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EXPERIMENTAL THESIS

Role of the ICOS–ICOSL Pathway in the Maturation and Differentiation of Plasma Cells and Megakaryocytes, and in Platelet Aggregation

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1. SUMMARY

The ICOS–ICOSL pathway is a central immune co-stimulatory axis that regulates T cell activation, germinal center formation, and B cell differentiation. Within the bone marrow, this pathway also influences plasma cell survival and megakaryocyte function, contributing to a specialized niche that supports long-lived plasma cells. In Multiple Myeloma, dysregulation of ICOS/ICOSL is observed: malignant plasma cells express high ICOSL and low ICOS, which might be involved in promoting tumor progression, immunosuppression, and bone disease through interactions with stromal cells, MKs, and platelets. Despite these observations in pathology, the physiological role of ICOS/ICOSL in plasma cell maturation, megakaryocyte differentiation, and platelet function remains poorly understood.

To address this gap, we used wild-type mice and mice deficient for ICOS, ICOSL, or both, analyzing plasma cells, megakaryocytes, and platelets under healthy conditions. Flow cytometric analysis revealed that ICOS is predominantly expressed on splenic plasma blasts and plasma cells, whereas ICOSL is enriched in bone marrow plasma cells, possibly resident Long Lived Plasma Cells, suggesting stage-specific regulation and a role in migration to and retention within the BM niche. ICOS/ICOSL-double-deficient mice exhibited reduced BM plasma cells and accumulation of splenic plasma cells, indicating impaired trafficking and maturation. Single deficiencies for ICOS or ICOSL further confirmed that ICOS and ICOSL cooperate to support the establishment of the BM plasma cell compartment.

In the megakaryocyte–platelet compartment, ICOSL deficiency reduced early-stage (2N and 4N) megakaryocytes in the bone marrow, while higher-ploidy mature MKs were unaffected. Platelet aggregation in response to ADP was impaired in ICOS-, ICOSL-, and double-deficient mice, linking the ICOS/ICOSL pathway to both MK maturation and platelet function. Immunofluorescence and qPCR confirmed that MKs express ICOS and ICOSL, highlighting direct involvement in hematopoietic regulation.

Overall, this study suggests that ICOS/ICOSL signaling orchestrates plasma cell maturation and homing to the BM niche, supports early megakaryocyte development, and contributes to platelet responsiveness. Future investigations in human plasma cells, MKs, and platelets will be essential to translate these insights and explore therapeutic modulation of the ICOS/ICOSL axis in hematological diseases.

2. INTRODUCTION

2.I. ADAPTIVE IMMUNE SYSTEM

The adaptive immune system, comprised of T and B lymphocytes generated in the bone marrow (BM) (1), mounts an antigen-specific response through complementary cell-mediated and humoral arms, which are critical for directing tissue inflammation and repair (2).

2.I.1 T cells

T cells originate in the BM and mature in the thymus, where they undergo positive and negative selection to ensure functional, self-tolerant effectors (3). Mature T cells differentiate into $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells, each with distinct roles in immune responses (3). Their activation is governed by co-stimulatory (and co-inhibitory) receptors of the CD28 family, including CD28, CTLA-4, PD-1, and ICOS (5). The ICOS/ICOSL axis delivers critical signals in inflamed tissues, modulating helper T cell cytokine production, cytotoxic activity, and Treg development. Notably, ICOSL triggering also exerts reverse signaling, inhibiting endothelial and tumor cell migration (4).

2.I.2 B cells

B lymphocytes originate in the BM, where a transcription factor hierarchy (E2A, EBF1, PAX5) governs lineage commitment and BCR diversification. Meanwhile, central tolerance eliminates or edits self-reactive clones before they are released as mature naïve B cells (6,7,8). Upon interaction with the antigen, follicular (FO) B cells interact with activated T cells, delivering cognate $CD4^+$ T-cell help through CD40–CD40L interactions and cytokine delivery, thereby initiating Germinal center formation (9,10,15). A pivotal regulator of this process is ICOS, which promotes the differentiation, migration, and maintenance of $CXCR5^+$ T follicular helper (Tfh) cells via PI3K activation and sustained T-cell motility. ICOS-mediated co-stimulation of Tfh cells induces IL-21, IL-10, and Bcl-6 expression in Tfh cells, enabling their localization within B-cell follicles and supporting somatic hypermutation, affinity-based selection, and antibody class switching in the GC (11,12,13). The GC reaction ultimately yields memory B cells and precursors of long-lived, high-affinity antibody-secreting plasma cells (PCs), establishing durable humoral immunity (13,14).

2.I.3 Plasma Cell Differentiation and Maturation

The generation of antibody-secreting cells in response to T cell-dependent (TD) antigens is a two-step process, providing immediate and long-term humoral protection. In the first step, known as the extrafollicular response, B cells receive antigen receptor-dependent signals that induce the formation of B lymphoblasts, which proliferate, may undergo immunoglobulin class-switch recombination, and differentiate into short-lived plasma blasts. These cells secrete antibodies rapidly but exhibit little somatic hypermutation, resulting in moderate-affinity antibodies that form the early wave of protection. Short-lived plasma blasts are initially immature, proliferating, and transient, persisting only a few days in peripheral lymphoid organs such as lymph nodes, spleen, Peyer's patches, and tonsils (28, 29).

In the second step, a subset of activated B cells re-enters the B cell follicle and, under the influence of specialized T follicular helper (Tfh) cells, proliferates vigorously to form a GC. Within the GC, B cells undergo extensive proliferation, somatic hypermutation, and selection based on affinity for antigen, ultimately generating high-affinity, long-lived plasma cells (LLPCs). GC-derived LLPCs upregulate IRF4 and Blimp-1 (PRDM1) following the downregulation of the GC regulator BCL6, which represses these key plasma cell determinants (9, 15, 21, 34). Blimp-1 orchestrates a comprehensive phenotypic switch by repressing the B cell gene program (e.g., PAX5) and activating the secretory machinery, including XBP-1, which drives endoplasmic reticulum expansion to support high-rate antibody synthesis (10, 16, 17). This transformation is coupled with metabolic rewiring and cell cycle exit (18).

LLPCs home via CXCR4 to specialized BM survival niches, where stromal signals such as IL-6, APRIL, and BAFF sustain their continuous secretion of high-affinity antibodies for decades, forming the cellular basis of long-term humoral immunity (10, 13, 17, 29). While the majority of LLPCs arise from TD responses involving germinal centers, short-lived PCs generated early in the extrafollicular response provide immediate protection, illustrating the temporal layering of humoral immunity. Notably, T cell-independent (TI) antigens can also induce SLPCs and, in some cases, long-lived antibody responses, further contributing to early and persistent protection (28).

