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Supplemental Information

Multiplexed Quantitation of Intraphagocyte *Mycobacterium tuberculosis* Secreted Protein Effectors

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Supplemental Information

Supplemental Figures

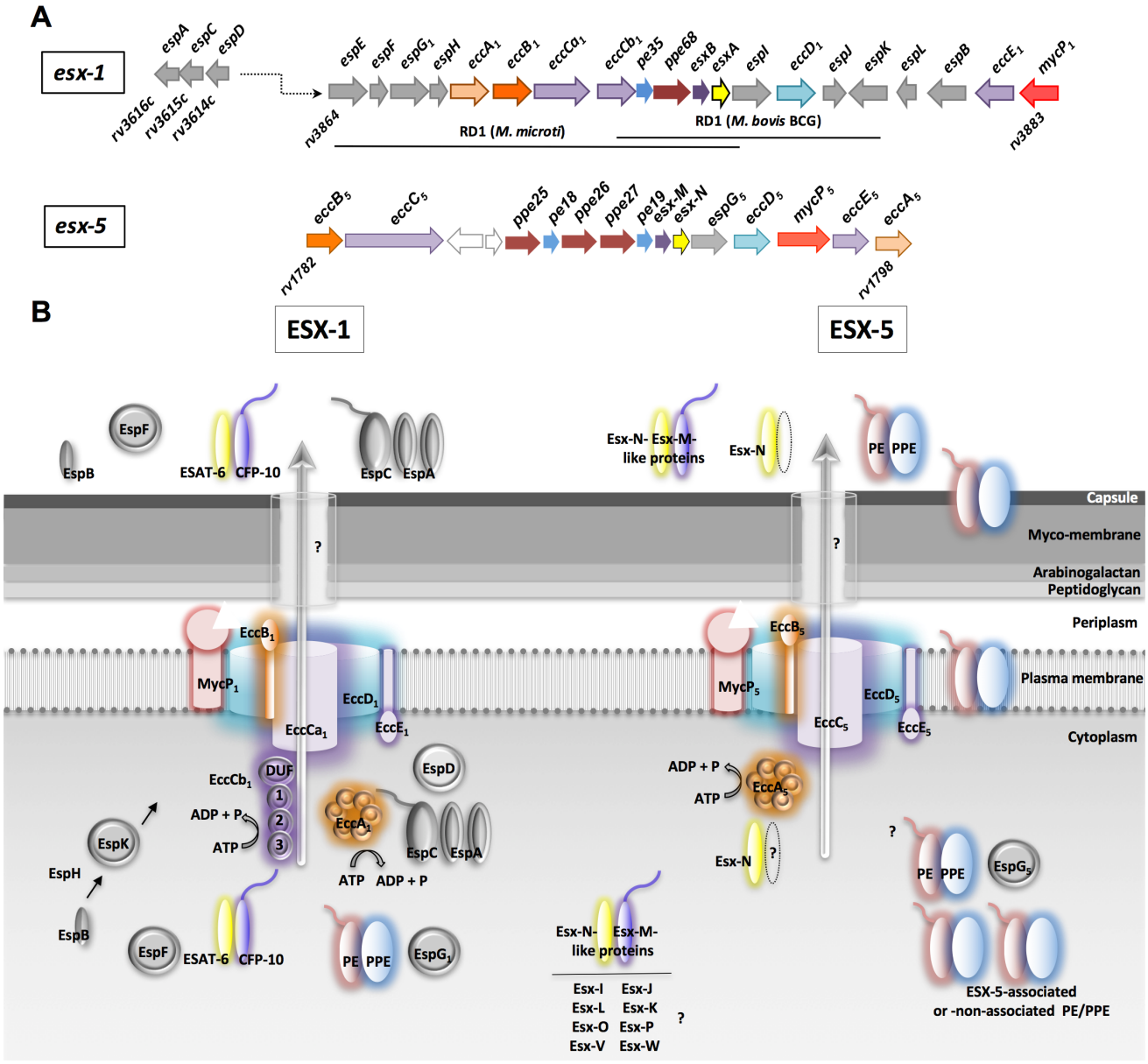

Figure S1. Working Model of ESX-1 and ESX-5 T7S machineries. Related to Figure 1. (A) The *Mtb esx-1* and *esx-5* genomic regions coding for ESX-1 and ESX-5 systems. **(B)** Model of so-far known role of different components of the secretion systems encoded by *esx-1* or *esx-5* genes and involved in the secretion of different members of Esx, Esp and PE/PPE protein families. Ecc = Esx-conserved components, MycP= extracellular membrane-bound proteases mycosins, DUF = Domain of Unknown Function. Adapted from (Majlessi et al., 2015).

