Associazione Italiana Sindrome di Shwachman-Diamond (AISS)
Via Pioveghetto 15, - 35136 Padova
Tel - FAX +39 049 8736130
E-mail: aiss@shwachman.it

Shwachman-Diamond Syndrome Italian Research Grant

Maximum Amount euro 10.000,00

Firm Deadline for Receipt of Applications: 31 December 2016

Eligibility: Persons applying for these grants if not in a faculty position need to provide a declaration by a supervisor with a position in the department (not a training position) and with authority to hold an independent research grant.

Terms of Support: Support may be provided for one (1) year in an amount not to exceed E 10,000. Indirect costs are permitted and are not to exceed 10% of the total costs.
The AISS will provide preference to those applications in which funds are used for supplies, equipment, technicians and other expenses and not for support of the salary of the PI or co-PIs.

Review: All applications will be reviewed by the AISS Scientific Committee (AISS-SC) or its designees.

Application: The application contains two sections.

Section 1, forms attached. The applicant and co-applicants must also include a current curriculum vitae.
Section 2: Research Plan, divided as indicated below. Parts A through D should not exceed 6 pages, using a font no smaller than 10 point.

Part A. Specific aims
Part B. Significance and background
Part C. Preliminary studies
Part D. Experimental design and methods
Part E. References (not included in the 6 page limit)
Part F. Relevance of the research to Shwachman-Diamond Syndrome
Part G. For junior faculty separate letter from supervisor or department head confirming commitment to project, and to provision of space and facilities
Part K. If human subjects and animals are involved, a statement by the PI or supervisor overseeing human or animal studies is compulsory. If considered as necessary by the AISS-SC, more information about ethical committee study approval may be asked.

Submission by email to the AISS: aiss@shwachman.it

1. Title of Proposal
Further insights into the leukocyte immunophenotype of SDS patients and extensive analysis of mTOR activation in leukocyte subsets

2. **Applicant Information:**

Name: Antonio Vella

Title and Degree(s):
Specialization in Clinical Biochemistry , University of Brescia (Italy), July 2000
Degree in Biology, University of Palermo (Italy), 1984.

Work Address:
Unit of Immunology, Azienda Ospedaliera Universitaria Integrata di Verona, Piazzale A. Scuro 10, Verona, Italy

Telephone: 045-8126445
FAX: 045-8027469

Email: antonio.vella@univr.it

3. **Applicant Curriculum Vitae**: beginning on the next page, with 2 page limit. This will form application pages 3 and 4.

Curriculum Vitae

Education and Training

Specialization in Clinical Biochemistry , University of Brescia (Italy), July 2000
Degree in Biology, University of Palermo (Italy) 110/110 summa cum laude, 1984.

Employment and Research Experience

Biologist manager AOUI Verona Immunology Section (since 1994) with responsibility for clinical and experimental flow cytometry. During last decade, focus was directed on experiencing the theory and the advanced application of analytical flow cytometry in support to clinical diagnostics and cell research in immunology including the maintenance and trouble shooting of equipment, as well as providing technical support and training to project scientists in the theory and application of flow cytometry.

Personal bibliography

1. New insights into the Shwachman-Diamond Syndrome-related haematological disorder: hyper-activation of mTOR and STAT3 in leukocytes. Bezzerri V, Vella A, Calcaterra E, Finotti A, Gasparello J, Gambari R, Assael BM, Cipolli M, Sorio C. SciRep. 2016 Sep 23;6:33165.

2. Low ozone concentrations stimulate cytoskeletal organization, mitochondrial activity and nuclear transcription. Costanzo M, Cisterna B, Vella A, Cestari T, Covi V, Tabaracci G, Malatesta M. Eur J Histochem. 2015 Apr 21;59(2):2515

3. Towards an MP Model for B Lymphocytes Maturation Alberto Castellini, Giuditta Franco, Vincenzo Manca, Riccardo Ortolani, and Antonio Vella. O.H. Ibarra et al. (Eds.): UCNC 2014, LNCS 8553, pp. 80–92, 2014. Springer International Publishing Switzerland 2014

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5. Lymphocyte subpopulations in active tuberculosis: association with disease severity and the QFT-GIT assay. Guglielmetti L, Cazzadori A, Conti M, Boccafoglio F, Vella A, Ortolani R, Concia E. Int J Tuberc Lung Dis. 2013 Jun;17(6):825-8

6. Colostrum-derived B and T cells as an extra-lymphoid compartment of effector cell populations in humans. Peroni DG, Chirumbolo S, Veneri D, Piacentini GL, Tenero L, Vella A, Ortolani R, Raffaelli R, Boner AL. J Matern Fetal Neonatal Med. 2013 Jan;26(2):137-42.

