. Total T cell, $V_{\beta}6^+$ or $V_{\beta}8^+$, and $V_{\beta}6^-$ and $V_{\beta}8^-$ cell counts in the peritoneal lavage of infected or uninfected (Uninf.) MrI^+ and $MrI^{-/-}$ mice. Representative of 3 independent experiments (* $p < 0.01$; Mann Whitney test). $n = 3$ to 4 per group.

(d) Proportion of activated (CD25⁺) cells in uninfected (Uninf.: diamond), MrI^+ (closed triangle) or $MrI^{-/-}$ (open triangle) mice, according to V_{β} expression: $V_{\beta}6^+$ or $V_{\beta}8^+$ cells and $V_{\beta}6^-V_{\beta}8^-$ cells. Representative of 2 independent experiments (Bars represent mean; * $p < 0.01$; Mann Whitney test) $n = 2$ to 12 per group.

Figure 8: MAIT cells anti-mycobacterium activity.

(a) BMDCs from MrI^+ or $MrI^{-/-}$ mice were infected with *M. abscessus* at various MOI. MAIT cell activation was assessed by CD69 up-regulation after overnight culture. Representative histogram plots and dose response out of 5 experiments.

(b) *M. abscessus* CFU counts in the spleen of wild type (WT), $iV_{\alpha}19$ or $V_{\beta}6$ Tg and $Tap^{-/-}Ii^{-/-}$ or $Cd3^{-/-}$ deficient mice in an MrI^+ (closed triangle) or $MrI^{-/-}$ (open triangles) background, as well as B6 controls (closed square) 15 days after i.v. injection of 10^6 *M. abscessus*. Pooled data of 2 independent experiments. Bars represent Median. (* $p < 0.05$ Mann Whitney test).

Methods

Mice:

Mice used in this study have been described previously⁸. Briefly, V β 6 Tg mice were generated on the B6 background and *iV α 19* Tg mice and MR1 deficient mice are backcrossed onto B6 background for more than 10 generations. TAP-Ii double deficient animals are on a mixed B6/129 background. All *iV α 19* transgenic animals are on a C α deficient B6 background to avoid endogenous V α expression. All experiments were conducted with littermates.

In addition, *MyD88*-, *Trif*-, *Nod1*-, *Nod2*-, *Nlrp3*-, *Asc*-, *Ips1*- knockout bone-marrow were kindly provided by Michel Chignard, Lena Alexopoulou, Dana Philpott, Jurg Tschopp and Mathew Alberts, respectively and have been described elsewhere^{15, 20, 43-45}. All mice were housed in our accredited SPF colony and genotyped by PCR or FACS staining, as appropriate. Live animal experiments were done in accordance with the guidelines of the French Veterinary Department.

Germ-free mice were housed at the (TAAM, CNRS, Orléans, France) and were fed with single bacteria as indicated. Colonization was verified several times during the experiments. Two to 3 months after reconstitution, MLN were harvested and MAIT cell numbers were measured by real time RT-QPCR as described⁷.

Cell preparation:

Cell suspensions were prepared from spleen, peripheral or MLN by mechanical disruption on cell strainers.

Cells were cultured in DMEM+Glutamax supplemented with 10% FCS, Penicillin and Streptomycin, non-essential amino-acids, HEPES and sodium pyruvate (all from Gibco).

BMDC were prepared as previously described⁴⁶. Briefly, bone marrow was obtained from legs of 6-12 weeks old mice and cultured for 7 days in the presence of 20% J558 culture supernatant or 20ng/mL recombinant GM-CSF (Peprotech). Cells were fed at day 3 and 5 with fresh media.

For *in vitro* T cell activation, splenocytes were incubated with magnetic beads (anti-CD11c and anti-CD19. anti-CD4 where added for cytokine secretion experiments) prior to magnetic separation using the MACS Pro system according to the manufacturer recommendation (Miltenyi). Purity and yield of the depletion or enrichment were checked by FACS analysis.

The cell lines WT3 and WT3-mMR1 (stably over-expressing the mouse MR1 molecule) have been described elsewhere²⁷.

