Supplemental Material

Platelets Play Differential Role during the Initiation and Progression of Autoimmune Neuroinflammation

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Supplemental Methods

Subjects

Peripheral blood samples were obtained after receipt of written informed consent from healthy controls and MS patients. The study was approved by the institutional review boards at Brigham and Women's Hospital and at the Chinese University of Hong Kong. MS patients participated as a part of the CLIMB study at Brigham and Women's Hospital (1) and the study at the Chinese University of Hong Kong consisted of a group of untreated relapsing-remitting and secondary progressive MS patients (**Supplemental Table I**).

Isolation of lipid rafts

To purify the lipid rafts from the human brain, unidentified frozen autopsy samples from New York tissue bank were used. The work with human autopsy samples was approved by Harvard Medical School review board committee. Brian tissues were separated from meninges the lipid rafts were isolated by the homogenization of the brain tissue in PBS with 0.5% Triton X-100. The homogenate was then centrifuged at low speed (250g) for 5 minutes and the supernatant was filtered through 0.4 μ and nylon filter (Millipore) and washed two times with PBS by the centrifugation at high speed (16,000g, 10 min) as described (2). The amount of lipid rafts was quantified using a kit for the measurement of the concentration of phospholipids (Wako Chemicals) and used at concentration of 5 μg of phospholipids in 1 ml.

Platelet and mononuclear cell isolation and analysis

For isolation and analysis of human platelets, peripheral blood samples were drawn using 5-10 ml collecting tubes with EDTA (BD Biosciences). Blood samples were kept at room temperature and platelets were analyzed within 2 hours of blood collection.

For the analysis of platelet-CD4 T cell aggregates, whole blood was diluted with PBS (1:10) and analyzed by multicolor flow cytometry after staining with anti-CD42a (human), or anti-CD41 (mouse) and anti-CD4 antibodies similarly as described (3). The samples were incubated with proper antibodies (dilutions are 1:200 for anti-mouse antibodies and 1:5 for anti-human antibodies) in the total volume of 250-300 μl for 15 min at room temperature and then fixed by addition of 1 ml of 1% paraformaldehyde in PBS. Platelet-CD4 T cell aggregates were analyzed by multicolor flow cytometry and the percentage of aggregates was identified by calculating the fraction of $CD4^{+}CD42a^{+}$ (human) or $CD4^{+}CD41^{+}$ (mouse) double positive aggregates out of all $CD4^{+}$ cells.

For assessment of Annexin V expression on platelets, platelet rich plasma was gained from peripheral blood by low speed centrifugation at 250g for 5 minutes at room temperature. After which, platelet rich plasma containing 1.5×10^{6} cells was diluted in Annexin-binding buffer (BS Bioscieces) and 200 μl of platelet rich plasma were incubated with 50 μl of anti-CD42-PerCP antibodies and 3 μl of Annexin V-PE

(both from BS Biosciences) for 20 min at room temperature. Then the samples were diluted with 1 ml of Annexin-binding buffer and analyzed by FACS within 30 min.

The analysis of Ca^{2+} influx in *ex-vivo* isolated platelets in response to brain lipid rafts was performed using Fura 2M probe as described in our previous studies (2, 4). Fura-2M probe, a membrane-permeable derivative of Fura-2, was purchased from Molecular Probes. Platelet rich plasma was diluted with HBSS to proper platelet concentration (10^7/ml) and platelets were loaded with 1 µM Fura-2M at 37°C for 30 min. Washed platelet suspensions were then prepared by centrifugation for 10 min at 10,000g and re-suspension of the pellet in HBSS buffer. Fura-2M fluorescence signal measurements at 340 and 380 nm excitation were conducted at room temperature using spectrofluorometer system (TECAN) after addition of lipid rafts (5 µl/well).

For isolation of peripheral blood mononuclear cells (PBMCs) from HC subjects or MS patients, blood samples were drawn from the same group of patients as for the platelet analysis by using 10 ml collecting tubes with heparin (BD Biosciences). PBMCs were isolated using the Ficoll density gradient (Ficoll-Paque Plus from GE Healthcare) according to the standard protocol (2, 3).

Mouse platelets were isolated from the peripheral blood as described previously (2). To isolate mouse platelets, blood was collected through cardiac puncture (0.38% sodium citrate was used as anticoagulant agent) using 1 ml syringe. Platelet rich plasma was obtained by a low speed centrifugation (250g, 5 min), while platelets were isolated by high speed centrifugation (10,000g, 10 min).

For the assessment of platelets and platelet-derived microparticles by FACS, the forward and size scatter parameters on the flow cytometer were set to a logarithmic scale and the $CD61^+CD42a^+$ gated cells in the "platelet" forward/size scatter gate were analyzed as platelets, and the $CD42a^+$ particles in the sub-platelet "microparticle" forward/size scatter gate were determined as platelet-derived microparticles as described (2).

Human Anti-human CD42a-PerCP, CD4-FITC and PE conjugated Annexin V were purchased form BD Biosciences. Anti-mouse CD4-FITC, CD61-FITC, CD41-PE, CD11b-PE, CD41-PE-Cy7 and CD4-APC antibodies were purchased form BD Biosciences. Anti-mouse CD45-APC-Cy7antibodies were purchased from Biolegend.

Platelet 5HT content and 5HT/PF4 and Release Assays

Serotonin was assessed in platelet lysate or in the supernatant. To assess 5HT in platelet lysate, platelet rich plasma was obtained and platelet numbers were counted. The volume was adjusted to have equal number of platelets in all samples ($2x10^8$) and platelets were centrifuged at 10,000g for 10 min.. Platelet pellet was resuspended in 100 µl of milliQ water and kept at -70°C before analysis. 5HT level was analysed by ELISA according to instructions for Serotonin ELISA kit (Abcam)

To assess 5HT or PF4 in supernatant after stimulation of platelets with brain lipid rafts, 300 µl of the platelet rich plasma were mixed with 60 µl of the brain lipid rafts or PBS and incubated for 30 minutes for serotonin and 1 hour for PF4 assessments at +37°C. Then the samples were centrifuged at 10,000 g for 10 minutes at +4°C and 100 µl supernatant (platelet-free plasma) was used for serotonin (GenWay and

Abcam), or mouse PF4 (Uscn Life Science) assessments using ELISA kits according to the manufacturer's recommendations.

