Supplementary Information

Table. S1:

NK Source	Method of selection	Feeder cells	Stimulation	Expansion	References
PBMC	Negative selection for NK	Autologous and allogeneic osteoclasts	IL-2, anti-CD16 mAb, sAJ2	21,000-132,000 fold expansion at 20 days, 0.3-5.1 million at 31 days; 17-21 population doublings with an average of 19 population doublings within 4 weeks	Current paper
PBMC	Whole PBMCs	None	IL-2, anti-CD16Ab-immobilized flask, OK432	637–5712 fold at 21 days	[6]
PBMC (negative selection)	Negative selection for NK	Irradiated autologous PBMC's (pretreated for 3-5 days with IL2, IL-15, and concanavalin A)	IL-2 and IL-15, (phytohemagglutinin (PHA) and ionomycin first 24 hours)	100 fold at 16 days	[7]
PBMC	CD3 depletion of PBMCs	Autologous irradiated PBMCs stimulated with OKT3	IL-2	79-322 fold at 21-26 days	[8]
PBMC	Whole PBMCs	Wilms tumor cell line (HFWT)	IL-2	58-401-fold at 10-21 days	[9]

PBMC	Whole PBMCs	K562-mb15-41BBL	IL-2	165 (4-567) fold at 14 days	[10]
PBMC	Whole PBMCs	K562-mb15-41BBL	IL-2	21.6 (5.1-86.6) fold at 7 days	[11]
PBMC	Whole PBMCs	K562-GM (transmembrane expression of IL-15, 4-1BBL, and IL-18)	IL-2	500 fold at 21 days	[12]
PBMC	CD3 depletion followed by CD56+ selection	Irradiated EBV-TM-LCL	IL-2	490 (+/- 260) at 21 days	[13]
Cord Blood (CB)	CD34+ positive selection	None	SCF, TPO, IL-7, Fit3L, IL-15, IL-2, G-CSF, GM-CSF, IL-6, LIF, MIP-1α	>15,000 fold at 5 weeks	[14]
Cord Blood (CB)	CD34+ positive selection	None	SCF, Fit3L, TPO, IL-7, GM-CSF, G-CSF, IL-6, IL-15, LMWH, and IL-2	>2,000 fold at 6 weeks (using GMP)	[15]
NK-92	N/A (cell line)	None	IL-2	218- 250 fold at 15-17 days	[16]
PBMC (genetically modified with TERT for immortalization)	Whole PBMCs used initially, residual T-cells removed after 7 days using anti-CD3 Dynabeads	K562-mb15-41BBL (continued stimulation)	IL-2	TERT transformed (130-227 population doublings over 1000 days) Non-transformed (11-20 population doublings at 8-15 weeks)	[17]

PBMCs (after 7 days of culture, retrovirally transduced with NKG2D-DAP10-CD3ζ)	Whole PBMCs used initially, residual T-cells removed after 7 days using anti-CD3 Dynabeads	K562-mb15-41BBL	IL-2	N/A	[18]

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Fig. S1A

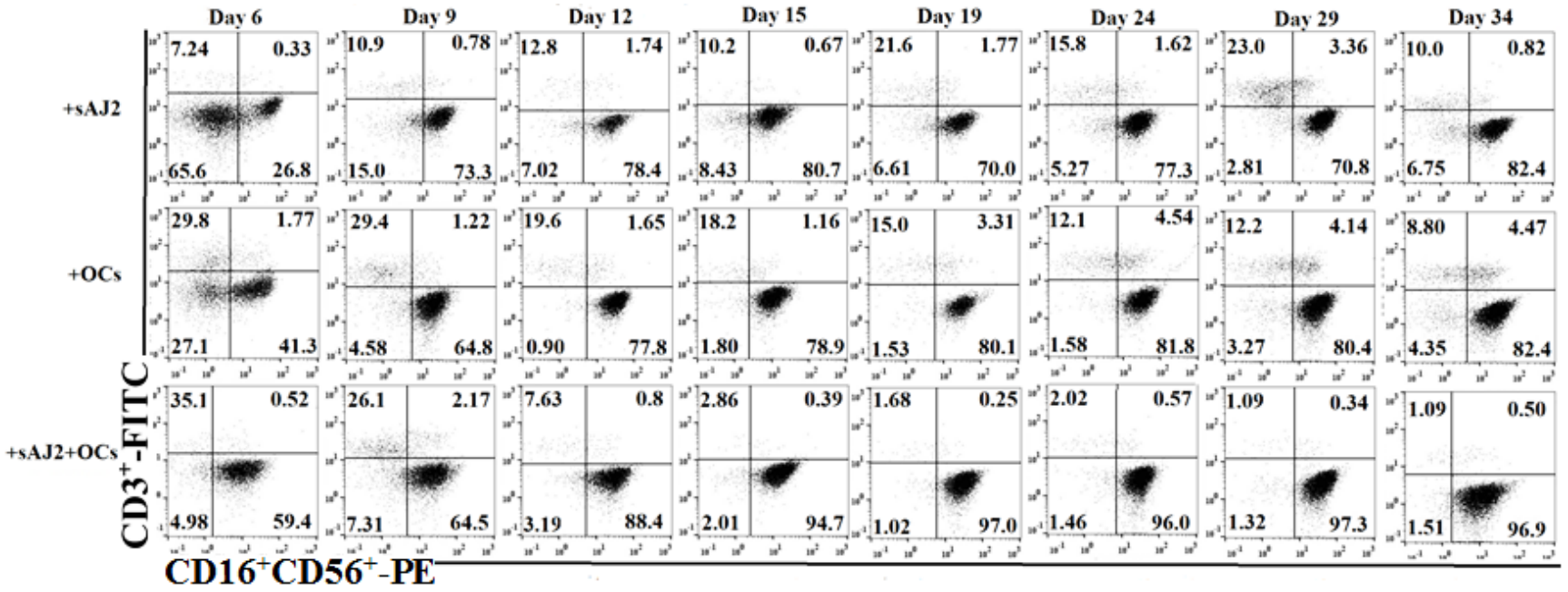

Day 6
Day 9
Day 12
Day 15
Day 19
Day 24
Day 29
Day 34
+sAJ2
+OCs
+sAJ2+OCs
7.24 0.33 65.6 26.8
10.9 0.78 15.0 73.3
12.8 1.74 7.02 78.4
10.2 0.67 8.43 80.7
21.6 1.77 6.61 70.0
15.8 1.62 5.27 77.3
23.0 3.36 2.81 70.8
10.0 0.82 6.75 82.4
29.8 1.77 27.1 41.3
29.4 1.22 4.58 64.8
19.6 1.65 0.90 77.8
18.2 1.16 1.80 78.9
15.0 3.31 1.53 80.1
12.1 4.54 1.58 81.8
12.2 4.14 3.27 80.4
8.80 4.47 4.35 82.4
35.1 0.52 4.98 59.4
26.1 2.17 7.31 64.5
7.63 0.8 3.19 88.4
2.86 0.39 2.01 94.7
1.68 0.25 1.02 97.0
2.02 0.57 1.46 96.0
1.09 0.34 1.32 97.3
1.09 0.50 1.51 96.9
CD3+-FITC
CD16+CD56+-PE

