Science Immunology

immunology.sciencemag.org/cgi/content/full/1/1/aaf8759/DC1

Supplementary Materials for

Donor exosomes rather than passenger leukocytes initiate alloreactive T cell responses after transplantation

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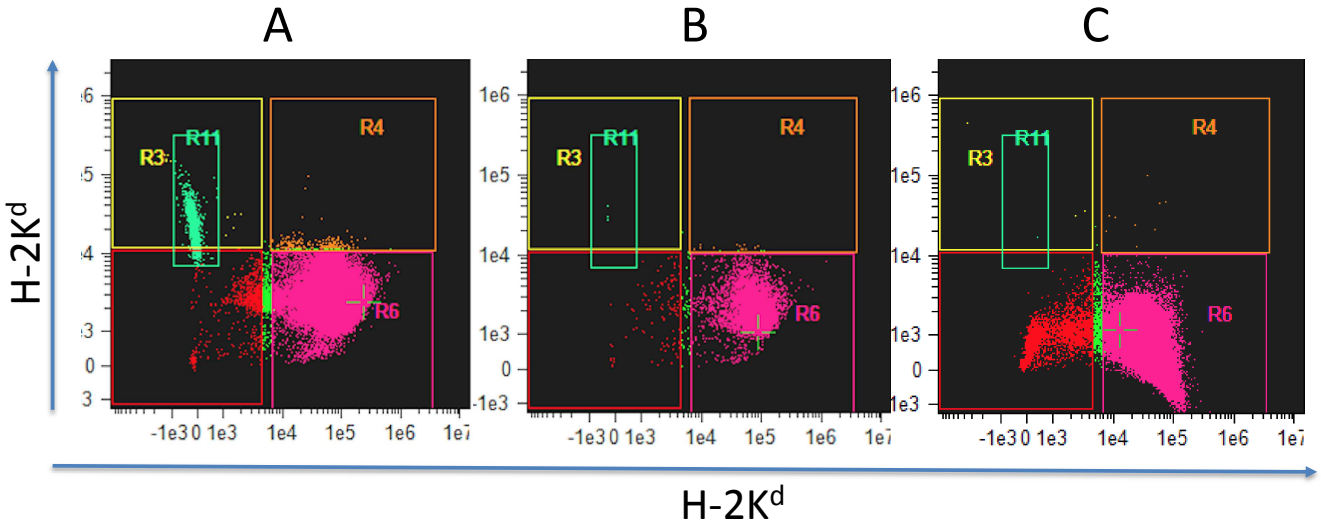
Published 14 July 2016, *Sci. Immunol.* **1**, aaf8759 (2016)
DOI: 10.1126/sciimmunol.aaf8759

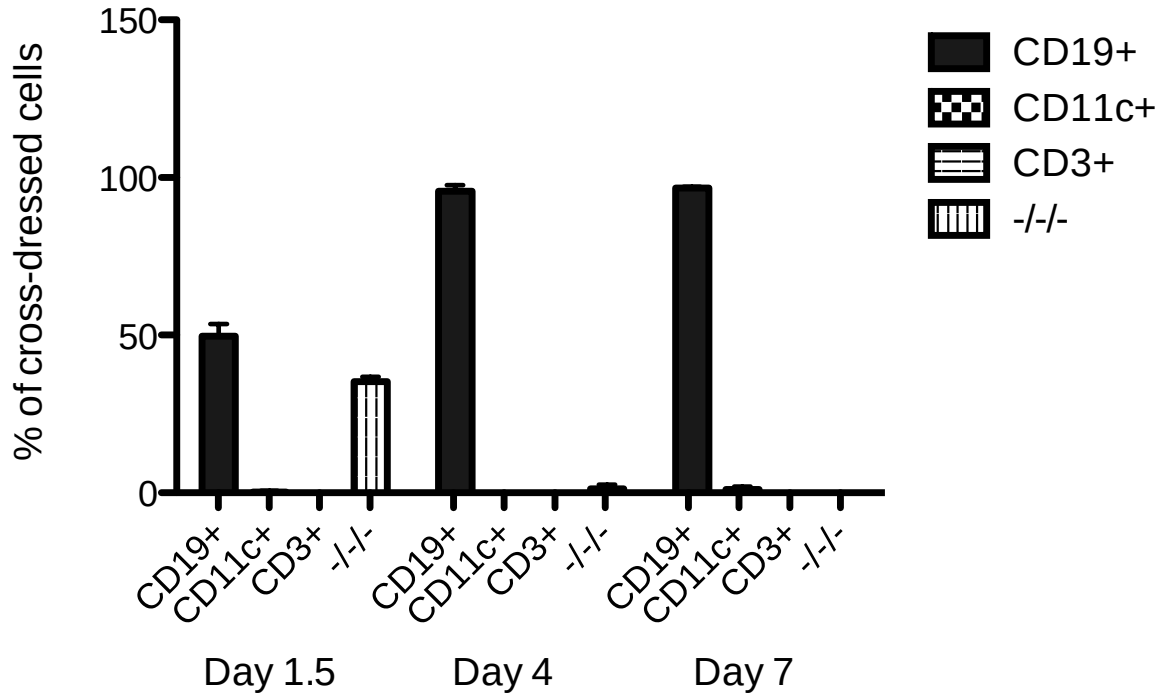
This PDF file includes:

