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Production of cells with activated immunogenic properties from hematopoietic and non-hematopoietic progenitors by activation specific protective pathways

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Abstract

Background: The possibility about derivation of immune cells from hematopoietic and non-hematopoietic progenitors was investigated. Pilot studies on the underlining mechanisms in both types of cellular progenitors were performed.

Methods: NK cells isolated from mouse spleens were incubated in the presence of different combinations of cytokines (IL-15/IL-18 + IL-12). Additional oncogene copy in normal mouse embryonic stem cells (mESCs) was inserted by transfection with appropriate recombinant DNA-constructs, based on the *AAV* DNA-genome. mESCs, both containing and non-containing additional copy of oncogene *Dcn1*, were pre-incubated in the presence of GM-CSF, and sub-populations of the derived initial myeloid and lymphoid progenitors were then co-cultivated with each other. General features in the protein electrophoretic profile of sperm plasma with these from different cellular, tissue and organ samples were proposed, and the presence of white blood cells in ejaculates from patients with proved inflammatory process (prostatitis) was observed. Several methods for assay were applied: light microscopy, transmission electron microscopy (TEM), electrophoresis in 1% Agarose Gel after standard PCR and Reverse Trancriptase PCR (RT-PCR) and SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) with subsequent Coomasie-blue staining.

Results: Despite the established activated proliferative activity of hematopoietic cellular progenitors, incubated in the presence of cytokines and cytokine combinations, statistically significant differences were noted only in the presence of IL-15 and IL-18, compared with the non-treated controls, and with the cells, incubated in the presence of other cytokines and cytokine combinations. The noted signs of initial myeloid and lymphoid progenitors, as well as of further phagocyte and plasmatic cell differentiation, respectively, confirmed additionally the preserved non-malignant characteristics and immunogenic capacity in *in vitro*-conditions of the so received cells, containing additional oncogene copy. Furthermore, a capability of non-myeloid and non-lymphoid cells to produce membrane receptor glycoproteins was suggested. On the other hand, a possibility for production of recombinant viral vaccines by exchange of nucleotide sequences between the used recombinant DNA-vector and cellular genome were proposed. In analogical way have been proved the preserved non-malignant characteristics and adequate immune response of human embryonic trophoblasts, immortalized by virus *SV40*. Besides the established morphological similarities, many signs of analogy in the electrophoretic profiles were established in the protein compositions between the separate biological samples, described above. Similarly to seminal plasma, synovia fluid and the extracts of each anatomic organ contain proteins, produced by different types of normal cells in various phases of maturation and differentiation.

Conclusions: Besides of cells with hematopoietic origin, a possibility non-hematopoietic cells, tissues and organs to acquire immunogenic properties of in appropriate conditions was also proposed. Future studies are necessary in this direction.

Background

The immune cells play an important role in the protection against different infections, but also against malignancies and metastases [1]. Investigations on the signal pathways activated by various cytokines, and clarification of the cellular events and functions induced by stimulation with combinations of them are required.

Widely investigated is the possibility about *in vitro*-incubation of viruses in cell cultures for development of viral recombinant vaccines, as well as for the aims of gene therapy. For this goal could be applied DNA- and RNA-viruses [2, 3], but also bacterial plasmids, yeasts and other eukaryotic cells [4–6]. Besides the respective gene(s) of interest, for which expression is necessary to be inserted appropriate promoter gene. Another component, which should be located near to the tested gene(s) of interest, is a marker gene, which is necessary about selection of the cells, positive of its presence and expression. Most often are used genes, coding color proteins (fluorescent protein, as GFP, YFP, RFP) or products, participating in a color reaction (enzymes luciferase or thymidine-kinase), or determining antibiotic resistance. The described gene sequences should be bind to each other by specific ligases, and thus, the initial vector constructs should contain specific restriction sites, which is necessary to be obtained the DNA-fragments by treatment with specific restriction enzymes (most often bacterial endo-nucleases). Other key components are the reverted end DNA-repeats. In this way could be inserted genes, for cellular receptors, cytokines, enzymes, complement activators, activators and/or inhibitors of apoptosis, surface antigens, tumor markers, etc. Polymerase chain reaction (PCR) of the received construction should be subsequently performed by use of specific oligonucleotide primers for insurance of respective restriction sites – *SfiI*-restriction site on the 5'-end, and *RsrII*-restriction site on the 3'-end of the PCR-product, obtained by digestion with respective restriction enzymes (particularly bacterial endonucleases), bound to the respective early or late promoter in the viral genome [2, 3, 5] or bacterial plasmid DNA [4, 6]. Taking in consideration all these data, several questions could arise: i) if any possibility of non-myeloid and non-lymphoid cells to acquire immunogenic properties in appropriate internal (genetic), epigenetic and external conditions exists; ii) if there is any possibility of the used about gene transfection viral strains to acquire viral vaccine (immunogenic) characteristics.