Fig. S1B

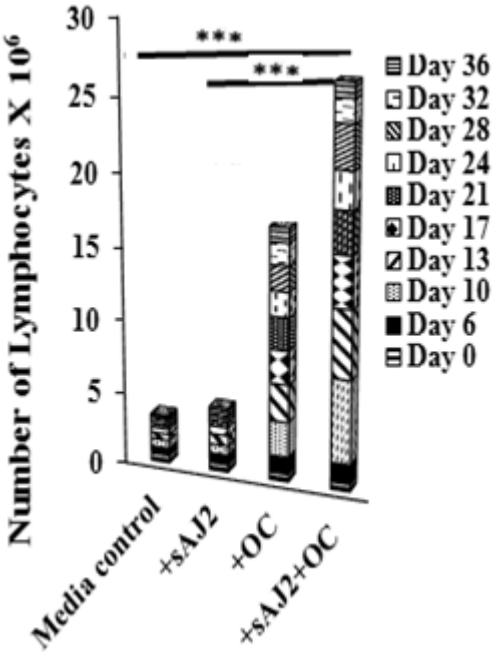


Fig. S1C

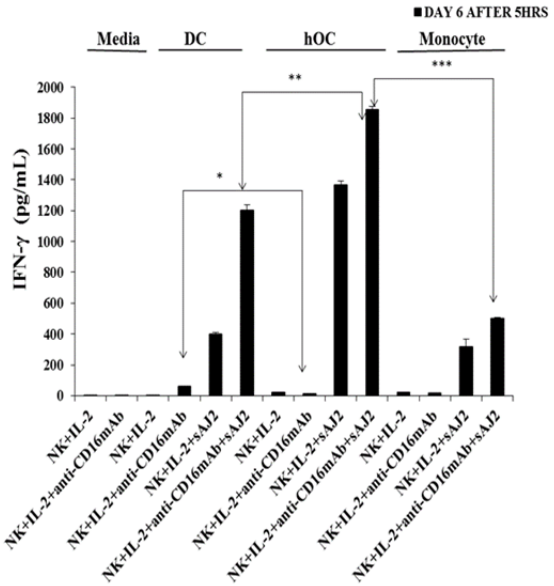


Fig. S1D

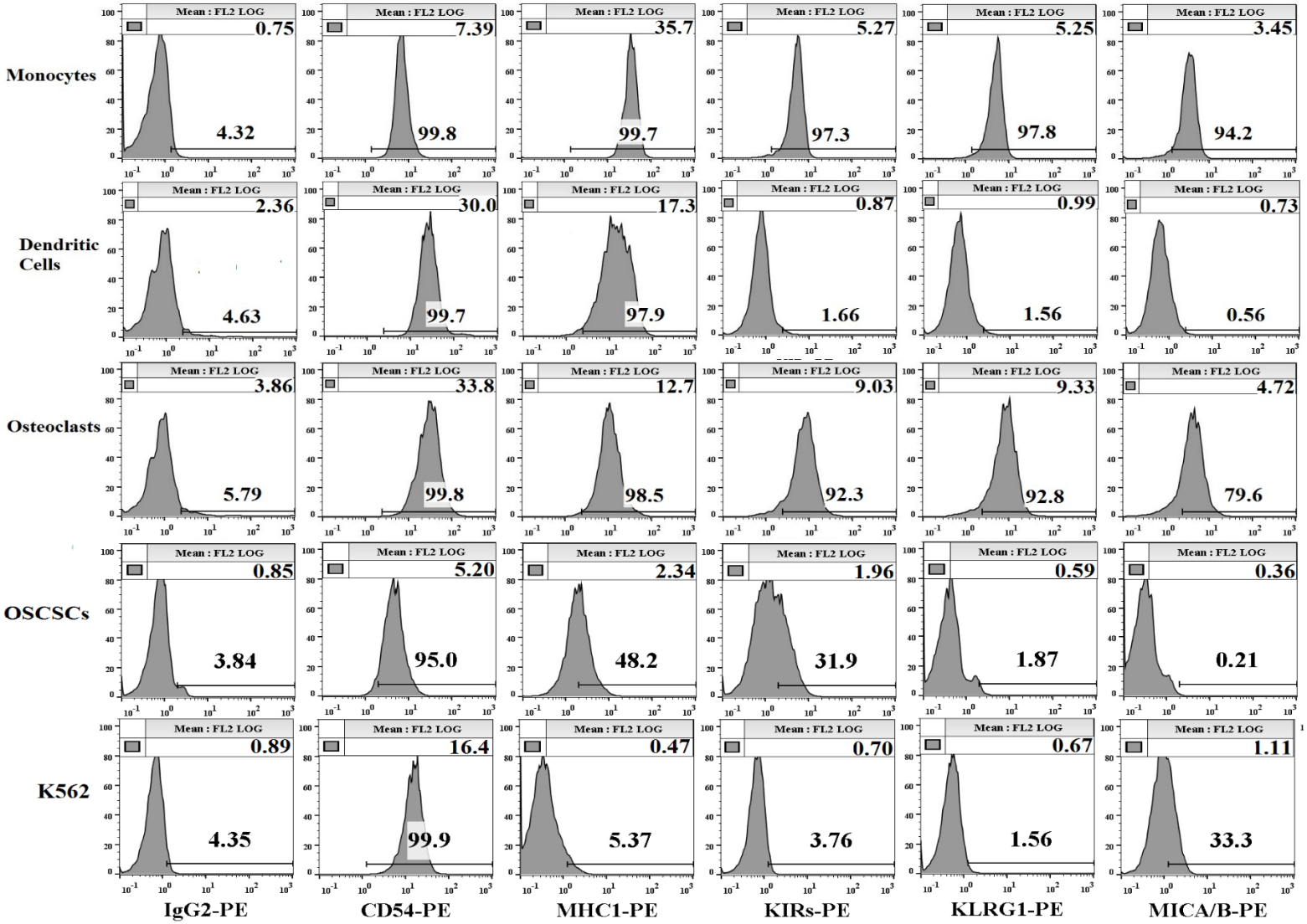

Monocytes
Dendritic Cells
Osteoclasts
OSCSCs
K562
Mean : FL2 LOG
0.75
4.32
7.39
99.8
35.7
99.7
5.27
97.3
5.25
97.8
3.45
94.2
2.36
4.63
30.0
99.7
17.3
97.9
0.87
1.66
0.99
1.56
0.73
0.56
3.86
5.79
33.8
99.8
12.7
98.5
9.03
92.3
9.33
92.8
4.72
79.6
0.85
3.84
5.20
95.0
2.34
48.2
1.96
31.9
0.59
1.87
0.36
0.21
0.89
4.35
16.4
99.9
0.47
5.37
0.70
3.76
0.67
1.56
1.11
33.3
IgG2-PE
CD54-PE
MHC1-PE
KIRs-PE
KLRG1-PE
MICA/B-PE

Fig. S1: Higher expression of NK activating ligands by Osteoclasts.

To generate osteoclasts (OCs), monocytes were cultured in medium containing M-CSF (25 ng/ml) and RANKL (25 ng/ml) for 21 days. Highly purified NK cells ($1x10^6$ cells/ml) were treated with the combination of IL-2 (1000 U/ml) and anti-CD16mAb (3ug/ml) for 18 hours before they were co-cultured with autologous OCs in the presence or absence of sAJ2 bacteria at 1:2:4 ratios (OCs:NK:sAJ2), respectively. Surface expression of CD3, CD16, and CD56 was analyzed in $1x10^4$ lymphocyte samples from co-cultures at days 6, 9, 12, 15, 19, 24, 29, and 34 using flow cytometry, and culture medium was refreshed and supplemented with rh-IL-2 (1000 U/ml) **(A).** Cells were co-cultured as described in Fig. S1A and expanded lymphocytes were counted manually using microscope **(B).** Monocytes were isolated from the PBMCs of a healthy donor. To generate dendritic cells, monocytes were cultured in medium containing GM-CSF (150 ng/ml) and IL-4 (50 ng/ml) for 8 days. Osteoclasts were generated and NK cells were purified as described in Fig. S1A, before they were co-cultured with autologous cells in the presence of sAJ2 bacteria at 1:2:4 ratios (target cells:NK:sAJ2), respectively. On day 6 of the culture, the culture media was removed and the NK cells were treated with rh-IL-2 for 5 hours before the supernatants were harvested, and IFN-γ secretions were determined using single ELISA **(C)**. Monocytes were isolated, dendritic cells and osteoclasts were generated as described in Figs. S1A and D, OSCSCs and K562 tumor cell lines were cultured as described in materials and methods, $1x10^4$ cells were used to analyze MHC-1, CD54, KIR2, KIR3, KLRG1 and MICA/B surface expressions, employing PE-conjugated antibodies and flow cytometry. IgG2 isotype was used as a control **(D).**