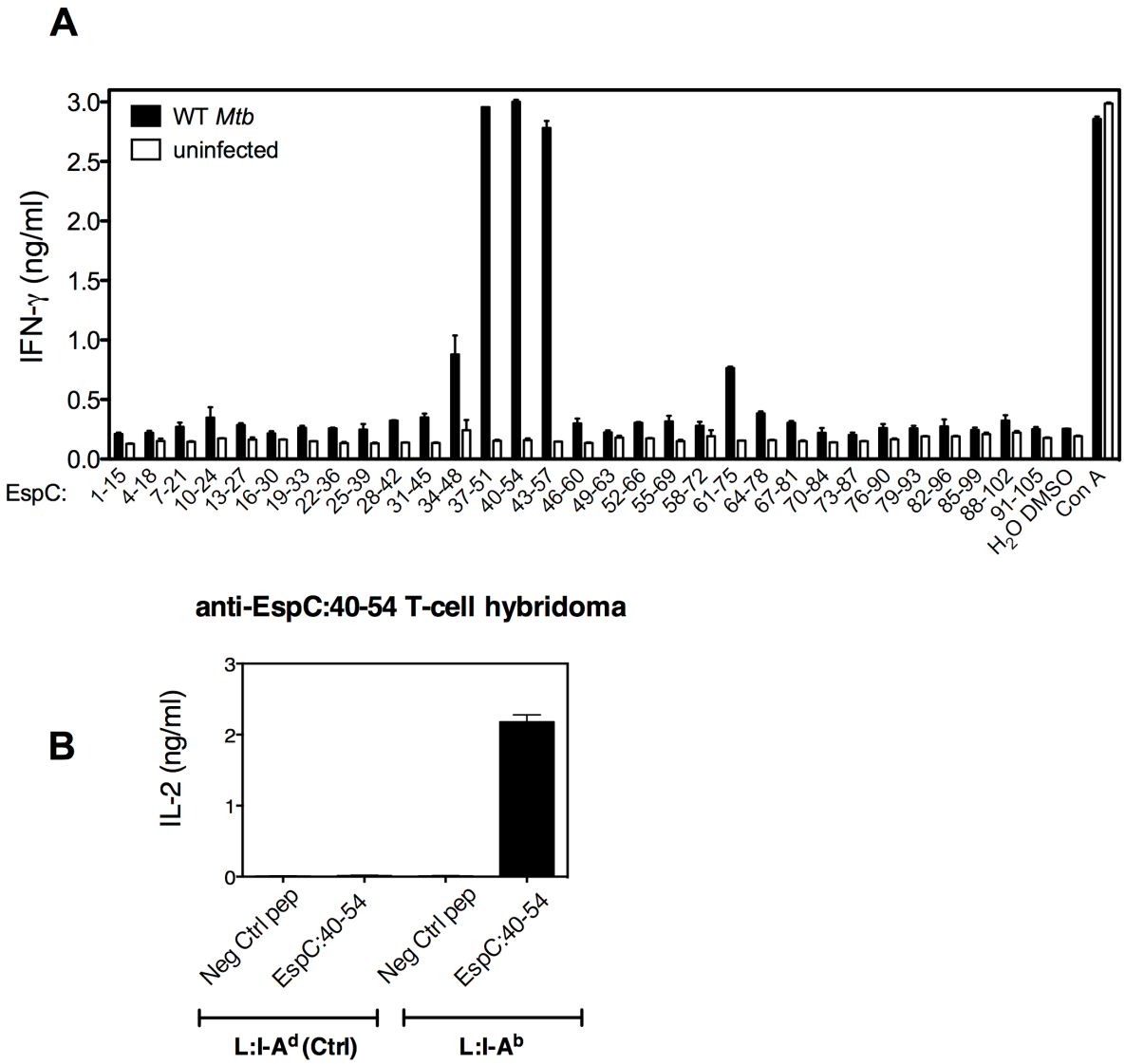

Figure S2. EspC T-cell epitope mapping. Related to Figure 1. (A) Total splenocytes from C57BL/6 (H-2^b) mice, unimmunized or immunized (s.c.) with 1×10^6 CFU/mouse of *Mtb* WT, were stimulated *in vitro* at 2 weeks p.i. with 10 µg/ml of individual synthetic 15-mers from EspC, offset by 4 AA. IFN-γ production in the culture supernatants was used as read out after 72 h incubation. **(B)** The immunogenic EspC:40-54 segment harbors an epitope restricted by I-A^b, as determined by use of L fibroblasts transfected with I-A^b or I-A^d (as a negative control), loaded with EspC:40-54 and co-cultured with the anti-EspC:40-54 "IF1" T-cell hybridoma.

Unexpectedly, no EspA-specific immunogenic region could be identified by epitope mapping of the 392-AA-long EspA protein, when using H-2^b, H-2^d, H-2^k or H-$2^{b/k}$ haplotypes in mice of different genetic backgrounds that were immunized with either *Mtb* H37Rv or Erdmann strains. Thus, it is likely that EspA cannot accede efficiently to the host MHC-II machinery during infection for yet unknown reasons, despite its upregulation in the acidic conditions of the host phagosomes (Ates et al., 2016a; Pang et al., 2013).

