



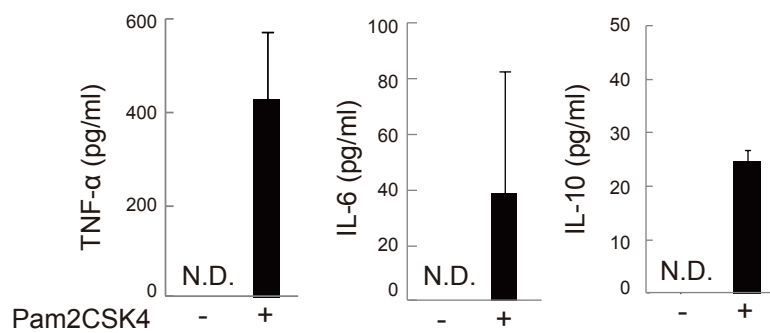
Title	Toll-like receptor 2 ligand and interferon- γ suppress anti-tumor T cell responses by enhancing the immunosuppressive activity of monocytic myeloid-derived suppressor cells
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Toll-like receptor 2 ligand and interferon- γ suppress anti-tumor T cell responses by enhancing the immunosuppressive activity of monocytic myeloid-derived suppressor cells

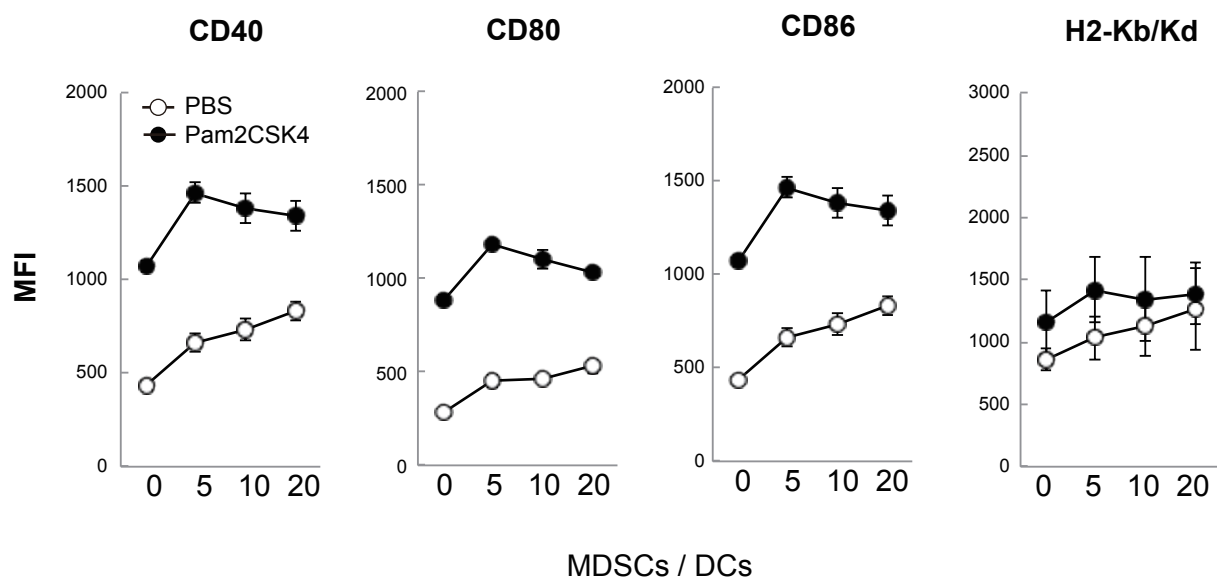
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Supplemental Figures and table

