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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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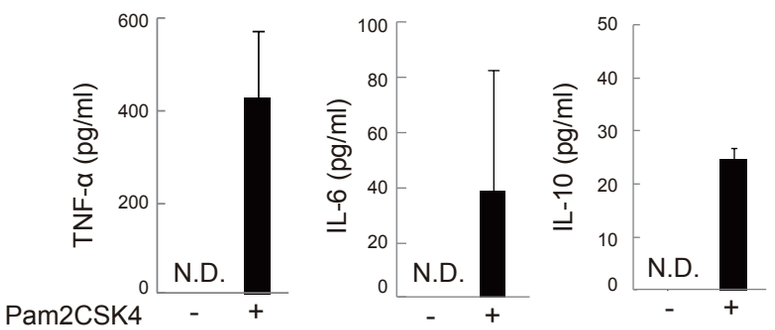
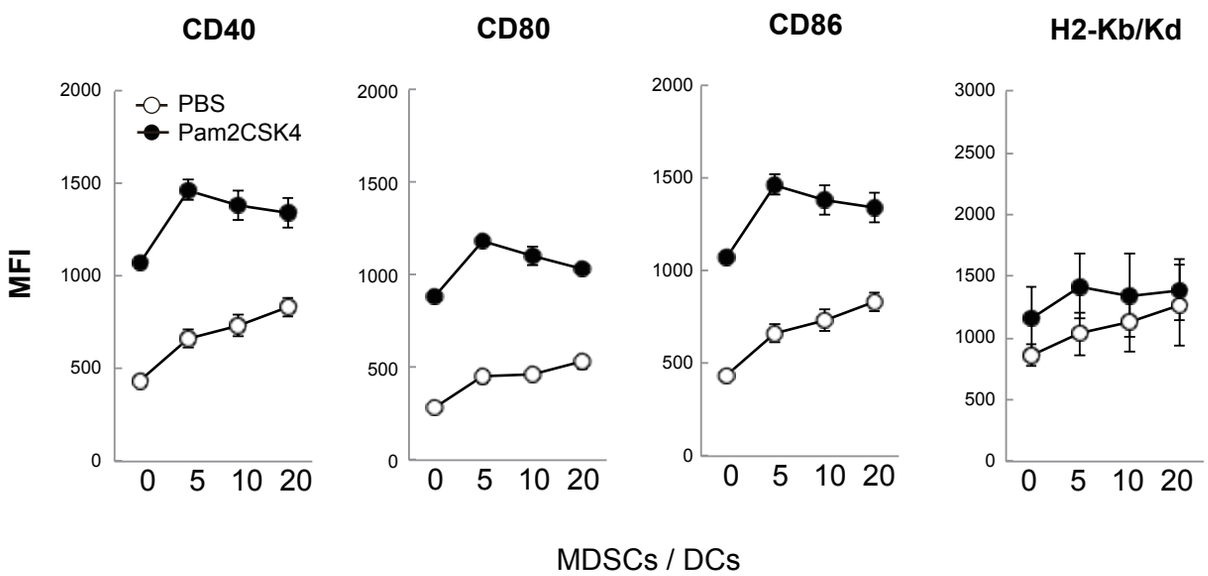
Supplemental Figures and table

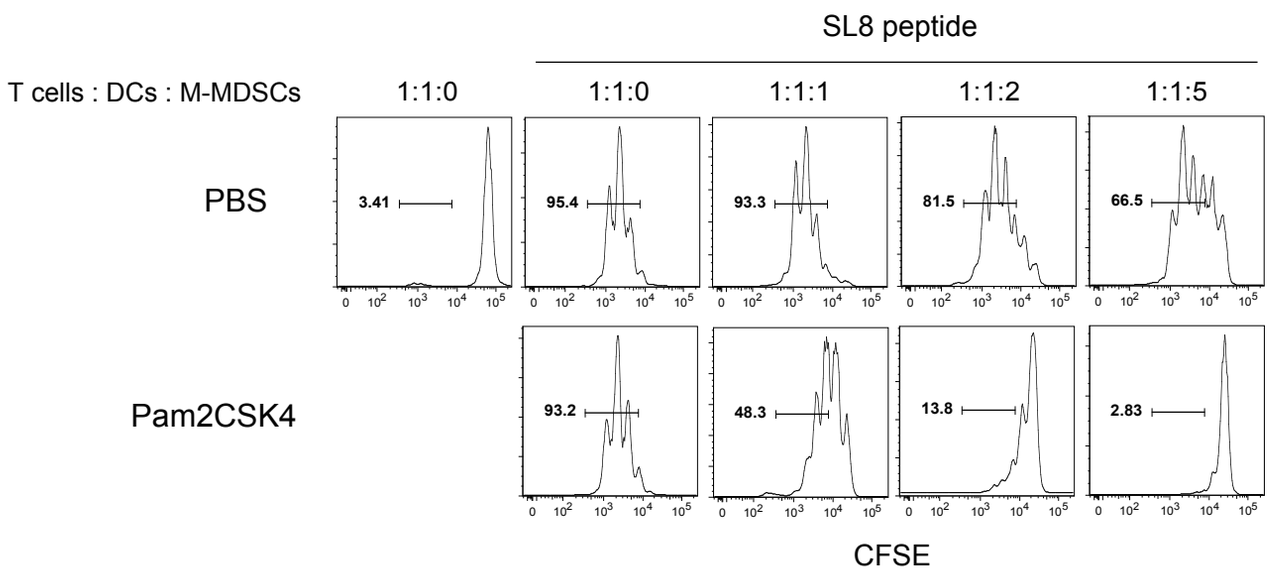


Supplementary Figure 1. Cytokine production by MDSCs in response to Pam2CSK4.

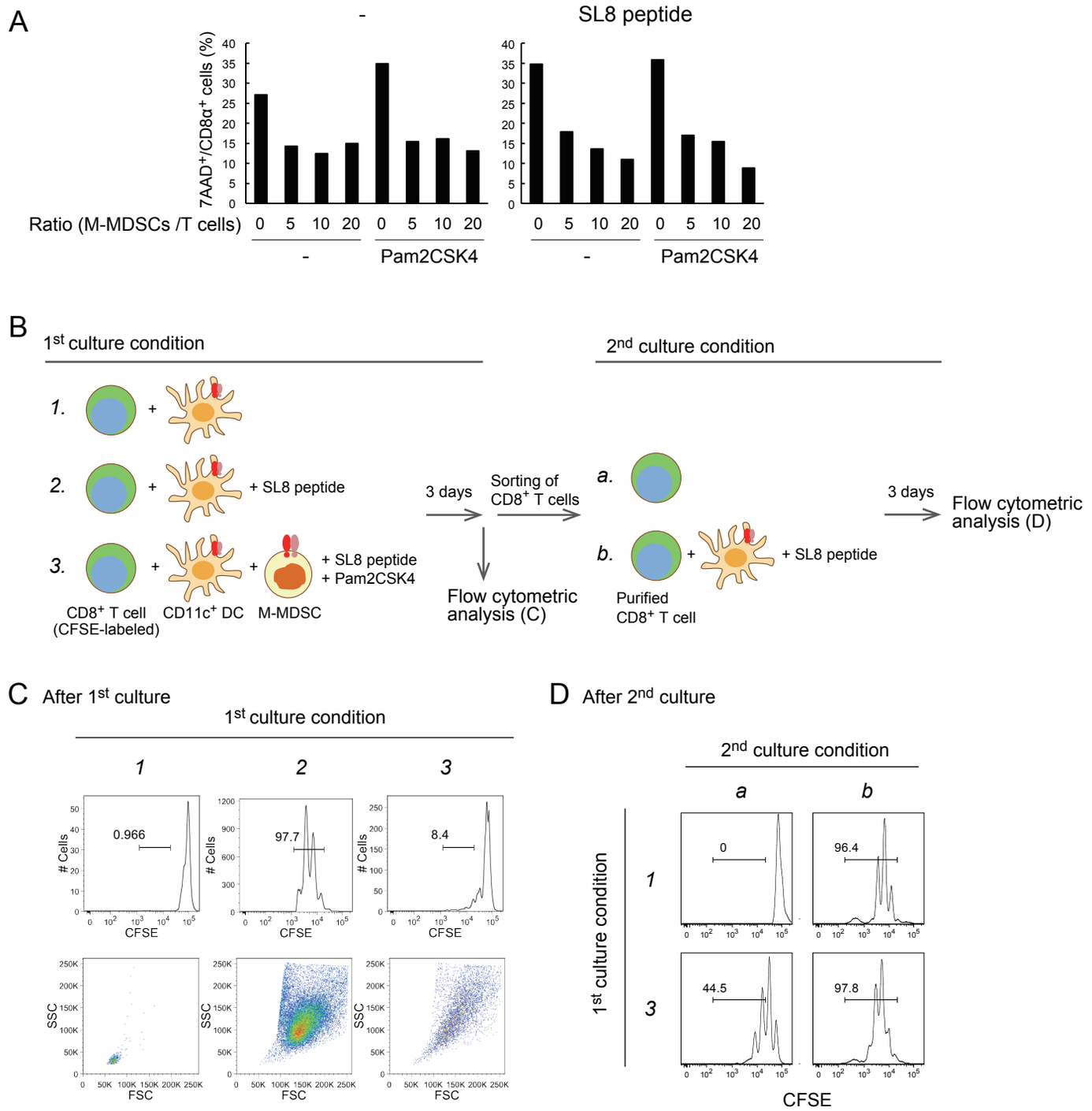