2. **II. The ICOS/ICOSL System**

The inducible T-cell co-stimulator (ICOS/CD278) is a pivotal co-stimulatory receptor and a member of the CD28 family. It is expressed at very low levels on naïve T cells but is rapidly upregulated following T-cell activation (4, 19, 20). ICOS is a transmembrane glycoprotein of the immunoglobulin superfamily, consisting of extracellular, transmembrane, and intracellular domains. Soluble ICOS (sICOS), containing the extracellular portion, can also be detected in plasma (24).

ICOS interacts with its ligand, ICOSL (CD275, also called B7-H2, B7h, or GL50), which is broadly expressed on antigen-presenting cells (APCs), activated endothelial cells (ECs), epithelial cells, fibroblasts, and keratinocytes. ICOSL is similarly a transmembrane glycoprotein (11, 19, 21).

The ICOS/ICOSL interaction plays a central role in adaptive immunity. In T cells, it critically promotes Tfh differentiation and modulates Th17 and Treg responses. This interaction is crucial for GC formation and drives the production of key cytokines such as IL-10, IL-17, IFN-γ in humans, and IL-4 in mice (11, 22, 23). Moreover, ICOS induces CD40L (CD154) expression on T cells, which enhances T cell/B cell interactions and enhances immunoglobulin production, including IgG, supporting humoral immunity (25).

2.III. Forward and Reverse Signaling of ICOSL

ICOS engagement on $CD4^+$ T cells triggers forward signaling, driving the differentiation and functional polarization of helper T cell subsets. ICOS enhances IFN-γ production in Th1 cells, IL-4 in Th2 cells, IL-17 in Th17 cells, IL-21 in T follicular helper (Tfh) cells, and immunosuppressive cytokines such as IL-10, IL-35, and TGF-β in regulatory T (Treg) cells. These signals support lineage-specific polarization and stabilize T cell activation and effector functions (24, 25).

In addition to its classical role, ICOS/ICOSL interaction initiates reverse signaling, whereby ICOSL transduces intracellular signals upon engagement by ICOS. This mechanism modulates the behavior of ICOSL-expressing cells in a cell-type-specific and context-dependent manner. In dendritic cells (DCs), ICOSL reverse signaling promotes partial maturation, enhances IL-6, IL-10, and IL-23 production, facilitates antigen cross-presentation, and inhibits migration (22, 26, 27). In endothelial cells (ECs) and tumor cells, ICOSL engagement reduces adhesiveness and migration, suppresses angiogenesis, and limits epithelial–mesenchymal transition (EMT), thereby restraining

tumor growth and metastasis (21–25, 27). In osteoclasts, ICOSL signaling inhibits differentiation and bone-resorbing activity both in vitro and in vivo (27). Notably, ICOSL reverse signaling can synergize with other innate pathways, such as NOD2-mediated cytokine secretion, further amplifying immune responses (26).

Overall, the ICOS/ICOSL axis exemplifies bidirectional signaling. Forward signaling primarily shapes T cell differentiation and effector functions, whereas ICOSL-mediated reverse signaling fine-tunes the migratory behavior, cytokine output, and functional properties of ICOSL-expressing cells, influencing immune regulation, angiogenesis, tumor progression, and bone homeostasis (21–27).

2.IV. Multiple Myeloma:

Multiple myeloma (MM) is the second most common hematological malignancy, accounting for ~1% of all cancers and 13% of hematological neoplasms (30, 32). It is a hematological malignancy of mature B cells, defined by clonal expansion of PCs in the BM, accompanied by myeloma-related organ damage (CRAB: hypercalcemia, renal insufficiency, anemia, bone lesions) and, in rare cases, extramedullary involvement. MM typically arises from premalignant stages, including monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM) (31, 32).

The disease is characterized by uncontrolled proliferation of clonal PCs, producing monoclonal immunoglobulins (M protein) detectable in serum or urine, and often excess kappa or lambda free light chains, resulting in an abnormal kappa/lambda ratio used to monitor disease progression and treatment. Other measures of tumor burden include the proportion of BM plasma cells, which can range from 10% to nearly 100%, the presence of focal lesions ≥5 mm on MRI, blood levels of β2 microglobulin, and occasionally circulating PCs in peripheral blood (33).

2.IV.1 Bone Marrow Niche and Microenvironment in Multiple Myeloma

The BM is a highly specialized organ responsible for hematopoiesis, immune regulation, and skeletal integrity, supported by a complex and dynamic microenvironment (35, 36). The bone marrow microenvironment (BMME) is classically organized into distinct but interconnected niches, including the vascular (perivascular) niche, the endosteal niche, and the perisinusoidal niche, which host an immune microenvironment that represents a functional compartment of differentiated immune cells within the BM stroma (34, 35). These niches are composed of

hematopoietic and non-hematopoietic cells—such as mesenchymal stromal cells (MSCs), osteoblasts, osteoclasts, endothelial cells, adipocytes, fibroblasts, and immune cells, together with extracellular matrix components and soluble factors, whose coordinated interactions maintain normal hematopoiesis (34, 36).

In MM, malignant PCs can localize to both endosteal and perivascular niches (34). MM cells preferentially localize to the endosteal niche, which provides a relatively protected environment that supports tumor cell dormancy and resistance to therapies targeting proliferating cells (34). MM cells exploit niche-derived signals, including IL-6, IGF-1, HGF, VEGF, SDF-1α, and APRIL, to sustain growth and survival (34,41). High expression of syndecan-1 (CD138) on MM cells further facilitates adhesion and signal integration by binding cytokines, chemokines, and growth factors within the niche (34).

A hallmark of MM progression is profound bone remodeling, driven by the disruption of the physiological balance between osteoblasts and osteoclasts (34, 37). MSCs play a central role in this process by promoting osteoclast differentiation and activation through the secretion of RANKL and M-CSF, while simultaneously impairing osteoblast function, resulting in osteolytic bone lesions (34, 37). Osteoclast-mediated bone resorption releases additional growth-promoting factors, such as TGF-β and IGF-1, which further reinforce MM cell survival and establish a self-sustaining vicious cycle (37).

In parallel, the BM niche actively supports angiogenesis, a critical process for MM progression. Endothelial cells respond to pro-angiogenic signals produced by MSCs and MM cells, including VEGF and FGF2, leading to the formation of new, often structurally abnormal blood vessels that supply nutrients and oxygen to the tumor (34, 37). Beyond structural support, MSCs also profoundly shape the immune microenvironment by interacting with T cells, dendritic cells, and natural killer cells through cytokines and extracellular vesicles, promoting immune tolerance and suppressing anti-tumor immune responses (35, 37). In MM, MSC-derived factors such as IL-6 and CCL2 contribute to immune evasion while directly enhancing myeloma cell survival and proliferation (37).