Oncogene *Dcn1* has been proved as highly conservative about all cellular types – from prokaryotic bacteria to eukaryotic mammalian cells. The protein product of this oncogene has been established to plays the role of kinase, which makes easier targets for degradation molecules, activating cellular ageing and death, by participation in cascade regulatory mechanisms [7–9]. This oncogene has been determined as actively expressed in all types of malignant cells, in early and late phases of differentiation and maturation, but also in germ cells [7, 10, 11].

Oncoprotein Dcn1 [12], albumin, as well as histones H2B and H3.3 [13], have been determined as proteins, actively expressed besides in stem/progenitor cells, also in germ cells. The histone-to-protamine transition process has been proved to play a key epigenetic role during the spermatogenesis. Analogical morphological similarities were established in smears from peripheral blood with rodent and human origin, but also between embryonic stem cells (ESCs), as well as other tissues and organs. As a key molecule, necessary about the packaging of protamines and DNA into chromatin and thus, for the

formation of the compact sperm nucleus, has been determined histone HANP1/H1T2, which is also essential in the fertilization process.

In this aspect, the main goal of the current study was directed to development of methods for stimulation of immunological properties of cells with both hematopoietic and non-hematopoietic origin, as well as about prove of general molecules in different biological samples, which could participate in various inter-molecular interactions and cascade regulatory mechanisms.

Methods

Derivation of immune cells from *in vitro*-incubated hematopoietic progenitors

Suspension of cultivated cells from mouse spleen was treated with buffer for erythrocytes lysis, and subsequently - CD4, CD8, CD19 MACS micro-beads, which are non-toxic bio-degradable nano-particles for detection and separation of cellular sub-populations. Before investigation on the cell proliferation or expression of the markers, the cells were harvested and their viability was monitored by Trypan Blue dye exclusion test. NK markers were proved by application of flow cytometry assay. The obtained population of NK cells was then *in vitro*-incubated and stimulated with different interleukins or combinations of them. The cells were picked-up and analyzed at day 4 post incubation (p. i.). The experiments were conducted in accordance with the ethical laws, and were approved by the Animal Research Committee of Hyogo College of Medicine. Animals had free access to food and water, and were kept in pathogen free environment with a 12 h/12 h light/dark cycle. A constant temperature of 25^{0}C was maintained. All cells were incubated for 4 days in RPMI1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% FBS (BioWest, Nuaillé, France), Lglutamine, 100 IU/mL penicillin and 100 mg/mL strepromycin, at 37^{0}C in a humidified atmosphere with 5% CO_2. A sub-population of the so incubated cells was pre-incubated in the presence of 10 ng/mL IL-15 and/or 100 ng/mL IL-18 (recombinant mouse IL-15 and IL-18 were commercially obtained by GlaxoSmithKline PLC (Research Triangle Park, NC), PEPROTECH Rocky Hill and NJ R&D, Minneapolis MN 55413, USA). At day 1, separate sub-populations of the so incubated cells were supplemented with 10 ng/ml IL-12 (R&D Systems). Statistical comparisons of the results were made with one-way analysis of variance (ANOVA) by application of JMP® Pro 13.1.0 (SAS Institute Inc. Cary, NC, USA). Statistical significance was defined as $P < 0.05$ [14].

In vitro-incubation and transfection of embryonic stem cells by recombinant DNA-viral vectors

Mouse ESCs were isolated from early mouse Balb/c embryos (2-3 months of the embryonic development), by trypsinization and resuspendation. After centrifugation of the received cell suspension, the supernatants were turned off and after resuspendation of the pellets, containing mouse ESCs, the last were incubated for 48 – 72 hours on previously formed monolayers of feeder primary mouse embryonic fibroblasts (MEFs) and/or from line 3T3, received in both cases from mouse embryos at later embryonic stages (4-5 months of the embryonic development). In the two cases, after formation of semi-confluent monolayers of mouse embryonic fibroblasts, they were treated by Mitomycin-c (mm-c) (Sigma-Aldrich),