$CD11b^{+}Gr1^{+}$ MDSCs (1×10^{5}) were incubated with Pam2CSK4 for 24 h. The concentration of TNF-α, IL-6, and IL-10 in the conditioned media was determined by ELISA. Mean ± SD were shonw. n=3, N.D., not detected..



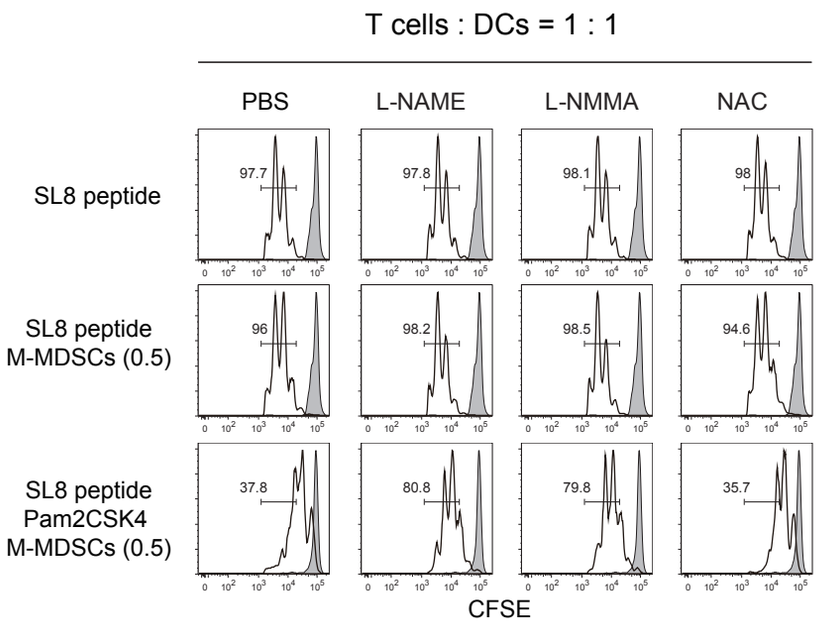
Supplementary Figure 2. MDSCs do not inhibit maturation of DCs. CD11c^{+} DCs were cocultured with MDSCs for 3 days in the presence or absence of Pam2CSK4. Expression levels of surface molecules CD40, CD80, CD86, and H2-K^{b}/K^{d}, were analyzed by flow cytometry. Each dot represents mean ± SD. n=3.