7. Long-term effects of amino-bisphosphonates on circulating γδ T cells. Rossini M, Adami S, Viapiana O, Fracassi E, Ortolani R, Vella A, Zanotti R, Tripi G, Idolazzi L, Gatti D. Calcif Tissue Int. 2012 Dec;91(6):395-9.

8. Lymphocyte phenotypic subsets in umbilical cord blood compared to peripheral blood from related mothers. Salvatore Chirumbolo, Riccardo Ortolani, Dino Veneri, Ricciarda Raffaelli , Diego Peroni, Roberta Bigozzi, Marco Colombatti, Antonio Vella. In press to: Part B - Clinical Cytometry B Clin Cytom. 2011 Jul-Aug;80(4):248-53.

9. CCR3 as a single selection marker compared to CD123/HLADR to isolate basophils in flow cytometry: Some comments. Chirumbolo S, Ortolani R., Vella A. Cytometry A. 2010 Dec 30

10. A comparative method for processing immunological parameters: developing an "Immunogram". Ortolani R, Bellavite P, Paiola F, Martini M, Marchesini M, Veneri D, Franchini M, Chirumbolo S, Tridente G, Vella A. Blood Transfus. 2010 Apr;8(2):118-25.

11. Circulating γδ T cells and the risk of acute-phase response after zoledronic acid administration. Rossini M, Adami S, Viapiana O, Ortolani R, Vella A, Fracassi E, Gatti D. J Bone Miner Res. 2012 Jan; 27(1):227-30.

4. **Applicant's Commitment as Investigator of the Project:**

I agree as the applicant to accept responsibility for the scientific management of this project as outlined in this application. I further agree to submit a report at the end of the granting period.

5. **Applicant's Affirmation:**

I certify that the investigations involving human subjects to be carried out in the application will have approval of the applicant's Institutional Ethical Committee

Approvals from the Institutional Ethical Committee must be included with the application.

6. **Research Results:**

Results of research may be made available to the public through appropriate scientific channels. All publications will bear the statement:

Signature of Applicant

20-12-16

Date

7. Applicant's Institution Certification and Commitment:

I certify that the statements herein and the Applicant's Affirmation are true, complete and accurate to the best of my knowledge and I agree to accept responsibility for the fiscal management of this project as outlined in this application. I further agree to commit this institution to comply with the Associazione Italiana Sindrome di Shwachman-Diamond (AISS) terms and conditions if a grant is awarded as a result of this application.

Name of Institution Official:
Prof. Vincenzo Bronte

Title:
Director of Unit of Immunology, AOUI Verona

Address: Piazzale Antonio Scuro, 10

Phone: 045-812-4007 Fax: 045-802-7469

E-mail Address: vincenzo.bronte@univr.it

Signature of Institution Official

22 - 12 - 16
Date

ABSTRACT OF RESEARCH PLAN

Within the space provided, summarize the long-term objectives, scientific aims and methodology of the proposal.

TITLE: Further insights into the leukocyte immunophenotype of SDS patients and extensive analysis of mTOR activation in leukocyte subsets