Human samples

Blood samples were obtained from healthy donors from the blood bank in accordance with institutional regulations.

PBMCs were obtained using a standard Ficoll gradient according to the manufacturer protocol (GE healthcare). Monocytes were isolated by adherence on plastic culture plates. MAIT cells were isolated by MACS sort using the biotinylated anti-V α 7.2 antibody and anti-biotin magnetic beads (Miltenyi) according to the manufacturer specifications.

Patient blood samples were obtained as leftover after hematological analysis from patients treated for infectious diseases in department of immunology of la Pitié-Salpêtrière Hospital. All patients with a known immunodeficiency or low CD4 counts were excluded from the analysis. Similarly, blood was obtained from patients followed for various solid tumors at Institut Curie and who had not received previous treatments (no chemo- nor radio-therapy). In our institution, all patients are informed that pathological specimens may be used for research purposes.

Microbes, infection and activation

All bacteria were kindly provided by the clinical microbiology laboratories of the Curie Institut and are ATCC reference strains. Bacteria were cultured overnight in Luria broth at 37°C, washed in PBS and diluted according to needs. Except *M. abscessus*, strain CIP 104536T-R, grown and used as previously described²⁸. Where indicated, bacteria were fixed in 1% PFA for 5 min and then washed extensively before use.

For *in vitro* infection, cells were washed and put in DMEM without supplement. Dilutions of bacteria in DMEM were added for 3 hours or other periods of time where indicated. At this point cells were washed two times in DMEM, 10% FCS, supplemented with penicillin and streptomycin. Purified T cells were added for an overnight co-culture or longer where indicated, then cells were harvested and stained for FACS analysis.

For drug treatments, cells were cultured for 1h in medium supplemented with Cytochalasin D (5 μ g/mL), Dynasore (80 μ M), Lactacystin (5 μ M) or DMSO and Chloroquine (50 μ M) or left untreated, prior to infection. After 3 hours of infection cells were washed. Where indicated, APCs were fixed with 0.05% glutaraldehyde for 2 min and quenched with

glycine and thoroughly washed with complete medium before adding responder cells or bacteria.

For *in vivo* infection, mice were injected i.p. or i.v. with 10^7 *E. coli* and 10^6 *M. abscessus* respectively, in PBS.

Flow cytometry

Flow cytometry was performed with directly conjugated antibodies (BD Pharmingen) according to standard techniques with analysis on a FACS Aria and LSRII flow cytometers (Becton Dickinson). DAPI and a 405 nm excitation were used to exclude dead cells. The following antibodies, mostly from BD Pharmingen or eBiosciences, were used in mice: anti- $V_{\beta}6$ -PE or FITC (RR4-7), anti- $V_{\beta}8$ -PE or FITC (F23.1) anti-CD44-PE or APC (IM-7), anti- TCR_{β} -PC5 (H57-597), anti-CD8 $_{\alpha}$ -APC-Cy7 (Ly-2), anti-CD4-Alexa-700 (L3T4), anti CD69-PE-Texas-Red or APC (H1.2F3), anti-CD25-PE-TexasRed (PC61), anti-CD11b-APC (MI/70), anti-CD40-PE (3/23), anti-CD11c-APC-Cy7 (HL3) and anti-CD86-PE (6L1).

For human cell staining, the following were used: anti-CD4-APC-Cy7 (RP4-T4), anti-CD3 ϵ -Alexa 700 (UCHT1), anti- $TCR_{\gamma\delta}$ -PC5 (IMMU510), anti-CD8 β -PE Texas Red (2ST8.5H7), anti-CD45RO-Fitc (UCHL1), anti-CD161-APC (DX12), anti-CD69-APC (CH/4) and anti-CD11b-FITC (B-Ly6). The antibody $V_{\alpha}7.2$ (3C10) has been described elsewhere⁸.

For quantification of cytokines, CBA (BD biosciences) technology was used according to the manufacturer specifications.

Statistical analysis:

All quantitative data were analyzed on Prism software using paired, unpaired t-test, non-parametric (U Mann-Whitney) or ANOVA where indicated.

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Figure 1 Le Bourhis et al.