Figure S1. Sensitivity of Imaging flow cytometry.



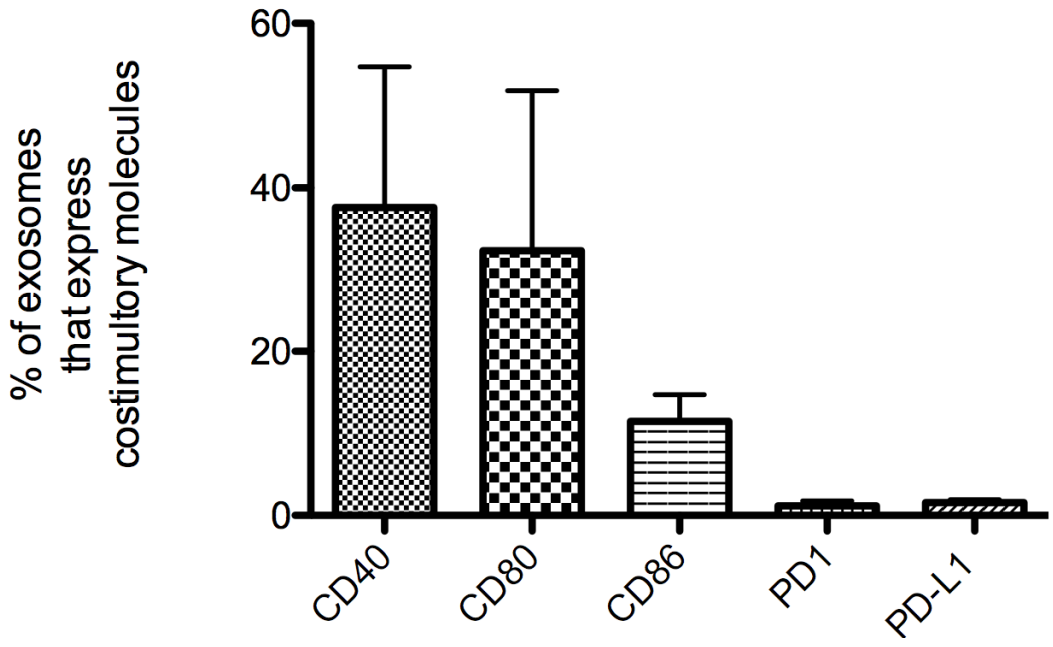
Different proportions of B6 cells were mixed with cells originated from BALB/c mice (Panel A: 1:100, Panel B: 1:100000, Panel C: 1:500000), labeled with anti-H-2K^d (APC, X axis) and anti-H-2K^b (FITC, Y axis). R3 represents the region of events tagged with anti-H-2K^b, R11 represents the number of viable cells expressing H-2K^b.

Figure S2. Markers expressed by cross-dressed cells after heart transplantation.



Spleen cells of B6 mice recipient of a BALB/c heart allograft were collected at different time points after transplantation. Recipient cells (H-2K^{b}+) cross-dressed with donor MHC class I K^{d} were stained with the following fluorochrome-bound antibodies: anti-CD3-Pacific Blue (for T cells), anti-CD19-PE (for B cells) and anti-CD11c-PECy7 (for dendritic cells). The results are representative of 3-6 mice tested individually at each time point.