2.IV.2 Role of Megakaryocytes and Platelets in Multiple Myeloma

Megakaryocytes are large, polyploid cells derived from hematopoietic stem cells (HSCs) that progressively migrate within the BM from the endosteal to the vascular niche during differentiation, where they ultimately release platelets into circulation. Within the BM, MKs are predominantly localized adjacent to sinusoidal vessels, a positioning that depends on chemokines produced by endothelial cells and perivascular mesenchymal stromal cells. Their primary physiological function is platelet biogenesis, a highly regulated process whereby mature endomitotic MKs extend long cytoplasmic extensions, known as proplatelets, which fragment into thousands of circulating platelets (38). Beyond platelet production, MKs actively contribute to BM homeostasis by regulating the hematopoietic vascular niche, bone remodeling, and inflammatory signaling (42).

Importantly, MKs constitute an important component of the plasma cell survival niche within the BM. PCs closely interact with MKs, which secrete key survival and growth factors, including interleukin-6 (IL-6) and APRIL, both of which are essential for plasma cell persistence. In line with this, mice deficient in the thrombopoietin receptor (c-Mpl), and therefore impaired in megakaryopoiesis, exhibit reduced numbers of immature and mature PCs in the BM and defective accumulation of antigen-specific PCs following immunization, whereas thrombopoietin administration promotes plasma cell persistence (39). In the context of MM, MKs have been implicated in early tumor growth within the BM niche, with eosinophils and MKs supporting initial myeloma cell expansion, and inflammatory cues such as neutrophil- and monocyte-derived S100A8/A9 proteins driving MM progression through MK expansion (42). Moreover, recent single-cell transcriptomic analyses have revealed marked heterogeneity among MK populations, suggesting specialized subsets with distinct immunoregulatory and inflammatory functions that may be co-opted during malignant transformation (40).

Platelets, the circulating progeny of MKs, have traditionally been viewed as mediators of hemostasis and wound repair; however, growing evidence indicates that they play an active role in tumor biology, including MM progression (44,45). Platelets act as reservoirs of numerous growths and pro-angiogenic factors, such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and basic fibroblast growth factor (bFGF), which are rapidly released upon platelet activation and contribute to the establishment of a tumor-supportive microenvironment (44). In MM, platelet activation is induced by malignant PCs and associated inflammatory cues, which secrete cytokines such as tumor necrosis factor-α

(TNF-α), and MM cell lines have been shown to trigger platelet aggregation and activation in vitro (43).

Functional studies have demonstrated that platelets enhance MM cell proliferation and BM engraftment. Co-culture of MM cell lines with platelets or platelet releases significantly increases tumor cell growth, while pre-exposure of MM cells to platelets promotes in vivo tumor engraftment and reduces host survival, a process critically dependent on platelet-mediated upregulation of interleukin-1β (IL-1β) in MM cells. Transcriptomic profiling further revealed enrichment of platelet-associated gene signatures in patients with smoldering MM and active disease, indicating disease-stage–dependent platelet reprogramming (43). Mechanistically, MM cells express platelet-derived growth factor receptors (PDGFR-α and -β), enabling responsiveness to PDGF released by activated platelets, a pathway strongly associated with angiogenesis and increased microvessel density. Additional interactions may occur through P-selectin and its ligand PSGL-1, as well as syndecan-1 (CD138), which binds angiogenic factors such as VEGF and FGF-2 and is linked to enhanced tumor growth and poor prognosis (32).

2.IV.3 Role of ICOS/ICOSL in Multiple Myeloma

The ICOS/ICOSL axis may play a role in MM by modulating immune interactions within the BM microenvironment and supporting tumor progression. Autologous bone marrow stromal cells (BMSCs), together with inflammatory cues such as tumor necrosis factor-α (TNF-α), have been shown to upregulate ICOSL and CD86 expression on malignant plasma cells. This, in turn, enhances IL-10 production by interacting T cells, contributing to the establishment of an immunosuppressive milieu and favoring regulatory T cell expansion (46). In MM patients, elevated circulating levels of soluble ICOS (sICOS) and soluble ICOSL (sICOSL) correlate with established markers of tumor burden, including β2-microglobulin, monoclonal protein levels, and bone marrow plasma cell infiltration, while increased sICOS levels are associated with advanced disease stage and reduced overall survival (47–49).

In addition to its immunoregulatory role, the ICOS/ICOSL pathway has been implicated in the control of bone homeostasis in MM. Reverse signaling through ICOSL, triggered by ICOS-Fc, inhibits osteoclast differentiation and RANKL-mediated bone resorption, whereas ICOSL expression on myeloma PCs has been associated with enhanced osteoclast activity and the development of osteolytic bone disease (47–49). These findings place ICOS/ICOSL signaling at the interface between immune regulation and bone remodeling in the myeloma BM niche.

Beyond published observations, our previous work has revealed that malignant PCs display aberrant expression patterns of ICOS and ICOSL compared with their normal counterparts. In particular, MM PCs exhibit high ICOSL expression accompanied by relatively low ICOS levels, while normal bone marrow PCs show a heterogeneous phenotype, including $ICOS^{+}ICOSL^{+}$ and $ICOS^{-}ICOSL^{+}$ subsets.

Within the BM niche, MKs represent an additional, functionally relevant component of plasma cell support. MKs secrete key plasma cell survival factors, including APRIL and IL-6, thereby contributing to myeloma cell persistence. Their circulating progeny, platelets, can further interact with MM cells, promoting proliferation and tumor engraftment through mechanisms involving IL-1β upregulation, PDGF and VEGF release, and P-selectin–mediated adhesion. The ICOS/ICOSL pathway may intersect with these processes by influencing immune cell activation as well as vascular and stromal components of the BMME, thereby linking immune costimulatory signaling to megakaryocyte–platelet biology (47).

Taken together, accumulating evidence highlights the ICOS/ICOSL axis as a multifaceted regulator in MM, influencing malignant plasma cell behavior, immune regulation, bone metabolism, and components of the BM niche, possibly including also MKs and platelets. Our previous work demonstrated that ICOS and ICOSL are expressed in myeloma PCs and that soluble forms of these molecules are closely associated with tumor burden, bone disease, and clinical outcome in MM patients. However, the physiological role of the ICOS/ICOSL system in plasma cell differentiation and in megakaryocyte–platelet biology under non-pathological conditions has remained unclear. In the present experimental work, which forms the basis of this thesis, we directly address this gap by investigating the ICOS/ICOSL axis in healthy settings, allowing a direct comparison with the alterations previously observed in MM.