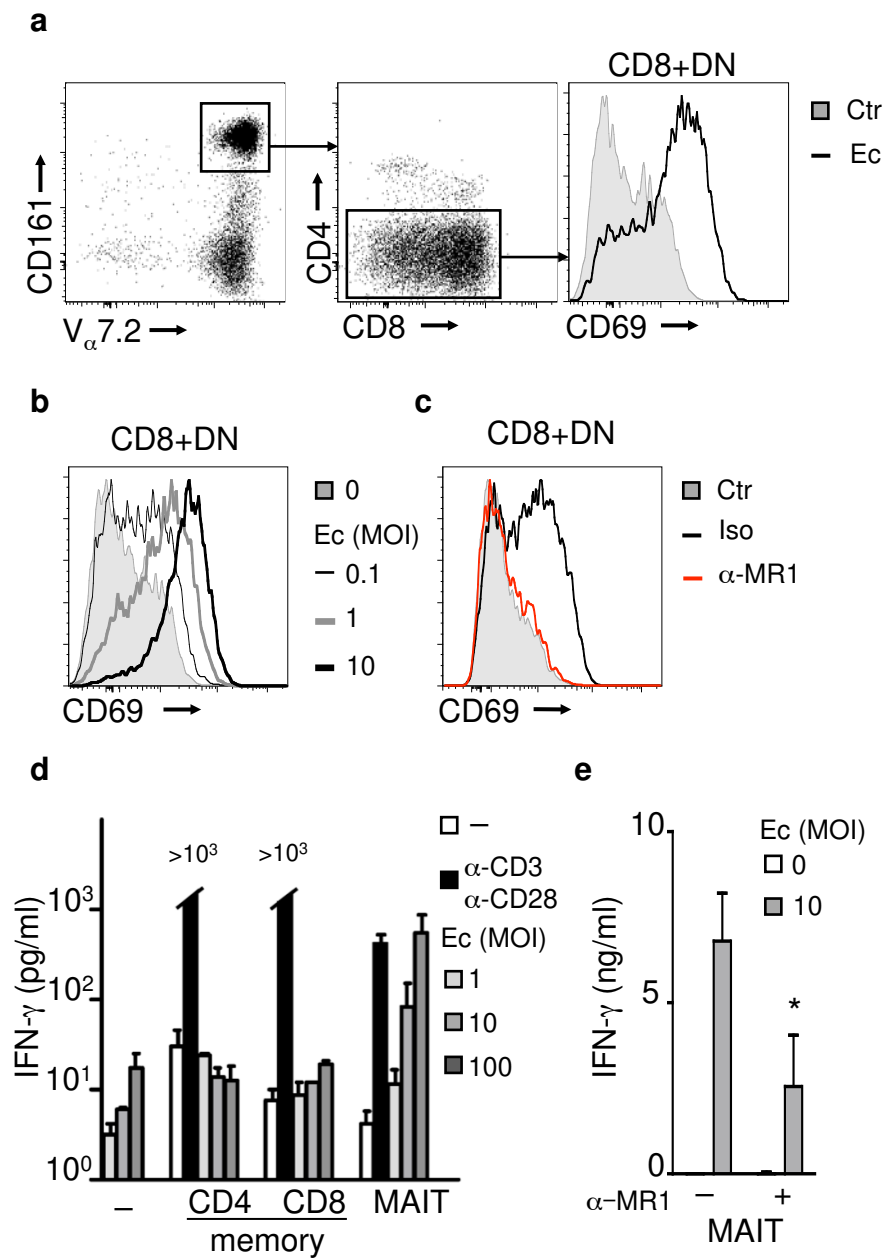


Figure 2 Le Bourhis et al.