about suppression of further fibroblasts' growth and proliferation. The isolated mouse ESCs seeded (5 x 10^6 cells/ml) on the so treated semi-confluent monolayers of fibroblasts. After formation of colonies of ESCs, the last were resuspended and subjected on transfection by recombinant DNA-genome from *adeno-associated virus* (*AAV*) (*Parvoviridae* family) [5], containing promoter for gene, coding Elongation Factor 1-alpha (EF-1α); copy of oncogene *Dcn1*, isolated from 3T3 fibroblasts of experimental mice Balb/c, as well as gene, determining the resistance to antibiotic Neomycin, isolated from bacterial DNA-plasmid. The cultivation media were supplemented with 10% FBS, 100 UI/ml penicillin, 0.25 mg/ml streptomycin and 0.25 mg/ml amphotericin-B. For this aim, the cultural fluid of the confluent mono-layer was changed with cultivation medium, containing Mitomycin-c (mm-c), for 2-3 hours at 37^0C. Stem cell medium was added, which besides of the described components, is supplemented with 20% FBS; 0.5% beta-Mercapto-ethanol; Leukemia-Inhibiting Factor (LIF) and Colonia-Stimulation Factor (CSF). Mouse ESCs (mESCs) are similarly isolated and proceeded, but in earlier studies of pregnancy, and they are seeded in the same initial density (5 x 10^6 cells/ml), after counting and determination of cell viability by Trypan Blue Dye Exclusion Test. Formation of stem cell colonies could be observed around the 3rd - 4th day after seeding, and they could be seen by phase-contrast light microscope. All transfected mESCs were incubated in the presence of the synthetic analogue of Neomycine – G418, in the presence of monolayer of feeder cells with previously suppressed further proliferation. Around the 28^{-th} day after the seeding in the presence of G-418, much more of the seeded mouse ESCs dead and only those, which possess the gene, connected with resistance to Neomycine, survived, continues to proliferate and formed colonies. After picking-up the so formed clones of transfected mESCs, possessing gene for Neomycine resistance and scaling-up of each one of them, they are subjected on genomic assay, by isolation total DNA was isolated. For this goal, after tripsinization of the transfected cells and their consequent treatment with mixture of phenolchlorophorm-isoamil alcohol (PCI) (Sigma-Aldrich), the so isolated nuclear material was treated with lysis buffer (Sigma-Aldrich) for isolation of genomic DNA. The last was subjected on standard PCR and Reverse-Transcriptase PCR (RT-PCR), respectively, of previously isolated nuclear DNA of them and its consequent electrophoresis on 1% agarose gel (Sigma-Aldrich), in the presence of specific DNA-primers, complementary to the used recombinant DNA-constructs (Sigma-Aldrich), mixture of the four types deoxy-nucleosid-tri-phosphates (dNTP - Sigma-Aldrich), enzyme Taq-polymerase (Sigma-Aldrich). Similarly transfected malignant cells from rat insulinoma cell line RIN-5F, containing additionally-inserted copy of rat tumor-suppressor gene *scgn*, coding the hormone-like protein Secretagogin (SCGN) [15-17], were similarly proceeded.

Separate sub-populations of mESCs, both containing and non-containing additional oncogene copy, were subjected on *in vitro*-differentiation initial myeloid and lymphoid progenitors in the presence of GM-CSF. Subsequently, sub-populations of the so received immune progenitors from both types were co-incubated for induction with each other for induction of further phagocyte and plasmatic cellular differentiation, respectively. All cells were cultivated at 37^0C in incubator with 5% CO_2 and 95% air humidification. The observation was performed by inverted light microscope (Leica), supplied with megapixel CCD-camera.

Determination of the protein composition in mammal seminal plasma

Seminal plasma with mammalian (ram) origin, as well as human synovial fluid from patient with rheumatoid arthritis, but also lysates from rat brain and rat pancreas were subjected on SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) separation and the gel was then stained by Coomasie-blue. The synovial fluid is known to contain different types of adult stem/progenitor cells. Besides the control samples of both rat anatomic organs, to separate aliquots of each one of them was added extract of transfected malignant cells from rat insulinoma line RIN-5F, containing additionally-inserted rat *scgn* gene plus *GSH*-tag, and the so prepared lysate mixtures were then subjected on affinity chromatography on GSH-Agarose columns. Each sample was previously subjected on protein precipitation in cold 100% ethanol overnight, after which the received pellets (precipitates) were washed by cold 80% ethanol. After centrifugation, the supernatants were taken-off, and the pellets/precipitates, eventually containing the proteins from each respective probe, were diluted in SDS-buffer.

Transmission electronic microscopy (TEM) study of biological material (human ejaculates) from infertile men (with chronic prostatitis) was performed according to the World Health Organization (WHO - 2010). The so prepared samples were observed by electronic microscope “Opton EM 109”, for eventual reveal of key characteristics and changes in the male reproductive tract on ultrastructural level.

Results

Proliferation of *in vitro*-incubated hematopoietic progenitor cells on the influence of different cytokines and cytokine combinations

In this study, the main goal was related with derivation of immune cells by appropriate *in vitro*- and *ex-vivo*-incubation of hematopoietic cellular progenitors. In all cases was observed activated proliferative activity of hematopoietic cellular progenitors, incubated in the presence of cytokines and cytokine combinations compared with the control cultures of the same cells (Fig. 1). Statistically significant differences, however, were assessed only in the presence of IL-15 and IL-18, compared with the non-treated controls, as well as with the cells, incubated in the presence of other cytokines and cytokine combinations.

Genetic assay for determination of cells, containing and non-containing additionally-inserted gene copy

Despite the near localization of the gene, determining the resistance to Neomycine in all selected clones of transfected cells, the results from both standard PCR and RT-PCR with subsequent electrophoresis on 1% agarose gel show that only in one of them the oncogene of interest - mouse *Dcn1*, is successfully inserted in the cellular genome after incubation in the presence of Neomycine analog G-418 (Fig. 2).