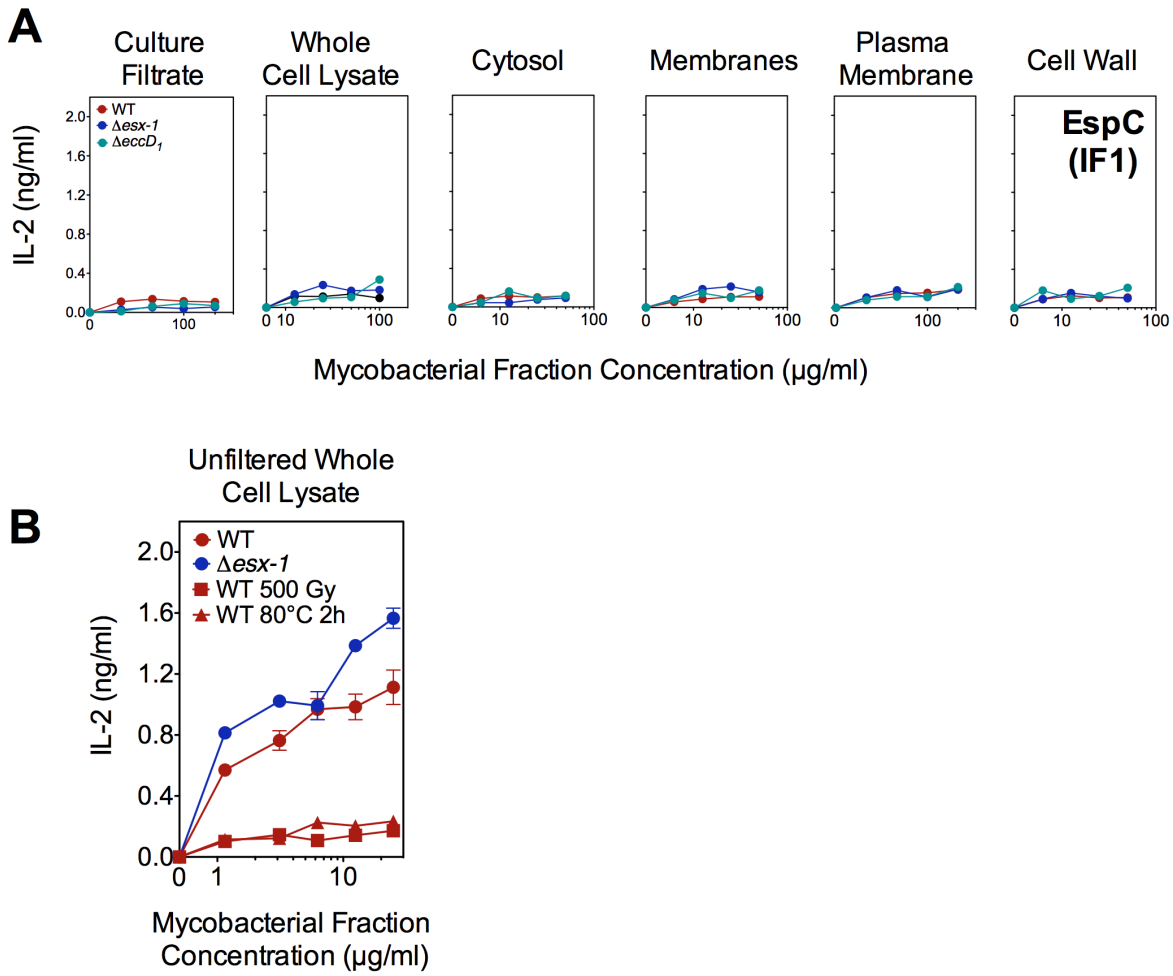

Figure S3. Attempt to localize EspC in different sub-mycobacterial fractions. Related to Figure 2. EspC has recently been shown to associate with EspA and to multimerize and self-assemble into long filaments during the secretion process. Electron microscopy studies also detected EspC as filamentous structure spanning the mycobacterial cell wall, possibly as a component of the ESX-1 secretion machinery (Lou et al., 2017). Here, with the T-cell based detection approach, an EspC-specific signal could not be detected neither in the *Mtb* culture filtrate, nor in different bacterial fractions prepared from the whole cell lysate (**A**). This was most likely due to the loss of multimerized EspC (Lou et al., 2017) during the sterilization of the whole cell lysate by filtration, required prior to mycobacterial fractionation. Indeed, EspC could be detected in the whole cell lysates of WT and *esx-1* mutants prior to the filtration. Various concentrations of culture filtrates, filtered whole cell lysates, or different fractions resulted from ultracentrifugation of the filtered whole cell lysates were added to the co-cultures of BM-DC with anti-EspC T-cell hybridoma. IL-2 was quantified in the co-culture supernatants at 24 h. (**B**) To try to overcome this difficulty, sterilization of the whole cell lysate was attempted by irradiation, which surprisingly also led to the loss of EspC signal. Moreover, heat inactivation of the whole cell lysate resulted in a notable precipitation and obvious EspC exclusion. Various concentrations of culture filtrates, filtered whole cell lysates, or different fractions resulted from ultracentrifugation of the filtered whole cell lysates were added to the co-cultures of BM-DC with anti-EspC T-cell hybridoma. IL-2 was quantified in the co-culture supernatants at 24 h. More investigations are thus needed to define particular fractionation/sterilization conditions required to map EspC in the mycobacterial compartments.

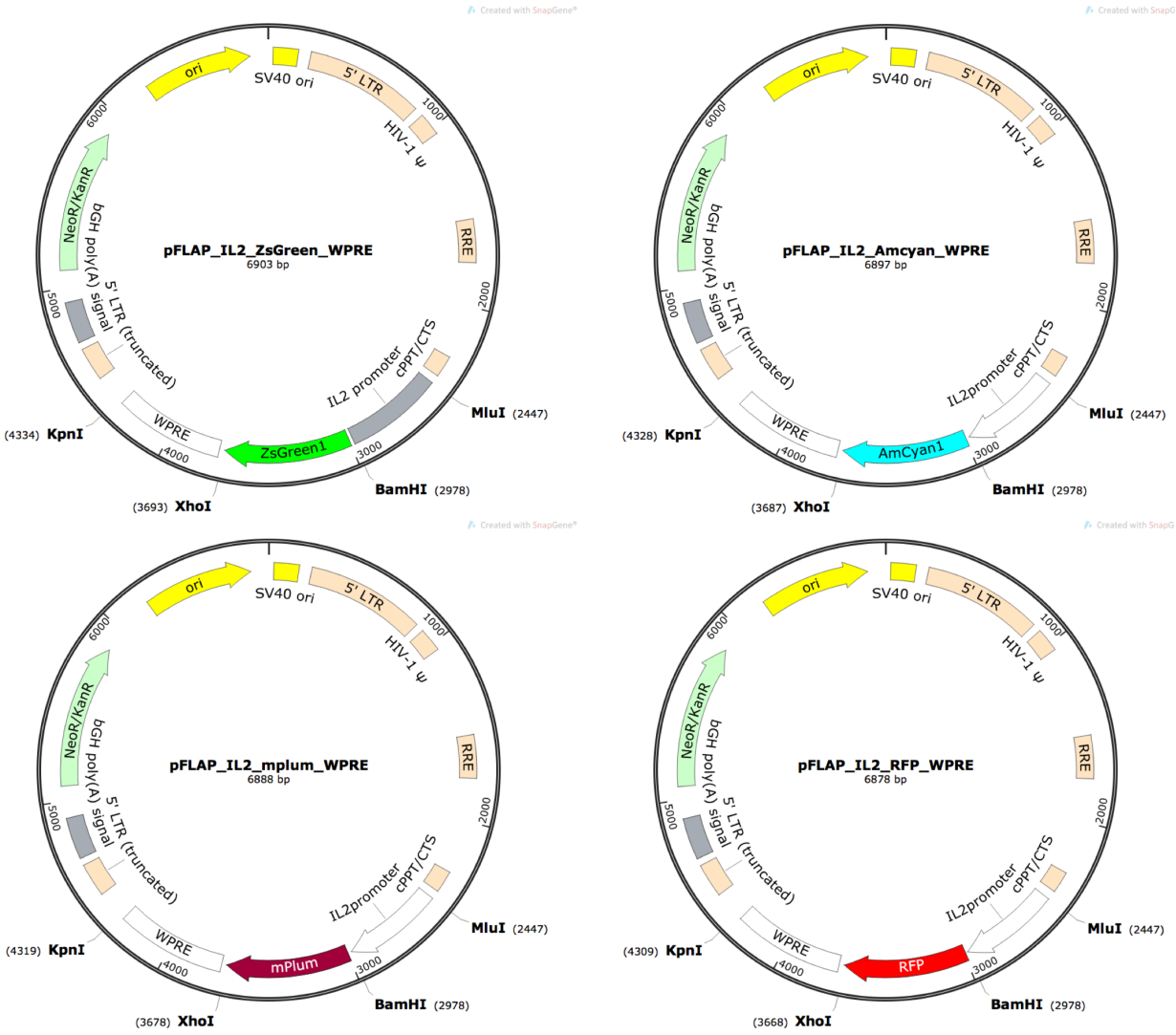

Figure S4. Lentiviral plasmids. Related to Figures 3 and 4. Map of different lentiviral integrative plasmids harboring individual fluorescence gene under *mil-2* promoter.