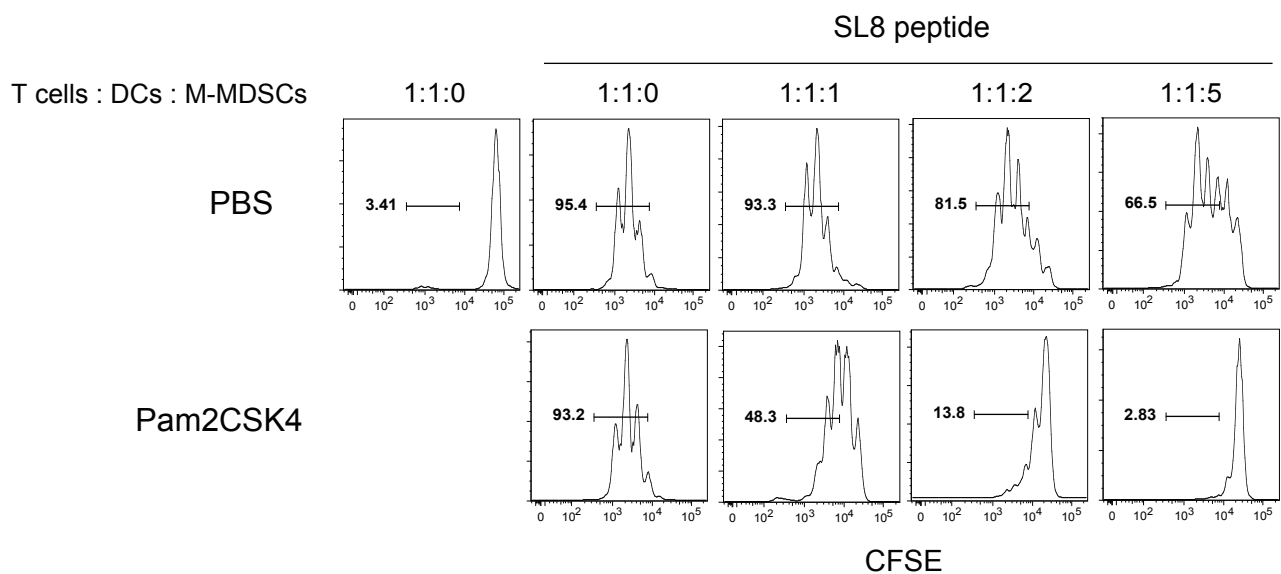


Supplementary Figure 1. Cytokine production by MDSCs in response to Pam2CSK4.