OUR OVERALL GOAL IS TO CHARACTERIZE THE LEUKOCYTE IMMUNOPHENOTYPE IN PERIPHERAL BLOOD OBTAINED FROM SDS PATIENTS AND ANALYZE MTOR SIGNALING PATHWAY WITHIN DIFFERENT LEUKOCYTE SUBPOPULATIONS, IN PARTICULAR IN GRANULOCYTES. IN ORDER TO ADDRESS THIS ISSUE, WE WILL PERFORM AN EXTENSIVE FLOW CYTOMETRY ANALYSIS OF IMMUNOPHENOTYPE ON AT LEAST TEN SDS PATIENTS, COMPARED WITH TEN HEALTHY DONORS MATCHED FOR AGE AND SEX. WE WILL FOCUS OUR ATTENTION IN PARTICULAR ON GRANULOCYTE SUBPOPULATIONS (NEUTROPHILS, BASOPHILS AND EOSINOPHILS), BUT ALSO ON CD4+, CD8+ T CELLS, B CELLS, MONOCYTES AND NK CELLS. OUR PRELIMINARY DATA, TOGETHER WITH PREVIOUSLY PUBLISHED DATA, INDICATE THAT MTOR PATHWAY IS UP-REGULATED IN SDS. IN THIS REGARD, WE WILL COMPARE THE MTOR PHOSPHORYLATION LEVEL IN SDS LEUKOCYTE SUBPOPULATIONS WITH HEALTHY DONOR LEVELS. THUS, WE WILL GAIN FURTHER INSIGHTS INTO THE ACTIVATION OF MTOR IN SEVERAL BLOOD CELL SUBTYPES EXTENDING THE STUDY TO GRANULOCYTES, LYMPHOCYTES AND NK CELLS. MOREOVER, AS WE REPORTED THAT RAPAMYCIN HAS ACTS AS A POTENT MTOR INHIBITOR ALSO IN SDS CELLS, WE WILL TEST THE EFFECT OF THIS MOLECULE IN ALL THE LEUKOCYTE SUBPOPULATIONS. NOTABLY, THE PRO-INFLAMMATORY CYTOKINE IL6 HAS BEEN REPORTED TO INDUCE MTOR ACTIVATION IN SDS LEUKOCYTES. AS WE OBSERVED AN INCREASED RELEASING OF IL6 IN EBV-TRANSFORMED B CELLS OBTAINED FROM SDS PATIENTS, WE WILL STUDY THE EXPRESSION LEVEL OF IL6 AND OTHER PRO-INFLAMMATORY CYTOKINES, IN TERMS OF RNA AND PROTEIN, IN DIFFERENT PRIMARY SDS LEUKOCYTE SUBTYPES AS WELL AS IN PLASMA SAMPLES COLLECTED BY FRESHLY WITHDRAWN PERIPHERAL BLOOD OF SDS PATIENTS, USING SINGLE-CELL RNA ANALYSIS AND BIOPLEX ASSAYS. THE IMPORTANCE OF THESE FINDINGS IS AMPLIFIED BY THE FACT THAT SEVERAL MTOR INHIBITOR HAVE BEEN RECENTLY DEVELOPED AND TESTED IN CLINICAL TRIALS DESIGNED TO COUNTERACT LEUKEMIAS AND OTHER HEMATOLOGICAL MALIGNANCIES. THUS, OUR RESULTS MIGHT OPEN A WIDER THERAPEUTIC SCENARIO WITHIN SDS PATHOLOGY.

BUDGET

List below a budget by categories for the support. The review committee will carefully consider the appropriateness of your budget. It must be well defined, justified, and realistic to complete the work proposed. The first column defines the total expenses that are expected to be necessary to realistically complete the project. The second column indicates the expenses requested from the AISS. Applicants **will not** be penalized in funding considerations for requiring additional funds beyond what is requested from the Foundation(AISS); however, the true costs of the project must be acknowledged.[This and the section on page 1 re: Other Funding need to be consistent]

EURO Amount Requested for:

	TOTAL COSTS REQUIRED TO COMPLETE PROJECT:	**COSTS REQUESTED FROM AISS:**(not to exceed E 10,000)
Personnel (including fringe benefits): PI: Name: Antonio Vella Co-I Name: Additional personnel (identify role): Name: Valentino Bezzerri, PhD Expert of signal transduction and cytokine expression	10,000	0
Equipment:		
Supplies:	15,000	7,000
Other Expenses:	5,000	2,000
Indirect Costs (not to exceed 10% of total)	1,000	1,000
TOTAL COSTS:	31,000	10,000

Justification: Define and justify expenses in each category. Explain the role of each of the individuals named in the Personnel section. The justification must include an explanation of what each category contributes to the project. Also explain any marked differences between the first- and second-year expenses in a particular category. The AISS will provide preference to those applications in which funds are used for supplies, equipment, technicians and other expenses and not for support of the salary of the PI or co-PIs. The AISS-SC may ask for further expense details.

Supplies (7,000 euro): this budget will be intended for reagents including flow cytometry buffers and antibodies, Bioplex Kit, PrimeFlow RNA probes, media for leukocyte cultures, plastic tubes and consumables. Supplies indicated above will be essential to address the proposed aims of the project (leukocyte immunophenotype, assessment of mTOR activation, cytokine Bioplex assays, RNA quantification in sigle-cell analysis).

