The bone marrow of myeloma patients is steadily inhabited by a normal-sized pool of functional regulatory T cells irrespective of the disease status

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SUPPLEMENTARY APPENDIX

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Foglietta M et al.

Supplementary Methods

Flow cytometry

Multicolor flow cytometry with the anti-CD4 PerCP (Caltag, Burlingame, CA, USA), anti-CD25 PE (Dako Cytomation, Milano, Italy), and anti-CD127 Al647 (Becton Dickinson, Mountain View, CA, USA) monoclonal antibodies (mAbs) was used to identify $CD4^{+}CD25^{hi}Foxp3^{+}$ and $CD4^{+}CD25^{+}CD127^{lo}Foxp3^{+}$ T cells. Intracellular Foxp3 expression was determined with the anti-Foxp3 FITC mAb and the Human Regulatory T cell staining kit (eBioscience, San Diego, CA, USA). Appropriate isotype controls were run for each sample. Samples were read with a FACS Calibur equipped with the CELLQuestPro software (Becton Dickinson).

In selected experiments, the anti-CCR4 APC, anti-CD27 FITC, and anti-CD45RA APC mAbs (Becton Dickinson) were also used to further characterize the Tregs phenotype, whereas intracellular staining of Foxp3 and IFN-γ or IL-17 after incubation for 6 hours with PMA (50 ng/ml), ionomycin (2 µg/ml) and monensin (2 µM) (St. Louis, MO, USA) was used to determine the production of IFN-γ and IL-17 at the single cell level.