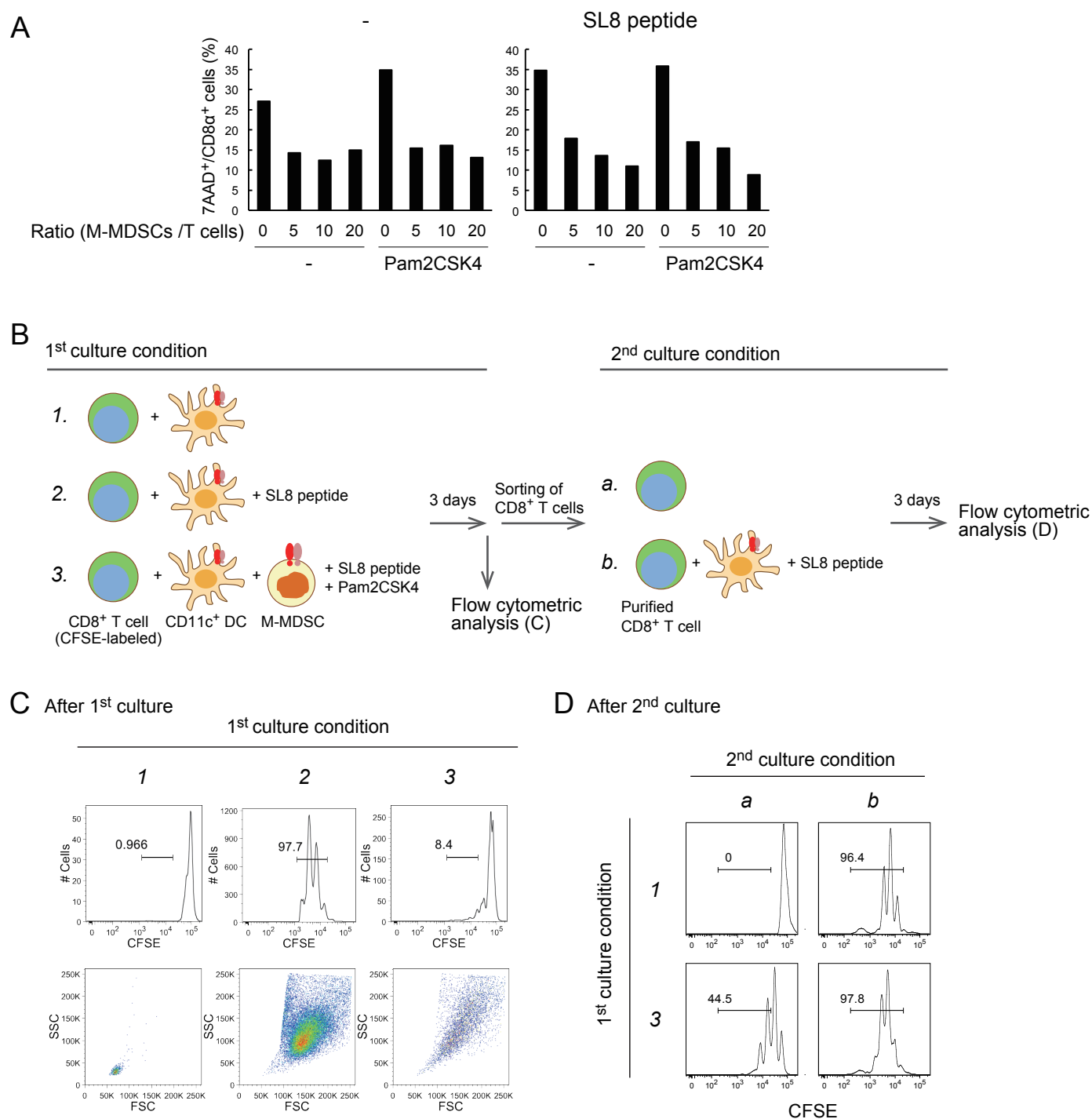
CD11b⁺Gr1⁺ MDSCs (1×10^5) were incubated with Pam2CSK4 for 24 h. The concentration of TNF-α, IL-6, and IL-10 in the conditioned media was determined by ELISA. Mean $\pm$ SD were shown. n=3, N.D., not detected..



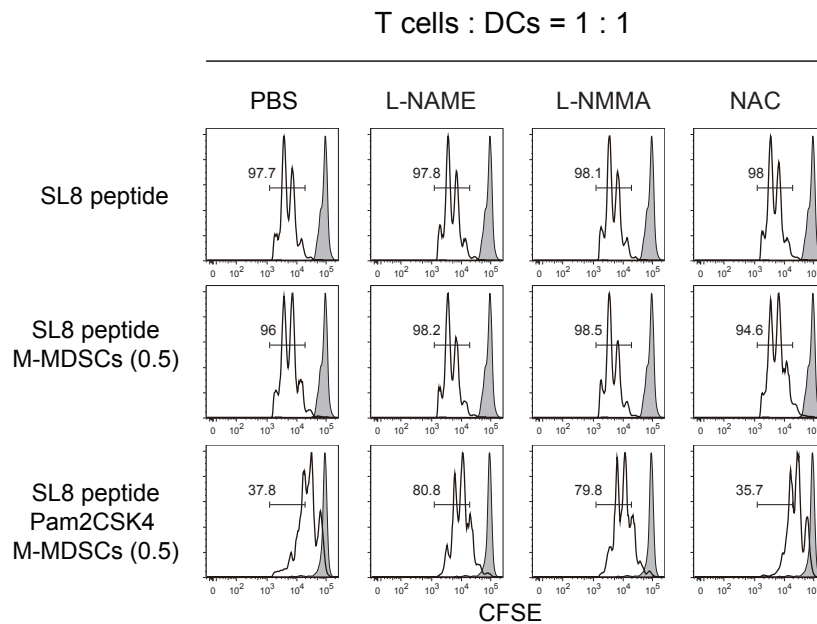
Supplementary Figure 2. MDSCs do not inhibit maturation of DCs. CD11c⁺ DCs were cocultured with MDSCs for 3 days in the presence or absence of Pam2CSK4. Expression levels of surface molecules CD40, CD80, CD86, and H2-K^b/K^d, were analyzed by flow cytometry. Each dot represents mean $\pm$ SD. n=3. MFI, mean fluorescence intensity.