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



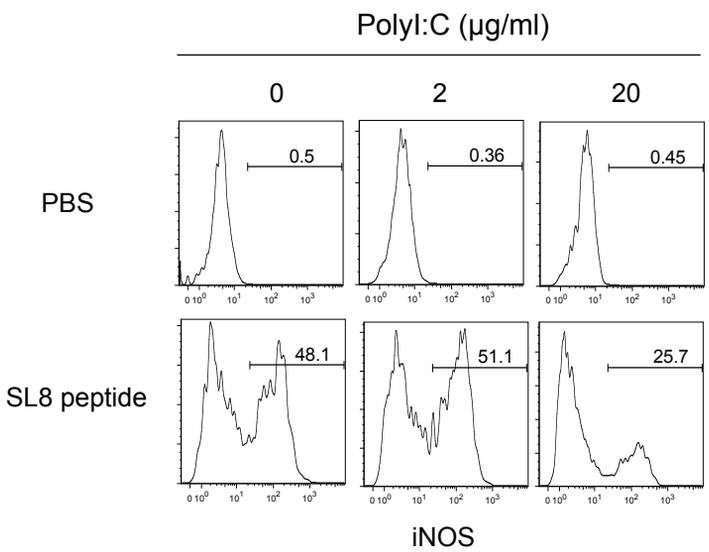
Supplementary Figure 3. Pam2CSK4 enhances the immunosuppressive activity of M-MDSCs isolated from mice bearing LLC-OVA tumors. M-MDSCs (1×10^5) isolated from LLC-OVA-implanted mice were co-cultured with CFSE-labeled $CD8^+$ OT-I T cells (0.5×10^5) and $CD11c^+$ DCs (0.5×10^5) in the presence of 100 nM Pam2CSK4 and 50 nM SL8 peptide. After 3 days, CFSE