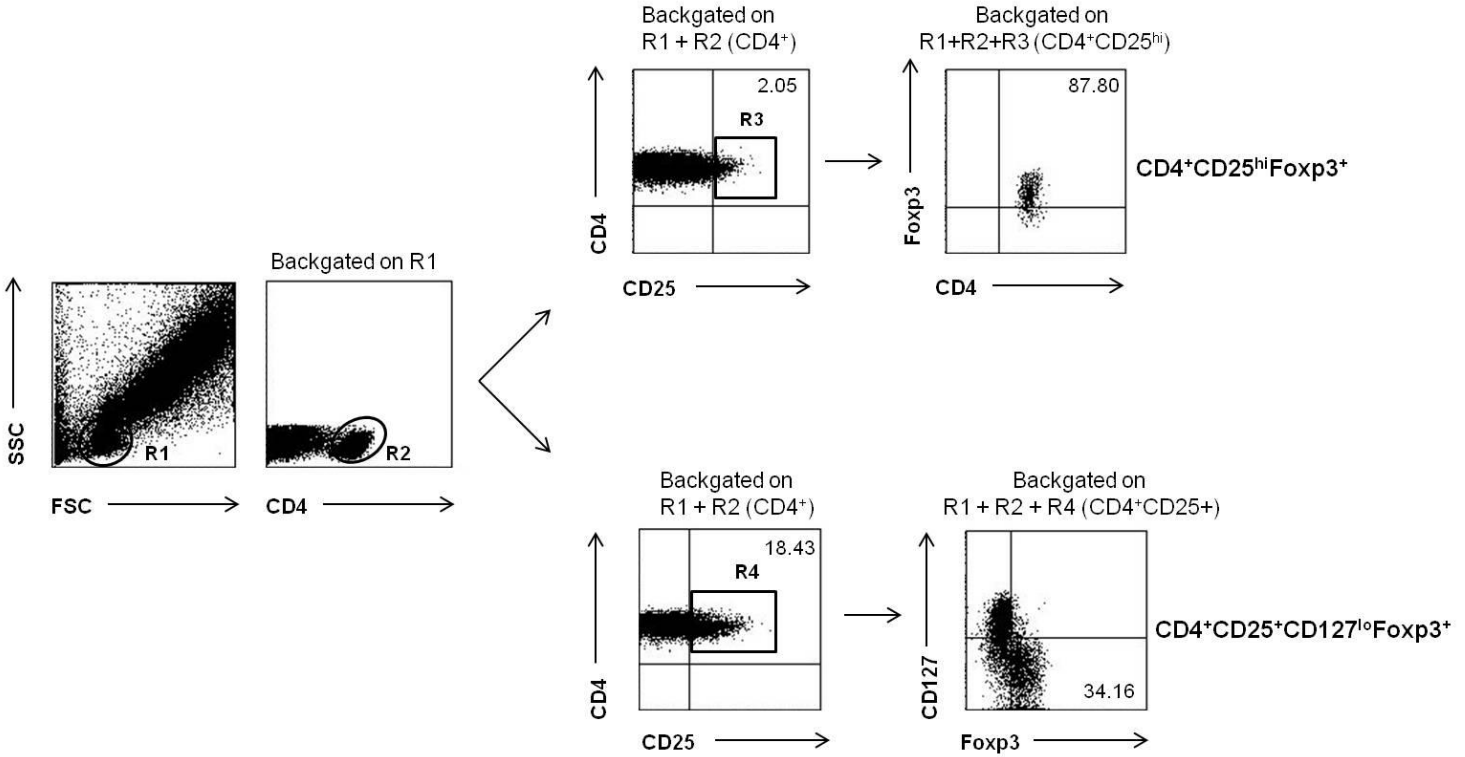
TCRBV repertoire analysis

TCR diversity of Tregs was determined by estimating the length distribution of the complementarity-determining regions 3 (CDR3) of β variable (BV) gene segments with a two-steps Multiplex PCR assay developed in our laboratory[17]. This assay allows the full representation of 22 BV subfamilies at the BV-BC and BV-BJ levels in samples containing as little as 80 ng of T-cell cDNA which is equivalent to approximately $1x10^5$ T cells.

Tregs isolation and suppression assay

Tregs were purified by immunomagnetic separation from PBMC and BMMC using the $CD4^+CD25^+$ T Regulatory Cells Isolation kit or the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T-Cell Isolation Kit II according to the manufacturer's instruction (Miltenyi Biotech). Tregs suppressor function was assessed using irradiated allogeneic PBMC as accessory cells, purified $CD4^+CD25^+$ or $CD4^+CD127^{lo}CD25^+$ cells as indicated, and autologous $CD4^+CD25^-$ as responder cells, and soluble anti-CD3 as previously reported[18]. Cell proliferation was evaluated by adding 1 µCi ^{3}HTdR (Amersham) to each well during the last 4 hours of culture and measuring its uptake with a scintillation counter. The percent inhibition of proliferation was calculated as follows: 1 - [(average cpm counts in T responders + Tregs wells)/(average cpm counts in T responders wells)] x 100.

Supplementary Tables and Figures



Supplementary Figure 1. Identification of Tregs in the bone marrow in MM patients.

Tregs were identified as $CD4^{+}CD25^{hi}Foxp3^{+}$ or $CD4^{+}CD25^{+}CD127^{lo}Foxp3^{+}$ T cells by using a multigating strategy as shown. Representative dot plots from an individual MM patient at diagnosis.

Supplementary Table 1. Frequencies and total counts of BM Tregs from MM patients stratified for disease status and CTRL