Other (2,000 euro): expenses for travels and results dissemination (conferences, seminars, posters, publications).

Indirect costs (1,000): overheads for the host institutional organization

Other Support for this Project:
Applicants are allowed to receive funding from other sources for parts of the project not funded by the AISS. Please, list all other funding sources.

None.

Research Plan

Part A. Specific aims

Our overall objective is to characterize the leukocyte immunophenotype in peripheral blood obtained from SDS patients. Moreover we will determine the mTOR signaling pathway activation in SDS leukocyte subpopulations, with particular attention to granulocytes. Finally, we will test the effect of rapamycin in reducing mTOR activation in peripheral blood cells.

Aim 1: Assessment of leukocyte immunophenotype in peripheral blood withdrawn from SDS patients. The immunophenotype of peripheral lymphocytes (T and NK cells) and neutrophils has been already reported (Dror Y, 2001), however further investigation on the entire leukocyte cell populations could be helpful to understand the role of SBDS protein in basophils, eosinophils, monocytes and lymphocytes subsets.

Aim 2: To investigate the role of m-TOR pathways in CD8+, CD4+ T cells, Natural Killer cells and granulocytes freshly isolated by SDS patients. We previously reported that SDS patients present mTOR hyper-activation in B cells, Monocytes and PMNs. We will perform a further analysis of mTOR activation in other granulocytes such as basophils and eosinophils as well as in T cells and NKs. Since IL6 has been reported to induce mTOR activation in monocytes (Bezzerri V, 2016), we will test the IL6-dependet mTOR activation also in other cells of the innate immune system (basophils, eosinophils and NKs). IL6 expression has been reported to be up-regulated in MSCs (Andrè V, 2012). Thus, we will check the IL6 expression in single cell analysis also in leukocyte subsets obtained from SDS patients.

Aim 3: Test the effect of mTOR inhibitor rapamycin on immune cell activation in SDS lymphocyte populations (T cells, NK, B cell subsets) and granulocytes. mTOR inhibitors have been recently developed to treat different forms of leukemia and solid tumors (Porta C, 2014). Since about 15% of SDS patients develop MDS or AML (Donadieu, 2005), an extensive study of the effect of rapamycin on the SDS-related mTOR hyper-phosphorylation could open a new therapeutic perspective for SDS pathology. Moreover we will measure the IL6 expression in single cell analysis in the presence or in the absence of rapamycin treatment.

Part B. Significance and background

Shwachman-Diamond syndrome (SDS) is a genetic disease caused by mutations affecting the Shwachman-Bodian-Diamond syndrome (SBDS) gene (Boocock GR, 2003). It has been reported that human SBDS protein is enriched in nucleolus and it seems to be associated with the ribosomal RNA (rRNA) biogenesis (Ganapathi KA, 2007). Consistently with this observation, it has recently postulated that SBDS together with elongation factor-like 1 (EFL1) are involved in the removal of eukaryotic initiation factor 6 (eIF6) during the maturation of the pre-60S ribosomal subunit, allowing the formation of the 80S ribosome (Finch AJ, 2011). However, the exact pathologic mechanism whereby defects in ribosome biogenesis could lead to the specific SDS pathology remains unclear. The pathology is characterized by a multiple-organ impairment involving bone marrow dysfunctions, exocrine pancreatic insufficiency, skeletal malformations, hepatic and cognitive