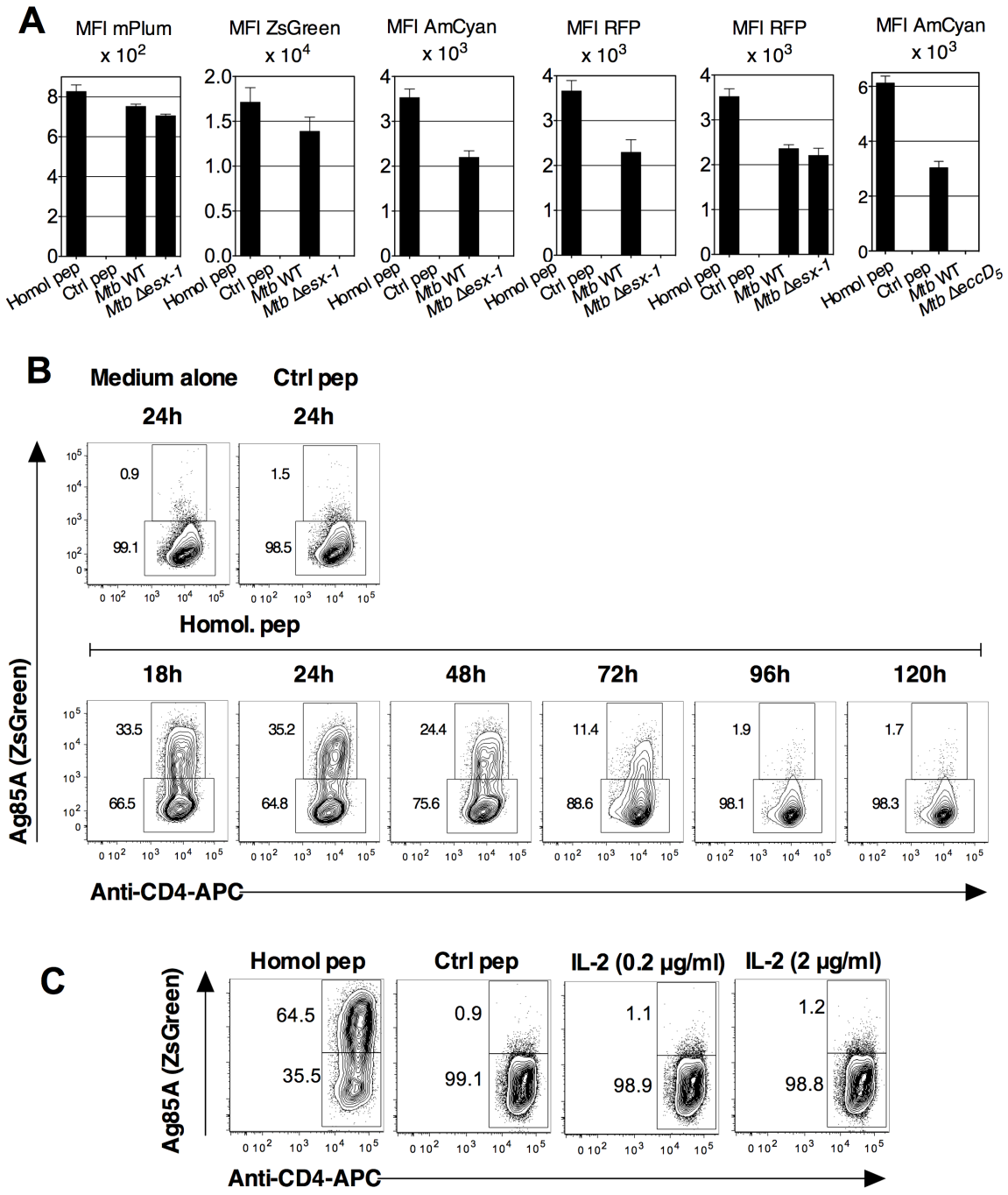

Figure S5. Characteristics of the specific reporter signal. Related to Figure 4. (**A**) Mean Fluoresence Intensity (MFI) of the reporter^{+} T cells shown in the Figure 4A, as an index indicative of the level of activation induced by the T7S-substrate-derived epitopes available to the phagocytes. Data are representative of at least two independent experiments. **(B)** Kinetics of fluorescent reporter expression by T cells in response to Ag presentation in vitro. Cytometric analysis of the ZsGreen signal of the transduced anti-Ag85A (DE10) T-cell hybridoma at different time points after its co-culture with BM-DC loaded with 1 µg/ml of homologous peptide. **(C)** The possibility of a by-stander effect, in which IL-2 produced by a given T cell could cross-activate P*mil-2* of neighboring T cells, was ruled out, as the addition of exogenous IL-2 to the transduced T cells induced no reporter signal.