Fig. S2A

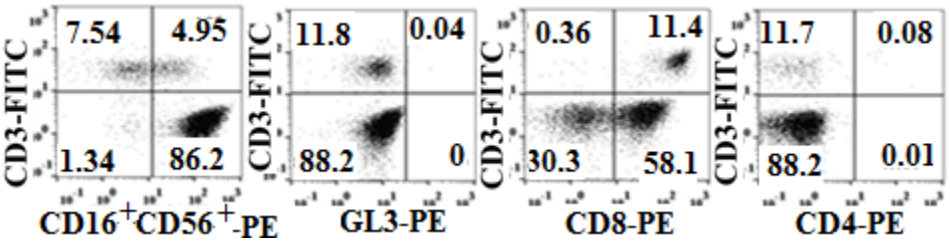


Fig. S2B

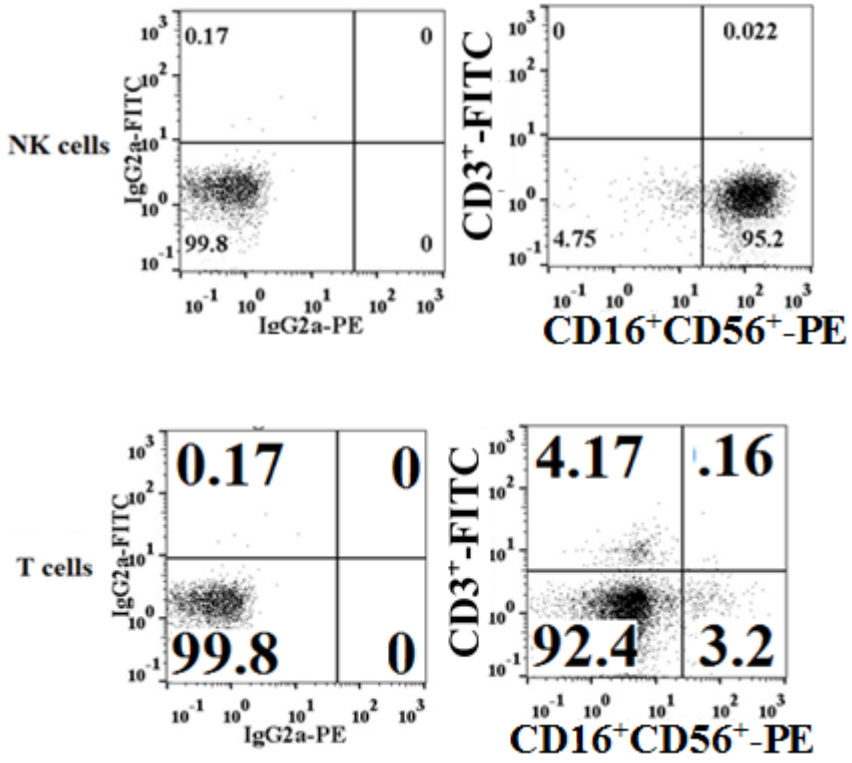


Fig. S2C

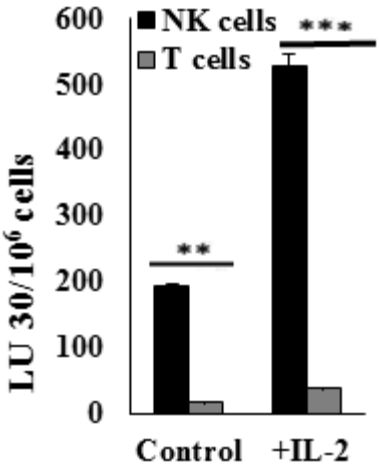


Fig. S2D

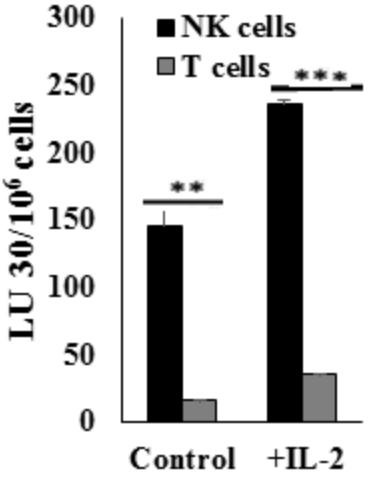


Fig. S2E

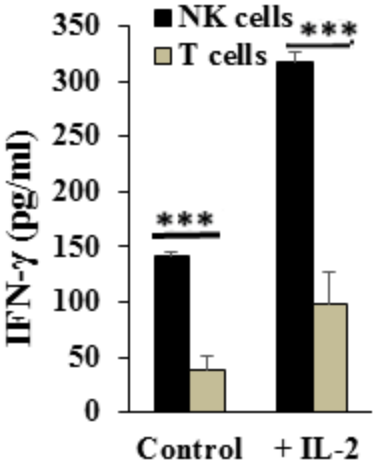


Fig. S2F

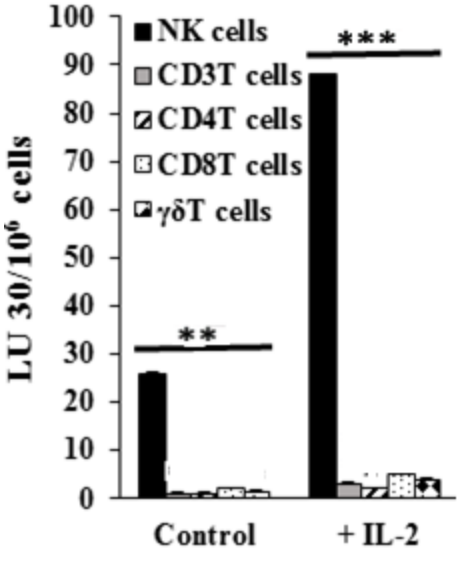


Fig. S2G

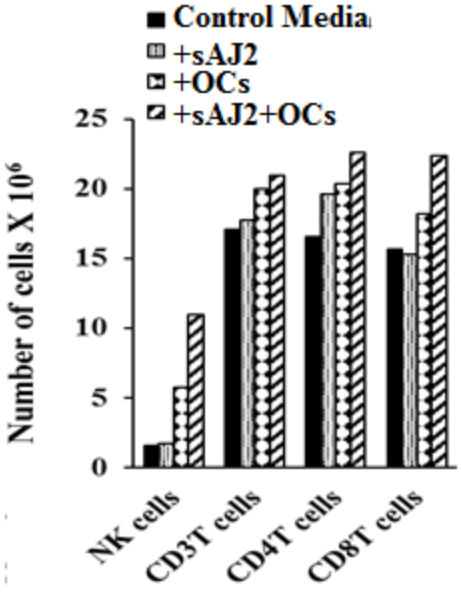


Fig. S2H

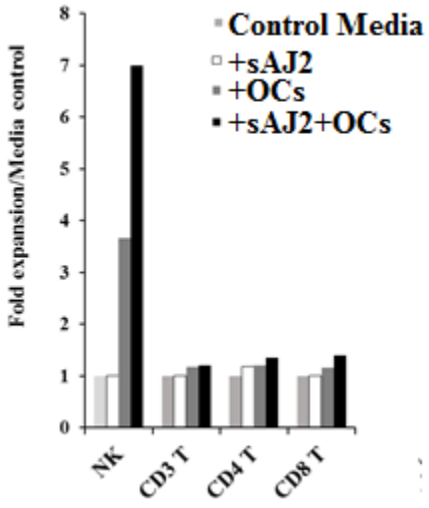


Fig. S2. Unlike NK cells, T cells purified from osteoclast-expanded NK cells do not mediate cytotoxicity against OSCSCs and secrete IFN-γ moderately.

Freshly purified NK cells were treated and co-cultured with monocyte-derived autologous osteoclasts as described in Materials and Methods. Surface expression of CD3, CD16, CD56, GL3 (TCR γ/δ), CD4 and CD8 was analyzed in lymphocyte samples from co-cultures at day 9 using FITC- and PE-conjugated antibodies and flow cytometry **(A)**. NK cells were treated and co-cultured with autologous osteoclasts as described in Fig. S1A and on day 9, CD3T-positive cells were sorted out using CD3T positive selection kit, purity of CD3T-negative (NK) cells was assessed using CD3, CD16, CD56 FITC and PE- conjugated antibodies and flow cytometry **(B).** CD3T-positive cells and CD3T-negative cells (CD16 positive cells) were treated with rh-IL-2 (1000 U/ml) for 18-20 hours before they were tested for cytotoxicity using a standard 4-hour ^{51}Cr release assay against the OSCSCs **(C)** and K562 **(D**) cell lines. The lytic units 30/10^6 cells were determined using the method described in Materials and Methods, for OSCSCs and K562 respectively. The supernatant was harvested from the culture and IFN-γ secretion was determined using single ELISA **(E).** NK cells, CD3T, CD4T, CD8T, and γδT cells were purified from PBMC as described in materials and methods, and were activated with rh-IL-2 for 18-20 hours, before they were tested for cytotoxicity using a standard 4-hour ^{51}Cr release assay against the OSCSCs **(F).** The lytic units 30/10^6 cells were determined using the inverse number of lymphocytes required to lyse 30% of OSCSCs x 100 **(F)**. NK and T cells were purified from PBMCs as described in materials and methods, NK cells were treated as described in Materials and Methods. T cells were activated with anti-CD3 (1 ug/ml) and anti-CD28 (3 ug/ml) 18-20 hours before they were cultured with autologous OCs, and expanded lymphocytes were counted manually using microscope day 4 after the culture **(G)**. NK and T cells were purified and cultured with OCs, and counted on day 4 as described in Fig. S2G, fold expansion of

lymphocytes expanded by the OCs were divided by fold expansion of lymphocytes without the OCs **(H)**.