The main goal of the performed transfer of additional oncogene copy was to be elongated the life cycle of the transfected ESCs, containing it. However, the preserved non-malignant characteristics of the transfected cells, containing additional oncogene copy should be tested and proved. So, the immunogenic properties of these cells in *in vitro*-conditions were investigated and compared with these

of non-containing additional oncogene copy normal cells, as well as of malignant cells, in the same conditions.

In vitro-derivation of cells with activated immunogenic properties

According to the results obtained, the derived transfected mESCs, containing additionally-inserted oncogene copy, have preserved their normal/non-malignant characteristics (Fig. 3). Furthermore, the presented data proved the safety and immunogenic efficiency of the transfected mESCs, containing additional copy of oncogene *Dcn1* in *in vitro*-conditions, as well as their influence on the further differentiation of normal myeloid and lymphoid progenitors, derived from non-transfected mESCs by their incubation in the presence of GM-CSF (Fig. 3A, B), to phagocyte and plasmacyte cells, in co-cultivation with transfected mESCs, containing additional oncogene copy (Fig. 3C).

The questions which arose were: 1) did the so derived transfected cells, containing additional oncogene copy (as experimental *in vitro*-model of cellular life cycle elongation and suppression of ageing), preserve their non-malignant characteristics (instead induction of their malignant transformation, which could be a reason of much faster and earlier organism death in their application), and 2) what makes the cells to be acceptable to viruses or other microbes, as well as of the normal cells to malignant cells in co-cultivation of cells from both types? So, additional studies about clarification were performed. Possibilities about production of recombinant viral vaccines by exchanging of nucleotide sequences between the used recombinant DNA-construct and cellular genome, but also in the opposite direction (from the cellular genome to the used recombinant vectors) were proposed. Additionally, a possibility for production of membrane receptor glycoproteins by non-myeloid and non-lymphoid cellular types in appropriate conditions was suggested. Future studies are necessary in this direction, including on the immunogenic potential of the so received products, both *in vitro* and *in vivo*.

Two different methods for assay of the availability of common characteristics of the reproductive components with all other biological samples (cells, tissues and organs, including experimental *in vitro*- and *in vivo*-models) were applied – SDA-PAGE for investigation of these features on molecular level, and TEM for prove of these characteristics on morphological and ultra-structural levels, respectively. The protein composition of male seminal plasma showed the strongest analogy with that of synovial fluid, which is known as rich of various types of adult stem/progenitor cells (Fig. 4, lanes 2 & 3), compared with the samples of brain and pancreas, containing a lot of mature cells (Fig. 4, lanes 2; 4 & 5). On the other hand, similarities in the protein composition of the seminal plasma with this of mixed extracts of synovial fluid and of both anatomic organs with lysate of malignant cells were noted (Fig. 4, lanes 2; 6 & 7). These results suggested a possibility about immunogenic properties of the sperm fluid. They were confirmed by the presence of leucocytes in such probes (Fig. 5).

The assay of the molecular composition in mixed biological samples with *in vitro*-incubated malignant cells, containing and non-containing additionally-inserted by recombinant DNA-vectors additional tumor-suppressor gene copy, was undertook for eventual decrease of the malignant cellular potential, but also about following the changes in the mechanisms of adequate immune reply, in mixed "*in vivo* + *in vitro*"

experimental models. The presence of different cellular types in various phases of differentiation and maturation in the composition of anatomic organs and other biological samples (as body fluids) was taken in consideration, which proposed the role of different inter-molecular interactions and cascade regulatory pathways in each separate biological system, including when general molecules participate.

Discussion

The combination of the two cytokines (IL-15 and IL-18) has been found to be particularly effective in induction of NK cell activation and proliferation [18]. IL-15 has been characterized as pivotal about the proliferation and survival of NK cells. Its signal has been found to be transduced via JAK/STAT pathway and probably also by activation of mTOR, because PI3K-Akt-mTOR pathway has been determined as critical for cytokine responses of primary NK cells to IL-15 [19]. The important role of IL-12 about the NK cells has also been assessed. It is considered as an NK cell stimulatory factor despite being unable by itself to promote the expansion of their population. NK cells constitutively express the IL-12 receptor β1 chain, which allows them to respond to IL-12 in early stages of infection [20]. In T-lymphocytes, the cytokine IL-12, especially in combination with IL-18, causes increased production of interferon gamma (IFN-γ). According to other data, the combination of IL-12 and IL-18 in T-cells strongly up-regulates the production of IFN-γ [21, 22]. Additionally, the role of IL-18 as a responder molecule against stresses caused by various risk signals has been established. Besides by various cells of the immune system, IL-18 has also been found to be secreted by non-hematopoietic cellular types including cardiac myocytes, keratinocytes, intestinal epithelial cells, retinal cells (especially retinal stem/progenitor cells) and some endocrine cells [23].