3. Aim of the Study

The research project was designed with two main objectives. First, to examine how ICOS and ICOSL are expressed across various subsets of differentiating PCs in wild-type (WT) mice, as well as in mice genetically deficient for ICOS, ICOSL, or both. Second, to explore the involvement of the ICOS/ICOSL pathway in regulating platelet aggregation and megakaryocyte differentiation.

4. MATERIALS AND METHODS

4.1 Animal model

Wild-type C57BL/6J mice and mice deficient (Charles River Laboratories, Wilmington, MA, USA) for ICOS ($ICOS^{-/-}$), ICOS ligand ($ICOSL^{-/-}$), or both ICOS and ICOSL ($ICOS/ICOSL^{-/-}$) were used in this study. Mice were bred under standard laboratory conditions with free access to food and water. Tissues were collected from 6-8 week-old mice that were not specifically bred or assigned for this study but were scheduled for euthanasia due to colony management reasons (e.g., surplus animals). Tissue collection was performed post-mortem following euthanasia carried out under approved institutional protocols. All animal procedures were carried out at the animal facility of Università del Piemonte Orientale and were approved by the local Ethical Committee and conducted in accordance with European guidelines for the care and use of laboratory animals (Authorization No.DB064.N.TFX).

4.2 Plasma cell isolation from spleen and bone marrow

PCs were isolated from the spleen and bone marrow of WT mice, and mice deficient for ICOS (ICOS-KO), ICOS ligand (ICOSL-KO), or both ICOS and ICOSL (ICOS/ICOSL-KO). Femurs, tibias, and spleens were collected under sterile conditions and maintained in phosphate-buffered saline (PBS).

Bone marrow cells were obtained from femurs and tibias by centrifugal flushing, resuspended in PBS, and passed through 70-µm cell strainers (Pluriselect, Germany). Spleens were smashed in petri dishes containing PBS and filtered through a 70-µm cell strainer. Lymphocytes were enriched by density gradient centrifugation using Lympholyte cell separation media (Lympholyte-H, Cedarlane Laboratories) at 800 for 20 minutes, after which the buffy coat layer was collected. Residual red blood cells were lysed using BD FACS™ Lysing Solution (BD Biosciences), and cells were washed with PBS.

4.3 Flow cytometric analysis of plasma cells

After red blood cell lysis and washing with PBS, cells were resuspended in staining buffer (PBS,1% fetal bovine serum (FBS), and 0.1% sodium azide). Cells were stained with a cocktail of

fluorochrome-conjugated antibodies including anti-CD138 (APC, BioLegend), anti-B220 (Alexa Fluor 700, Invitrogen), anti-CD19 (PE-Cy7, Invitrogen), anti-CXCR4 (APC eFluor 780, Invitrogen), anti-ICOS (PE-Cy5, Invitrogen), anti-ICOSL (PE, Invitrogen), and a lineage exclusion cocktail containing antibodies against CD3, IgD, CD16, and CD14, all conjugated to FITC (Invitrogen). Cells were incubated with the antibody cocktail at 4 °C for 20 minutes in the dark, washed with staining buffer, and resuspended for acquisition. Data were acquired on an Attune™ NxT flow cytometer (Thermo Fisher Scientific) and analyzed using FlowJo software (BD Biosciences).

4.4 Isolation of mature primary megakaryocytes

Mature primary megakaryocytes were isolated from mouse bone marrow by size-exclusion filtration. Femurs and tibias from WTmice were collected in catch buffer (12.5 mM HEPES, 2 mM EDTA, 1.75% BSA, 2.5% FBS in PBS). Muscles were removed, and bones were flushed by centrifugation for 1 min at 2600 × g at room temperature. Bone marrow cells were collected, resuspended in catch buffer, and filtered through a 70-µm cell strainer to remove debris. The cell suspension was then passed twice through a 20-µm cell strainer (Pluriselect, Germany). The retained cells (megakaryocyte fractions I and II) were recovered by inverting the filter into a new conical tube, while the flow-through was discarded. The collected fractions were subsequently filtered through a 15-µm cell strainer (Pluriselect, Germany) to enrich for large mature megakaryocytes. The retained cells (megakaryocyte fraction III) were recovered by inverting the filter into a new tube. Megakaryocyte fraction III was centrifuged at 300 × g for 10 minutes, while the flow-through containing smaller non-megakaryocytic cells was collected as the NON-MK fraction. Cells were resuspended in PBS, fixed by dropwise addition of 75% ethanol, and stored at 4 °C overnight.

4.5 Assessment of megakaryocyte ploidy by propidium iodide staining

After fixation, cells were centrifuged at 400 × g for 5 minutes, washed with PBS, and centrifuged again at 200 × g for 5 minutes. The pellet was resuspended in staining buffer and incubated with anti-mouse CD41 (APC-Cy7, BioLegend) for 30 minutes at 4 °C to identify megakaryocytes. Cells were washed and permeabilized using PBS containing 0.2% Triton X-100.

DNA content was measured by staining with propidium iodide (50 µg/mL; Invitrogen) in the presence of RNase A (0.1 mg/mL; Thermo Fisher Scientific). Samples were incubated for 30

minutes at room temperature in the dark, acquired on Attune™ NxT flow cytometer (Thermo Fisher Scientific), and analyzed with FlowJo software (BD Biosciences) to determine megakaryocyte ploidy.

4.6 RNA extraction and quantitative real-time PCR

Cultured primary murine megakaryocytes were collected, and the cell pellet was lysed for total RNA extraction using TRIzol reagent (Ambion, Life Technologies) according to the manufacturer's instructions. The extracted RNA was retro-transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen).

Relative expression levels of ICOS, ICOSL, and GATA1 (a megakaryocyte-specific marker) were measured using TaqMan gene expression assays (Assay-on-Demand; Applied Biosystems, Foster City, CA). GAPDH was used as the endogenous control to normalize cDNA input. Quantitative real-time PCR was performed on a CFX96 system (Bio-Rad Laboratories). Each reaction was run in duplicate in a final volume of 10 µL, containing 1 µL of diluted cDNA, 5 µL of TaqMan Universal PCR Master Mix (Applied Biosystems), and 0.5 µL of the corresponding assay mix. Gene expression levels were calculated using the ΔΔCt method.