Platelet-T cell co-culture

Human CD4 T cells were isolated from PBMCs preparations by negative selection using a magnetic separation kit (BD Biosciences), and stimulated for six days with plate-bound anti-CD3/CD28 antibodies (1 µg/ml; BD Biosciences) in RPMI media with 10% FBS (5) without or with various numbers of syngeneic or allogeneic washed platelets that were obtained from platelet-rich plasma by centrifugation as described previously (2). Cytokine production in culture supernatants were measured by ELISA similarly as described (5) using kits from BD Biosciences. Syngeneic and allogeneic co-cultures of CD4 T cells with platelets demonstrated similar results (not shown). In several co-culture experiments, anti-PF4 (25 µg/ml, "PeproTech") mAbs and PAF receptor inhibitor WEB2086 (10^{-5} M, "Sigma") were used

Mouse CD4 T cells were isolated from the spleen by negative selection using a BD Biosciences kit similarly as for human CD4 T cells and stimulated with soluble or immobilized anti-CD3 (2-4µg/ml for soluble; 10 µg/ml for immobilized; BD Biosciences) with or without soluble anti-CD28 (4µg/ml; BD Biosciences) activating antibodies. Stimulation with soluble anti-CD3 was done in the presence of irradiated syngeneic splenocytes (at the ratio of 1:5) used as antigen presenting cells, with or without TB- or ADP- activated and washed mouse autologous platelets (1:20) for three days for analysis of T cell activation markers CD25-PerCP and CD44-APC on CD4 T cells.

TB and ADP were purchased for Sigma. Blocking anti-mouse CD62P antibodies (30 µg/ml) were purchased from BD Biosciences, polyclonal anti-mouse CD166 antibodies (25µg/ml) were purchased from R&D Systems, and rabbit polyclonal anti-serotonin antibodies (1:500) were purchased from GenWay. Before addition to the culture anti-serotonin antibodies or control serum were washed with PBS using Centricon 50kDa membrane filter units (Millipore). Activation of human and mouse platelets with thrombin (TB) or ADP was performed as previously described (2).

Mouse models

C57BL/6 mice were purchased from Jackson Laboratories. B6.MOG-TCR transgenic 2D2 mice were maintained in our colony. All animal protocols were approved by Harvard Medical School and the Chinese University of Hong Kong Institutional Animal Ethics Committees.

For CD4 T cell recall response, MOG-TCR transgenic mice were immunized with 150 µg MOG_{35-55} (American peptide) in 4 mg/ml CFA (Diffco)[26]. Platelet depletion was performed similarly as described earlier (2). On days 0, 2, 4, 6 and 8 post-immunization 30 µl of anti-TS or CS was injected i.p., and on day 10 post-immunization CD4 T cells were analyzed for cytokine expression and proliferation.

For analysis of CD4 T cell apoptosis, *ex-vivo* isolated stimulated T cells were stained for CD4-FITC and Annexin V-PE, washed with Annexin V binding buffer (BD Bioscieces) and were analysed by three-color flow cytometry within 30 min in the presence of 7-Aminoactinomycine D (7AAD, BD Biosciences).

For EAE induction, 8-12–week-old B6 mice were immunized with 150 µg MOG in 4 mg/ml CFA with administration of Pertussis toxin (Sigma, 150 ng/mouse) on day 0 and day 2 as described previously (6, 7). The mice were observed for the signs of disease starting on day five post-transfer, and the disease severity was scored on a numerical scale 0–5 as follows: 0) no disease; 1) weak tail or wobbly walk; 2) hind limb paresis; 3) hind limb paralysis; 4) hind and forelimb paralysis; and 5) death or euthanasia due to humane reasons.

Rabbit anti-mouse thrombocyte polyclonal antibodies (serum; anti-TS) was purchased from Accurate Chemical & Scientific Corp. Anti-CD62P NA/LE grade antibodies were purchased from BD Biosciences For depletion of platelets or blocking of cell contact interactions of activated platelets with CD4 T cells during late stage of EAE, 20-30 µl of anti-TS or 50 µl of anti-CD62P mAb (1 mg/ml), or CS was injected i.p. or i.v. as indicated (2). Platelets counts were decreased with anti-TS from 5–8 to 0.1–0.3 ($\times 10^5/\mu l$) as measured 24 hours after i.p injection.

Flow cytometry analysis of expression of intracellular molecules

For intracellular detection of IL-17, IFN-γ, IL-4, or FoxP3 expression, mouse or human T cells were activated with phorbol myristate acetate (50ng/ml) and ionomycin (1 µg/ml; both from Sigma) in the presence of GolgiStop (1µl/ml, BD Biosciences) for four hours. Cells were immediately stained for surface markers (CD4 only for assessment of CD4 T cells, or CD4 and CD25 for assessment of Tregs, or CD4 and CD42a (human) or CD41 (mouse) for assessment of cytokine expression in CD4 T cells aggregated vs. not aggregated with platelets), fixed/permeabilized and stained for intracellular antigens with proper antibodies directly conjugated with fluorophores. For human surface markers anti-CD4-FITC, CD25-PE, CD42a-PerCP were used (BD Biosciences). For mouse surface marker analysis CD4-FITC, CD4-APC, CD41-PE, CD41-PE-Cy7 monoclonal antibodies were used (BD Biosciences). Antibodies for intracellular cytokine staining analysis (anti-human IL-17-PE, IL-17-AF647, and IFN-γ-APC; anti-mouse IL-17-PE, and IFN-γ-PE) were purchased from BD Biosciences. APC conjugated antibodies for intracellular FoxP3 staining were purchased eBioscences. The data was acquired on LSR II and LSR Fortessa cytometers (BD Biosciences) and was analyzed with FlowJo software (TreeStar Inc.). Isolation of mononuclear cells from the CNS and spleen of mice with EAE and FACS analysis was performed as described previously (6, 8, 9).