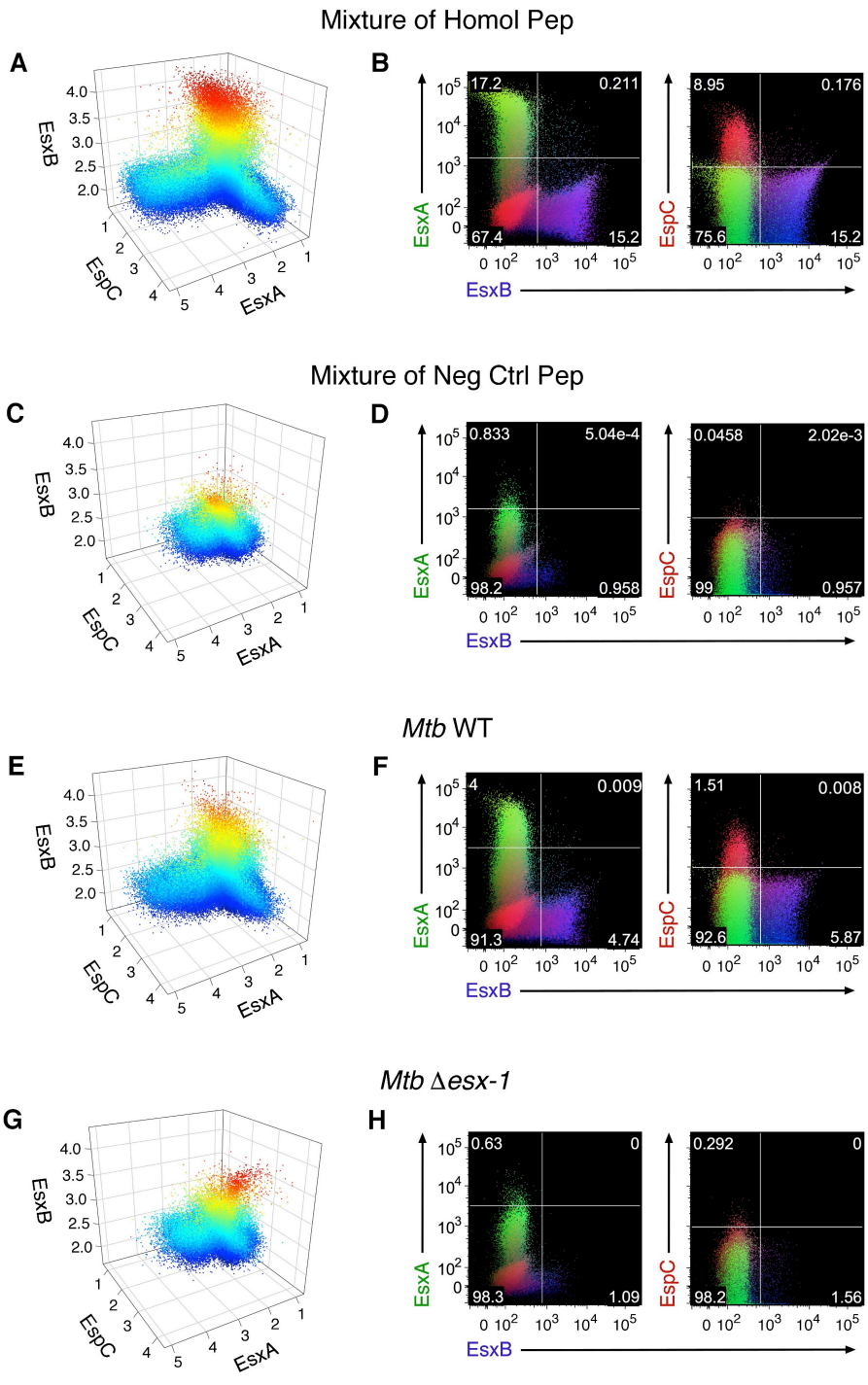

Figure S6. Cytometric analysis of the MASSTT data. Related to Figure 4. BM-DC from C57BL/6 × CBA F1 (H-$2^{k/b}$) were pre-loaded with a mixture of the homologous **(A)** or negative **(C)** control peptides, or infected with WT **(E)** or *Δesx-1 Mtb* **(G)**, and then co-cultured with a pool of the EsxA-, EsxB- and EspC-specific transduced T-cell hybridomas. 3D representation of the cytometric plots for the three reporter signals detected at 24h after T-cell addition were generated by using the scatter3D function from the R package plot3D. (**B, D, F, H**) Conventional dot plot analyses showing the MASSTT data obtained with BM-DC (H-$2^{b/k}$), loaded with homologous **(B)** or negative **(D)** control, or infected with WT **(F)** or *Δesx-1 Mtb* **(H)**. Note that no reporter signal was recorded in the absence of specific antigen or infection which showed that in the mixed cultures, no P*mil-2* activation linked to H-2^{b} - H-2^{k} allo-reactivity was observed between various T-cell hybridomas originating from C57BL/6 and C3H genetic backgrounds.

Supplemental Tables

Table S1. MHC-II-restricted T-cell hybridomas specific to various mycobacterial antigens*. Related to Figure 1.

ESX substrate	Secretion systems	MHC-II epitope	Epitope sequence	T-Cell clone	Restric-ting element**	Ag recognized
EsxA	ESX-1	1-20	MTEQQWNFAGIEAAASAIQG	NB11	I-A^b	
EsxB		11-25	LAQEAGNFERISGDL	XE12	I-A^K	
EspC		40-54	VAITHGPYCSQFNDT	IF1	I-A^b	
				5A8		both EsxH and EsxR
EsxH	ESX-3	74-88	**S**THE**A**NTMAM**M**ARD**T**[***]	1H2	I-A^d	EsxH > EsxR
EsxR		74-88	**G**THE**S**NTMAM**L**ARD**G**	1G1		EsxH but not EsxR
PE18	ESX-5	1-18	MSFVTTQPEALAAAA**GS**L	IF6	I-A^b	*esx5*-encoded PE18/19
PE19		1-18	MSFVTTQPEALAAAAA**N**L	IB12		but not other PE homologs
Ag85A	Tat	284-303	QDAYNA**G**GGHN**G**VF**D**FP**DS**G	DE10	I-A^b	Ag85A and Ag85B
Ag85B		281-300	QDAYNA**A**GGHN**A**VF**N**FP**PN**G			
Ag85A	Tat	144-163	LTSELP**G**WL**Q**ANR**H**VKPTGS	2A1	I-E^d	Ag85A and Ag85B
Ag85B		141-160	LTSELP**Q**WL**S**ANR**A**VKPTGS			

*The panel included the previously described EsxA- and Ag85A/B-specific (Frigui et al., 2008; Majlessi et al., 2006) and the newly generated T-cell hybridomas specific for EsxB, EspC, EsxH/R or PE18/19.

**As determined by the capacity of L fibroblasts, transfected with I-A^b, I-A^d, I-A^k or I-E^d and loaded with homologous peptides, to trigger IL-2 production by specific T-cell hybridomas.