4.7 Immunofluorescence

Primary megakaryocyte (MK) cells were seeded onto Matrigel-coated coverslips and incubated for 1 hour at 37°C to allow adherence. Following incubation, cells were fixed with 3% paraformaldehyde and 4% sucrose. Fixed cells were then pre-blocked with goat serum (5%) for 1 hour in a humidified chamber at room temperature to reduce nonspecific binding. Cells were subsequently incubated overnight at 4°C with primary antibodies against ICOS and ICOSL (in Goat anti-ICOS and anti-ICOSL, Thermo Fisher Scientific, PA5-47161). After washing, cells were incubated with Alexa Fluor 546-conjugated/ Alexa Fluor 488-conjugated secondary antibodies (Invitrogen) and counterstained with DAPI (Invitrogen). Coverslips were mounted using SlowFade™ Light Antifade Mounting Medium (Molecular Probes, Invitrogen) and imaged using a confocal microscope. Image acquisition and analysis were performed using Leica LAS X 2.6 software.

4.8 Platelet aggregation assay

Platelet-rich plasma (PRP) was obtained from the peripheral blood of WT, ICOS-KO, and ICOSL-KO mice. Blood was collected into 2-mL tubes containing sodium citrate (3.8%) as an anticoagulant (400 μL blood with 50 μL sodium citrate) and centrifuged at 600 rpm for 10 minutes to separate PRP. The upper PRP phase was carefully collected and used for aggregation assays. Platelet concentration in PRP was determined and diluted in 1x Tyrode buffer (HEPES, NaCl, KCl, NaHCO3, pH ≈ 7.4) to a final concentration of 6×10^5 platelets/μL. For each measurement, 225 μL of diluted PRP was transferred into the aggregometry cuvette, and aggregation was initiated by the addition of 25 μL of ADP (final concentration 2 μM). Platelet aggregation was measured using a PAP-8E aggregometer (Bio/Data Corporation, Horsham, PA, USA).

4.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 6.01). Comparisons between two independent groups were performed using the non-parametric Mann–Whitney *U* test. A p-value < 0.005 was considered statistically significant. All graphs were generated using GraphPad Prism.

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5. RESULTS

1. Expression of ICOS and ICOSL during plasma cell differentiation in WT mice

Flow cytometry was used to investigate ICOS and ICOSL expression in plasma cell subsets from WT mice, including spleen plasma blasts, spleen plasma cells, and bone marrow plasma cells, as plasma blasts and PCs arise within secondary lymphoid tissues and subsequently mature into LLPCs that home to the bone marrow. Plasma blasts were gated as Lin^- (CD3, IgD, CD14, and CD16) $CD138^+$ $B220^+$ cells, whereas PCs were identified as Lin^- (CD3, IgD, CD14, and CD16) $CD138^+$ $CXCR4^+$ cells (Fig. 1.a). In the spleen, approximately 10.5% of plasma blasts expressed ICOS, while ICOSL expression was negligible. A higher frequency of ICOS expression was observed in spleen plasma cells, with approximately 18% of cells being $ICOS^+$, whereas ICOSL remained largely undetectable (Fig. 1. b). In contrast, bone marrow PCs displayed a distinct expression profile, characterized by the presence of ICOSL in approximately 7.3% of cells, while smaller subsets expressed ICOS alone or co-expressed ICOS and ICOSL (Fig. 1. c).

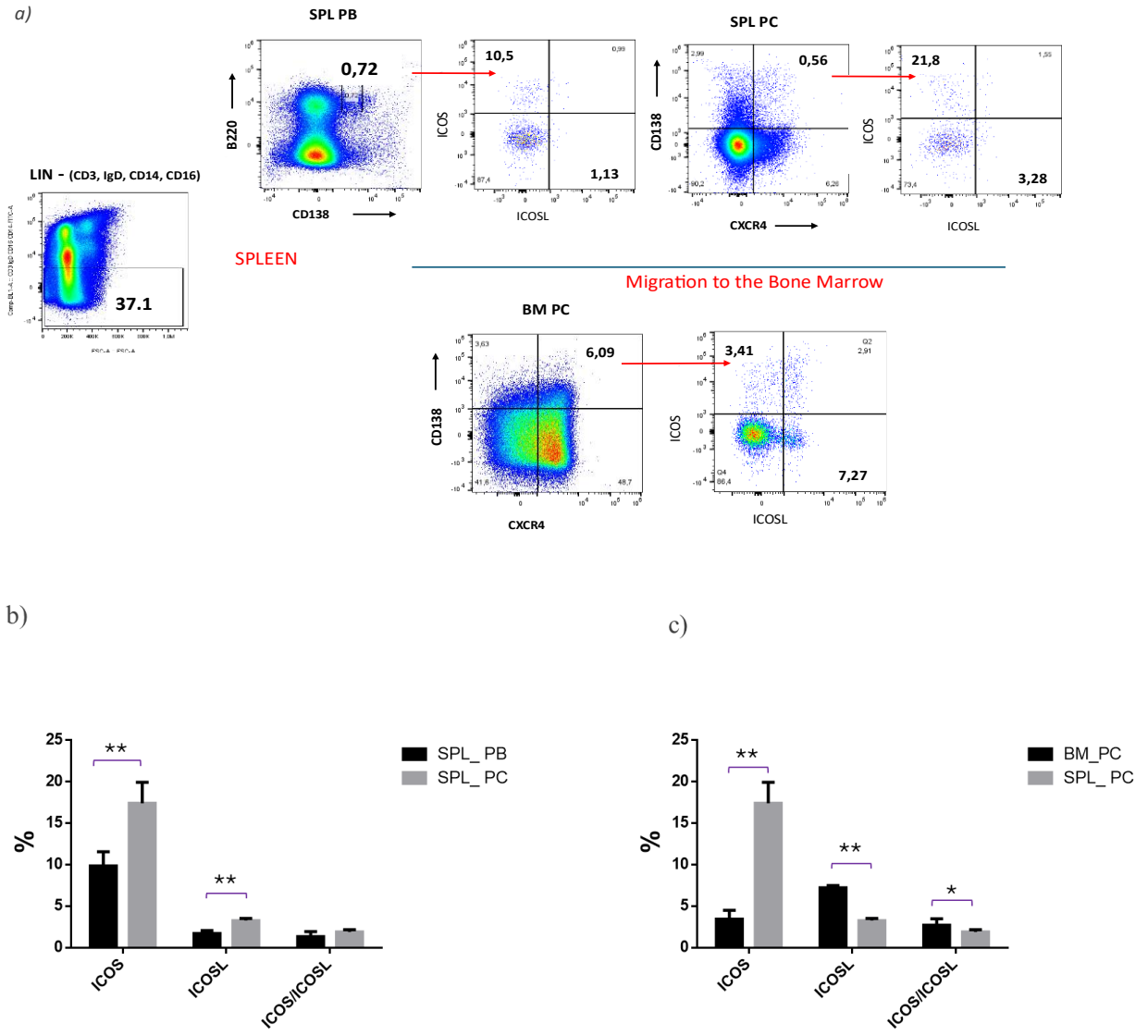

Figure 1. Flow cytometric gating strategy of WT BM and splenic plasma blasts and plasma cells*: a) Schematic and flow cytometry analysis showing ICOS and ICOSL expression in WT bone marrow and splenic plasma blasts and plasma cells. WT mice display a distinct expression pattern at each differentiation stage, indicating a possible pathway from plasma blasts to LLPC. Plasma blasts were gated as CD138⁺/B220⁺, and PCs as CD138⁺/CXCR4⁺. b) Bar graphs showing the percentage of ICOS⁺, ICOSL⁺, and ICOS⁺/ICOSL⁺ cells among splenic plasma blasts (SPL-PB), splenic plasma cells (SPL-PC), and bone marrow plasma cells (BM-PC) in WT mice. Data was collected from five individual experiments. Values are represented as *, P < 0.05; **, P < 0.005 vs control, calculated by the Mann-Whitney test.*