For isolation of mononuclear cells from mouse peripheral blood, blood was collected through cardiac puncture using 1 ml syringe (0.38% sodium citrate was used as anticoagulant). For isolation of mononuclear cells from mouse spleens, splenocytes were isolated by homogenization of whole spleen using 40µ Cell strainer (BD Biosciences). To isolate mononuclear cells from blood or spleen, the cell suspensions were overlayed over 5 ml of Ficoll-Paque Plus (GE Healthcare) and centrifuged at cell culture centrifuge at 2000 rpm for 30 min. To isolate mononuclear cells from the CNS, mice were perfused intracardially with PBS, brains and spinal cords were dissected and homogenized. CNS mononuclear cells were further isolated using 40%/70% Percoll (Sigma) density gradient. Calculation of absolute numbers of the cells in the CNS or periphery was also performed as previously described (2). The absolute numbers of IL-17$^+$CD4$^+$ T cells, and IFN-γ$^+$CD4$^+$ T cells, CD3$^+$CD4$^+$ T cells, CD11b$^-$CD45^{hi} lymphocytes, and CD11b$^+$CD45^{hi} macrophages were calculated by multiplying the total cell count obtained using a hemocytometer by the percentage of these cells determined by flow cytometry.

Proliferation assays

BrdU incorporation assay was used to assess proliferation of CD4 T cells *in vitro* or *in vivo* as was described in our earlier studies (6). To perform this analysis we used BrdU kit from BD Biosciences following manufacturer's instructions. BrdU was added to cultures (10 µM) or injected i.p. (2 mg/mouse) 14 hours prior to analysis. For assessment of the level of proliferation of CD4 T cells aggregated vs. not aggregated with platelets, the cells were co-cultured with the same numbers of non-activated/washed or TB-activated/washed platelets for 3 days (human) or isolated ex-vivo (mouse), stained for surface markers

CD4 and CD42a (human) or CD41 (mouse), fixed/permeabilized and stained with FITC conjugated anti-BrdU antibodies according manufacturer's instructions for BrdU kit. Analysis of T cell proliferation using ^{3}H- thymidin (^{3}H- Td) incorporation analysis was performed as described (10). The cells were stimulated with MOG peptide (2-10 µg/ml) and pulsed with ^{3}H- Td 48 hours later. The cells were analyzed on cell harvester 24 hours after addition of ^{3}H- Td.

Scanning electron microscopy

Mouse CD4 cells were stimulated with immobilized anti-CD3 antibodies for two days. The alive cells were separated from dead cells using Ficoll gradient, washed with PBS and placed on coverslips pre-coated with anti-CD3 antibodies and co-cultured with syngenic washed platelets (ratio 1:15) for eight hours. After that the cells on coverslips were rinsed with 0.1M Sorensen' phosphate buffer (PB, pH=7.2), fixed with 2.5% glutaraldehyde in PB for 1hour at room temperature. Fixed specimens were washed with PB three times for 10 min for each wash and further fixed with 2% osmium tetroxide for 1 hour. Then the cells were washed three times in milliQ water (10 min each wash). The samples were then dehydrated in graded ethyl alcohols (EtOH): 70-75%, 80-85%, 90-95% (15 minutes for each step), then dehydrated in 95% EtOH and in absolute EtOH (2 changes, 15 min each step) and placed in LADD Critical Point Dryer. Finally the samples were mounted on specimen stubs and coated with gold-palladium on Edwards Sputter Coater right before examination under the Hitachi SU8010 Scanning Microscope.

Statistical Analysis

The results are presented as mean and individual data-point, mean ± S.E., or mean (S.D.). Unpaired Student's t-test and Mann–Whitney U test were used to determine significance between groups. P values of less than 0.05 were considered significant. When appropriate the Mann–Whitney U test was used in addition to Unpaired Student's t-test to further confirm the significance between groups with semi-normal distributions (e.g. Fig. 1A). Prism GraphPad and SigmaPlot software were used for creating graphs and performing statistical analysis.

Supplemental Table I. **Demographic and clinical characteristics of multiple sclerosis (MS) patients and healthy control (HC) subjects used in this study**[1]

	MS (all)		RRMS[2]		SPMS[3]		HC	
N	27		22		5		21	
Age: Mean (SD)	47.38	(13.9%)	43.4	(12.0%)	64.4	(6.5%)	35.9	(10.5%)
Male *N* (%)	7	(25.9%)	5	(22.7%)	2	(40.0%)	5	(23.8%)
EDSS Median (range)	1.5	(0 - 6.5)	1	(0 - 6.5)	6.5	(3 - 6.5)	-	

[1]See *Supplemental Methods* section for details.

[2]RRMS, Relapsing remitting multiples sclerosis.

[3]SPMS, Secondary progressive multiples sclerosis.

<u>Supplemental Table II</u>. Comparison of the levels of secretion of 5HT and PF4 by platelets from healthy control subjects vs. MS patients in response to stimulation with brain lipid rafts (LR) and ADP[1]

Exp #		Healthy Control Subjects			MS patients		
		Background level	**LR**	**ADP**	**Background level**	**LR**	**ADP**
1.	**5HT**	1.9[2]	6.4 (**3.4**)[3]	N.T.[4]	1.0	1.3 (**1.3**)	N.T.
	PF4	2.2[5]	3.6 (**1.6**)	3.2 (**1.4**)	2.6	2.9 (**1.1**)	2.3 (**0.9**)
2.	**5HT**	0.5	1.5 (**3.0**)	N.T.	3.5	3.6 (**1.0**)	N.T.
	PF4	2.0	2.6 (**1.3**)	2.8 (**1.4**)	2.6	2.6 (**1.0**)	2.7 (**1.0**)
3.	**5HT**	1.2	3.3 (**2.8**)	N.T.	3.2	3.4 (**1.1**)	N.T.
	PF4	2.0	2.4 (**1.2**)	3.0 (**1.5**)	2.5	2.5 (**1.0**)	2.8 (**1.1**)