Fig. S3A

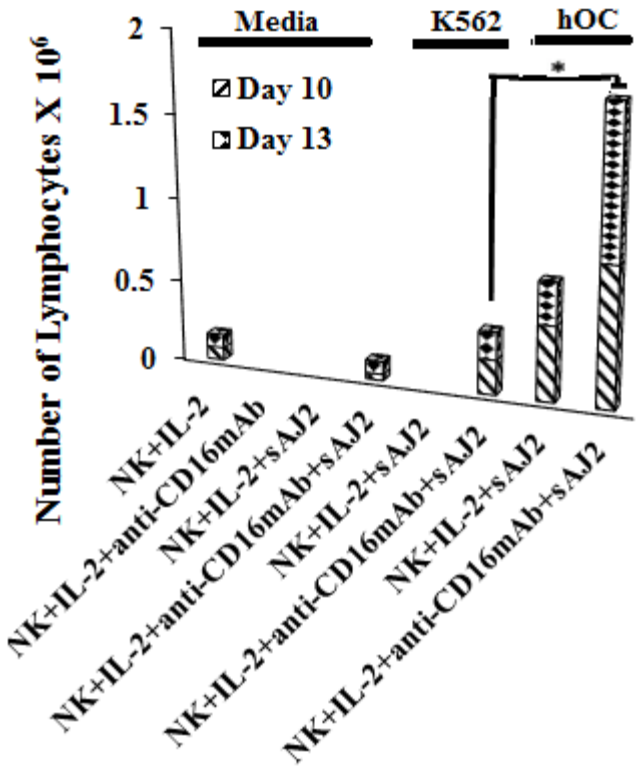


Fig. S3B

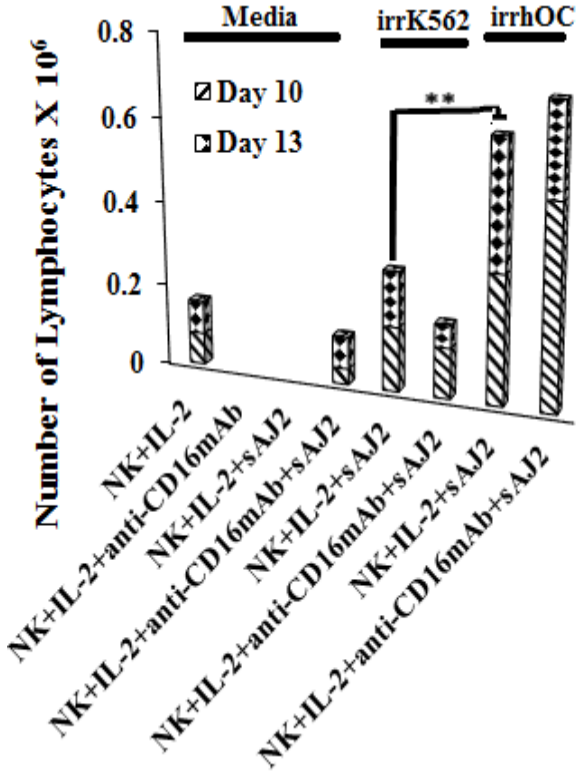


Fig. S3C

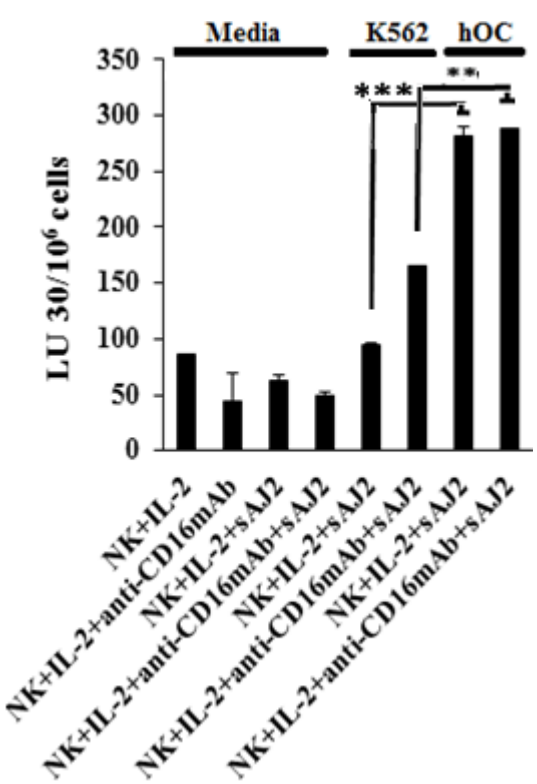


Fig. S3D

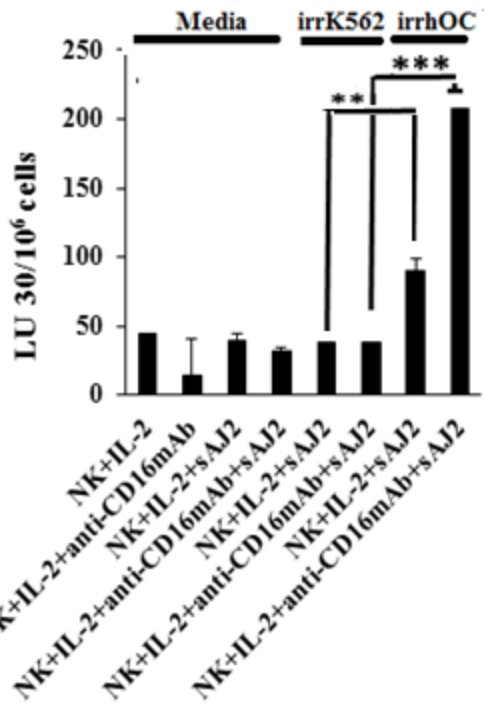


Fig. S3E

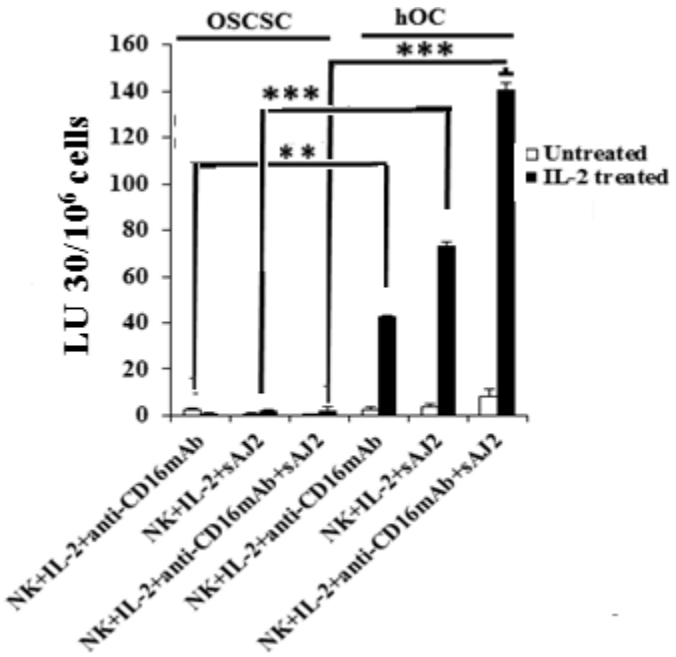


Fig. S3F

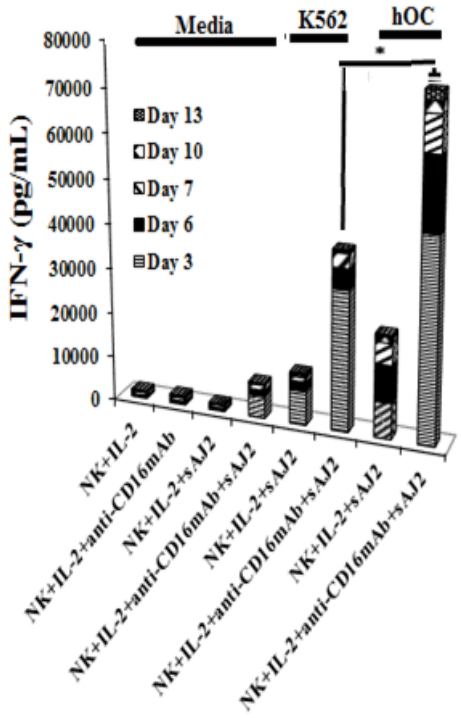


Fig. S3G

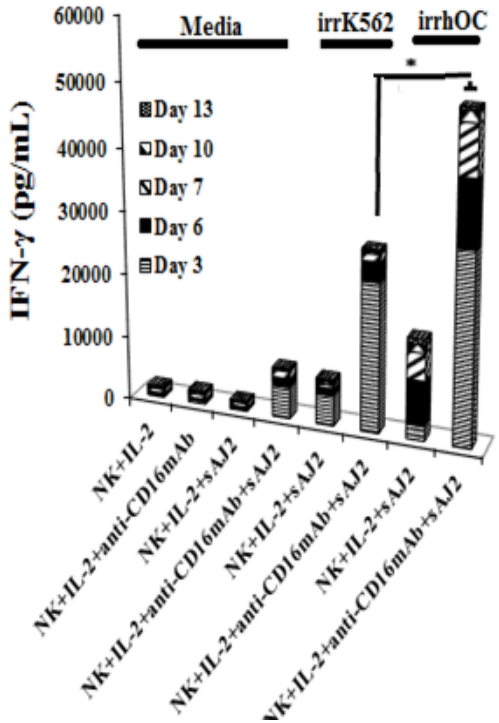


Fig. S3H

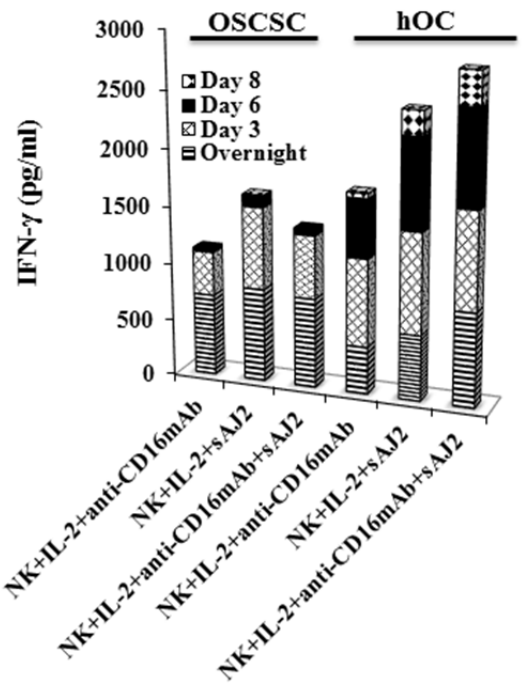


Fig. S3 Osteoclasts, but not K562 or OSCSCs, expand NK cells and increase NK cell function substantially.

To generate osteoclasts, monocytes were cultured in medium containing M-CSF (25 ng/ml) and RANKL (25 ng/ml) for 21 days, K562 tumor cell lines were cultured as described in materials and methods. Highly purified NK cells ($1x10^6$ cells/ml) were treated with the combination of IL-2 (1000 U/ml) and anti-CD16mAb (3ug/ml) for 18 hours before they were co-cultured with K562 and autologous OCs in the presence of sAJ2 bacteria at 1:2:4 ratios (OCs:NK:sAJ2), respectively. Cells from the cultured were counted manually using microscope on day 6, 10 and 13 **(A)**. The osteoclasts generated as described in Fig. S1A and K562 tumor cells lines were irradiated at 40 grays (Gy) as described in the materials and methods. NK cells were purified and treated as described in Materials and Methods, before they were co-cultured with irradiated K562 and irradiated autologous OCs in the presence of sAJ2 bacteria at 1:2:4 ratios (OCs:NK:sAJ2), respectively. Cells from the cultured were counted manually using microscope on day 6, 10 and 13 **(B)**. NK cells were purified and cultured with OCs and K562 as described in Fig. S1A, cytotoxicity of lymphocytes co-cultured for 6 days was determined using a standard 4-hour ^{51}Cr release assay against OSCSCs. The lytic units $30/10^6$ cells were determined using method described in Fig. S2F **(C)**. NK cells were purified and cultured with OCs and K562 as described in Fig. S3B, cytotoxicity of lymphocytes co-cultured for 6 days was determined using a standard 4-hour ^{51}Cr release assay against OSCSCs. The lytic units $30/10^6$ cells were determined using method described in Fig. S2F **(D)**. NK cells were purified and cultured with OCs and OSCSCs as described in Fig. S3A, cytotoxicity of lymphocytes co-cultured for 6 days was determined using a standard 4-hour ^{51}Cr release assay against OSCSCs. The lytic units $30/10^6$ cells were determined using method described in Fig. S2F **(E)**. NK cells were purified and cultured with OCs and K562 as described in Fig. S2A,

supernatant was harvested on day 3, 6, 7, 10 and 13, and IFN-γ secretion was determined using single ELISA (F). NK cells were purified and cultured with irradiated OCs and irradiated K562, as described in Fig. S3B, supernatant was harvested on day 3, 6, 7, 10 and 13, and IFN-γ secretion was determined using single ELISA (G). NK cells were purified and cultured with OCs and OSCSCs as described in Fig. S3A, supernatant was harvested on day 1, 3, 6 and 8, and IFN-γ secretion was determined using single ELISA (H).