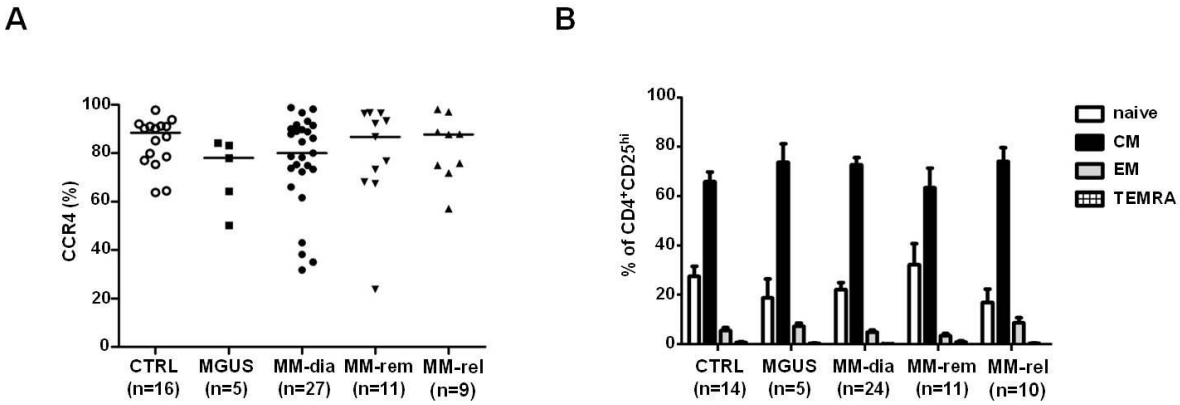
	Phenotype	CTRL Median (range),n	MGUS Median (range),n	MM at diagnosis Median (range),n	MM in remission Median (range),n	MM at relapse Median (range),n
Percentage*	$CD4^+CD25^{hi}Foxp3^+$ $CD4^+Foxp3^+$ $CD4^+CD25^+CD127^{lo}Foxp3^+$	0.85 (0.13-4.10),20 4.90 (1.53-8.77),20 3.55 (1.68-5.54),10	0.98 (0.09-4.27),12 5.28 (1.41-12.6),12 4.18 (0.73-5.34),6	1.04 (0.06-9.83),71 5.74 (0.94-22.78),71 2.91 (0.50-12.13),20	1.32 (0.49-3.75),23 8.23 (3.02-22.6),23 2.55 (1.68-6.8),9	1.36 (0.09-2.49),14 4.36 (0.99-9.10),14 3.31 (0.75-6.28),7
Absolute number(/µl)	$CD4^+CD25^{hi}Foxp3^+$ $CD4^+Foxp3^+$ $CD4^+CD25^+CD127^{lo}Foxp3^+$	16.44 (0.20-45.20),9 89.76 (1.05-230.89),9 72.01 (0.53-198.6),9	12.17 (0.81-57.22),12 63.03 (8.68-147.5),12 44.93 (3.47-69.30),6	10.47 (0.93-135.3),71 58.61 (6.29-591.9),71 22.13 (1.65-221.4),20	6.14 (0.62-53.35),22 71.35 (7.02-301.5),22 10.59 (4.57-111.20),9	12.5 (1.00-47.00),14 58.5 (17.00-253.00),14 51.64 (9.15-141.00),7

***of total $CD4^+$ T cells**

Supplementary Table 2. Frequencies and total counts of PB Tregs in MM patients at diagnosis and CTRL

	Phenotype	Controls Median (range),n	MM at diagnosis Median (range),n
Percentage*	$CD4^+CD25^{hi}Foxp3^+$ $CD4^+Foxp3^+$ $CD4^+CD25^+CD127^{lo}Foxp3^+$	1.45 (0.23-5.56),41 5.86 (1.9-12.08),41 4.33 (2.34-8.73),10	1.77 (0.28-6.94),47 7.28 (1.87-15.06),47 4.01 (0.96-11.22),17
Absolute number(/µl)	$CD4^+CD25^{hi}Foxp3^+$ $CD4^+Foxp3^+$ $CD4^+CD25^+CD127^{lo}Foxp3^+$	13.47 (1.59-72.89),36 56.03 (6.76-124.5),36 33.94 (20.75-66.23),10	17.32 (3.08-53.96),34 48.81 (8.37-206),34 39.28 (6.22-67.73),10

***of total $CD4^+$ T cells**



Supplementary Figure 2. CCR4 expression and Tregs subsets distribution in the BM of CTRL, MGUS, and MM patients according to the disease status

A) Percentages of $CCR4^+$ Tregs in the BM of CTRL, MGUS, MM-dia, MM-rem, and MM-rel. Results are expressed as dot density plots with median lines. Differences between medians are not statistically significant ($p > 0.05$).

B) Percentages of naïve ($CD27^+CD45RA^+$), CM ($CD27^+CD45RA^-$), EM ($CD27^-CD45RA^-$), and TEMRA ($CD27^-CD45RA^+$) Tregs in BM were determined. The Tregs subsets distribution in the BM of CTRL, MGUS, MM-dia, MM-rem, and MM-rel is shown. CM was the predominant subset in all groups ($p > 0.05$). Bars represent the mean ± SE from 5 MGUS to 24 MM-dia.