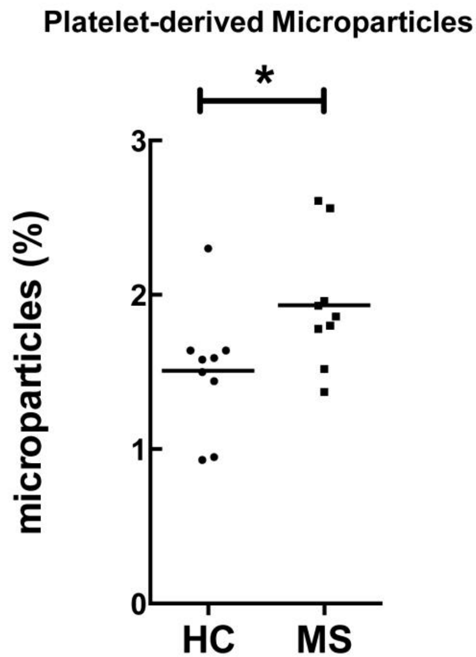
[1]The level of 5HT and PF4 was assessed by ELISA after stimulation of platelets with LR or ADP as described in *Supplemental Methods* section.

[2]Serononin (5HT) concentration (ng/ml)

[3]Fold difference from the background level is shown in the parentheses

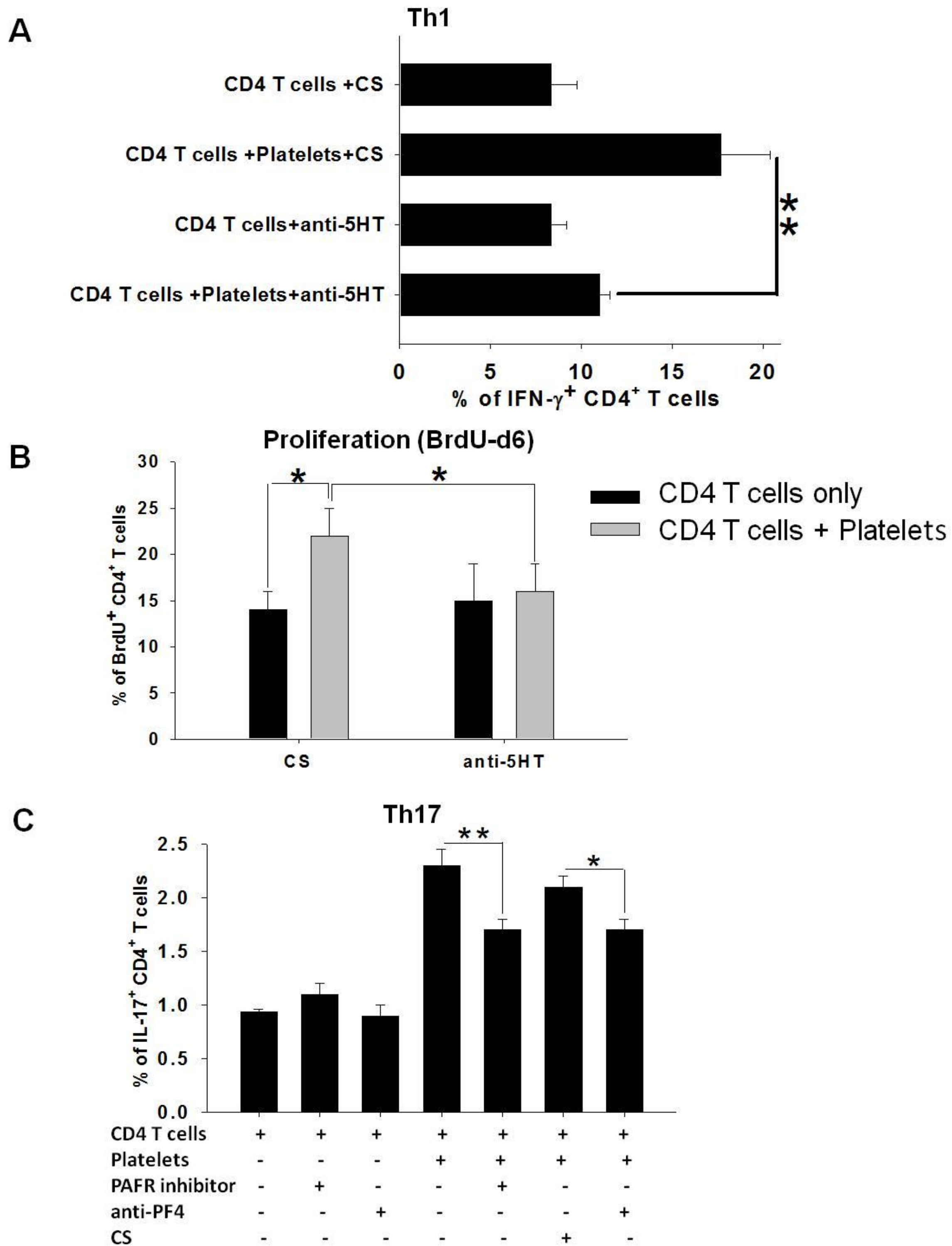
[4]N.T., not tested.

[5]PF4 concentration (ng/ml)



<u>Supplemental Figure I</u>. Analysis of platelet-derived CD42a^{+} microparticles in platelet rich plasma of untreated patients diagnosed with multiple sclerosis (MS) or healthy control (HC) subjects.

Flow cytometry analysis of platelet-derived microparticles from platelet rich plasma isolated from multiple sclerosis patients vs. healthy control subjects. Percentages of FSC^{low}CD42a^{+} microparticles of all CD42a^{+} events are shown**.** Individual values and mean are indicated (*p<0.05).



Supplemental Figure II.