Fig. S4A

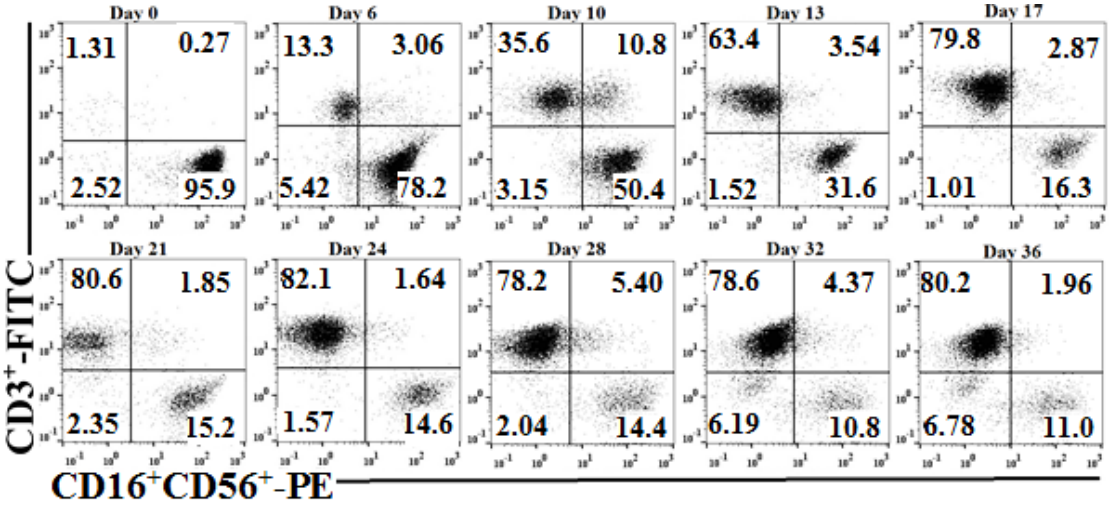


Fig. S4B

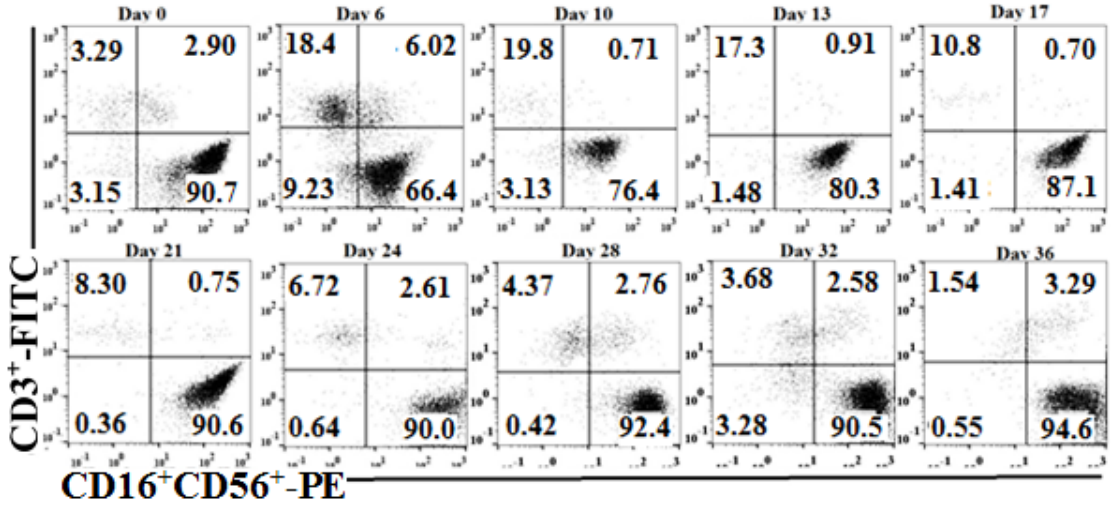
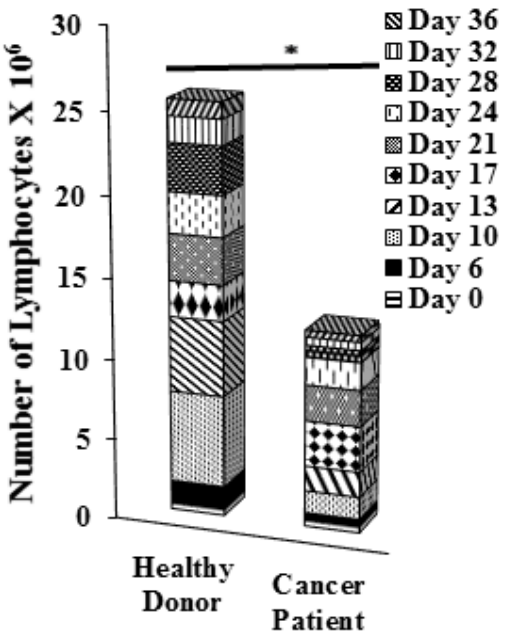


Fig. S4C



Fig, S4D

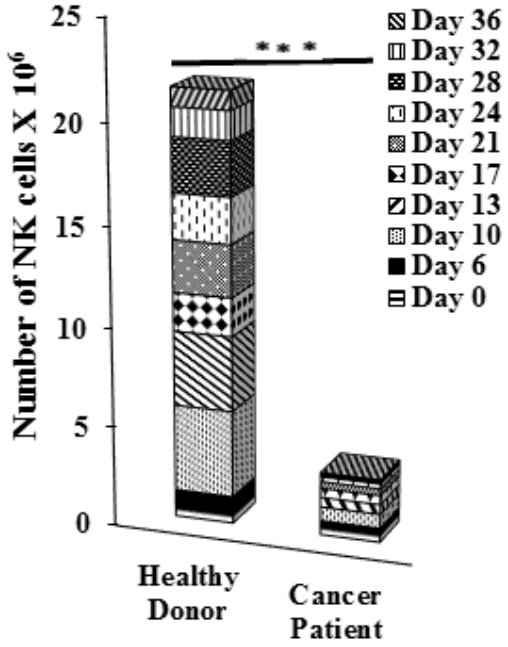


Fig. S4E

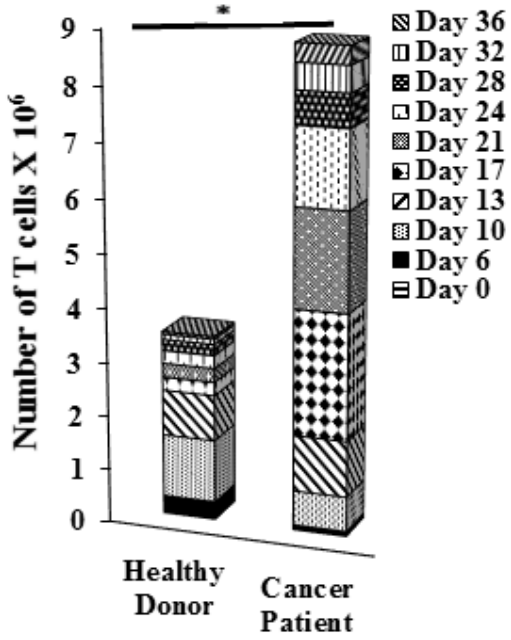
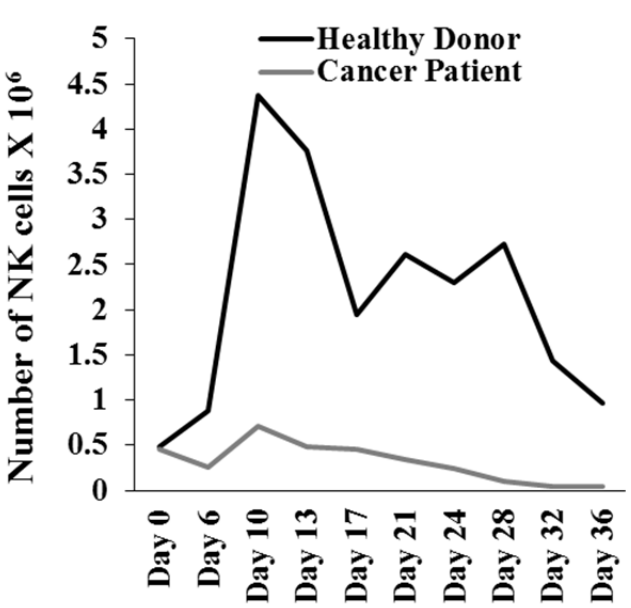


Fig. S4F


Healthy Donor
Cancer Patient
Number of NK cells X 10⁶
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Day 0
Day 6
Day 10
Day 13
Day 17
Day 21
Day 24
Day 28
Day 32
Day 36

