

Supplementary Material

DN2 thymocytes activate a specific robust DNA Damage response to IR-induced DNA double-strand breaks

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1 Supplementary Methods

The ‘Plastic Thymus’ Culture System

The ‘Plastic Thymus’ culture system was set up as previously described (1). In short, wells of a 6-well culture plate were coated overnight at 4°C with 2 ml of a 10 µg/ml solution of mouse monoclonal anti-human IgG₁ Fc antibody (HUF5.4) diluted in sterile PBS (Sigma-Aldrich) (Supplementary Figure 1). The PBS containing HUF5.4 was then removed and wells washed twice with 5 ml of IMDM growth medium prior to the addition of DL4-Fc diluted in growth medium at a final concentration of 2 µg/ml. 2 ml of this DL4-Fc solution were added per well and incubated overnight at 4°C. The wells were then washed twice with growth medium in order to eliminate any free DL4-Fc molecules not bound to the well surfaces. Sorted DN2 thymocytes were cultured in 2 ml Serum Free-Iscove’s Modified Dulbecco’s Medium (SF-IMDM) (Gibco) supplemented with 5% HyClone FCS (Thermo Scientific), 10% IL-7-containing supernatant, 0.1 µg/ml SCF and 0.2% Ciproxin® (Bayer Pharmaceutical) per well at 37°C in 21% or 5% O₂.

Isolation and sorting of mouse CD4/CD8 double negative (DN) 1-3 sub-populations

For the DN cell isolation, thymi were crushed through a 70µm pore size cell strainer in sterile IMEM (Gibco) to generate a thymocyte suspension. CD4+ and CD8+ T lymphocytes depletion was achieved

by incubating the thymocyte suspension with culture supernatants of rat IgM anti-mouse-CD4 (RL172.4) and rat IgM anti-mouse-CD8 monoclonal antibodies (3.168.8.1) (2,3) and lysing them using Low-Tox®-M Rabbit Complement (Cedarlane®). Viable DN thymocytes were then separated from the cell debris using Ficoll-Hypaque density media (Sigma-Aldrich) and stained with anti-CD25, anti-CD44 and anti-CD117 antibodies at 4°C for 30 minutes in IMDM culture medium. DN thymocytes were then re-suspended in 500 µl of IMDM following a washing step and filtered prior to sorting using a BD FACS Aria II® (BD Biosciences). Dead cells were excluded using propidium iodide staining.

Antibodies

For western blotting, anti-phospho-Histone H2A.X (Ser139) mouse monoclonal antibody (Millipore), anti-H2AX rabbit polyclonal antibody, anti-ATM [2C1(1A1)] mouse monoclonal antibody, anti-DNA-PKcs (Y393) rabbit monoclonal antibody, anti-DNA Ligase IV rabbit polyclonal antibody, anti-Rad51 rabbit polyclonal antibody (Abcam), anti-ATR (N-19) goat polyclonal antibody, anti-Chk2 (H-300) rabbit polyclonal antibody, anti-p53 (1C12) mouse monoclonal antibody, anti-Bcl-XL (54H6) rabbit monoclonal antibody, anti-Bcl-2 (D17C4) rabbit monoclonal antibody, anti-Bim (C34C5) rabbit monoclonal antibody, anti-Puma (E1S7A) rabbit polyclonal antibody, anti Mcl-1 (D35A5) Rabbit monoclonal antibody, anti-β-Actin rabbit polyclonal antibody (Sigma-Aldrich), Pierce horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody, and Immuno-Pure HRP-conjugated goat anti-rabbit IgG antibody (Thermo Scientific), were used. For immunofluorescence staining, anti-phospho-Histone H2A.X (Ser139) mouse monoclonal antibody (Millipore) and fluorescein (FITC)-conjugated AffiniPure F(ab0)2 Fragment goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories Inc.) were used. For flow cytometry, anti-5-bromodeoxyuridine (BrdU) mouse monoclonal antibody (BD Biosciences), FITC-conjugated anti-mouse IgG (whole molecule) antibody (Sigma-Aldrich), PE-conjugated anti-CD3ε (145-2C11) (Biolegend), PE- or APC-conjugated anti-CD117 (cKit) (2B8) (BD Biosciences), and PE-Cy7- or APC-conjugated anti-CD44 (IM7) (BD Bioscience) were used.

2 Supplementary Figure Legends

Supplementary Figure 1: *DN2 and HSC in vitro culture systems.* (A) “The plastic thymus” culture system: Tissue culture wells used for DN2 thymocyte culture were coated with DL4-Fc fusion protein bound to the well surface using with anti-human IgG1-Fc antibody (HUF5.4) In addition, culture medium was supplemented with Interleukin-7 (IL-7) and Stem Cell Factor (SCF) (1). (B) NH-HSC generation and culture system: retroviral transduction of the NUP98-HOXB4 fusion construct allows long-term expansion of HSCs in vitro. Culture medium is supplemented with Interleukin-6 (IL-6) and Stem Cell Factor (SCF) (4).

Supplementary Figure 2: *DN2 phenotype remains stable over time in culture.* Representative cytograms of DN2 cells stained for their surface expression of (A) CD25 and CD117 (cKit); and (B) CD25 and CD44, after 5 and 12 days of normoxic (21% O₂) culture.

Supplementary Figure 3: *Isolation and analysis of DN pro-T cells following IR treatment.* (A) DN2 pro-T cell isolation and enrichment protocol [adapted from (5)] and (B) sorting strategy used for the purification of mouse DN pro-T cells from control and irradiated mice.

Supplementary Figure 4: *Cell cycle progression of DN2 pro-T cells and NH-HSC cells.* Representative cytograms of control un-irradiated DN2 and NH-HSC cells, 0-24h after BrdU labelling and stained for BrdU incorporation and DNA content (propidium iodide). Representative gating strategy for the identification of G₁, S and G₂/M phase cells is shown.

Supplementary Figure 5: *NH-HSCs display a higher radio-resistance in hypoxia.* Clonogenic survival assays of NH-HSCs γ -irradiated at 0.5-4 Gy and cultured for 3 days in normoxia (21% O₂) or hypoxia (5% O₂). Error bars represent mean \pm SD, n=4. * $p < 0.05$, Two-way ANOVA analysis.

Supplementary Figure 6: *DN1/2 pro-T cells downregulate surface levels of cKit upon irradiation.* (A) Representative cKit staining and (B) MFI of DN1/2 thymocytes 0 to 12h after irradiation with 9Gy.

3 Supplementary References

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