(See next page for the *Result Description* and the *Legend*).

Supplemental Figure II (*Result Description*). Platelet-derived serotonin (5HT), PF4, and PAF contributes to CD4 T cell proliferation and production of IFN-γ and IL-17.

Result Description: It was demonstrated that 5HT contributed to the activation of CD4 T cells and stimulated the proliferation and the production of IFN-γ by CD4 T cells (11, 12). Therefore we proposed that platelet-derived serotonin promoted the proliferation and differentiation of human CD4 T cells towards pathogenic phenotypes. To test this hypothesis we added anti-5HT polyclonal antibodies (serum) or control serum (CS) to our co-cultures and found that anti-5HT serum partially abrogated platelet-induced proliferation and differentiation of CD4 T cells towards Th1 as indicated by BrdU incorporation assay and IFN-γ production (**Supplemental Fig. IIA,B;** see the **Legend** below).

However, anti-5HT serum did not affect IL-17 production (not shown) indicating that other factors contributed to Th17 differentiation. It was reported that other platelet-derived soluble factors such as PF4 and PAF could also contribute to Th17 differentiation (13-16). By using anti-PF4 antibodies and PAF receptor inhibitor, we found that both PF4 and PAF contributed to Th17 differentiation during co-culture of platelets with CD4 T cells (**Supplemental Fig. IIC).**

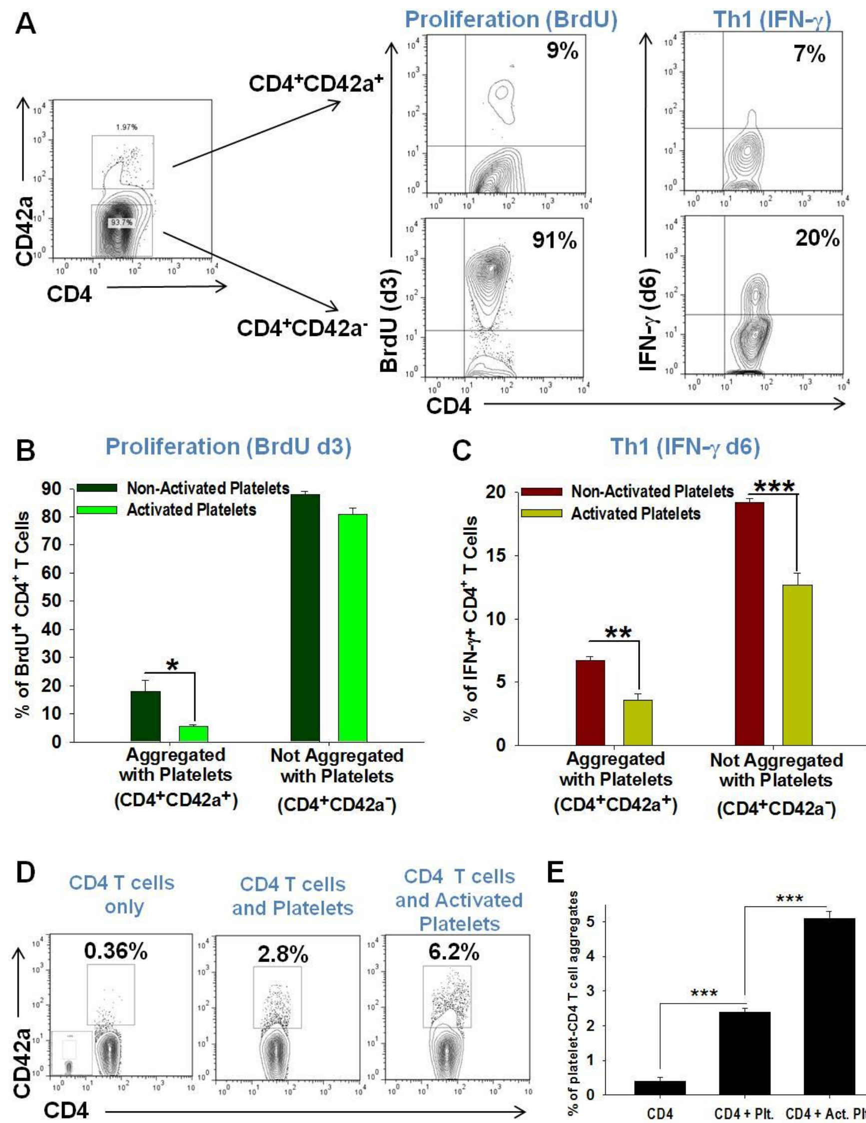
Thus platelet-derived 5HT contributed to increased proliferation of CD4 T cells and differentiation towards Th1, while PF4 and PAF contributed to Th17 differentiation.

Supplemental Figure II (*Legend*). Platelet-derived serotonin (5HT), PF4, and PAF contributes to CD4 T cell proliferation and production of IFN-γ and IL-17.

(A-B) Human CD4 T cells were co-cultured with platelets in the presence of control rabbit serum (CS) or rabbit anti-serotonin serum (anti-5HT) as described in *Supplemental Methods*. Proliferation (BrdU incorporation) and IFN-γ production were assessed on d6 and analyzed by flow cytometry as described in *Supplemental Methods.* Percentages of IFN-γ^+ (**A**) and $BrdU^+$ (**B**) CD4 T cells are shown.

(**C**) Human CD4 T cells were cultured alone or co-cultured with platelets in the presence of anti-PF4 antibodies (anti-PF4), control serum (CS), or PAF receptor inhibitor (PAFR inhibitor) as described in *Supplemental Methods.*

In (**A-C**), mean ± S.E. of 4-5 separate experiments is shown (*, $p<0.05$; **, $p<0.01$).