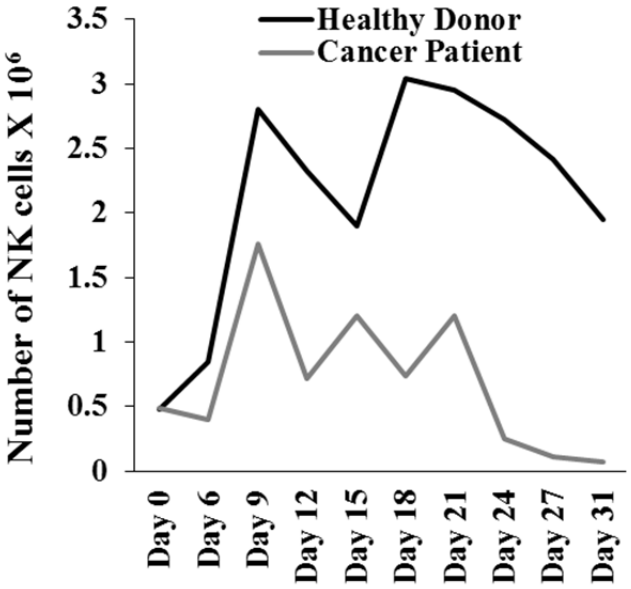

Healthy Donor
Cancer Patient
Number of NK cells X 10⁶
3.5
3
2.5
2
1.5
1
0.5
0
Day 0
Day 6
Day 9
Day 12
Day 15
Day 18
Day 21
Day 24
Day 27
Day 31

Fig. S4G

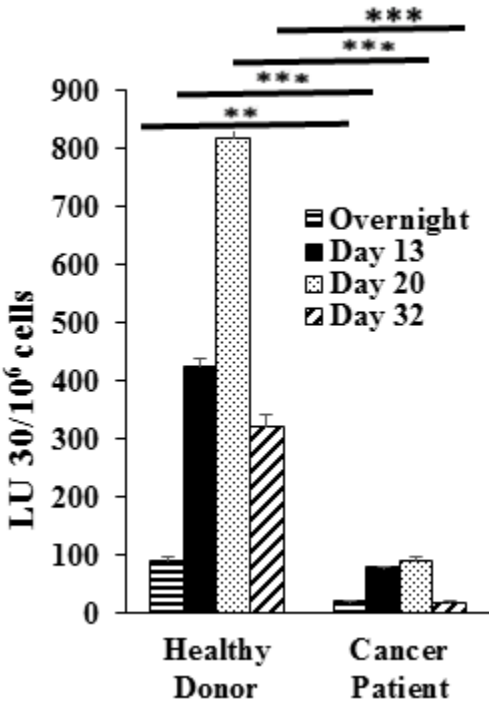


Fig. S4H

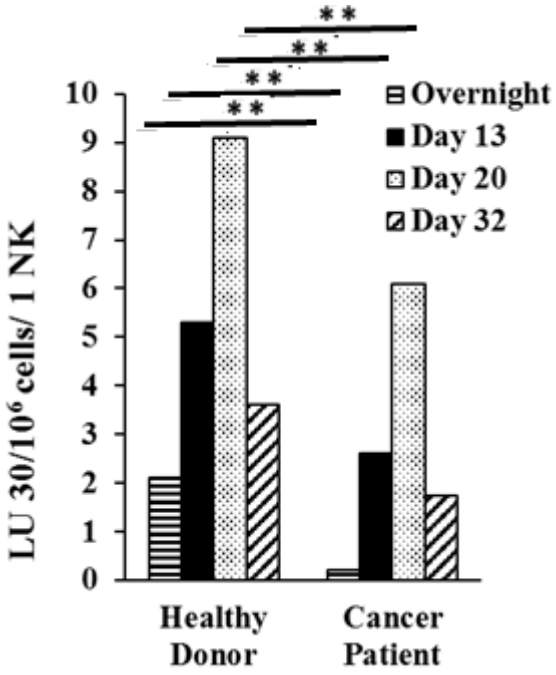


Fig. S4I

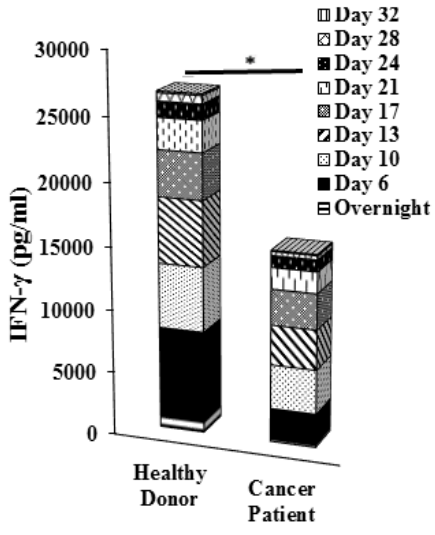


Fig. S4J

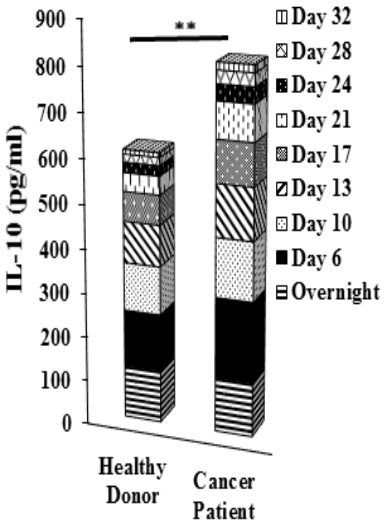


Fig. S4K

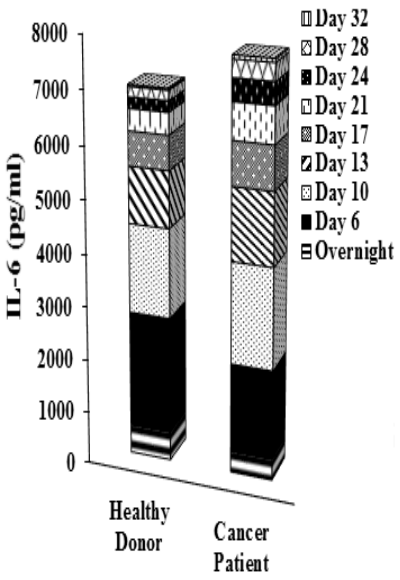


Fig. S4. Small fraction of contaminating T cells within purified NK cells from cancer patient expand faster and crowd out NK cells likely due to decreased NK cell function.

Freshly purified NK cells from a healthy donor and a pancreatic cancer patient were treated and co-cultured with monocyte-derived allogeneic (from different healthy donor) osteoclasts as described in Fig. S1A. Surface expression of CD3, CD16 and CD56 was analyzed in $1x10^4$ lymphocyte samples from co-cultures at days 6, 10, 13, 17, 21, 24, 28, 32 and 36 of cancer patient (**A**) and healthy donor (**B**) using FITC- and PE-conjugated antibodies and flow cytometry. After 6, 10, 13, 17, 21, 24, 28 and 32 days of co-culture, expanded lymphocytes were counted manually using microscope **(C).** Cells counted as mentioned in Fig. S4C and were adjusted based on the surface expression analyzed in Fig. S4A and S4B to determine the number T/NKT cells **(D)** and NK cells at each day **(E and F).** Cytotoxicity of lymphocytes co-cultured for 18-20 hours, 13, 20 and 32 days was determined using a standard 4-hour ^{51}Cr release assay against OSCSCs. The lytic units $30/10^6$ cells were determined using method described in Fig. S2F **(F)**. Lytic units from Fig. S4F was adjusted based on surface expression analyzed is Fig. S4A and S4B to determine the cytotoxicity mediated by 1 NK cell against OSCSCs **(G)**. The supernatant was harvested from the overnight, 6, 10, 13, 17, 21, 24, 28 and 32 days of co-culture and IFN-γ **(H)**, IL-10 **(I)**, and IL-6 **(J)** secretion was determined using single ELISA.

Fig. S5A **Fig. S5B**

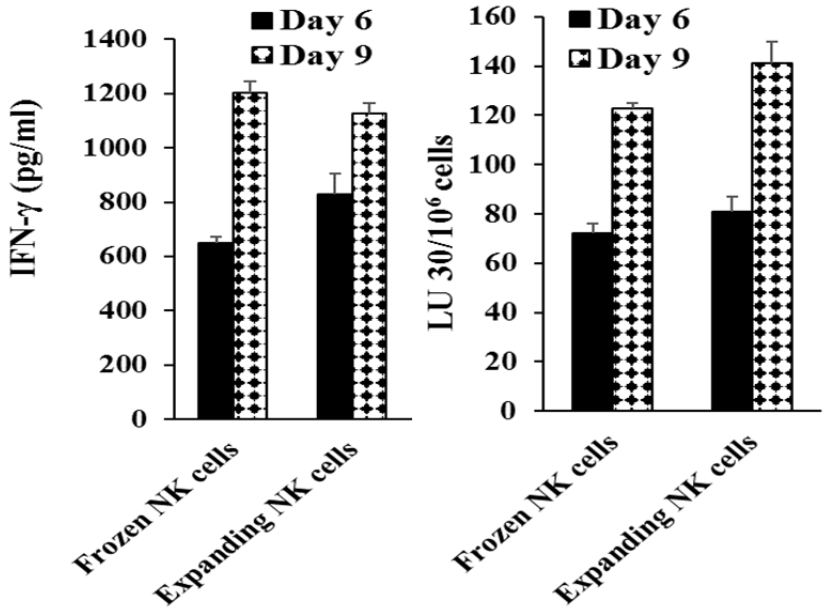


Fig. S5. Osteoclast-expanded NK cells retain their cytokine secretion and cytotoxic function after freezing.