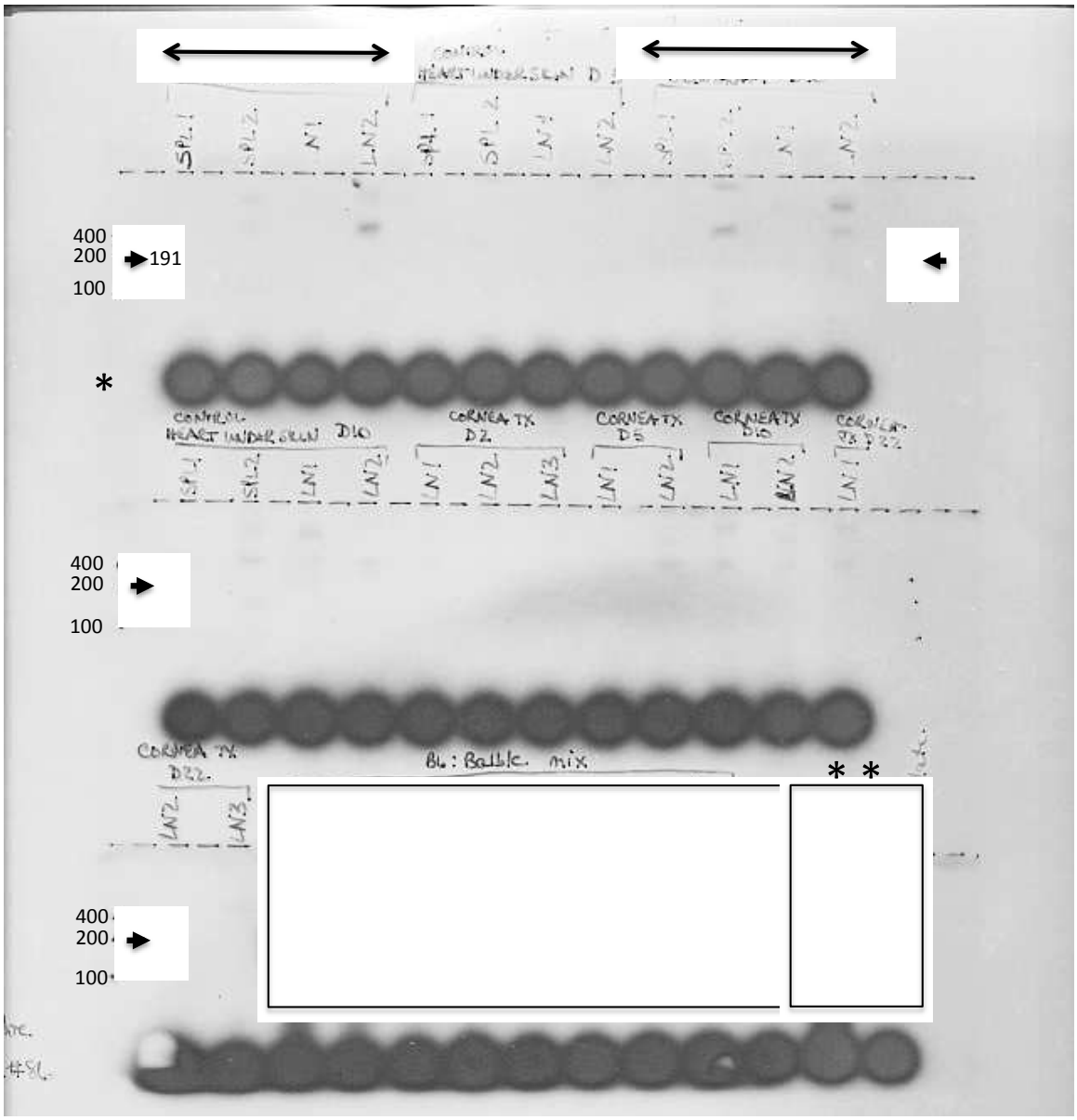
Figure S3. Detection of costimulatory molecules on donor exosomes that cross-dress recipient cells.



Lymph node cells (ipsilateral axillary and brachial LNs) from B6 mice recipient of a BALB/c skin allograft were collected at different time points after transplantation. The donor vesicles that were bound to recipient cells were stained with anti-CD40, anti-CD80, anti-CD86, anti-PD1 and anti-PD-L1 antibodies and analyzed by imaging flow cytometry.