***Mismatched residues are indicated in bold characters.

Table S2. Analysis sequence using Columbus system (version 2.3.1, PerkinElmer). Related to Figure 3.
Properties of an in-house multi-parameter script which was developed for the analysis of images obtained for transduced T cells co-cultured with DCs loaded with homologous peptide or infected with different *Mtb* or BCG strains.

Input Image	**Stack Processing:** Individual Planes **Flat field Correction:** None	**Method**	**Output**
Find Nuclei	Channel: Exp1Cam1 ROI: None	C Common Threshold: 0.4 Area: > 30 µm² Split Factor: 7 Individual Threshold: 0.4 Contrast: > 0.1	Nuclei
Calculate Morphology Properties	Population: Nuclei Region: Nucle	Standard (Area, Roundness)	Nucleus
Calculate Intensity Properties	Channel: Exp2Cam2 Population: Nuclei Region: Nucleus	Standard (Mean, Maximum)	Intensity Nucleus ZsGreen
Select Population	Population: Nuclei	Filter by Property Intensity Nucleus ZsGreen Mean: > 150 Intensity Nucleus ZsGreen Maximum: >300 Boolean Operations: F1 and F2	Activated Hybridomas
Calculate Intensity Properties	Channel: Exp1Cam2 Population: Activated Hybridomas Region: Nucleus	Standard (Mean)	Intensity ZsGreen Exp1Cam2
Calculate Image		By Formula Formula: iif(A>100,1,0) Channel A: Exp1Cam1 Negative Values: Set to Zero Undefined Values: Set to Local Average	Hoechst modified
Calculate Image		By Formula Formula: iif(A>300,B,0) Channel A: Exp2Cam2 Channel B: Hoechst modified Negative Values: Set to Zero Undefined Values: Set to Local Average	Activated ZsGreen
Find Image Region	Channel: Exp1Cam1 ROI: None	Whole Image Region	Whole Image Whole Image Region
Calculate Intensity Properties	Channel: Hoechst modified Population: Whole Image Region: Whole Image Region	Standard (Sum)	Area Hoechst
	Channel: Activated ZsGreen Population: Whole Image Region: Whole Image Region	Standard (Sum)	Area Activated ZsGreen
	Channel: Activated ZsGreen Population: Whole Image Region: Whole Image Region	Standard (Mean)	Intensity Whole Image Region Activated ZsGreen
Define Results	Method: List of Outputs Number of Objects Population: Activated Hybridomas Number of Objects		

	Intensity Nucleus ZsGreen Mean: Mean Population: Whole Image Method: Formula Output Formula: a/b*100 Population Type: Objects Variable A: Whole Image - Area Activated ZsGreen Sum Variable B: Whole Image - Area Hoechst Sum Output Name: Activated ZsGreen Area/ Hoechst Area (in %) Method: Formula Output Formula: a/b*100 Population Type: Objects Variable A: Activated hybridomas - Number of Objects Variable B: Nuclei - Number of Objects Output Name: % Activated hybridomas

Table S3. T-cell hybridomas transduced with lentiviral vectors in such a way that to each antigen specificity corresponds a given fluorescence reporter. Related to Figures 3 and 4.

ESX Substrate	T-Cell Clone	Fluorescent Reporter	$\lambda_{exc}/\lambda_{em}$ (nm)
EsxA	NB11	ZsGreen*	493/505
		AmCyan**	458/489
EsxB	XE12	AmCyan	458/489
EspC	IF1	RFP***	555/584
EsxH	1G1	RFP	555/584
PE18/19	IF6	AmCyan	458/489
Ag85A	DE10	ZsGreen	493/505
		mPlum****	590/649
Ag85A	2A1	AmCyan	458/489

* Zoanthus Green Fluorescent Protein
** *Anemonia majano* Cyan fluorescent protein
*** Red Fuorescent Protein from *Entacmaea quadricolor*
**** A far RFP mutant derived from the tetrameric *Discosoma* sp. RFP, DsRed

Table S4. Mycobacterial variants used in this study. Related to Figures 5 and 6.

Mycobacteria	Variant	locus
Mtb	H37Rv WT	
	H37Rv Δ*esx-1*	RD1
	H37Rv Δ*espF*	*rv3865*
	H37Rv Δ*espG$_1$*	*rv3866*
	H37Rv Δ*eccCb$_1$*	*rv3871*
	H37Rv Δ*pe35*	*rv3872*
	H37Rv Δ*ppe68*	*rv3873*
	H37Rv Δ*esxB*	*rv3874*
	H37Rv Δ*phoP*	*rv0757*
	H37Ra::*phoP*	*rv0757*
	H37Ra::*pRpsl*	*rv0682*
	H37Ra::*phoP*	*rv0757*
	H37Ra::*pRpsl*	*rv0682*
	H37Rv Δ*ppe25-pe19*	*rv1787-rv1791*
	H37Rv Δ*ppe25-pe19::esx-5*	*rv1787-rv1791*
	H37Rv Δ*esxM*	*rv1792*
	H37Rv Δ*esxM::esx-5*	*rv1792*
	H37Rv Δ*eccD$_5$*	*rv1795*
	Erdman WT	
	Erdman Δ*espA*	*rv3616c*
M. bovis	*M. bovis* WT	
	BCG::pYUB	
	BCG::*esx-1*	RD1
	BCG::*esx-1* Δ*ppe68*	*rv3873*
	BCG::*esx-1*Δ*esxB*	*rv3874*
	BCG::*esx-1* Δ*espI*	*rv3876*
	BCG::*esx-1* Δ*espJ, K, L, B*	*rv3878-3881*
M. microti	OV254::*esx-1*	
	OV254 Δ*espH*	*rv3867*
	OV254 Δ*eccB$_1$*	*rv3869*
	OV254 Δ *eccCb$_1$*	*rv3871*
	OV254 Δ*esxA*	*rv3875*
	OV254 Δ*espI*	*rv3876*

Table S5. Mutations in the *esx-3* and *esx-5* regions, or in genes coding for Ag85A/B or TatABC recovered in *Mtb* clinical isolates. Related to Figure 6.