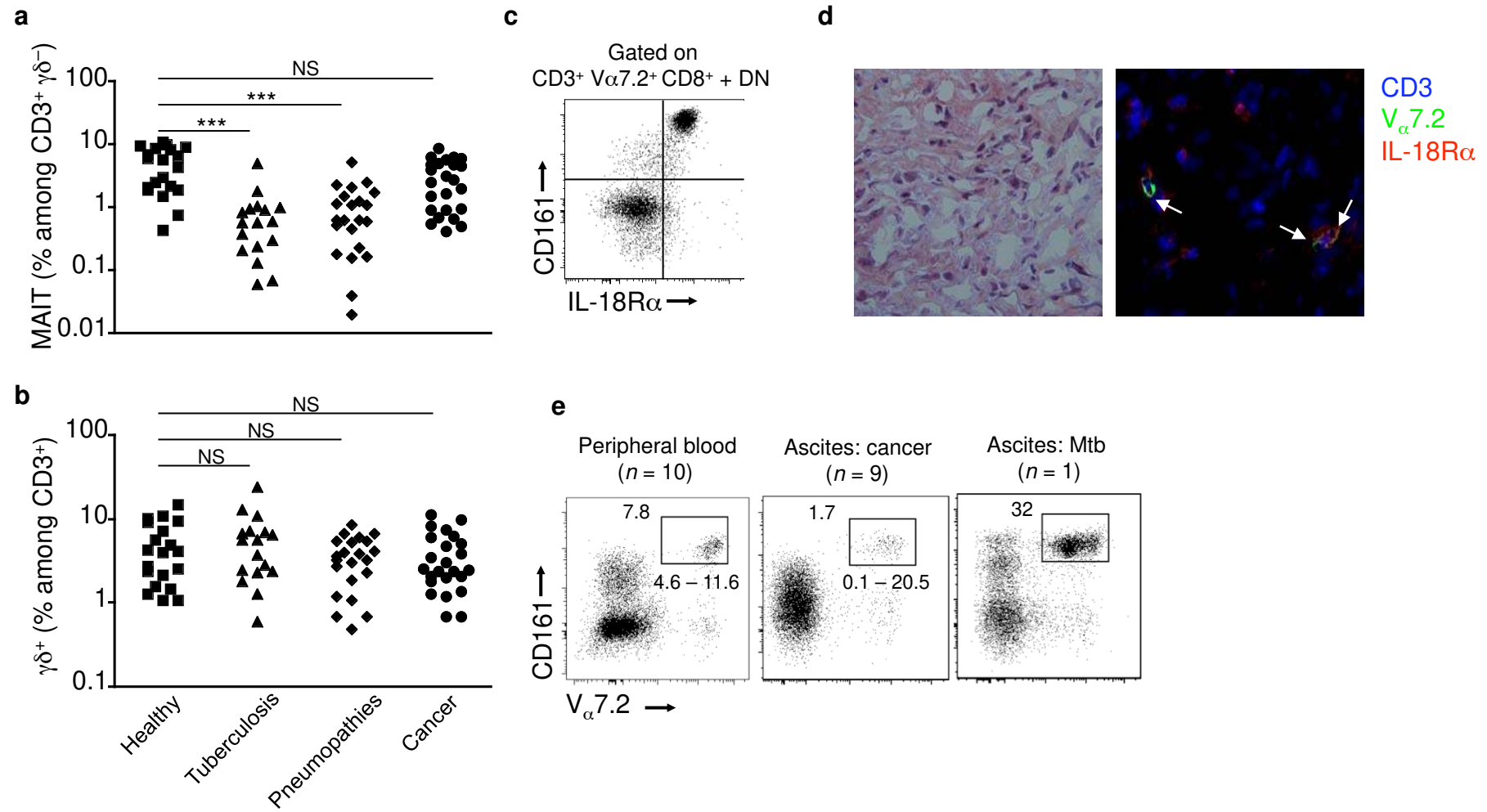


Figure 3 Le Bourhis et al.