fluorescence of $CD8\alpha^+TCR\nu\beta5.1/5.2^+$ cells was analyzed by flow cytometry.



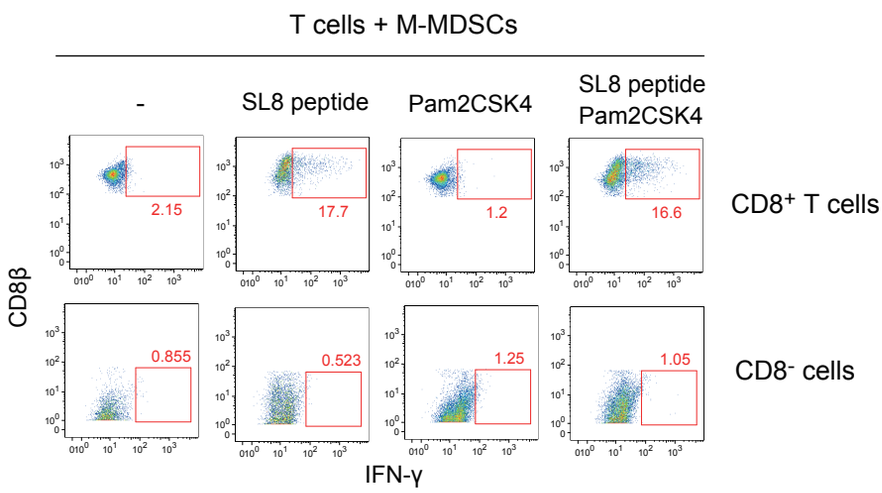
Supplementary Figure 4. Proliferation of CD8$^+$ T cells is reversibly inhibited by Pam2CSK4-activated M-MDSCs. (A) Viability of CD8$^+$ T cells after coculture with M-MDSCs and Pam2CSK4 was determined by 7AAD staining and the following flow cytometric analysis. (B, C, D) CFSE-labeled CD8$^+$ T cells 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).