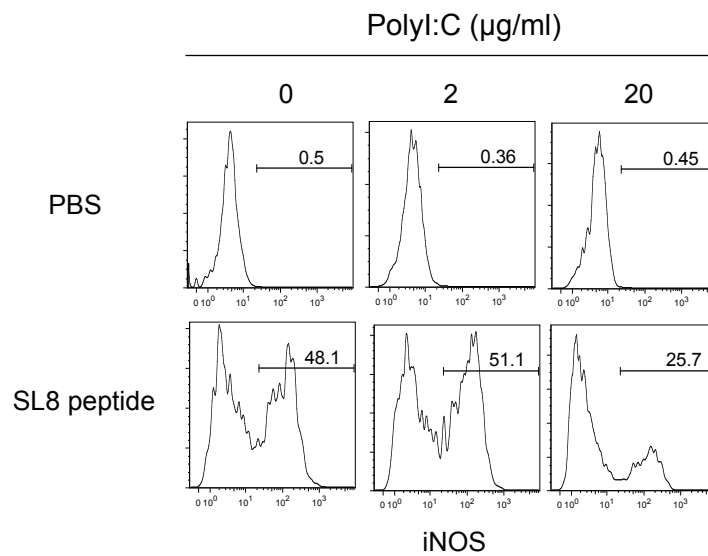
Supplementary Figure 3. Pam2CSK4 enhances the immunosuppressive activity of M-MDSCs isolated from mice bearing LLC-OVA tumors. M-MDSCs (1×10^5) isolated from LLC-OVA-implanted mice were co-cultured with CFSE-labeled CD8⁺ OT-I T cells (0.5×10^5) and CD11c⁺ DCs (0.5×10^5) in the presence of 100 nM Pam2CSK4 and 50 nM SL8 peptide. After 3 days, CFSE fluorescence of CD8 α ⁺TCRv β 5.1/5.2⁺ cells was analyzed by flow cytometry.



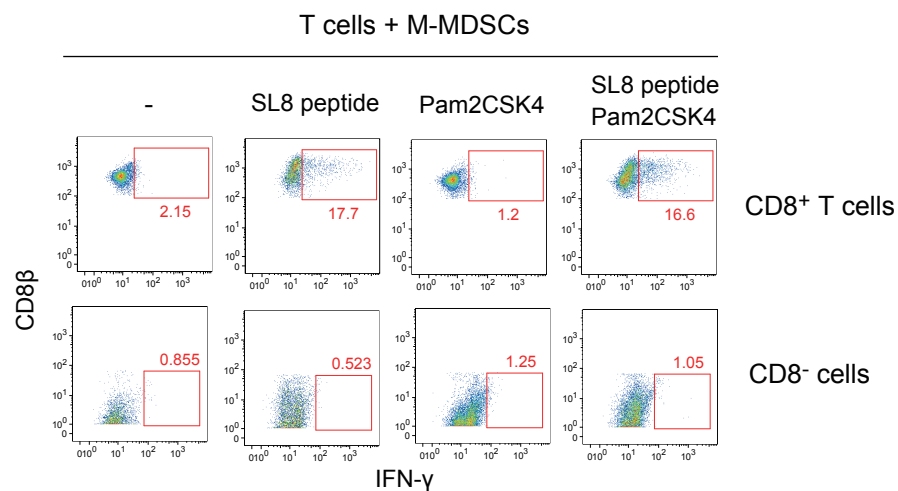
Supplementary Figure 4. Proliferation of CD8⁺ T cells is reversibly inhibited by Pam2CSK4-activated M-MDSCs. (A) Viability of CD8⁺ T cells after coculture with M-MDSCs and Pam2CSK4 was determined by 7AAD staining and the following flow cytometric analysis. (B, C, D) CFSE-labeled CD8⁺ T cells were cultured with CD11c⁺ DCs in the presence or absence of 50 nM SL8 peptide, M-MDSCs, or Pam2CSK4 for 3 days (condition 1-3 of 1st culture). CFSE fluorescence (upper) and forward and side scatter (lower) of total CD8α⁺TCRvβ5.1/5.2⁺ T cells were shown (C). Single cell-sorted CD8α⁺TCRvβ5.1/5.2⁺ cells (1×10^5) were cultured with or without CD11c⁺ DCs (0.5×10^5) and 50 nM SL8 peptide for 3 days (condition a or b of 2nd culture), then their CFSE fluorescence was analyzed (D).