Clinical Isolate: strain No., lineage (MLVA-Mtbc 15-9), R*	Position	Reference	Sample	Region	AA Exchange
	2135154	G	T	Rv1886c (Ag85B)	silent (Pro238)
No.78	342146	A	C	Rv0282 (eccA3)	Glu6Ala
Beijing	346275	C	G	Rv0284 (eccC3)	Pro214Arg
(204-32)	353309	G	A	Rv0290 (eccD3)	Ser76Asn
R isoniazid,	353365	G	A	Rv0290 (eccD3)	Ala95Thr
sreptomycin,	356528	A	G	Rv0292 (eccE3)	Asn217Asp
ethambutol	2022868	T	C	Rv1783 (eccC5)	silent (Ser1204)
	2023628	C	G	Rv1785c (cyp143)	Gly334Ala
	2030848	A	G	Rv1793 (esxN)	Glu52Gly
	2037015	C	T	Rv1798 (eccA5)	Leu106Leu
	1367484	T	G	Rv1224 (tatB)	Trp8Gly
	2135154	G	T	Rv1886c (Ag85B)	silent (Pro238)
No.25343	342146	A	C	Rv0282 (eccA3)	Glu6Ala
Beijing	346275	C	G	Rv0284 (eccC3)	Pro214Arg
(10762-32)	353309	G	A	Rv0290 (eccD3)	Ser76Asn
R isoniazid,	353365	G	A	Rv0290 (eccD3)	Ala95Thr
ethionamide	356528	A	G	Rv0292 (eccE3)	Asn217Asp
	2022868	T	C	Rv1783 (eccC5)	silent (Ser1204)
	2023628	C	G	Rv1785c (cyp143)	Gly334Ala
	1367484	T	G	Rv1224 (tatB)	Trp8Gly
	2135154	G	T	Rv1886c (Ag85B)	silent (Pro238)
No.68243	342146	A	C	Rv0282 (eccA3)	Glu6Ala
Beijing	346275	C	G	Rv0284 (eccC3)	Pro214Arg
(100-32)	352334	G	C	Rv0289 (espG3)	silent (Pro62)
Susceptible to all	353309	G	A	Rv0290 (eccD3)	Ser76Asn
antituberculous drugs	353365	G	A	Rv0290 (eccD3)	Ala95Thr
	356528	A	G	Rv0292 (eccE3)	Asn217Asp
	2020255	A	C	Rv1783 (eccC5)	Gln333His
	2020420	C	T	Rv1783 (eccC5)	silent (Asp388)
	2022868	T	C	Rv1783 (eccC5)	silent (Ser1204)
	2023628	C	G	Rv1785c (cyp143)	Gly334Ala
	2030848	A	G	Rv1793 (esxN)	Glu52Gly
	1367484	T	G	Rv1224 (tatB)	Trp8Gly
	342146	A	C	Rv0282 (eccA3)	Glu6Ala
	343281	G	C	Rv0282 (eccA3)	silent (Ala384)
	344288	C	G	Rv0283 (eccB3)	silent (Ser89)
No.47927	346275	C	G	Rv0284 (eccC3)	Pro214Arg
EAI	350489	G	C	Rv0286 (PPE4)	silent (Ala185)
(?-47)	351876	C	T	Rv0288 (esxH)	Ala10Val
monoR-ofloxacin	352572	C	T	Rv0289 (espG3)	Arg142Trp
	356528	A	G	Rv0292 (eccE3)	Asn217Asp
	2019236	T	G	Rv1782 (eccB5)	silent (Pro499)
	2022868	T	C	Rv1783 (eccC5)	silent (Ser1204)
	2023211	G	T	Rv1783 (eccC5)	Val1319Phe
	2026025	A	G	Rv1787 (PPE25)	Gln242Arg
	2026029	C	T	Rv1787 (PPE25)	silent (Phe243)
	2026030	T	C	Rv1787 (PPE25)	Phe244Leu
	2026032	C	T	Rv1787 (PPE25)	silent (Phe244)
	2026033	G	A	Rv1787 (PPE25)	Ala245Thr
	2028269	G	A	intergenic	
	2028967	A	C	Rv1790 (PPE27)	silent (Ala181)
	2029087	G	C	Rv1790 (PPE27)	Leu221Phe
	2030487	C	T	intergenic	
	2030488	A	C	Intergenic	
	2030489	T	A	Intergenic	
	2030521	T	C	Intergenic	

	2030848	A	G	Rv1793 (esxN)	Glu52Gly
	2035937	G	A	Rv1797 (eccE5)	Arg152His
	2035986	G	T	Rv1797 (eccE5)	Val168Val
	1367484	T	G	Rv1224 (tatB)	Trp8Gly
	346275	C	G	Rv0284 (eccC3)	Pro214Arg
No.99205	348210	G	C	Rv0284 (eccC3)	Cys859Ser
Ural	350738	C	T	Rv0286 (PPE4)	silent (Thr268)
(12364-15)	353766	T	C	Rv0290 (eccD3)	silent (Ile228)
R isoniazid,	353767	C	T	Rv0290 (eccD3)	Pro229Ser
streptomycin,	355803	G	T	Rv0291 (mycP3)	Ala436Ser
ethambutol	356528	A	G	Rv0292 (eccE3)	Asn217Asp
	2022868	T	C	Rv1783 (eccC5)	silent (Ser1204)
	2025848	T	C	Rv1787 (PPE25)	Val183Ala
	2025913	T	C	Rv1787 (PPE25)	Ser205Pro
	346275	C	G	Rv0284 (eccC3)	Pro214Arg
No.103788	349200	G	A	Rv0284 (eccC3)	Arg1189His
NEW1	356528	A	G	Rv0292 (eccE3)	Asn217Asp
(?-223)	2022868	T	C	Rv1783 (eccC5)	silent (Ser1204)
R rifampicin,	2030521	T	C	intergenic	
isoniazid, ethionamide,	2030634	G	C	intergenic	
streptomycin	2035893	G	T	Rv1797 (eccE5)	silent (Ser137)
	2353070	G	A	Rv2094c (tatA)	silent (Asp76)

*R = antibiotic resistance profile.

MLVA Mtbc 15-9 codes were assigned using the MIRU-VNTR*plus* database (http://www.miru-vntrplus.org/).