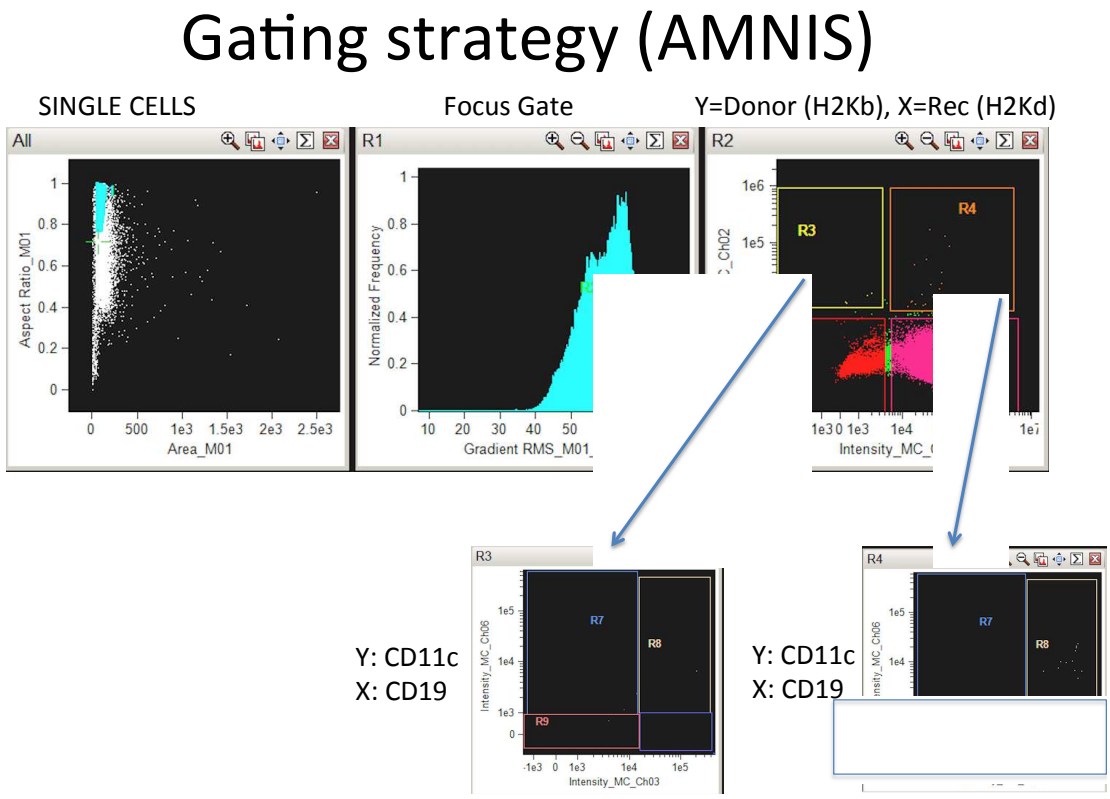
Figure S4. Detection of donor MHC class I mRNA.

ORIGINAL AUTORADIOGRAPHY OF GELS PROBED WITH 32P- PROBE.
Figure 3 C and D



Two-day exposure autoriadiograph of RT-PCR analysis of splenocytes (SPL) and lymph nodes (LN) from recipients of various B6 → BALB/c graft combinations. Skin graft alone (Control SG) at Day 5 and Day 10; Heart under skin (Control Heart under Skin) day 5 and day10 and cornea (Cornea Tx) at Day 2, 5, 10 and 22. Expected amplified band is at 191 bp (arrow head). Double head arrow and boxes indicate the portions of autoradiograph selected in Figure 3D and 3C, respectively. Because of overexposure, control data from the second box (**) were from an overnight exposure. Dark band at bottom of gel (*) corresponds to excess ^{32}P-probe.

Figure S5. Gating strategy used in imaging flow cytometry.



Analysis gates were based on aspect ratio, area and the root mean square of the rate of change of the image intensity profile of the bright field image for each event, using this strategy, events that corresponded to non-cellular events or debris as well as out-of-focus images were gated out of the analysis. Subsequent gates were based on the intensity of the fluorescence associated to each event and it's morphological distribution in the cell.

Figure S6. Kinetics of alloresponse by T cells after skin transplantation.