Freshly purified NK cells were treated and co-cultured with monocyte-derived autologous osteoclasts as described in Fig. S1A, day 9 after the cultures, expanded NK cells were frozen. NK cells were thawed and treated with rh-IL-2 (1000 U/ml), day 6 and day 9 after the culture, the supernatant was harvested and IFN-γ secretion was determined using single ELISAs **(A)**. NK cells were cultured as described in Fig. S1A, and the cytotoxicity of lymphocytes day 6 and 9 after the culture was determined using a standard 4-hour ^{51}Cr release assay against OSCSCs. The lytic units 30/10^6 cells were determined using method described in Fig. S2F **(B)**

Fig. S6

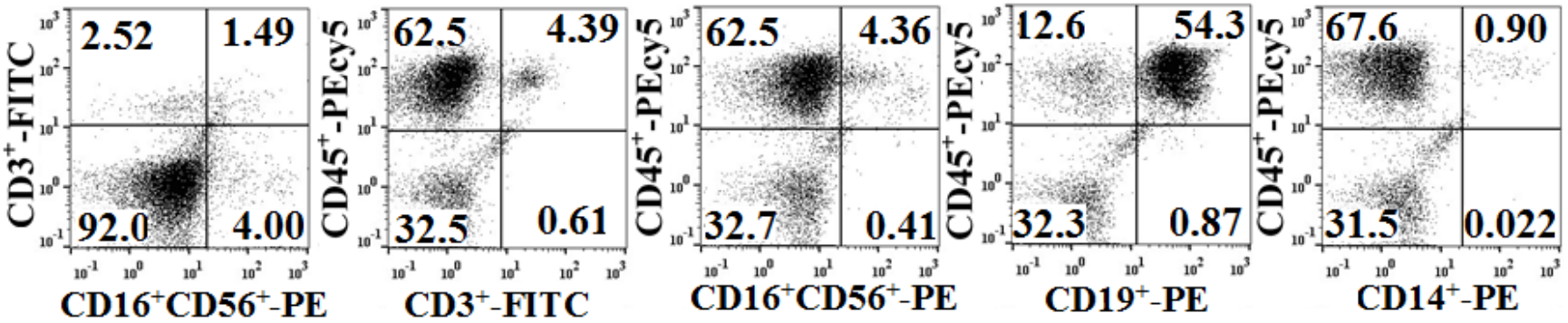


Fig. S6. Phenotype of CD3 T cell depleted lymphocytes from the splenocytes of hu-BLT mice.

Humanized-BLT (hu-BLT; human bone marrow/liver/thymus) mice were generated by surgical implantation of human fetal liver and thymus tissue under the renal capsule of 6-8 weeks old immunocompromised NOD.CB17-Prkdcscid/J and NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice. 4-6 weeks post tissue transplant, mice were sub-lethally irradiated and intravenously injected with $CD34^+$ cells isolated from fetal liver to support full reconstitution of human bone marrow. 8-12 weeks after injection with $CD34^+$ cells, reconstitution of human immune system was analyzed using blood specimens. At the end of this experiment engraftment of human immune cells was confirmed by staining splenocytes and bone marrow cells with anti-human CD45, CD3, CD4 and CD8 antibodies and analyzed by flow cytometry (data not shown). Successfully reconstituted hu-BLT mice (levels and lineages of T cells comparable to healthy donors) were orthotopically injected with $1x10^6$ of human OSCSCs into the floor of the mouth. Disease

progression and weight loss was monitored for another 3-4 weeks. Animals were sacrificed, spleens were harvested from the sacrificed animals, and single cell suspensions were obtained as described in materials and methods. CD3T cells were sorted out using human CD3T positive selection kit. Flow through cells (CD3-negative cells) were analyzed for surface expression of human CD3, CD16, CD56, CD45, CD19, CD14, after staining with the respective PE-conjugated, PE-Cy5-conjugated and FITC-conjugated antibodies. Isotype control antibodies were used as a control **(Fig. S6)**.

Fig. S7A

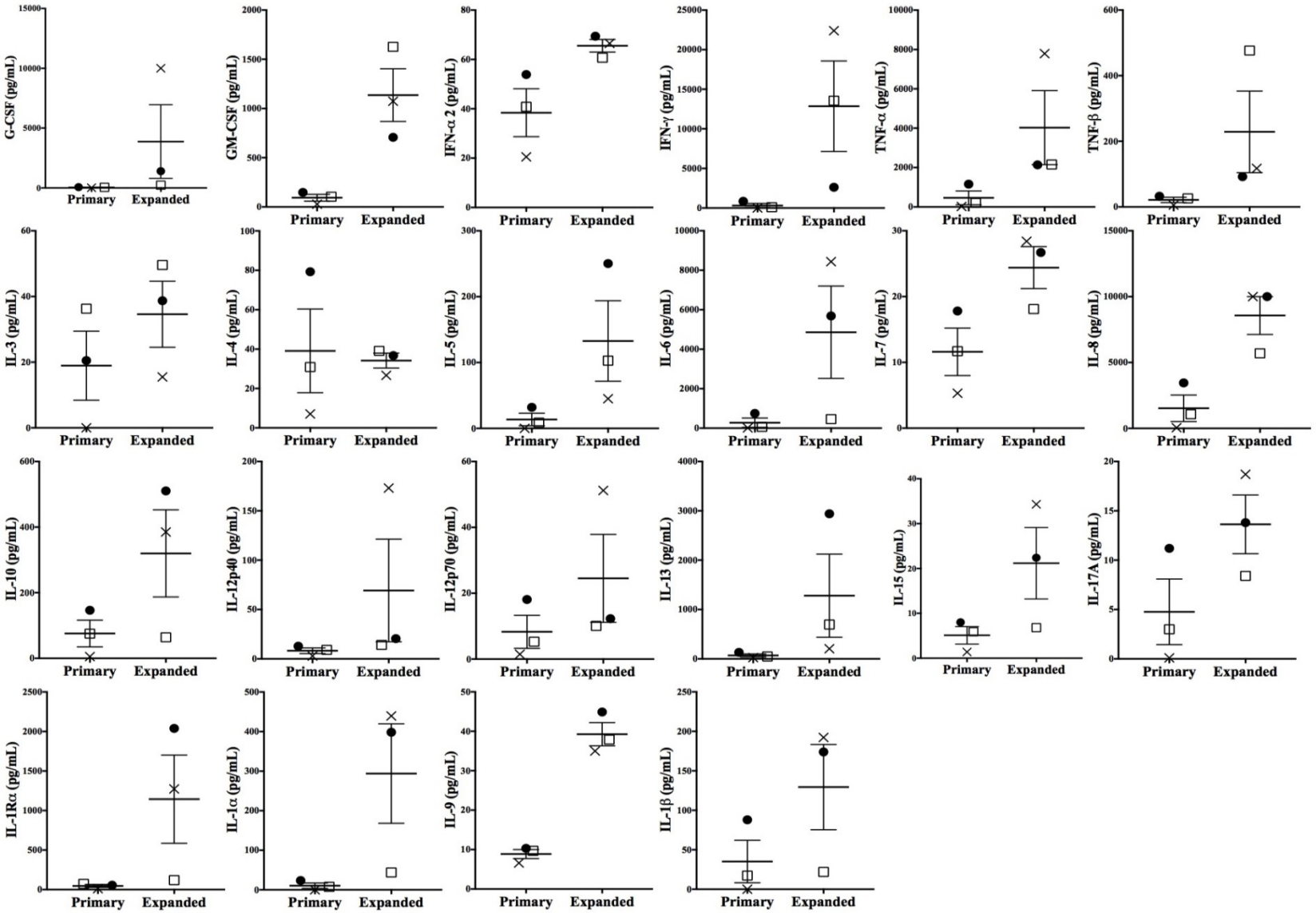
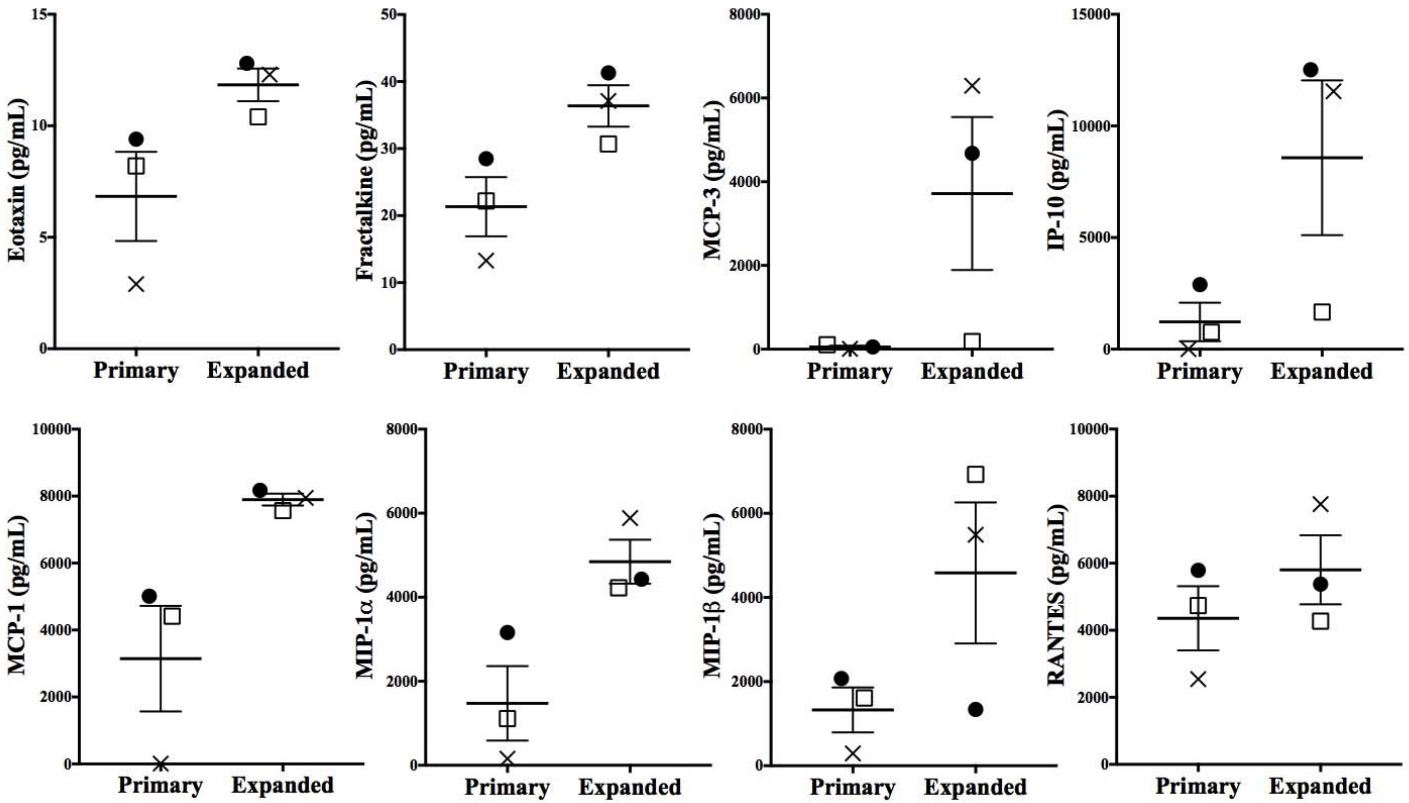