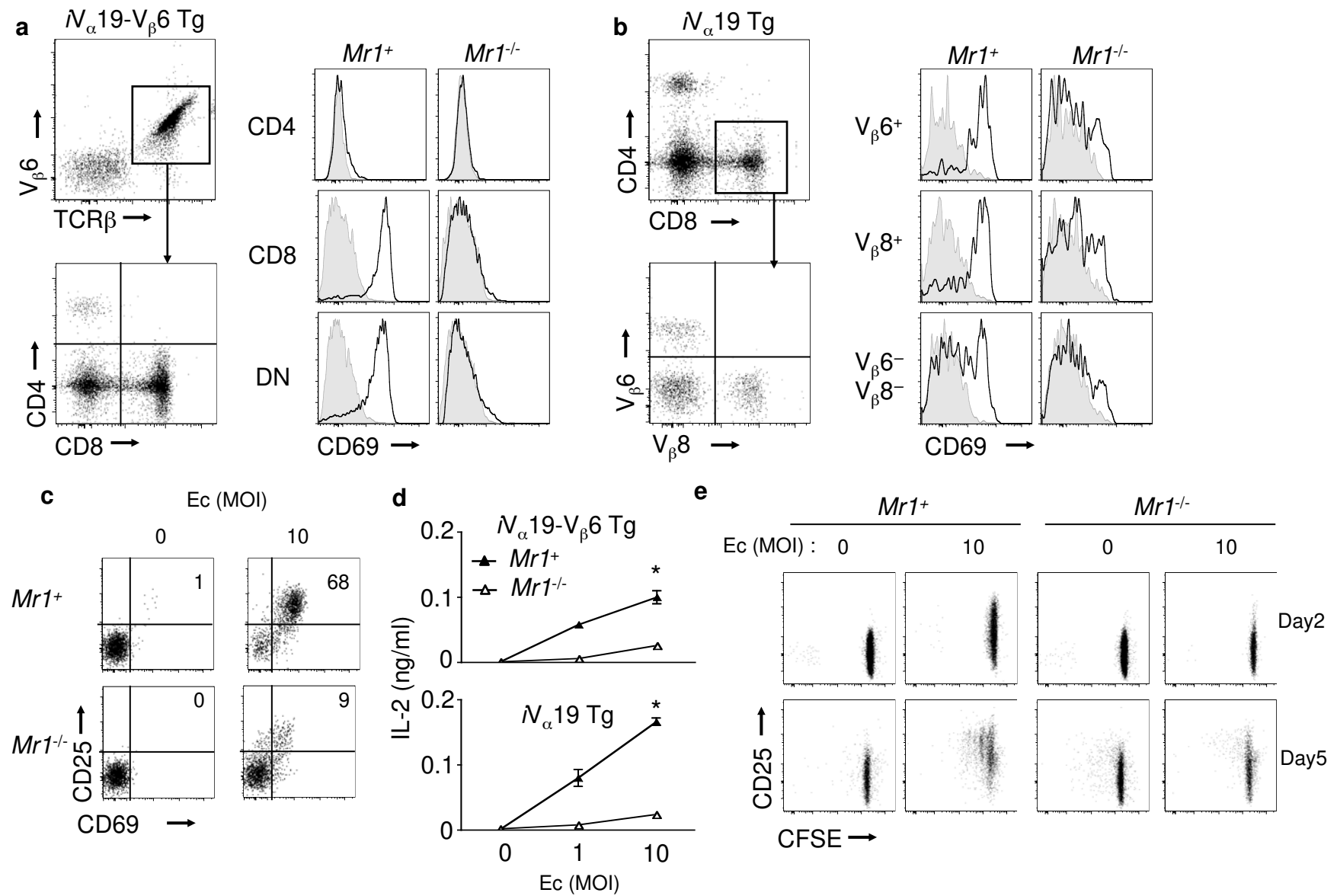


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