disorders (Cipolli M, 2001). Other complications including several immunological abnormalities have been found in patients affected by SDS, in particular low percentage of circulating B-lymphocytes, lack of antibody production, low percentage of circulating CD3+/CD4+ T-cells and natural killer cells and decreased in vitro proliferation of T-cells (Dror Y, 2001). SDS patients presents severe hematologic disorders. Neutropenia and impaired neutrophil chemotaxis play a critical role as contributors to recurrent infections reported in young children (Stepanovic V, 2004, Kuijpers TW, 2005). SBDS co-localizes with the mitotic spindle in granulocyte progenitors contributing to cell division process, and leading to a decreased proliferation capacity of SDS-patient bone marrow CD34+ hematopoietic progenitor cells and finally contributing to neutropenia in SDS patients (Orelio C, 2009). Moreover, SDS patients develop myelodysplastic syndrome (MDS) with increased risk to the progression to leukemia, in particular acute myeloid leukemia (AML) (Donadieu J, 2005). The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that belongs to the phosphoinositide 3 (PI3K)-related kinase family and resides in at least two multi-protein complexes, namely m-TORC1 and m-TORC2 (Chapuis N, 2010). The m-TORC1 promotes rDNA transcription increasing the activity of RNA polymerase I and leads to rRNA processing to its mature form (Iadevaia V, 2012). Recently, we reported that the pro-inflammatory cytokine IL6 is able to induce mTOR activation in leukocytes (Bezzerri V, 2016). mTOR has been reported to regulate neutrophil chemotaxis (Bao Y, 2015) and has been associated with basophil activation (Gernez Y, 2012). SDS pathology seems affect the CD34+ hematopoietic progenitor expansion. mTOR plays a role in the innate immune response driving granulocyte differentiation (Hua W, 2015). Our hypothesis is that all granulocyte subpopulation metabolism might be impaired in SDS. Thus, besides the well-known reduced neutrophil maturation and subsequent neutropenia observed in SDS, we will gain further insights into the basophil and eosinophil maturation in SDS patients, as these cells play a key role in the pro-inflammatory immune response. Furthermore, the mTORC1 signaling pathway has been found to be deregulated in a wide range of hematological malignancies and has been designed as a major target for tumor therapy (Chapuis N, 2010). We recently reported that mTOR and Signal Transducer and Activation of Transcritpion (STAT)-3 are hyper-activated in B cells, Monocytes and neutrophils (PMNs) isolated from SDS patients (Bezzerri V, 2016). Interestingly, IL6 is a well-known activator of STAT3 pathway (O'Shea JJ, 2013), and its expression has been reported to be up-regulated in mesenchymal stem cells obtained from SDS patients (Andrè V, 2012).We found that loss of SBDS gene expression is sufficient to induce a strong up-regulation of mTOR phosphorylation in S2448 residue. Nevertheless, using the well-known mTOR inhibitor rapamycin, we strongly reduced the over-activation of mTOR and STAT3 observed in SDS cells (Bezzerri V, 2016). However, no results have been so far reported regarding the role of mTOR phosphorylation in T cells, NK and granulocyte subpopulations. Several mTORC1 and dual PI3k-m-TOR inhibitors have been developed and are currently being evaluated within clinical trials for the treatment of several malignancies, including AML (Porta C, 2014). Rapamycin (Sirolimus) analogs are small molecules which inhibit m-TOR by forming a complex with the FK506 binding protein-12 (FKBP-12) and leading to inhibition of cell cycle progression, survival, and angiogenesis. Among the Sirolimus analogs, Everolimus (RAD001) has been already approved by FDA and EMA for the treatment of advanced renal cell carcinoma (Motzer RJ, 2008). Interestingly, RAD001 has been recently tested for the therapy in a Phase I clinical trial on refractory multiple myeloma (Gunther A, 2015). A new generation of dual m-TOR/PI3K inhibitors has been developed, starting from the consideration that the CAT sites of phosphatidylinositol-3-Kinase (PI3K) and m-TOR share a high degree of sequence homology (Porta C, 2014).

Part C. Preliminary studies

Recently, we reported that mTOR S2448 phosphorylation is increased in EBV-transformed lymphoblastoid cells (LCLs) as well as in primary B cells, monocytes and neutrophils obtained from SDS patients (Bezzerri V, 2016). Here we preliminary report a strong up-regulation of mTOR phosphorylation also in CD4+ and CD8+ T cells (figure 1). Moreover, results indicate that low doses of rapamycin (350nM) are able to reduce mTOR S2448 phsphorylation both in CD4+ and CD8+ T cells (figure 1). These findings indicate that SBDS impairment affects myeloid lineages as well as lymphoid cell lineages, suggesting a immune-deficiency and/or a dysregulation of the entire immune system. We also recently show that the pro-inflammatory cytokine IL6 is able to induce mTOR and STAT3 pathways in SDS leukocytes (Bezzerri V, 2016). Notably, IL6 has been also reported to be regulated by STAT3 (Wake MS, 2015). Here we show that IL6 expression is up-regulated in SDS EBV-transformed LCLs (Figure 2). This finding may indicate that SDS condition promotes an autocrine loop of activation involving STAT3 and IL6. Furthermore, here we show that also pro-inflammatory cytokines IL8 and IL13 are significantly up-regulated in SDS cells (Figure 2). Since IL8 and IL13 expression has been also reported to be regulated by STAT3, these findings seems strengthen the hypothesis of a STAT3 hyper-activation in SDS leukocytes.