G-CSF (pg/mL)
GM-CSF (pg/mL)
IFN-α 2 (pg/mL)
IFN-γ (pg/mL)
TNF-α (pg/mL)
TNF-β (pg/mL)
IL-3 (pg/mL)
IL-4 (pg/mL)
IL-5 (pg/mL)
IL-6 (pg/mL)
IL-7 (pg/mL)
IL-8 (pg/mL)
IL-10 (pg/mL)
IL-12p40 (pg/mL)
IL-12p70 (pg/mL)
IL-13 (pg/mL)
IL-15 (pg/mL)
IL-17A (pg/mL)
IL-1Rα (pg/mL)
IL-1α (pg/mL)
IL-9 (pg/mL)
IL-1β (pg/mL)
Primary
Expanded

Fig. S7B


Eotaxin (pg/mL)
15
10
5
0
Primary
Expanded
Fractalkine (pg/mL)
50
40
30
20
10
0
Primary
Expanded
MCP-3 (pg/mL)
8000
6000
4000
2000
0
Primary
Expanded
IP-10 (pg/mL)
15000
10000
5000
0
Primary
Expanded
MCP-1 (pg/mL)
10000
8000
6000
4000
2000
0
Primary
Expanded
MIP-1α (pg/mL)
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6000
4000
2000
0
Primary
Expanded
MIP-1β (pg/mL)
8000
6000
4000
2000
0
Primary
Expanded
RANTES (pg/mL)
10000
8000
6000
4000
2000
0
Primary
Expanded

Fig. S7C

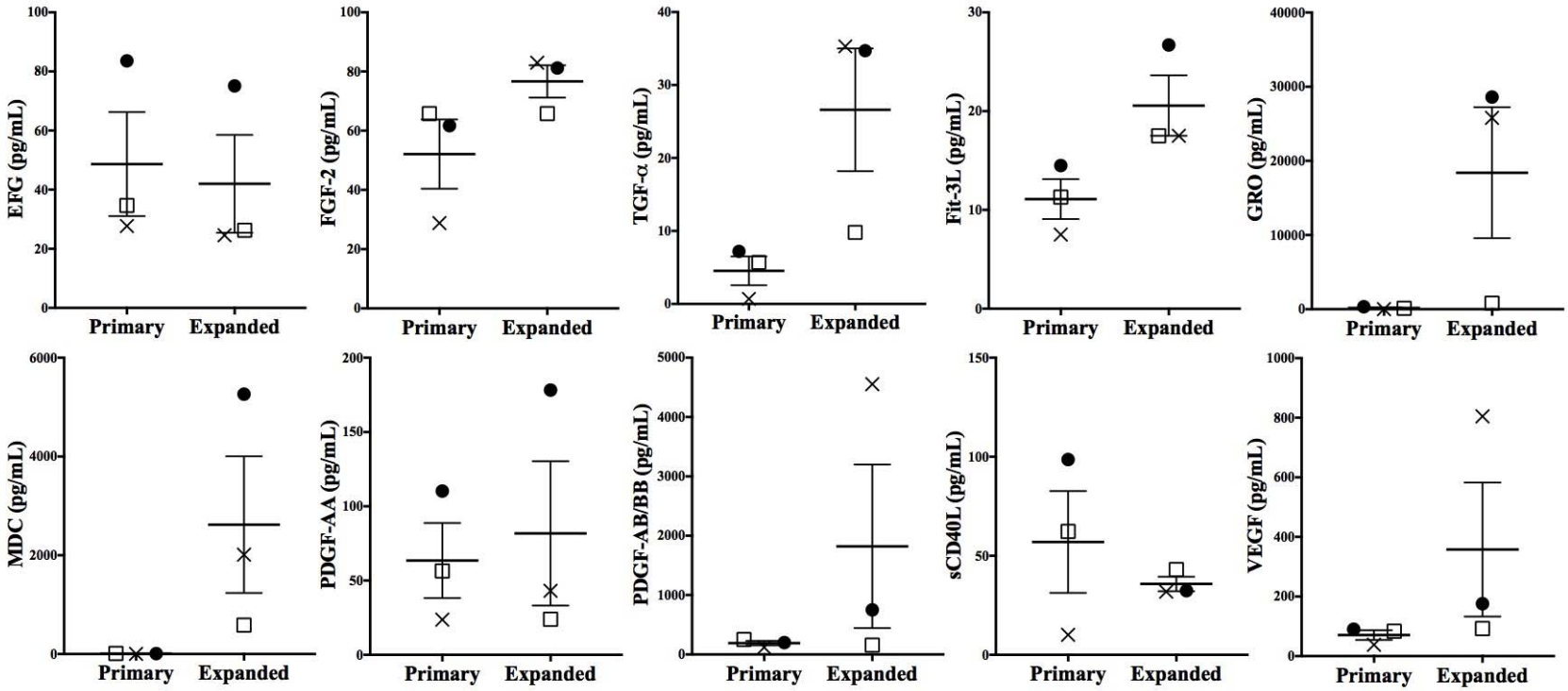

Fig. S7. Cytokines, chemokines and growth factors and ligands secreted by primary and osteoclast-expanded NK cells

Highly purified NK cells and monocytes were obtained from peripheral blood mononuclear cells (PBMCs) of healthy donors and NK cells were treated ($1x10^6$ cells/ml) with IL-2 (1000 U/ml) for 18 hours before the supernatant was harvested. To generate osteoclasts, monocytes were cultured in alpha-MEM media containing M-CSF (25 ng/ml) and RANKL (25 ng/ml) for 21 days. For expansion,

purified NK cells (1x10^6 cells/ml) were treated with the combination of IL-2 (1000 U/ml) and anti-CD16mAb (3ug/ml) for 18 hours before they were co-cultured with autologous osteoclasts in the presence of sAJ2 bacteria at 1:2:4 ratios (OC:NK:sAJ2), respectively. The supernatant was harvested after 6 days of co-culture and multiplex assay was used to determine cytokines **(A)**, chemokines **(B)** and growth factors **(C)** levels secreted by the primary and expanded NK cells.

Fig. S8

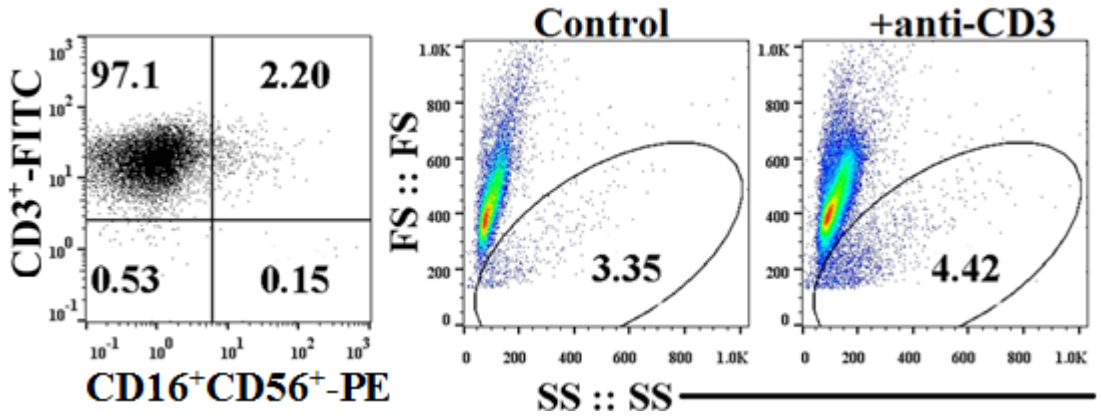

Fig. S8. Purified T cells treated with anti-CD3mAb in the absence of NK cells did not lose forward and side scatter.

Highly purified T cells and monocytes were obtained from peripheral blood mononuclear cells (PBMCs) of healthy donors and T cells were treated ($1x10^6$ cells/ml) with IL-2 (100 U/ml) and anti-CD3 (1ug/ml) for 18 hours before they were co-cultured with autologous osteoclasts in the presence of sAJ2 bacteria at 1:2:4 ratios (OC:T cells:sAJ2), respectively. The cells were analyzed for CD3, CD16 and CD56 on day 9 after the culture. Anti-CD3 treated T cells did not lose Forward and side scatter in the absence of NK cells **(Fig. S8)**.