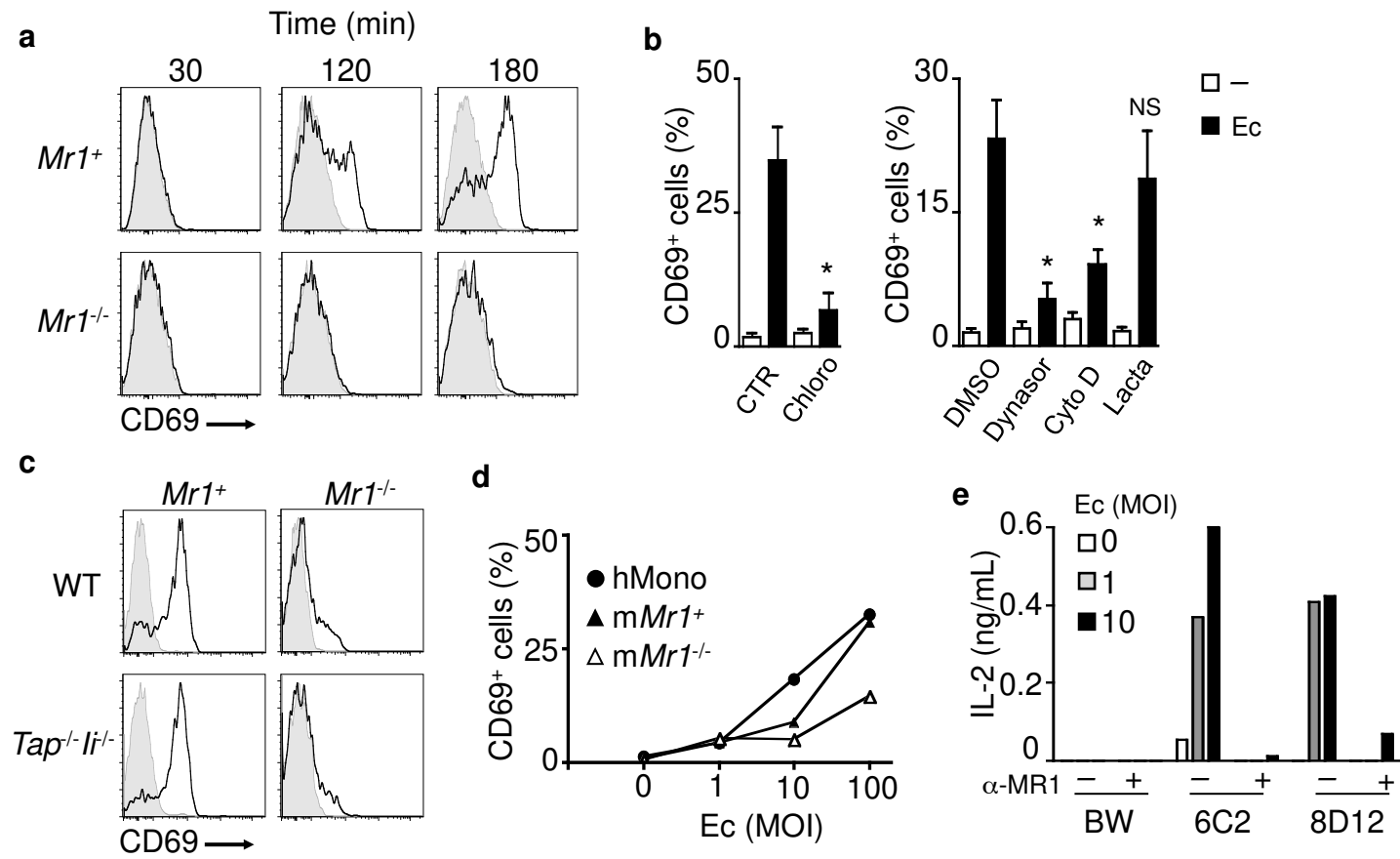