2. Altered Distribution of Plasma Blasts and Plasma Cells in KO Mice

To investigate the role of ICOS and ICOSL in plasma cell maturation, the distribution of plasma blasts and PCs was compared in the spleen and bone marrow of WT, ICOS-KO, ICOSL-KO, and ICOS/ICOSL-KO mice. The frequencies of splenic plasma blasts and plasma cells, as well as bone marrow plasma cells, were largely comparable among WT, ICOS-KO, and ICOSL-KO mice, except for a mild increase of splenic plasma blasts and bone marrow plasma cells in ICOS-KO mice. In contrast, ICOS/ICOSL-KO mice displayed a mild reduction in plasma blasts together with a marked increase of plasma cells in the spleen, and a marked reduction of plasma cells in the bone marrow. This altered distribution suggests that the combined loss of ICOS and ICOSL disrupts normal plasma cell differentiation and/or trafficking to the bone marrow, resulting in impaired establishment of the bone marrow plasma cell compartment (Fig. 2).

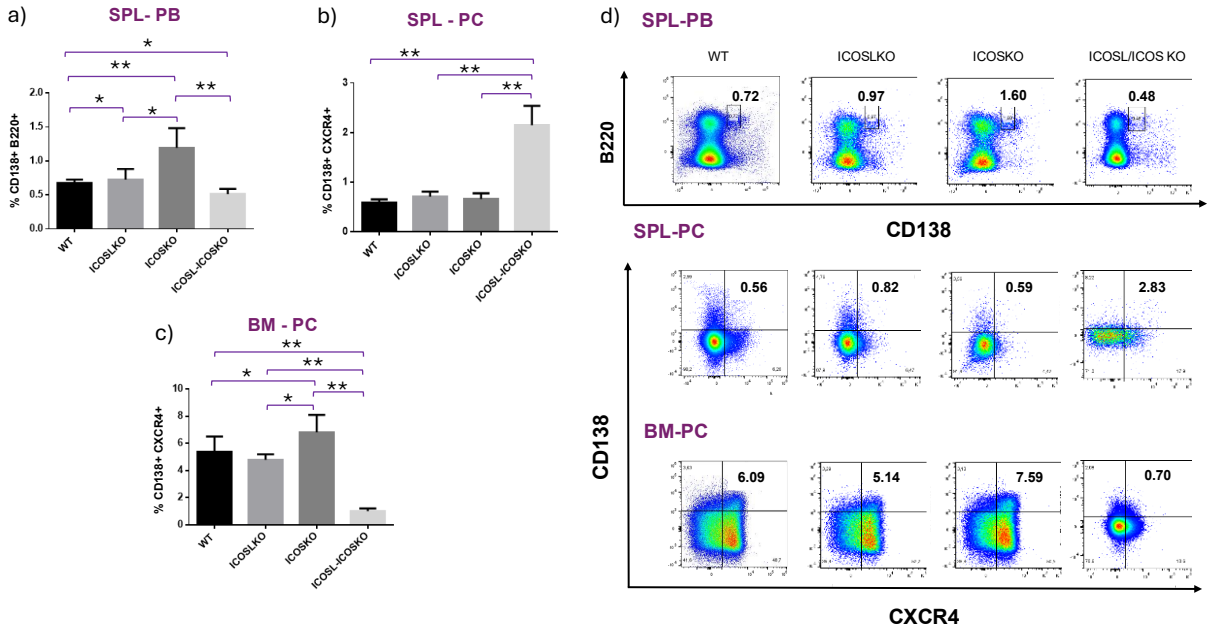

Figure 2: Effect of ICOS and ICOSL Deficiency in Plasma Cell Maturation and Distribution Across Spleen and Bone Marrow: *a) the percentage of plasma blasts in the spleen ($CD138^+/B220^+$), b) the percentage of PCs in the spleen ($CD138^+/CXCR4^+$), c) the percentage of PCs in the bone marrow ($CD138^+/CXCR4^+$), d) Representative flow cytometry plots of plasma blasts and PCs in spleen and bone marrow across WT, ICOSL KO, ICOS KO, and ICOS/ICOSL KO mice. Data was collected from five individual experiments. Values are represented as *, $P < 0.05$; **, $P < 0.005$ vs control, calculated by the Mann-Whitney test.*

Analysis of ICOS and ICOSL expression revealed marked differences among genotypes. As expected, ICOSL was downregulated in ICOSL-KO and ICOS/ICOSL-KO mice, and ICOS in ICOS-KO and ICOS/ICOSL-KO mice.

Comparison of ICOSL expression in WT and ICOS-KO mice showed that, in ICOS-KO mice, the proportion of ICOSL$^+$ plasma blasts and plasma cells was substantially increased in the spleen, which was possibly due to a compensatory upregulation of ICOSL secondary to the lack of ICOS; no substantial difference in the frequency of ICOSL$^+$ PCs was observed in the bone marrow (Fig. 3). By contrast, no substantial difference was detected in ICOS expression between ICOSL-KO and WT mice