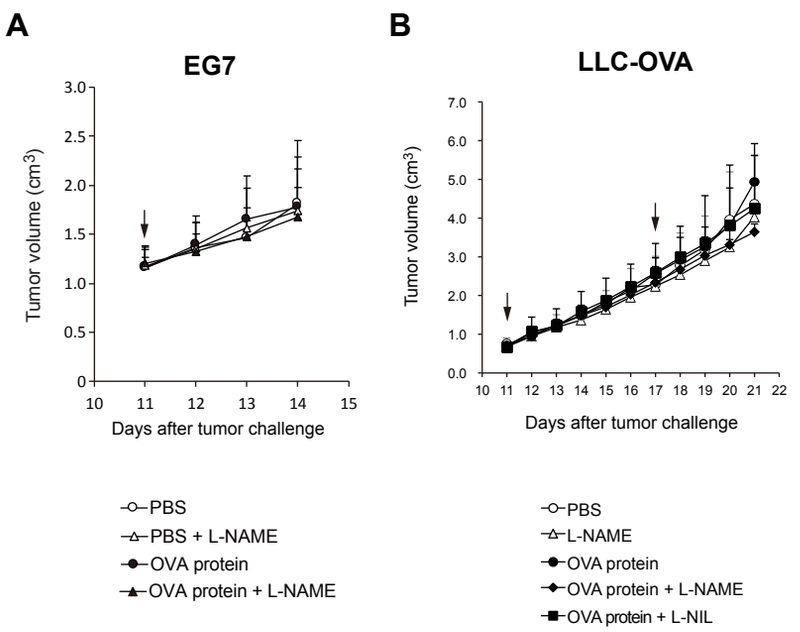
Supplementary Figure 5. iNOS inhibitors restore T cell proliferation inhibited by Pam2CSK4-activated M-MDSCs. CFSE-labeled $CD8^+$ T cells (0.5×10^5) were co-cultured with $CD11c^+$ DCs (0.5×10^5) and M-MDSCs (0.25×10^5) in the presence of 50 nM SL8 peptide with or without 100 nM Pam2CSK4. L-NAME (1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



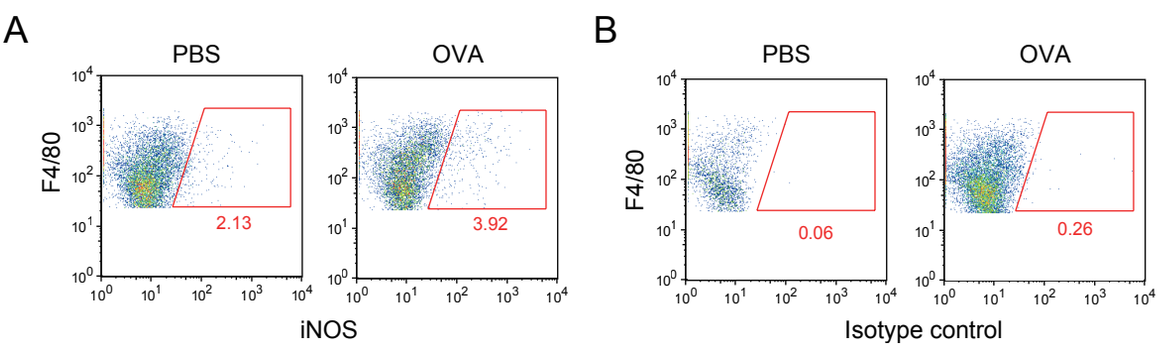
Supplementary Figure 6. Polyl:C does not induce iNOS expression in F4/80$^+$ macrophages derived from M-MDSCs. M-MDSCs (1 × 10^5) isolated from EG7 tumor-bearing mice were co-cultured with CD8$^+$ OT-I T cells (0.5 × 10^5) in the presence of polyI:C and 50 nM SL8 peptide. After 24 h, intracellular iNOS 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 μg/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 μg 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
Gapdh	GCCTGGAGAAACCTGCCA	CCCTCAGATGCCTGCTTCA
IL-10	GGCGCTGTCATCGATTTCTC	TGCTCCACTGCCTTGCTCTTA
TNF-α	AGGGATGAGAAGTTCCCAAATG	GCTTGTCACTCGAATTTTGAGAAG
IL-6	GTGCATCATCGTTGTTCATACAATC	CTGGGAAATCGTGGAAATGAG
Bcl2l1	CACTGTGCGTGGAAA GCATA	AAAGTGTCCCAGCCGCC
Bcl3	CGCAGCCGCAGGGTCATTGAT	TTGGCGAGGAC TGGAGGCCA
Ccnd1	CACAACG CACTTTCTTTCCA	GACCAGCCTCTTCCTCCAC
Ccnd2	CGCTCTGTGCGCTACCGACTT	GCCCACAGATGGC TGCTCCC
Ier3	TCCACCGCGCGTTTGAACACT	GTAGCTGGCGCCGGACCACTC
c-Myc	TGAGCCCCTAGTGCTGCAT	AGCCCGACTCCGACCTCTT