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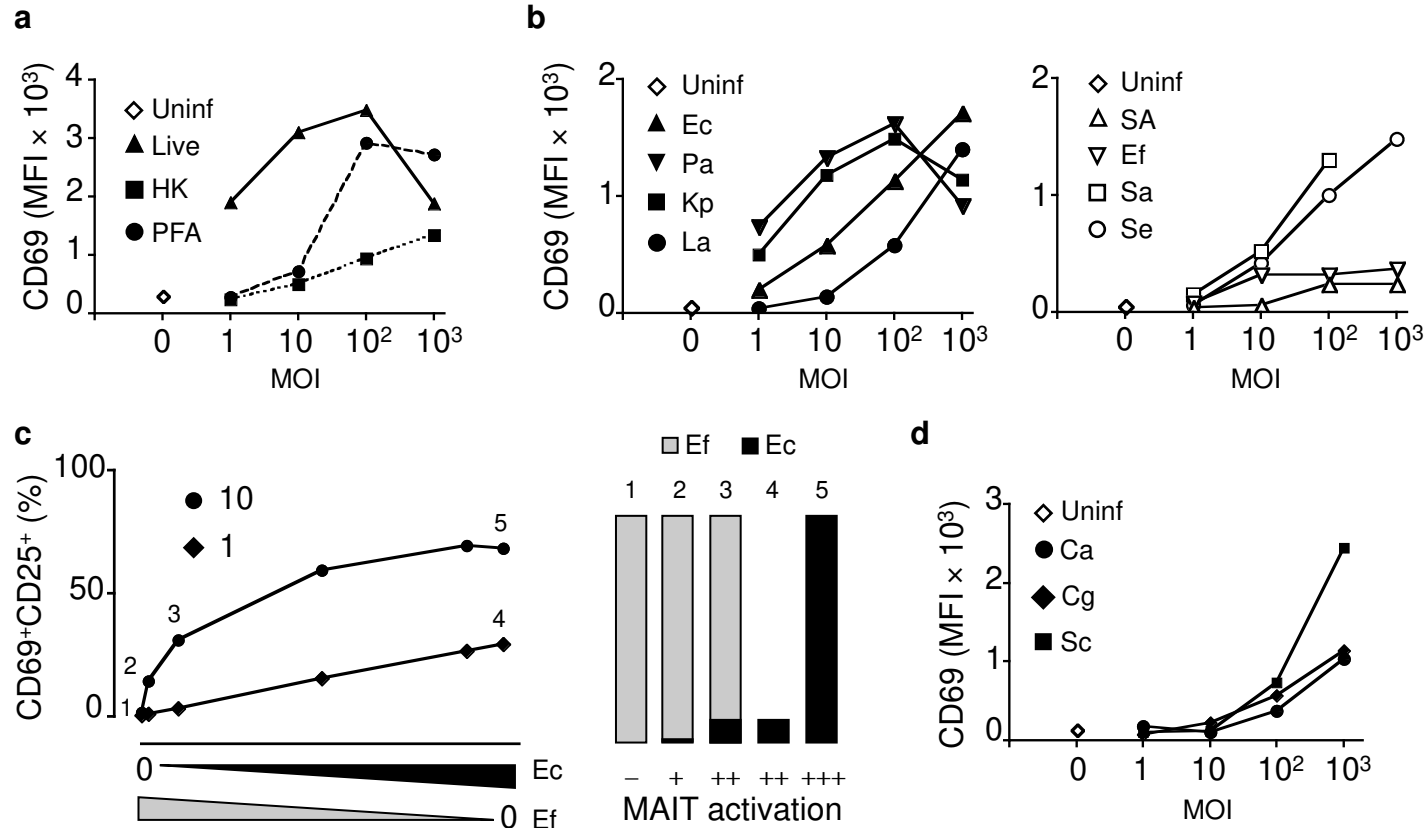


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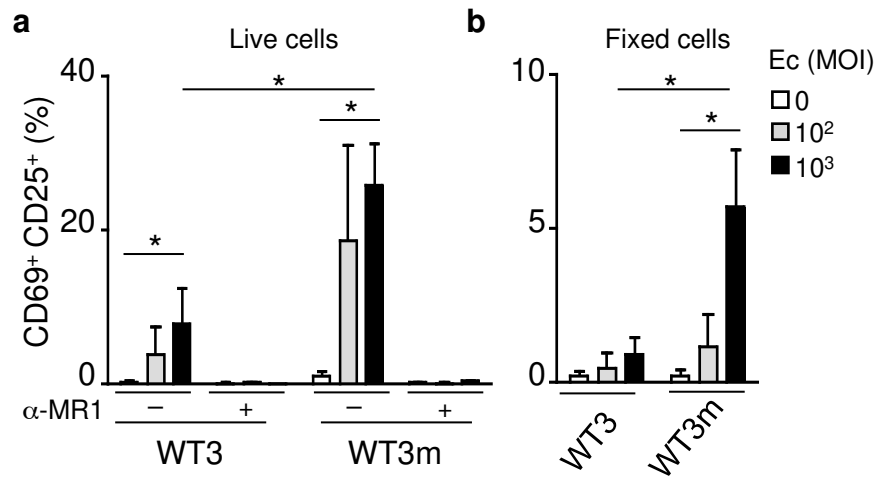