Figure 1

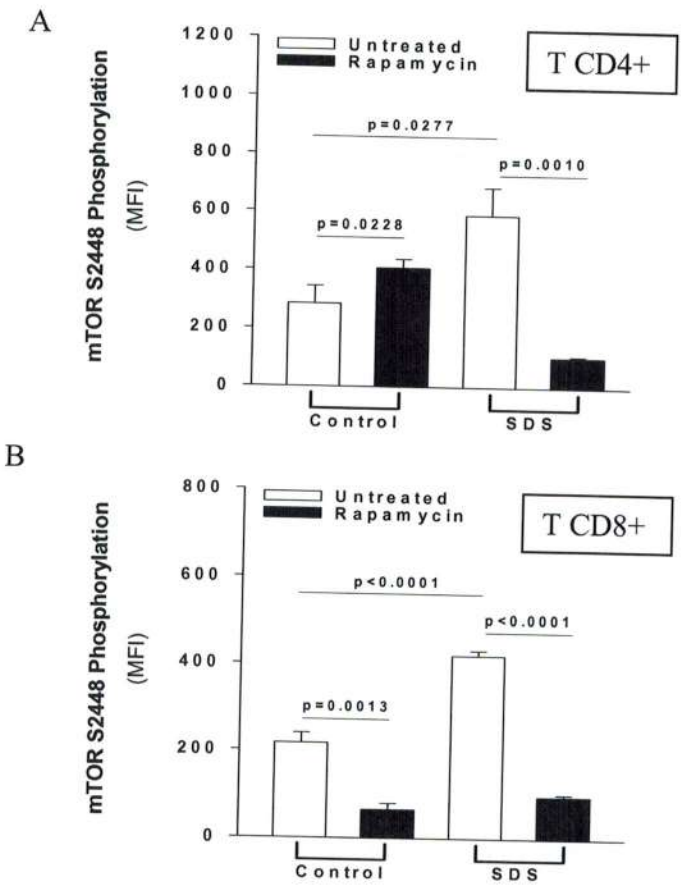

Figure 1. mTOR (S2448) phosphorylation is increased in SDS CD4+ and CD8+ T cells and rapamycin treatment is able to strongly reduce level of phosphor-mTOR. CD4+ (**A**) and CD8+ (**B**) T cells were isolated from peripheral blood withdrawals obtained from both healthy controls and SDS patients, using a CD8+ versus CD3+ gating strategy in lymphocyte subpopulation by Phospho-flow analysis. Rapamycin (350 nM) was added to total leukocytes and incubated for 1 hour before analysis. Data are mean ± SEM of five independent experiments performed in peripheral blood samples obtained from five SDS patients versus five healthy controls. Statistical analysis was performed using Student's t-test and p values are indicated. Results indicate that, similarly to data obtained in B cells, monocytes and PMNs, SDS patients present mTOR hyper-phosphorylation also in T cells. Moreover, rapamycin is able to significantly reduce S2448 phospho-mTOR level in SDS cells.

Figure 2

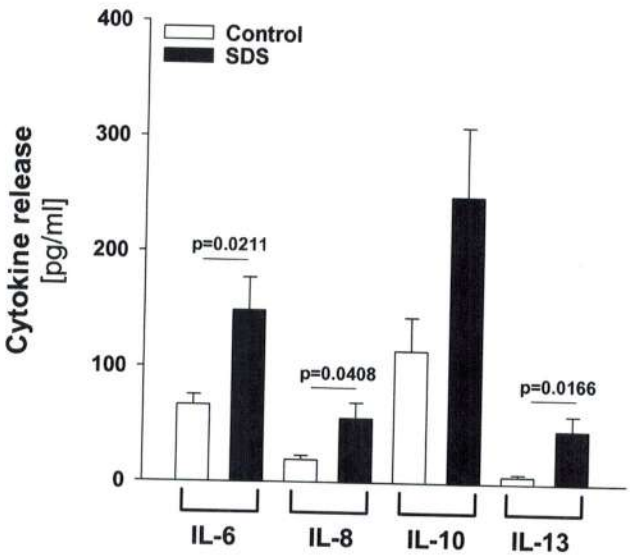

Figure 2. Comparison of cytokine expression between healthy donor and SDS LCLs. Lymphoblastoid cell lines were incubated for 16 hours in RPMI-1640 medium containing low doses (0.5%) of FBS in order to synchronize cell cycle. Subsequently, cells were incubated in RPMI-1640 medium containing 10% FBS. Culture supernatants were collected and protein levels of 27 different cytokine released into media were measured by Bioplex assay. Data are mean ± SEM of 10 independent experiments performed in duplicate. Statistical analysis was performed using Student's t-test and p values are indicated. Results indicate that IL6, IL8 and IL13 expression is significantly up-regulated in SDS patients.