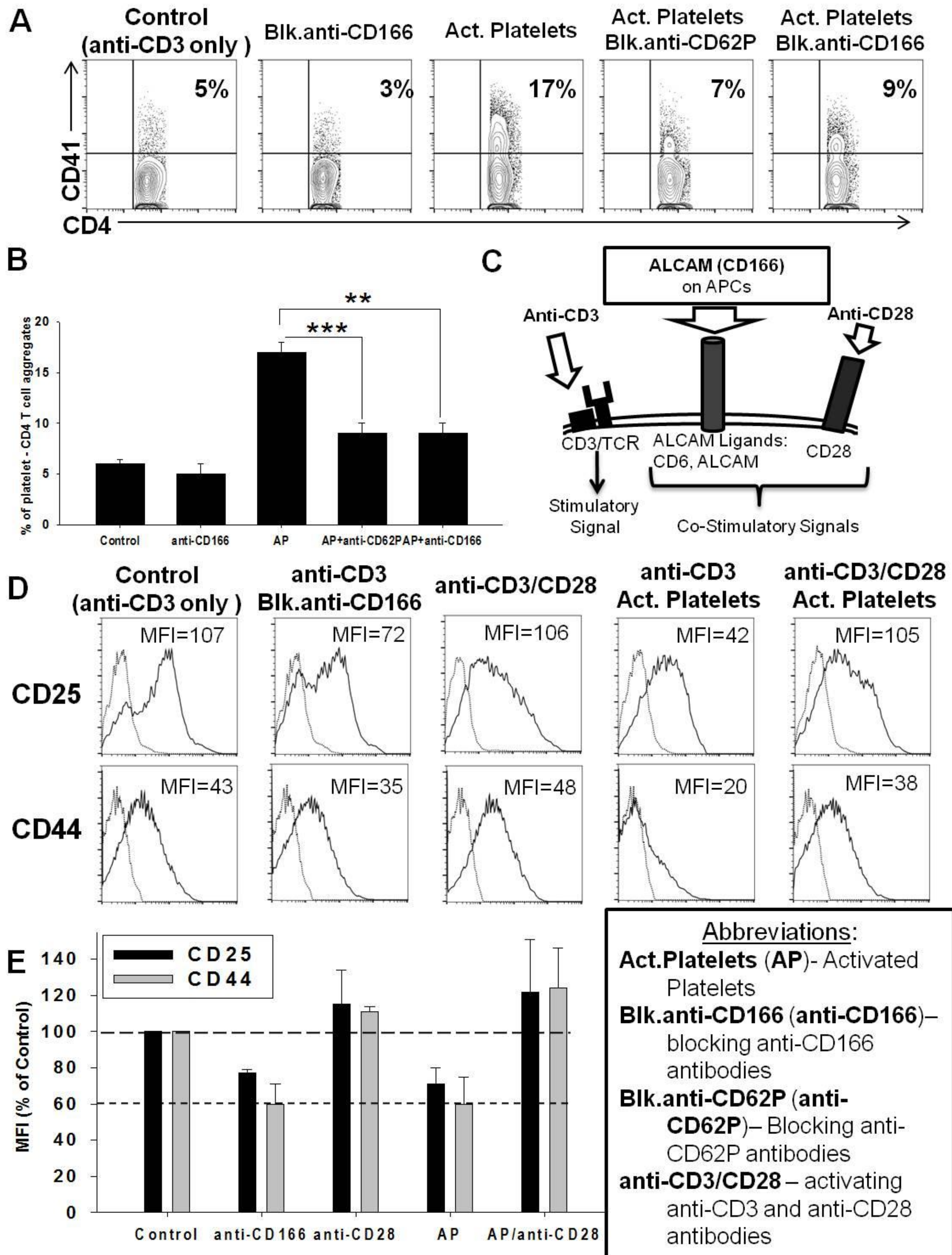


Supplemental Figure III. Influence of platelet-CD4 T cell formation on proliferation and differentiation of human CD4 T cells and analysis of abilities of activated vs. non-activated platelets to form aggregates with CD4 T cells. Non-activated or activated platelets were co-cultured with CD4 T cells for 3-6 days and then the cells were stained for surface markers CD4 and CD42a and then analysed for intracellular IFN-γ expression or BrdU incorporation as described in *Supplemental Methods.* The cells were analysed by three-color follow cytometry for simultaneous expression of three markers (CD4, CD42a, and IFN-γ) or (CD4, CD42a, and BrdU). Gated $CD4^+CD42a^+$ and $CD4^+CD42a^-$ subsets were analysed BrdU or IFN-γ.

(**A-C**) Comparison of abilities of CD4 T cells that were aggregated ($CD4^+CD42a^+$) vs. non-aggregated ($CD4^+CD42a^-$) with platelets to proliferate and produce IFN-γ. Representative contour-plots for $CD4^+BrdU^+$ and $CD4^+$IFN-γ^+ cells are shown in (**A**) and statistics is shown in (**B-C**). Proliferation and platelet-CD4 T cell aggregates were assessed on d3 using BrdU incorporation assay (**A,B**) and IFN-γ production was assessed on d6 by intracellular cytokine staining (**A,C**) as described in *Supplemental Methods*.

(**D-E**) CD4 T cells were incubated alone (*CD4*) or co-cultured with non-activated (*CD4 + Plt.*) or TB-activated platelets (*CD4 + Act.Plt*) and percentages of CD4 T cells aggregated by platelets were analyzed as described in *Supplemental Methods*. Isotype-matched control staining for CD42a on gated CD4 T cells is shown in the left histogram in the left lower corner (**D**).

In (**B,C,E**) mean ± S.E. of 3-5 separate experiments is shown (*, $p<0.05$, **, $p<0.01$; ***, $p<0.005$).



Supplemental Figure IV.

(See next two pages for the *Result Description* and the *Legend*).

<u>Supplemental Figure IV (*Result Description*)</u>. Activated mouse platelets bind to autologous CD4 T cells via interaction of P-selectin (CD62P) on platelets with ALCAM (CD166) on activated CD4 T cells, which results in decrease in expression of CD4 T cell activation markers CD25 and CD44.