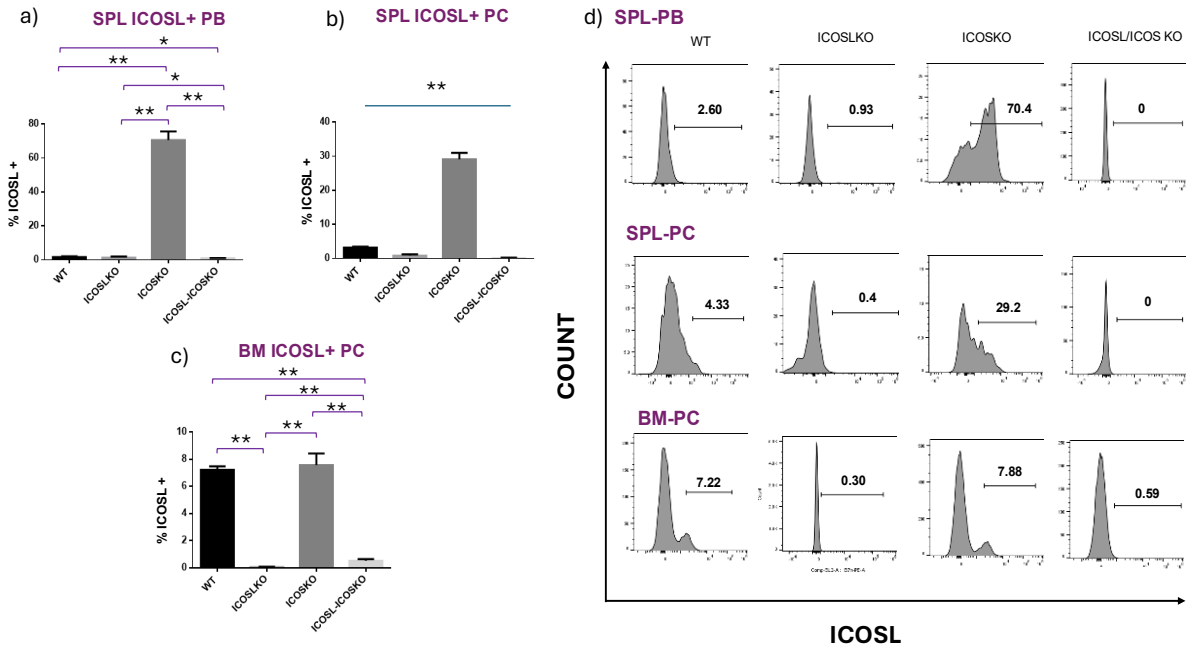

Figure 3: ICOSL expression on PCs in the spleen and bone marrow of ICOS/ICOSL-deficient mice. *a) Percentage of ICOSL+ splenic plasma blasts, b) Percentage of ICOSL+ splenic plasma cells, c) Percentage of ICOSL+ bone marrow plasma cells, d) Flow cytometry histograms plots of ICOSL expression in spleen plasma blasts and PCs and BM PCs. Data was collected from five individual experiments. Values are represented as *, P < 0·05; **, P < 0·05 vs control, calculated by the Mann-Whitney test.*

3. Involvement of ICOS and ICOSL in platelet function

To assess the involvement of ICOS and ICOSL in platelet function, we compared aggregation induced by ADP (2 µM) in platelet-rich plasma (PRP) from WT, ICOS-KO, ICOSL-KO, and ICOS/ICOSL-KO mice. The results showed that platelet aggregation was significantly lower in PRP isolated from ICOS-KO, ICOSL-KO, and ICOS/ICOSL-KO mice compared to that from WT mice (Fig. 4).

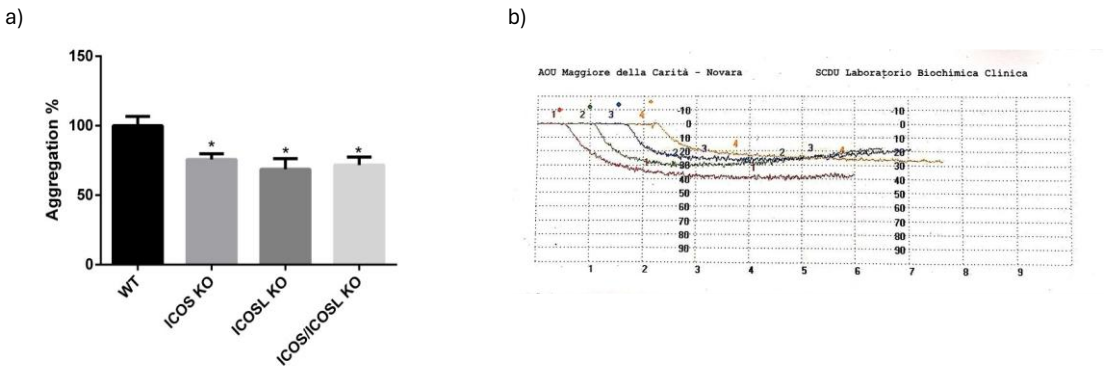

Figure 4: *a: Bar graph showing the quantification of platelet aggregation (%) in platelet-rich plasma (PRP) from WT, ICOS-KO, ICOSL-KO, and ICOS/ICOSL-KO mice following stimulation with ADP (20 µM). b) Representative real-time aggregation traces for WT (trace 1), ICOS-KO (trace 2), and ICOS/ICOSL-KO (trace 3), ICOSL-KO (trace 4) mice. Data was collected from five individual experiments. Values are represented as *, P < 0.05 calculated by the Mann-Whitney test.*

Analysis of expression of ICOS and ICOSL in platelets by immunofluorescence was inconclusive because of apparent non-specific staining. Therefore, expressions of ICOS and ICOSL were assessed by immunofluorescence and quantitative Real-Time PCR in primary WT megakaryocytes. Results showed that megakaryocytes expressed both ICOS and ICOSL as detected by both immunofluorescence and PCR analysis (Fig 5a-b).

To investigate the role of the ICOS/ICOSL interaction in megakaryocyte development, we compared the DNA ploidy in WT and ICOSL-KO bone marrow megakaryocytes. Results showed that the 2N and 4N populations were significantly reduced in ICOSL-KO mice compared to WT, while higher ploidy populations (≥8N) remained unaffected (Fig. 5b). This suggests a role for ICOSL in the early stages of megakaryocyte survival and maturation.