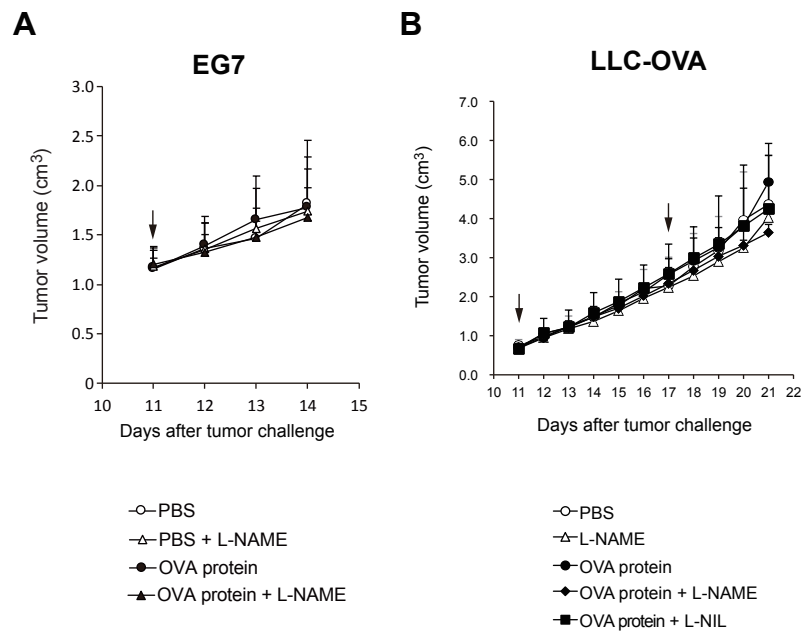
Supplementary Figure 5. iNOS inhibitors restore T cell proliferation inhibited by Pam2CSK4-activated M-MDSCs. CFSE-labeled CD8⁺ T cells (0.5×10^5) were co-cultured with CD11c⁺ DCs (0.5×10^5) and M-MDSCs (0.25×10^5) in the presence of 50 nM SL8 peptide with or without 100 nM Pam2CSK4. L-NAME (1 mM), L-NMMA (0.1 mM), or NAC (1 mM) were added to the cultures. After 3 days, CFSE fluorescence of CD8 α ⁺TCR ν β 5.1/5.2⁺ cells was analyzed by flow cytometry. Gray histogram represents T cells cocultured without SL8 peptide.