Part D. Experimental design and methods

Patient recruitment

Ten volunteer SDS patients and ten healthy donors will be recruited during the programmed Day Hospital visits for clinical evaluation at Cystic Fibrosis Centre of Ancona, in collaboration with Dr. Marco Cipolli, Director of the Centre. Within the outpatient visit performed in the centre for routine blood chemistry, one additional blood sample (5 ml) will be obtained. Samples and data will be obtained and used for analysis only after that informed consent will be signed according to the guidelines approved by the local Ethical

Committee.

Flow cytometry

Total leukocytes isolated from peripheral blood will be incubated at 37 °C in the presence or in the absence of 350 nM Rapamycin (Sigma-Aldrich, St Louis, MO) for 1 hour. Then cells were stimulated with 10 ng/ml IL-6 or 100 nM N-Formyl-Met-Leu-Phe (fmlp) (Sigma-Aldrich) for further 30 minutes. Cells will be stained with following fluoro-conjugated antibodies: anti-CD3, anti-CD4, anti-CD8, anti-CD45, anti-CD63, anti-CD123, anti-CD203c, anti-HLA-DR, anti-CD19, anti-CD56, anti-CD16 (all supplied by Beckman-Coulter, Indianapolis, IN). Leukocytes will be fixed and permeabilized with Intracellular Fixation and Permeabilization Buffer Set (eBioscience, San Diego, CA), following the manufacturer's protocol. After permeabilization, primary leukocytes will be washed once in flow buffer and stained with anti p-S2448-mTOR-PE or isotype control-PE conjugated antibodies for 30 minutes. Cells will be washed and acquired on a 10 color, 3 laser (Blue Solid State Diode: 488nm, 22mW, Red Solid State Diode: 638nm, 25mW, Violet Solid State Diode: 405nm, 40mW), Navios flow cytometer (Beckman Coulter, Indianapolis, IN). All acquired data files will be analyzed using the "Navios" or Kaluza software, version 1.3 (Beckman Coulter, Indianapolis, IN). CD45 versus SS gating strategy will be used to recovery lymphocytes, monocytes, neutrophils and eosinophils. Lymphocytes CD45-bright region will be plotted on CD3 versus CD19 dotplot to isolate B cells population, on CD3 versus CD4 and CD8 dotplot to isolate T CD4 and CD8 cells respectively, on CD3 versus CD56/16 dotplot to isolate NK cells. CD45-dim region will be plotted on CD123 versus HLA-DR to isolate basophils.

Bioplex assay

We will perform a Bioplex assay (Bio-rad laboratories) able to detect the presence of 27 different cytokines, chemokineas and growth factors. Whole blood samples, drawn in citrate-containing tubes, will be incubated in the presence and in the absence of 10 ng/ml IL-6 or 100 nM fmlp at 37 °C for 30 min. Plasma derived from both SDS patients and control blood samples will be analyzed by BioPlex station using Illumina technology. Briefly, 12.5 μl of plasma will be incubated with an immunoassay formatted on magnetic beads. Capture antibodies against each cytokine/chemokine/growth factor are covalently coupled to the magnetic beads. After the reaction of samples with beads, a series of washes will be performed to remove the excess of unbounded biomarkers and a biotinylated antibody will be added to create a sandwich complex. The final detection complex will be formed by the addition of a streptavidin-phycoerythrin conjugate. The fluorescence will be measured by Bio-Plex station following the manufacturer's procedures.