<u>Result Description</u>: It was reported that CD62P, which is expressed on activated platelets, played an important role in the formation of platelet-leukocyte aggregates including CD4 T cells in humans and mice (17-19). Indeed we found that the addition of activated platelets to activated mouse CD4 T cells resulted in relatively high levels of platelet-CD4 T cell aggregates (**Supplemental Fig. IVA,B;** see the **<u>Legend</u>** next page). At the same time, binding of activated platelets to CD4 T cells was decreased 2-fold after the addition of blocking anti-CD62P antibodies to co-cultures of platelets with CD4 T cells (**Supplemental Fig. IVA,B**). We have previously shown that CD62P can directly bind to ALCAM *in vitro* (2). ALCAM (CD166) is an adhesion molecule that is expressed on activated CD4 T cells, APCs, activated endothelial cells and plays an important role in CD4 T cell activation and EAE pathogenesis (20, 21). We found that the addition of blocking anti-CD166 antibodies to co-cultures of platelets with CD4 T cells resulted in ~2-fold decrease in the percentages of platelet-CD4 T cell aggregates (**Supplemental Fig. IVA,B**). It was reported that ALCAM on APCs provides a co-stimulatory signal to T cells by interacting with ALCAM ligands such as CD6 and other ALCAM molecules expressed on T cells (20, 22) (**Supplemental Fig. IVC**). CD6 gene polymorphism was also shown to be associated with high susceptibility to MS (23). We hypothesized that activated platelets, which express high levels of CD62P, bind to ALCAM on APCs and activated CD4 T cells and block co-stimulatory signals for CD4 T cells. It was previously reported that ALCAM-CD6 interactions significantly contributed to co-stimulation of CD4 T cells and expression of T cell activation markers (22). To test this we activated mouse CD4 T cells through TCR using soluble activating anti-CD3 mAbs in the presence of antigen presenting cells that provided co-stimulatory signals to CD4 T cells through ALCAM-CD6 and ALCAM-ALCAM interactions (**Supplemental Fig. IVC**). We found that in this system the addition of blocking anti-CD166 antibodies or activated platelets significantly decreased expression of activation markers CD25 and CD44 on CD4 T cells (**Supplemental Fig. IVD,E**). However in the presence of soluble anti-CD28 antibodies that provide a co-stimulatory signal in addition to antigen presenting cells (**Supplemental Fig. IVC**), activated platelets in co-cultures did not cause the decrease in expression of CD25 and CD44 on CD4 T cells (**Supplemental Fig. IVD,E**). Thus we proposed that activated platelets have a high capacity to bind CD4 T cells and APCs via CD62P-ALCAM interactions and interfere with the delivery of co-stimulatory signals from antigen presenting cells to T cells leading to a decreased level of CD4 T cell activation.

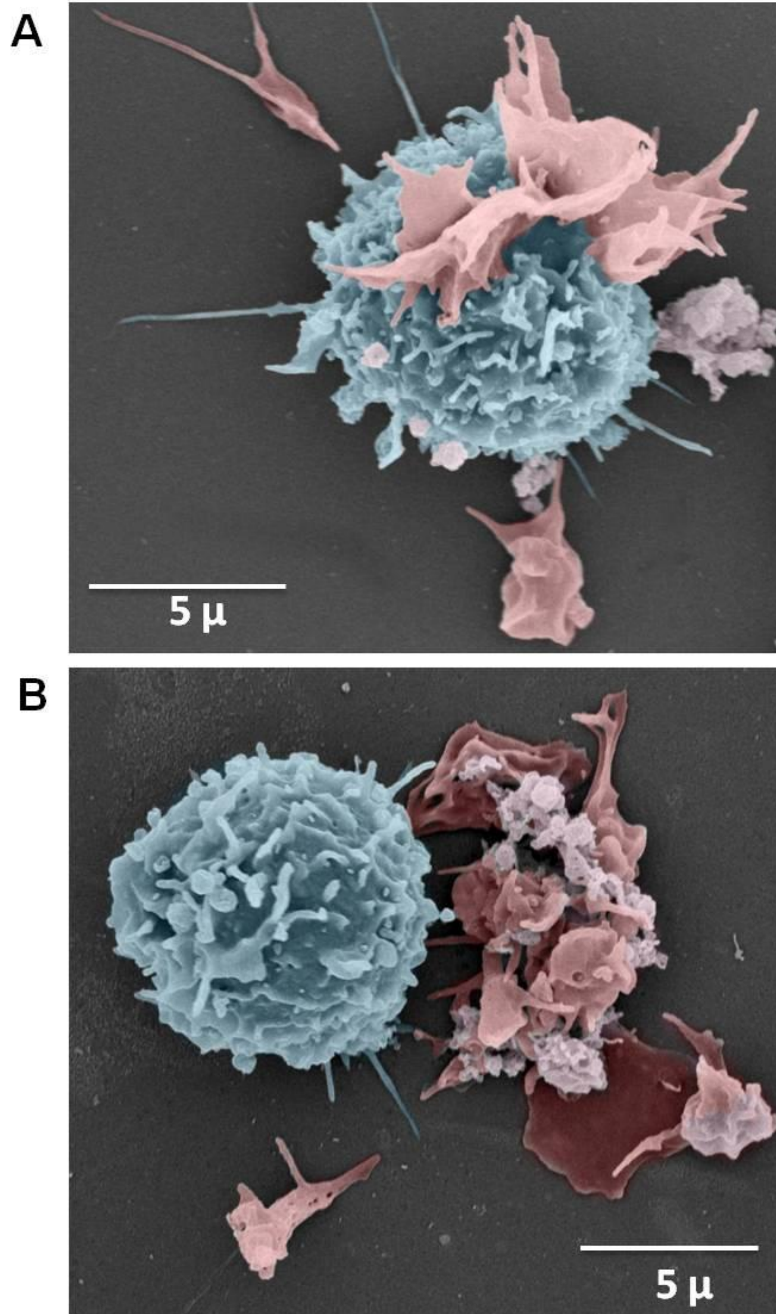
Supplemental Fig. IV (*Legend*). Activated mouse platelets bind to autologous CD4 cells via interaction of P-selectin (CD62P) on platelets and ALCAM (CD166) on activated CD4 T cells, which results in decrease in expression of T cell activation markers CD25 and CD44.