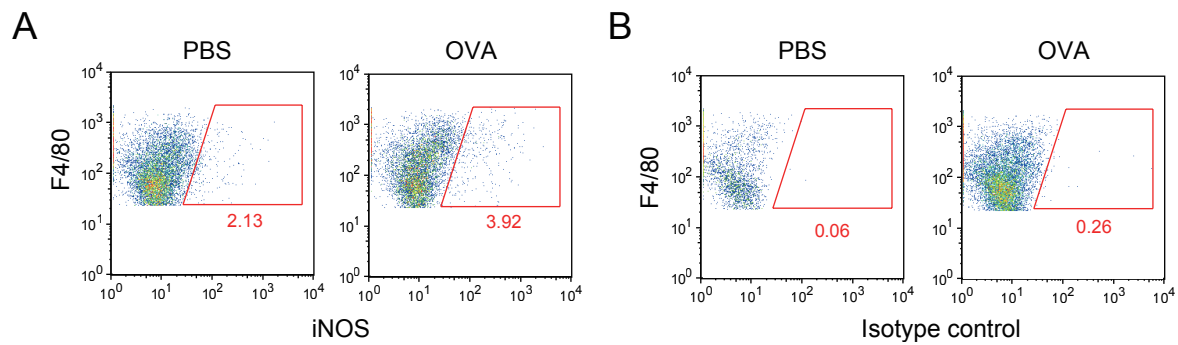
Supplementary Figure 6. PolyI:C does not induce iNOS expression in F4/80⁺ macrophages derived from M-MDSCs. M-MDSCs (1×10^5) isolated from EG7 tumor-bearing mice were co-cultured with CD8⁺ OT-I T cells (0.5×10^5) in the presence of polyI:C and 50 nM SL8 peptide. After 24 h, intracellular iNOS expression in CD11b⁺F4/80⁺ cells was analyzed by flow cytometry.



Supplementary Figure 7. CD8⁺ T cells produce IFN- γ in the cocultures with M-MDSCs and antigen peptide. CD8⁺ T cells (0.5×10^5) and M-MDSCs (1×10^6) were cocultured in the presence of 50 nM SL8 peptide and/or 100 nM Pam2CSK4. After 24 h, cells were further cultured for 5 h in the presence of 10 μ g/ml Brefeldin A and then IFN- γ production in CD8⁺ T cells was analyzed by intracellular cytokine staining.



Supplementary Figure 8. Treatment with OVA protein, L-NAME, and L-NIL does not affect tumor growth. B6 mice bearing EG7 tumors (A) or LLC-OVA tumors (B and C) were injected s.c. with PBS, 100 µg OVA protein, 25 nmol Pam2CSK4 on days indicated by arrows. Mice were daily injected i.p. with PBS, L-NAME (2 mg), or L-NIL (0.5 mg) from day 11 (A and B) or day 13 (C). n=3-4. Data represent mean ± SD.



Supplementary Figure 9. Monotherapy with OVA protein does not induce iNOS expression in tumor-infiltrating macrophages. EG7 tumor-bearing mice were injected s.c. with PBS or 100 μ g OVA protein. After 24 hours, single cell suspension of tumors were stained with APC-anti-F4/80 Ab and PE-anti-iNOS Ab (A) or PE-isotype control Ab (B) and analyzed by flow cytometry.

Supplementary Table 1. Real-time PCR primer sequences (5' to 3')

Gene	Forward primer	Reverse primer
<i>Gapdh</i>	GCCTGGAGAAACCTGCCA	CCCTCAGATGCCTGCTTCA
<i>IL-10</i>	GGCGCTGTCATCGATTTCTC	TGCTCCACTGCCTTGCTCTTA
<i>TNF-α</i>	AGGGATGAGAAGTTCCCAAATG	GCTTGTCACTCGAATTTTGAGAAG
<i>IL-6</i>	GTGCATCATCGTTGTTTCATACAATC	CTGGGAAATCGTGGAAATGAG
<i>Bcl2l1</i>	CACTGTGCGTGGA AAA GCATA	AAAGTGTCCCAGCCGCC
<i>Bcl3</i>	CGCAGCCGCAGGGTCATTGAT	TTGGCGAGGAC TGGAGGCCA
<i>Ccnd1</i>	CACAACG CACTTTCTTTCCA	GACCAGCCTCTTCCTCCAC
<i>Ccnd2</i>	CGCTCTGTGCGCTACCGACTT	GCCCACAGATGGC TGCTCCC
<i>Ier3</i>	TCCACCGCGCGTTTGAACACT	GTAGCTGGCGCCGGACCACTC
<i>c-Myc</i>	TGAGCCCCTAGTGCTGCAT	AGCCCGACTCCGACCTCTT