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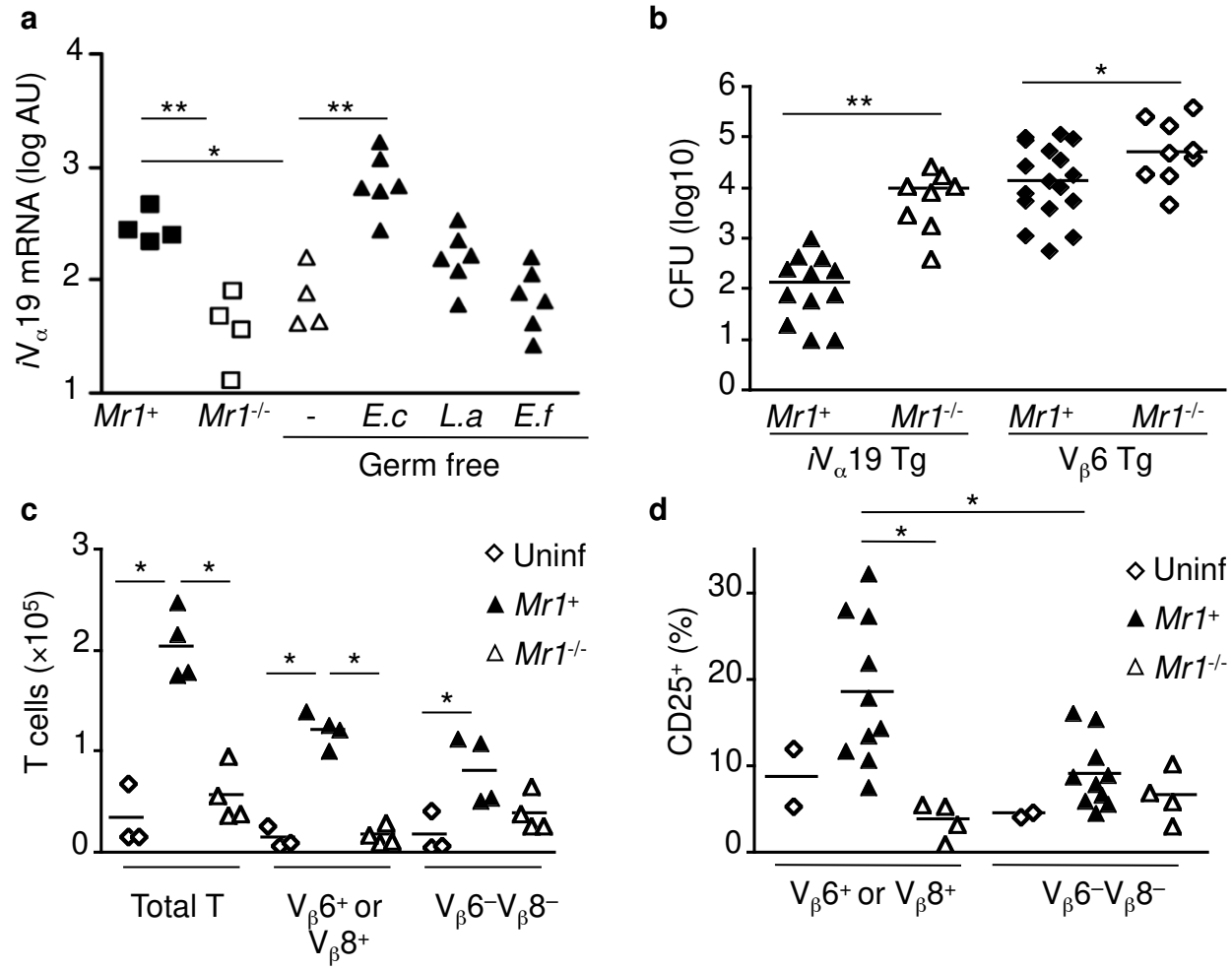


Figure 8 Le Bourhis et al.