Single cell PrimeFlow RNA Assay

PrimeFlow RNA Assay incorporates a proprietary oligonucleotide probe set design and branched DNA (bDNA) signal amplification technology to analyze RNA transcripts by flow cytometry. bDNA technology provides a unique approach to RNA detection and signal amplification by amplifying the reporter signal rather than the target sequence (e.g. PCR). In the PrimeFlow RNA Assay, target-specific probe sets contain 20-40 oligonucleotide pairs that hybridize to the target RNA transcript. Signal amplification is achieved through specific hybridization of adjacent oligonucleotide pairs to bDNA

structures, formed by pre-amplifiers, amplifiers and fluorochrome-conjugated label probes, resulting in excellent specificity, low background and high signal-to-noise ratio. Single-cell suspensions will be stained for cell surface markers and fixable viability dyes, as before the cells will be fixed and permeabilized. Subsequently, the cells will be stained with a target-specific Alexa-Fluor-conjugated probe set contains 20-40 oligonucleotide pairs that hybridize to specific regions across the target IL6 RNA sequence. Signal amplification using bDNA technology will be achieved through a series of sequential hybridization steps, that forms a "tree" like structure. Pre-amplifier molecules hybridize to their respective pair of bound oligonucleotide probes to form the "trunk" of the tree. Multiple amplifier molecules hybridize to their respective pre-amplifier to create the "branches." Finally, multiple Label Probes hybridize to the Amplifiers and form the "leaves" of the "tree." A fully assembled signal amplification tree contains 400 label probe binding sites. If all target-specific oligonucleotides in the probe set bind to the target IL6 RNA transcript, an 8,000 fold amplification can be achieved. Upon completion of the assay protocol, IL6 RNA data will be detected in single-cell by analyzing the sample on a 10 color Navios flow cytometer (Beckman Coulter, Indianapolis, IN).

Part E. References

1. André V, Longoni D, Bresolin S, Cappuzzello C, Dander E, Galbiati M, Bugarin C, Di Meglio A, Nicolis E, Maserati E, Serafini M, Warren AJ, Te Kronnie G, Cazzaniga G, Sainati L, Cipolli M, Biondi A, D'Amico G. Mesenchymal stem cells from Shwachman-Diamond syndrome patients display normal functions and do not contribute to hematological defects. Blood Cancer J. 2:e94 (2012).
2. Bao, Y. et al. mTOR and differential activation of mitochondria orchestrate neutrophil chemotaxis. J Cell Biol. 210:1153-64 (2015).
3. Bezzerri V, Vella A, Calcaterra E, Finotti A, Gasparello J, Gambari R, Assael BM, Cipolli M, Sorio C. New insights into the Shwachman-Diamond Syndrome-related haematological disorder: hyper-activation of mTOR and STAT3 in leukocytes. SciRep. 6:33165 (2016).
4. Boocock, G. R. et al. Mutations in SBDS are associated with Shwachman-Diamond syndrome. Nat Genet 33:97-101 (2003).
5. Chapuis, N. et al. Perspectives on inhibiting mTOR as a future treatment strategy for hematological malignancies. Leukemia 24: 1686-1699 (2010).
6. Cipolli, M. Shwachman-Diamond syndrome: clinical phenotypes. Pancreatology 1: 543-548 (2001).
7. Donadieu, J. et al. Analysis of risk factors for myelodysplasias, leukemias and death from infection among patients with congenital neutropenia. Experience of the French Severe Chronic Neutropenia Study Group. Haematologica 90: 45-53 (2005).
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13. Iadevaia, V., Zhang, Z., Jan, E. & Proud, C. G. mTOR signaling regulates the processing of pre-rRNA in human cells. Nucleic Acids Res 40: 2527-2539 (2012).
14. Motzer, R. J. et al. Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. Lancet 372: 449-456 (2008).
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Part F. Relevance of the research to Shwachman-Diamond Syndrome

Up-regulation of mTOR pathway has already been reported to be associated with MDS and AML progression in several hematological diseases (Sandhofer N, 2015; Porta C, 2014). The well-known mTOR inhibitor rapamycin and its derivatives are currently tested on hematological malignancies such as AML and other forms of leukemia in several clinical trials (Porta C, 2014). Thus, our study might open a wider scenario within the current therapeutic approaches designed to counteract SDS pathology.

Part K.

Ethics Statement

All human samples used in this work were analyzed only after that written informed consent was obtained from all subjects. Methods were carried out in accordance with the approved guidelines of Ethics Committee of the Azienda Ospedaliera Universitaria Integrata di Verona (protocol CRCFC-LymphoSDS038). All experimental protocols were approved by the Ethics Committee of the Azienda Ospedaliera Universitaria Integrata di Verona (approval nr. 658CESC).