Investigations on the stem cells' biology are directed mainly on their self-renewal and differentiation capabilities and thus, to differentiate to respective mature cellular lineages. [24, 25]. These characteristic propose possibilities about application of the stem cells in various fields of medical practice, including about different therapeutic and regenerative procedures. It is important, however, the functions of the oncogenes and coded by them oncoproteins, to be activated by ways, by which eventual neo-plastic cellular transformations and metastatic process, to be escaped, for instance by generation of adequate anti-malignant immune response on the influence of the protein products of oncogenes in their role of active antigens. Additionally, according to literature data "intrinsic antiviral immunity" changes as internal protection of the cell in response to its infection has been proved [26, 27]. Besides presence of leucocytes in non-immune tissues and organs, the presented results suggested a possibility for production of membrane receptor glycoproteins on the surface of non-myeloid and non-lymphoid cellular types in appropriate conditions as for instance viral or malignant antigens, as well as different cytokines and other immunomodulators. These results were in agreement with the literature findings in this direction [28–30]. One of the hypotheses was based on the proved in the scientific literature possibility about appearance of initial lymphoid and myeloid differentiation signs in sub-populations of immature stem-like cells, which are able to differentiate to various directions depending of the respective environment conditions. Another explanation could be related with the proved action of some cell-produced proteins as

glycoprotein receptors to specific bio-molecules [31]. Furthermore, regulation of target genes expression by enhancers, located to far of them, has also been found [32].

Taking in consideration the proved key role of glycosylation in many cellular processes as cell growth and differentiation, the importance of this process to reflect the physiological status of the cell has been proposed [33]. Besides glycoproteins, as the other main participants in this process have been determined glycosphingolipids, particularly gangliosides [34, 35]. As surface markers, located at the membrane surface, a key role of these molecules in the invasion of malignant cells and metastatic process has been proved. Additionally, the current data proposed a possibility for preservation of stem-like characteristics and preserved non-malignant nature of the transfected cells, containing additional oncogene copy, and thus, eventual life elongation in the presence of additional oncogene copy. Analogically preserved non-malignant characteristics and adequate immune response of human embryonic trophoblasts, immortalized by virus *SV40*, have been established [36, 37].

Besides of mature spermatozoa in the seminal plasma, many other cellular types have been proved as sperm precursors in different phases of maturation, but also various types of leucocytes (granulocytes, monocytes/macrophages, etc.) [38]. The determination of the different cellular types in ejaculate material and their differentiation to spermatogenic and/or to non-spermatogenic directions has been proposed as important for development of novel diagnostic and therapeutic strategies [39–41].

Different mechanisms of production, secretion and influence of immune molecules by cells, derived from hematopoietic and non-hematopoietic progenitors have been proposed [42]. These features could vary in the separate cells, tissues and organs. In this way, the current data supported the importance of various intra- and extra-cellular inter-molecular interactions and cascade regulatory pathways, underlining the cellular functions and differences between the separate cells, tissues and organs. Further studies should be performed.

Conclusions

Besides by cells with hematopoietic origin, possibilities about expression and production immunogenic molecules by non-hematopoietic cells, tissues and organs in appropriate conditions were suggested. Probably these differences are due to the presence of cells in various phases of differentiation and maturation. Different cascade mechanisms could be underlining these processes, in dependence of the cellular type and environmental conditions. Further investigations are necessary in this direction.

Declarations

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All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

References

1. Waldhauer I, Steinle A. NK cells and cancer immunosurveillance. Oncogene. 2008;27(45):5932–5943. doi: 10.1038/onc.2008.267
2. Barrette S, Douglas JL, Seidel NE, Bodine DM. *Lentivirus*-based vectors transduce mouse hematopoietic stem cells with similar efficiency to moloney murine leukemia virus-based vectors. Blood. 2000;96(10):3385-3391. doi: 10.1182/BLOOD.V96.10.3385.H8003385_3385_3391
3. Borysiewicz LK, Flander A, Numako M, Man S, Wilkinson GW, Westmoreland D, Evans AS, Adams M, Stacey SN, Boursnell MEG, Rutherford E, Hickling JK, Inglis SC. A recombinant *vaccinia* virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer. Lancet. 1996;347(9014):1523-1527. https://doi.org/10.1016/S0140-6736(96)90674-1
4. Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD. Designer deletion strains derived from *Saccharomyces cerevisiae S288C*: a useful set of strains and plasmids for PCR mediated gene disruption and other applications. Yeast. 1998;14(2):115-132. doi: 10.1002/(SICI)1097-0061(19980130)14:2<115::AID-YEA204>3.0.CO;2-2
5. Chen S, Agarwal A, Glushakova OY, Jorgensen MS, Salgar SK, Poirier A, Flotte TR, Croker BP, Madsen KM, Atkinson MA, Hauswirth WW, Berns KI, Tisher CC. Gene delivery in renal tubular epithelial cells using recombinant *adeno-associated viral* vectors. J Am Soc Nephrol. 2003;14(4):947-958. doi: 10.1097/01.asn.0000057858.45649.f7
6. Domi A, Moss B. Engineering of a *vaccinia virus* bacterial artificial chromosome in *Escherichia coli* by bacteriophage λ−based recombination. Nat Meth. 2005;2(2):95−97. doi: 10.1038/nmeth734
7. Colaluca IN, Tosoni D, Nuciforo P, Senic-Matuglia F, Galimberti V, Viale G, Pece S, Di Fiore PP. NUMB controls *p53* tumor suppression activity. Nat Letters. 2008;451(7174):76-80. doi: 10.1038/nature06412
8. O-charoenrat P, Sarkaria I, Talbot SG, Reddy P, Dao S, Ngai I, Shaha A, Kraus D, Shah J, Rusch V, Ramanathan Y, Singh B. SCCRO (DCUN1D1) induces extracellular matrix invasion by activating matrix metalloproteinase 2. Clin Cancer Res. 2008;14(21):6780-6789. doi: 10.1158/1078-0432.CCR-08-0719
9. Kurz T, Chou YC, Willems AR, Meyer-Schaller N, Hecht ML, Tyers M, Peter M, Sicheri F. Dcn1 functions as a scaffold-type E3 ligase for cullin neddylation. Molec Cell. 2008;29(1):23-35. doi: 10.1016/j.molcel.2007.12.012