Expt # 1	Days post-transplantation	No graft			Syngeneic skin graft			Allogeneic skin graft		
	2	89	123	65	134	176	98	598	764	632
	4	112	67	80	78	90	112	865	998	765
	6	145	110	98	65	134	187	998	1025	1113
	8	45	97	76	88	94	111	987	1231	1123
	10	127	67	87	78	91	159	1034	1432	1248
Expt # 2	2	56	98	96	199	214	122	432	389	511
	4	78	67	78	132	187	176	765	654	620
	6	90	76	64	91	101	156	765	891	875
	8	ND	112	60	75	145	149	1287	1236	998
	10	124	132	99	87	98	112	1245	1543	1124
Expt #3	2	145	134	145	123	122	176	321	540	432
	4	111	98	88	192	114	98	455	517	489
	6	97	143	74	201	145	125	672	784	653
	8	134	154	135	129	164	127	998	764	872
	10	89	122	ND	ND	148	163	1679	1244	1476

Expt #1	Day of skin graft removal	Medium			Syngeneic APCs			Allogeneic APCs		
	No Graft	2	4	5	1	3	2	211	125	199
	Day 2	3	4	7	1	2	4	432	670	398
	Day 6	1	0	0	1	0	0	654	876	987
	Day 9	2	1	0	0	2	0	789	854	910
Expt #2	No Graft	0	0	2	2	1	0	97	88	112
	Day 2	0	0	0	4	1	1	324	212	230
	Day 6	1	0	0	2	2	0	431	378	478
	Day 9	0	0	0	3	1	2	567	499	698
Expt #3	No Graft	0	0	1						
	Day 2	0	0	0	0	0	2	54	77	105
	Day 6	1	0	2	2	2	0	257	321	209
	Day 9	2	0	0	3	4	0	524	621	709

Top Table. C57Bl/6 (B6, H-2^b) mice received a skin graft from a fully allogeneic BALB/c (H-2^d) or a syngeneic B6 mouse. At different time points after transplantation, recipient lymph node T cells were isolated and cultured in vitro for 48 hours with donor irradiated spleen cells. **Bottom Table**. B6 mice were transplanted with a skin patch from a BALB/c mouse. Skin allografts were removed at different time points after transplantation. Recipient lymph node T cells were isolated at day 10 post-transplantation and cultured for 48 hours with medium or with either allogeneic (BALB/c) or syngeneic (B6) spleen cells (APCs). In both panel A and B, the frequencies of γIFN-producing cells were measured by ELISPOT. The results show the numbers of γIFN spots per million T cells (triplicate wells) isolated obtained in three separate experiments performed with mouse lymph nodes collected and pooled from 3-5 animals.

Figure S7. Cross-dressing results obtained with skin-grafted mice.

Fig 2D: N=6 per group	Day post-tx		Day 1.5						Day 4						Day 7							
	Stage of cross-dressing	Stage 1	85	72	76	70	71	90	35	50	40	0	15	7	8	10	10	7	5	10	% of recipient cross-dressed cells	
		Stage 2	10	15	14	11	15	18	55	30	55	40	42	52	10	10	10	6	4	1		
		Stage 3	1	2	6	10	5	2	10	15	3	3	6	10	80	75	90	84	82	91		
Fig 3B: N=6-7 per group, per time point	Days post skin graft		Day 1.5						Day 7													
	Size of beads	0.5 um	108	120	104	92	128	89	140	178	158	140	215	186	129	Number of beads in recipient's LN						
		2.2 um	0	0	0	0	0	0	0	55	30	55	15	65	62							
		6.0 um	0	0	0	0	0	0	0	17	10	20	0	2	20							
Fig 4B: N=5 per group, per time point	Phenotypic markers		CD19+					CD11c+					CD3+					-/-/-				
	Days post tx	Day 1.5	5.7	1.3	3	3.1	3.4	45.9	70.9	71.3	51.3	52.9	0	0	0	0	0	0.5	0.4	0	0	0	Proportion of phenotypic markers among recipient cross-dressed cells
		Day 4	4.8	9.4	17.8	10.2	16.2	80	63	57	70	65	0	0	0	0	0	6.9	13	15.1	7	8.6	
		Day 7	14.6	8.4	25.3	20.4	8.6	77.1	69.9	55.3	54	71.2	0	0	0	0	0	12.2	7.6	1.4	5.3	4.9	
Fig 4C: N=5 per group, per time point	Days Post Tx		Day 1.5					Day 4					Day 7									
	Recipient/Donor MHC	R MHC I, D MHC I	782	705	840	800	830	1100	1230	1150	990	970	1540	2105	1205	1010	1400	Number of recipient's cross-dressed cells per million in LN					
		R MHC II, D MHC I	1190	1040	1000	980	980	330	310	270	280	290	350	330	320	360	320						
		R MHC II, D MHC II	970	1150	950	940	1010	600	590	570	505	570	1200	1005	1305	985	1020						
Fig 5B: N=5 per group, per time point	Phenotypic markers		CD19+					CD11c+					CD3+					-/-/-				
	Days post tx	Day 1.5	0	0	5	0	0	33	0	10	20	25	0	15	25	20	20	100	75	80	80	75	Proportion of phenotypic markers among recipient donor-derived exosomes
		Day 4	0	30	10	10	40	0	25	5	0	5	5	10	33	20	25	50	60	33	50	50	
		Day 7	10	20	20	25	25	10	12	14	10	20	20	20	30	30	25	50	10	70	20	50	