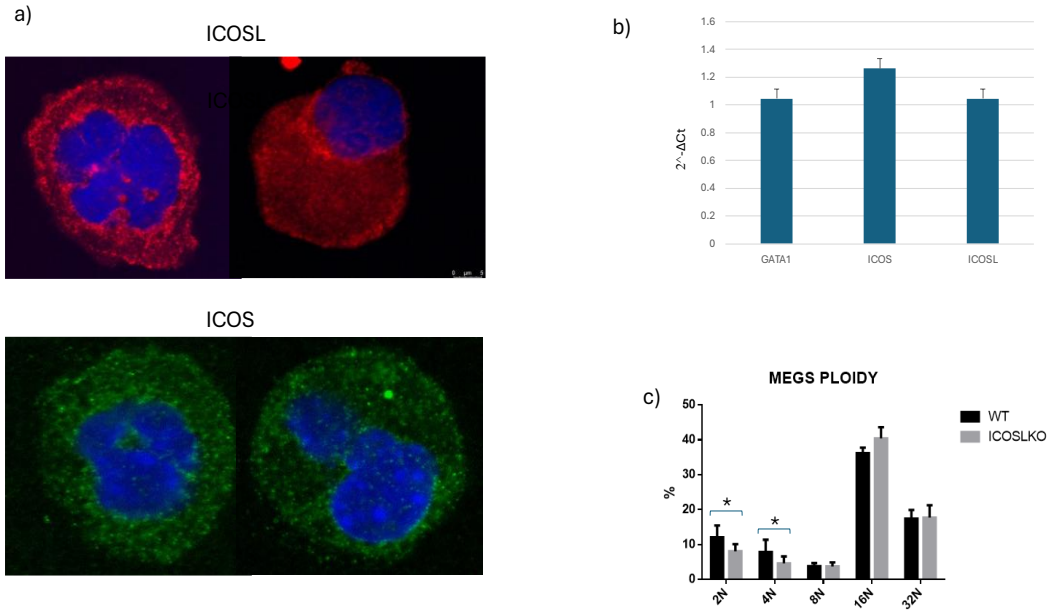

Figure 4: ICOSL deficiency impairs early megakaryocyte maturation and confirms receptor-ligand expression. *a)* ***ICOSL*** *(red) and* ***ICOS*** *(green) expression were assessed by confocal microscope (63X). b) Quantitative Real-Time PCR analysis of GATA-1, ICOS, and ICOSL expression in primary WT megakaryocytes, c) DNA ploidy levels in primary megakaryocytes from WT and ICOSL-KO mice. Data was collected from 5 individual experiments. Values are represented as *, P < 0.05 calculated by the Mann-Whitney test.*

6. DISCUSSION

Our previous work in Multiple Myeloma (MM) showed that malignant PCs express both ICOSL (at high levels) and ICOS (at low levels), which was unexpected since ICOS is mainly considered a T cell marker. Subsequent analysis of normal human bone marrow plasma cells detected two subsets: one expressing only ICOSL, the other expressing both ICOS and ICOSL. Altogether, these data suggested that $ICOS^+ICOSL^+$ plasma cells include LLPCs, which are likely normal counterparts of myeloma cells. In contrast, $ICOS^-ICOSL^+$ cells might correspond to plasma blasts or short-lived plasma cells, consistent with the notion that activated B cells upregulate ICOSL but not ICOS. Thus, ICOS/ICOSL expression appeared linked to the differentiation stage, not malignancy (47).

To further investigate ICOS and ICOSL role in plasma cell differentiation, in this thesis, we used WT, ICOS, ICOSL, and ICOS/ICOSL-deficient mice to study role of this pathway in plasma cell development and distribution.

Our results of WT mice were surprising since we found that, in the spleen, both plasma blasts and plasma cells comprise two subsets: one negative for both ICOS and ICOSL, the other expressing only ICOS, which contradicts our previous hypothesis in humans that plasma blasts and/or short-lived plasma cells may display an $ICOS^-ICOSL^+$ phenotype. By contrast, bone marrow plasma cells comprise three subsets expressing either the $ICOS^+$ or $ICOSL^+$ or $ICOS^+ICOSL^+$ phenotypes. These data indicate that the $ICOSL^+$ and $ICOS^+ICOSL^+$ phenotypes are a hallmark of bone marrow plasma cells and may comprise long-lived plasma cells in mice. One possibility is that a subset of splenic plasma blasts expresses ICOS, differentiates into $ICOS^+$ plasma cells, and migrates to the bone marrow, where they further mature into $ICOSL^+$ long-lived plasma cells, passing through an intermediate $ICOS^+ICOSL^+$ phenotype.

In this maturation pathway, expression of both ICOS and ICOSL seems to be crucial since, in ICOS/ICOSL double-knockout mice, bone marrow PCs are reduced while splenic PCs accumulated, indicating impaired migration from secondary lymphoid organs to the bone marrow. By contrast, single deficiencies of either ICOS or ICOSL had minor effects on plasma cell distribution in the spleen and bone marrow, suggesting the intervention of compensatory mechanisms. Indeed, ICOS deficiency caused appearance of $ICOSL^+$ plasma blasts and plasma cells in the spleen which

were absent in WT mice. This increased expression of ICOSL might compensate for the ICOS deficiency by increasing the interaction of ICOSL with other known ligands such as osteopontin and αvβ3 integrin.

Beyond plasma cells, the ICOS/ICOSL system seems to play a role also in platelets function since platelet aggregation induced by low doses of ADP is significantly reduced in ICOS-KO, ICOSL-KO, and ICOS/ICOSL KO mice, with blunted and delayed responses, indicating reduced platelet responsiveness.

The possibility that the ICOS/ICOSL system may be directly involved in platelet aggregation would assume that these molecules are expressed on platelets. However, we have not been able to prove this possibility, to date, since several types of immunofluorescence analysis failed to give convincing results because of high background staining, which is a known problem in platelets.

However, we showed that both ICOS and ICOSL are expressed in megakaryocytes as detected by immunofluorescence and RT PCR, which may indirectly support their possible expression in platelets. Moreover, analysis of megakaryocyte maturation in WT and ICOSL-KO mice by assessment of ploidy showed that ICOSL-KO mice display decreased proportions of immature 2N and 4N megakaryocytes, while higher-ploidy mature cells were mostly unaffected, which suggests that ICOSL plays a role in early megakaryocyte differentiation. These data indicate preservation of terminal megakaryocytic maturation with possible reduction or accelerated consumption of the progenitor compartment. This picture may be compatible with an increased thrombopoietic stimulus or a maturational shift, which may be a compensatory response to the decreased platelet function in KO mice.

These findings may have implications for the MM bone marrow niche. Physiologically, ICOS/ICOSL signaling may support plasma cell maturation and trafficking to the bone marrow, where interactions with MKs and stromal cells sustain LLPC survival. The reduction of bone marrow PCs in KO mice mirrors the dependence of PCs on niche support, suggesting that disrupted ICOS/ICOSL signaling could impair plasma cell persistence.

Moreover, since MKs and platelets contribute to a myeloma-supportive microenvironment through the secretion of APRIL, IL-6, pro-angiogenic factors, and direct interactions with malignant plasma cells, the observed defects in MK maturation and platelet aggregation in KO mice

suggest that this pathway may influence niche functionality, potentially affecting tumor survival and progression.

Overall, these data extend the role of the ICOS/ICOSL axis beyond immune costimulation, linking it to maintenance of the bone marrow plasma cell niche and identifying mechanisms by which altered ICOS/ICOSL signaling could contribute to MM development and progression. Future studies in human plasma cells, megakaryocytes, and platelets will be essential to validate these mechanisms and clarify their relevance to MM pathology.

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