10. Bowerman B. *C. elegans* aging: proteolysis cuts both ways. Curr Biol. 2007;17(13):R514-R516. doi: 10.1016/j.cub.2007.05.011
11. Yuen BTK, Bush KM, Barrilleaux BL, Cotterman R, Knoepfler PS. Histone H3.3 regulates dynamic chromatin states during spermatogenesis. Development. 2014;141(18):3483-3494. doi: 10.1242/dev.106450
12. Zalensky AO, Siino JS, Gineitis AA, Zalenskaya IA, Tomilin NV, Yau P, Bradbury EM. Human testis/sperm-specific histone H2B (hTSH2B). Molecular cloning and characterization. J Biol Chem. 2002;277(45):43474-43480. doi: 10.1074/jbc.M206065200
13. Akmal M, Aulanni'am A, Widodo MA, Sumitro SB, Purnomo BB. The important role of protamine in spermatogenesis and quality of sperm: a mini review. Asian Pacif J Reprod. 2016;5(5):357-360. https://doi.org/10.1016/j.apjr.2016.07.013
14. Oka N, Markova Tz, Tsuzuki K, Li W, El-Darawish Y, Pencheva-Demireva M, Yamanishi H, Sakagami M, Tanaka Y, Okamura H. IL-12 regulates the expansion, phenotype, and function of murine NK cells activated by IL-15 and IL-18. Cancer Immunol Immunother. 2020;69(9):1699-1712. doi: 10.1007/s00262-020-02553-4
15. Gartner W, Vila J, Daneva T, Nabokikh A, Koc-Sarah F, Ilhan A, Majdic O, Luger A, Wagner L. New functional aspects of the neuroendocrine marker secretagogin based on the characterization of its rat homolog. Am J Physiol Endocrinol Metab. 2007;293(1):E347-E354. doi: 10.1152/ajpendo.00055.2007
16. Wagner L, Oliyarnyc O, Gartner W, Nowotny P, Groeger M, Kaserer K, Waldhäusl W, Pasternack MS. Cloning and expression of secretagogin, a novel neuroendocrine- and pancreatic islet of Langerhans-specific Ca^{2+}-binding protein. J Biol Chem. 2000;275(32):24740-24751. doi: 10.1074/jbc.M001974200
17. Rogstam A, Linse S, Lindqvist A, James P, Wagner L, Berggard T. Binding of calcium ions and SNAP-25 to the hexa EF-hand protein secretagogin. Biochem J. 2007;401(1):353-363. doi: 10.1042/BJ20060918
18. French AR, Holroyd EB, Yang L, Kim S, Yokoyama WM. IL-18 acts synergistically with IL-15 in stimulating natural killer cell proliferation. Cytokine. 2006;35(5-6):229-234. doi: 10.1016/j.cyto.2006.08.006
19. Nandagopal N, Ali AK, Komal AK, Lee S-H. The critical role of IL-15-PI3K-mTOR pathway in natural killer cell effector functions. Front Immunol. 2014;5(187). doi: 10.3389/fimmu.2014.00187
20. Carbone S, Lee PJH, Mauro AG, Mezzaroma E, Buzzetti R, Tassell BT, Abbate A, Toldo S. Interleukin-18 mediates cardiac dysfunction induced by Western diet independent of obesity and hyperglycemia in the mouse. Nutrition and Diabetes. 2017;7(4):e258. doi: 10.1038/nutd.2017.1
21. Tsung K, Meko JB, Peplinski GR, Tsung YL, Norton JA. IL-12 induces T helper 1-directed antitumor response. J Immunol. 1997;158(7):3359-3365.
22. Okamura H, Kashiwamura S, Tsutsui H, Yoshimoto T, Nakanishi K. Regulation of interferon-gamma production by IL-12 and IL-18. Curr Opin Immunol. 1998;10(3):259-264. doi: 10.1016/s0952-