Legend: Mouse CD4 T cells were stimulated with soluble activating anti-CD3 antibodies in the presence of antigen presenting cells (irradiated splenocytes) and cultured alone (*Control*) or co-cultured with thrombin-activated platelets (*Act. Platelets*) for three days as described in *Supplemental Methods.* Stimulating anti-CD28 (*Anti-CD28*), or blocking anti-CD166 (*Blk.Anti-CD166*), or blocking anti-CD62P (*Blk.Anti-CD62P*) antibodies were added to cultures at day 0 as indicated.

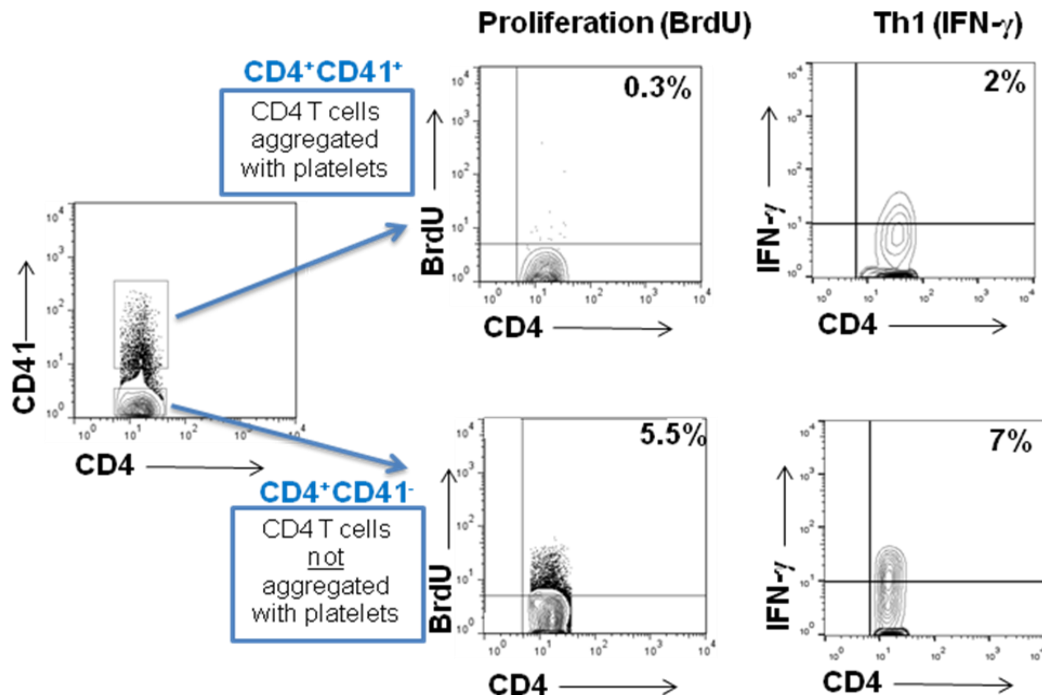
(**A-B**) Influence of blocking anti-CD62P and blocking anti-CD166 antibodies on formation of platelet-CD4 T cell aggregates *in vitro.* Percentages of platelet-CD4 T cell aggregates ($CD4^+CD41^+$ population) were analyzed by flow cytometry as described in *Supplemental Methods.* One representative experiment is shown in (**A**) and mean ± S.E. of four separate experiments is shown in (**B**) (**, $p<0.01$, ***, $p<0.001$).

(**C**) Schematic diagram of pathways of CD4 T cell activation in the current *in vitro* system. For proper T cell activation stimulatory signal through CD3/TCR is provided by soluble activating anti-CD3 antibodies, while co-stimulatory signal through CD6 and ALCAM is provided by ALCAM on antigen presenting cells (APCs) that are present in the culture. Additional co-stimulatory signal is provided by soluble activating anti-CD28 antibodies.

(**D-E**) Analysis of expression of T cell activation markers CD25 and CD44 on CD4 T cells cultured alone or co-cultured with activated platelets. Expression of activation markers was analyzed by flow cytometry on $CD4^+$ gated CD4 T cells as described in *Supplemental Methods.*
One representative experiment is shown in (**D**), mean ± S.E. of 3-4 separate experiments is shown for relative MFI levels (percentages of the controls) is shown in (**E**).



<u>Supplemental Figure V</u>. Visualization of interactions of CD4 T cells with platelets.
Mouse CD4 T cells were stimulated with anti-CD3 antibodies in the presence of platelets and analyzed by scanning electron microscopy as described in *Supplemental Methods.* Interactions of platelets of activated morphology (marked with red pseudocolor) with CD4 T cells (marked with blue pseudocolor) were observed as a formation of platelet-CD4 T cell aggregates (**A**) and paracrine interaction of clustered activated platelets with CD4 T cells without obvious platelet-CD4 T cell aggregate formation (**B**). Platelet-derived microparticles were also evident and marked with pink pseudocolor (size of the bar is 5 μ; X 10,000 (**A**) and X 9,000 (**B**) magnifications). Representative images are shown.



Supplemental Figure VI. Influence of CD4-platelet aggregate formation on proliferation and Th1 differentiation of mouse CD4 T cells *in vivo*.

On day 35 after EAE induction, mononuclear cells were isolated from spleen using Ficoll gradient and CD4 T cells that were aggregated with platelets ($CD4^+CD41^+$ subset) vs. not aggregated with platelets ($CD4^+CD41^-$ subset) were analyzed similarly as described in *Supplemental Methods*. For BrdU analysis, BrdU was injected i.p. 14 hours prior to isolation of splenocytes (see *Supplemental Methods*). For IFN-γ analysis, *ex-vivo* isolated splenocytes were activated with phorbol myristate acetate and ionomycin as described in *Supplemental Methods*. Representative contour-plot of four individual mice is shown.

Supplemental References

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