Supplemental Experimental Procedure

DNA constructs, lentiviral vector production and titration

Plasmids containing genes coding *Mus musculus* IL-2 promoter (P*mil-2)*, AmCyan1-N1 or mPlum-N1, were purchased from Addgene (Teddington, UK). The plasmid containing gene coding for the constitutive human Elongation Factor-1α promoter (*EFIα P*) was derived from vectors described in (Sirven et al., 2001). The P*mil-2* gene was amplified from the plasmid by PCR using Pfu polymerase (Stratagene) and Forward: 5'-ACGCGTTCTATCACCCTGTGTGCAATTAGC-3' and Reverse: 5'-GGATCCCTTAGCAAGGGTGATAGGCAGC-3' primers. Underlined sequences are MluI and BamHI restriction sites, added respectively for the further cloning of the P*mil-2* PCR fragment into the unique MluI/BamHI site of a non-replicative lentiviral pFLAPΔU3 plasmid which contains the *cis*-acting sequences required for formation of the central DNA Flap (Zennou et al., 2000) and a WPRE (Woodchuck Posttranscriptional Regulatory Element) sequence to increase gene transcription (S4 Fig). The genes coding for different fluorochromes were amplified by use of Forward: 5'-GGATCCACCGGTCGCCACCATGG-3' and Reverse: 5'-GCTGATTATGATCTCGAGTCGCGGCCG-3' primers. Underlined sequences are BamHI and XhoI restriction sites added respectively for cloning of the fluorochrome PCR fragments into the unique BamHI/XhoI site of the pFLAPΔU3 plasmid, downstream of the P*mil-2* or *EF1α* promoter to monitor gene transduction (S4 Fig).

Non-replicative lentiviral vector particles were produced in Human Embryonic Kidney (HEK)-293T cells, grown in DMEM medium supplemented with 10% FBS, by transient tripartite co-transfection by: (i) 10 µg of vector plasmids, (ii) 10 µg of an encapsidation plasmid (p8.74) containing the HIV-1 genes *gag*, *pol*, *tat*, and *rev*, and (iii) 10 µg of an envelope expression plasmid encoding the glycoprotein G from vesicular stomatitis virus (VSV) in presence of calcium phosphate, as previously described (Zennou et al., 2000). At 24 h post-transfection, the culture medium was replaced by fresh DMEM containing 10% FBS. Supernatants of the transfected cells were harvested at 48 h post-transfection, clarified by centrifugation at 2500 rpm, aliquoted and stored at -80°C. The average titers of the lentiviral vector stocks was 1 x 10^8 Transducing Unit (TU)/ml as determined by real-time PCR on total lysates from transduced HEK-293T cells, as described elsewhere (Iglesias et al., 2006). 60-70% of each T-cell hybridoma were practically transduced with lentiviral vectors, as judged by the quantitation of fluorescent cells subsequent to their co-culture with peptide-loaded BM-DC. The fluorescence positive cell populations were sorted on a MoFlo Astrios Cell Sorter (Beckman Coulter) in order to reach up to 99% of positively transduced cells. T-cell hybridomas were then cultured and amplified until they came back to the steady state and until clearance of the fluorescence signal for stock preparation. T-cell hybridomas were also transduced with lentiviral vectors, harboring each of the fluorescent reporter genes under the constitutive *EFIα* promoter and used as mono-stained cells for cytometer settings. The transduced T-cell hybridomas have been deposited at the "Collection Nationale de Cultures de Microorganismes" CNCM (https://www.pasteur.fr/en/cncm) at the Institut Pasteur Collection.

Bacterial fractionation

Mtb strains were cultured in ADC-supplemented Dubos broth without agitation until exponential phase (OD_{600} 0.6-1.2) was reached. Bacteria were collected from 200 ml of culture by centrifugation at 4000 rpm. The bacterial material was washed in 20 ml of Dubos completed with protease inhibitor cocktail ("cOmplete-EDTA-free", Roche), resuspended in 1 ml of this medium and bead-beated at 30 Htz for 8 minutes in a Mill Mixer (Qiagen) to disrupt cells. The lysate was then filtered through a 0.2 µm PVDF (Millipore) filter to eliminate remaining bacteria. Fractionation was performed at 45,000 rpm 30 min in order to pellet the cell wall, which is likely a combination of outer membrane, periplasmic and insoluble protein complexes. The cell wall fraction was washed once in PBS containing protease inhibitor cocktail. The supernatant was further centrifuged at 100,000 rpm 105 min to collect the bacterial cytosol and to pellet plasma membrane, washed once in PBS containing protease inhibitor cocktail.

An alternative method (Lou et al., 2017; Sani et al., 2010) was applied to recover total membrane fraction. *Mtb* strains were pre-cultured in 7H9 medium supplemented with ADC and 0.05% Tween-80 until exponential phase was reached. Bacteria were washed and inoculated in 100 ml Sauton's medium with 0.002% Tween-80 at 0.1 OD_{600}/ml and incubated without agitation. Six days later, bacteria were collected by centrifugation and aliquots of the culture supernatant were filtered through a 0.2 µm PVDF filter to eradicate remaining bacteria. The culture filtrate was concentrated 70-fold by centrifugation in centrifugal filter units (Amicon Ultra – 15®, Ultracel 3K®, Merck, Germany). Cellular material was treated with 0.25% Genapol X-080 detergent in PBS for 20 minutes at 37°C under mild agitation to extract capsular proteins. Remaining cellular material was washed gently with PBS and bead-beated to disrupt cells. Further fractionation was performed by ultracentrifugation at 100,000 rpm for 60 min. Insoluble proteins, considered as "membrane fractions" were resuspended in PBS. Fractionation of *M. marinum* was performed as described above with the exception of culture conditions which were adapted from Ates et al. 2016 (Ates et al., 2016b). Briefly, after pre-culture bacteria were inoculated at 0.3 OD_{600}/ml in 100 ml 7H9 medium without Tween-80 or ADC supplement, but with 2 mg/ml dextrose. Strains were grown 40 h at 30°C without agitation.

Protein detection by Western blot

Proteins were separated by SDS-PAGE and blotted on nitrocellulose membranes. Proteins were visualized by primary anti-EsxA (hyb76-8) (Harboe et al., 1998) or anti-EsxN (Alderson et al., 2000) mAbs and polyclonal anti-EsxB or anti-SigA antibodies, followed by electro-chemiluminesence detection of appropriate secondary antibodies.

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