7915(98)80163-5

23. He X-S, Draghi M, Mahmood K, Holmes TH, Kemble GW, Dekker CL, Arvin AM, Parham P, Greenberg HB. T cell-dependent production of IFN-γ by NK cells in response to influenza A virus. J Clin Invest. 2004;114(12):1812-1819. doi: 10.1172/JCI22797
24. Smith AG. Embryo-derived stem cells: of mice and men. Annu Rev Cell Dev Biol. 2001;17:435-462. doi: 10.1146/annurev.cellbio.17.1.435
25. Smith GH, Boulanger CA. Mammary stem cell repertoire: new insights in aging epithelial populations. Mech Ageing Dev. 2002;123(11):1505-1519. doi: 10.1016/s0047-6374(02)00114-8
26. Murray MJ, Peters NE, Reeves MB. Navigating the host cell response during entry into sites of latent *cytomegalovirus* infection. Pathogens. 2018;7(1):30. doi: 10.3390/pathogens7010030
27. Yan N, Chen ZJ. Intrinsic antiviral immunity. Nat Immunol. 2012;13(3):214-222. doi: 10.1038/ni.2229
28. Keller GM. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes & Dev. 2005;19(10):1129-1155. doi: 10.1101/gad.1303605
29. Kyba M, Perlingeiro RCR, Hoover RR, Lu C-W, Pierce J, Daley GQ. Enhanced hematopoietic differentiation of embryonic stem cells conditionally expressing Stat5. Proc Natl Acad Sci USA. 2003;100(suppl.1):11904-11910. doi: 10.1073/pnas.1734140100
30. Okubo T, Yanai N, Watanabe S, Arai K-I, Obinata M. Effect of human granulocyte-macrophage colony stimulating factor (hGM-CSF) on lymphoid and myeloid differentiation of sorted hematopoietic stem cells from hGM-CSF receptor gene transgenic mice. J Biochem. 2000;127(4):591-596. doi: 10.1093/oxfordjournals.jbchem.a022645
31. Furlong EEM, Levine M. Developmental enhancers and chromosome topology. Science. 2018;361(6409):1341-1345. doi: 10.1126/science.aau0320
32. Popi AF, Motta FLT, Mortara RA, Schenkman S, Lopes JD, Mariano M. Co-ordinated expression of lymphoid and myeloid specific transcription factors during B-1b cell differentiation into mononuclear phagocytes *in vitro*. Immunology. 2009;126(1):114-122. doi: 10.1111/j.1365-2567.2008.02883.x
33. Schöler HR, Hatzapoulis AK, Balling R, Suzuki N, Gruss P. A family of octamer-specific proteins present during mouse embryogenesis: evidence for germline-specific expression of an Oct factor. EMBO J. 1989;8(9):2543-2550. doi: 10.1002/j.1460-2075.1989.tb08392.x
34. Bretscher MS, Raff MC. Mammalian plasma membranes. Nature. 1975;258(43). doi: 10.1038/258043a0
35. Yu RK, Tsai Y-T, Ariga T, Yanagisawa M. Structures, biosynthesis, and functions of gangliosides - an overview. J Oleo Sci. 2011;60(10):537-544. doi: 10.5650/jos.60.537
36. Ali H, Kasper SJ, Arai T, DeCaprio JA. Cul7/p185/p193 binding to *Simian Virus 40* large T antigen has a role in cellular transformation. J Virol. 2004;78(6):2749-2757. doi: 10.1128/JVI.78.6.2749-2757.2004
37. Southern PJ, Berg P. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the *SV40* early region promoter. J Mol Appl Genet. 1982;1(4):327-341.

38. Fedder J. Nonsperm cells in human semen: with special reference to human leucocytes and their possible influence on fertility. Arch Androl. 1996;36(1):41-65. doi: 10.3109/01485019608987883
39. Ilieva I, Tzvetkova P, Kacarov M, Sainova I, Taushanova P, Vladov I, Zvetkova E. Round immature spermatogenic cells in semen fluids of infertile men with diagnosis “migrating testis”. Two casuistic cases in adults. Acta morphol anthropol. 2015;22:15-23.
40. Ilieva I, Tzvetkova P, Sainova I, Taushanova P, Kacarov M, Zvetkova E. Immature spermatogenic cells in semen fluids of infertile men. Compt rend del’Acad Bulg Sci. 2016;69(1):85-94.
41. Johansson E, Campana A, Luthi R, de Agostini A. Evaluation of “round cells” in semen analysis: a comparative study. Hum Reprod Update. 2000;6(4):404-412. doi: 10.1093/humupd/6.4.404
42. Stanley AC, Lacy P. Pathways for cytokine secretion. Physiology. 2010;25(4):218-229. doi: 10.1152/physiol.00017.2010