This table shows the results obtained using imaging flow cytometry for each skin grafted individual mouse used in our study (each number corresponds to one mouse).

Figure S8. Activation of allospecific T cells by allogeneic exosomes and allo-MHC cross-dressed cells.

In vitro data (Fig. 6B)

	Media	Self	Allogeneic APCs	CD with allogeneic Splenocytes exosomes	CD with allogeneic B cells exosomes
Expt #1	**0**	**7**	**79**	**86**	**74**
	4	**2**	**79**	**52**	**79**
	3	**1**	**108**	**66**	**42**

	Self	Balb/c (self) exosomes	B6 Splenocytes Exosomes	B6 B cells Exosomes	B6 APCs
Expt #2	**2**	**0**	**2**	**1**	**36**
	0	**0**	**0**	**0**	**28**
	6	**1**	**4**	**0**	**88**
					77

	Media	Syngeneic exosomes	Allo exosomes	Crossdressed cells with allo	Allo Cells	ConA
Expt #3	0	0	10	204	680	948
	0	13	0	212	623	950
	1	14	15	140	412	1022
Expt #4	0	0	22	146	514	854
	2	20	12	185	581	963
	5	10	15	149	432	1025

In vivo data (Fig. 6C)

	Naïve Balb/c	Syngeneic exosomes	B6 splenocytes	Allograft	Allogeneic exosomes mouse 1	Allogeneic exosomes mouse 2	Allogeneic exosomes mouse 3
Expt # 1	3	22	447	407	88	91	84
	11	30	423	437	16	113	53
	23	44	260	359	50	87	66
	59	48	267	407	22	47	47
	40	31	404	265			
	53	40	424	381			
Exp #2	33	51	340	426	70	102	
	29	40	263	426	109	131	
	35	40	272	438	86	89	
	31	78	269	423	92	88	
			293	354			
			286	433			
Expt # 3	16	28	425	1000	75	47	25
	20	29	295	467	82	48	23
	33	32	366	1000	66	79	33
	28	20	294	667	71	33	27
	12	16	343	729	53	82	11
	22	14	372	1000	58	69	43
		27		756			
		26		727			
		22		741			
Expt # 4	37	18	398	487	25	76	32
	33	30	418	500	27	52	38
	16	35	425	505	26	56	42
	53	11	442	481	22	77	25
	35	31	410	468	52	39	35
	41	26	391	477	59	84	40
Expt #5	41	65	68	606	129	156	
	35	62	67	649	108	124	
	47	29	99	759	106	123	
	54	47	56	605	163	122	
	49	73	73	697	117	138	
	41	55	87	706	146	112	

Top panel shows in vitro activation of T cells with exosomes or cross-dressed cells. BALB/c T cells (5 x 10^5 cells/well) were cultured with B6 allogeneic exosomes or BALB/c cells cross-dressed with allogeneic (H-2^b) or control syngeneic (H-2^d) MHC (5 x 10^5 cells/well) for 48 hours. The frequencies of activated T cells producing □IFN were measured using ELISPOT. **Bottom panel**: BALB/c mice were injected i.p with allogeneic (B6) or syngeneic (self, BALB/c) exosomes (2 x 10^8-10^9 vesicles). Fourteen days later, spleen T cells from these mice as well as control naïve mice were collected and stimulated in vitro with irradiated allogeneic B6 APCs for 72 hours. The frequency of T cells secreting γIFN was measured using ELISPOT. BALB/c mice recipient of a B6 skin graft or injected with B6 spleen cells were tested as positive controls. In panel B and C, the results are expressed as number of γIFN-producing spots per million T cells obtained from 13 mice tested individually.