Figures

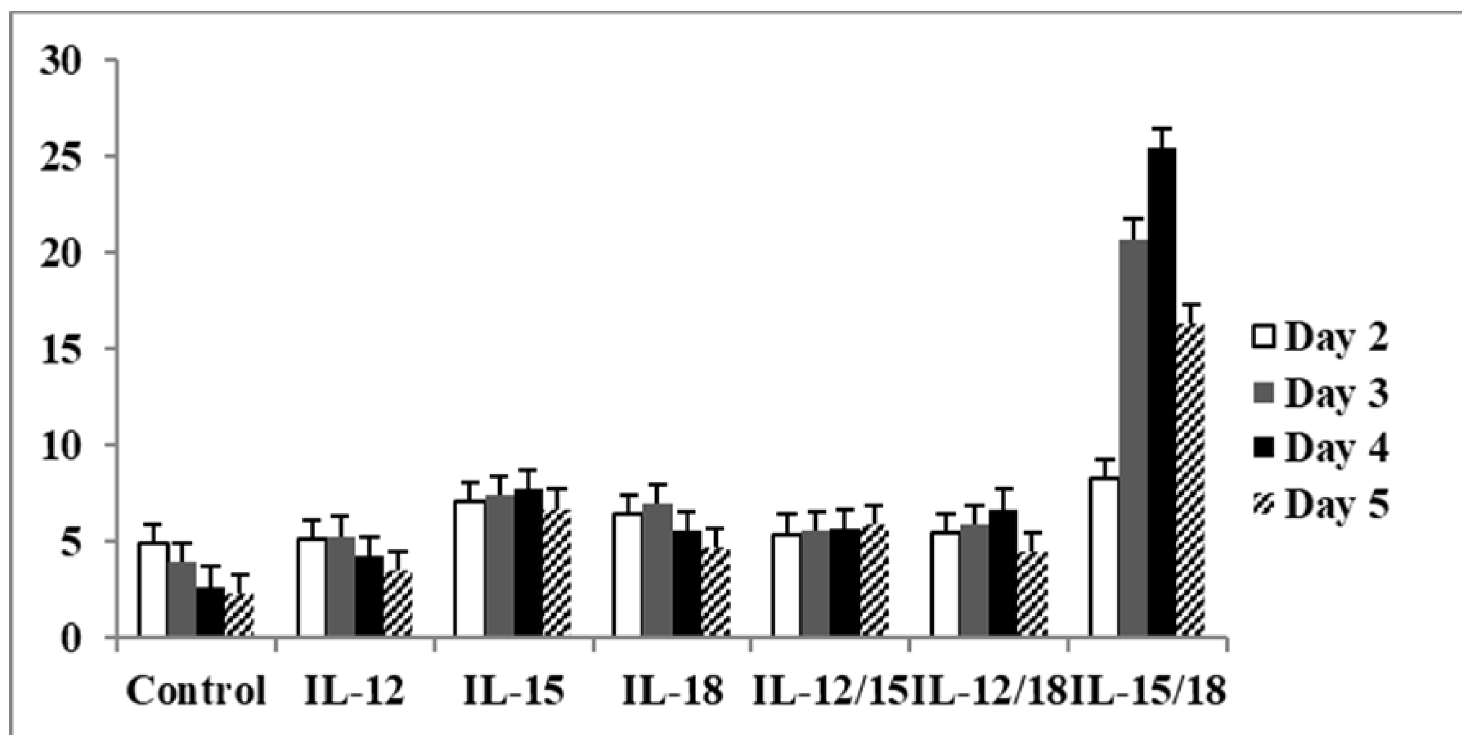

Figure 1

Proliferative activity of immune progenitors, derived from *ex vivo*-incubated hematopoietic stem cells in the presence of appropriate cytokines and cytokine combinations.

Standard PCR RT- PCR

M M
1 2 3 4 5 6 7 8 9 10

Figure 2

1% agarose gel electrophoresis for prove the presence or absence of additionally-inserted copy of mouse oncogene *Dcn1* in transfected mESCs, by standard PCR (lanes 2-5) and RT-PCR (lanes 6-9), by application of specific primers, complementary of the used recombinant DNA-construct: respectively, M – marker (lanes 1 and 10)

A B C

Figure 3

Initial stages of myeloid and lymphoid differentiation of mESCs: non-containing additional oncogene copy, incubated in the presence of GM-CSF (**A**); containing additional oncogene copy, incubated in the presence of GM-CSF (**B**); co-cultivated sub-populations of containing and non-containing additional oncogene copy initial myeloid-like and lymphoid-like progenitors, derived from both types of mESCs (**C**) (Fixed light-microscopy preparations, stained by Giemsa-dye, magnification: 100 x)

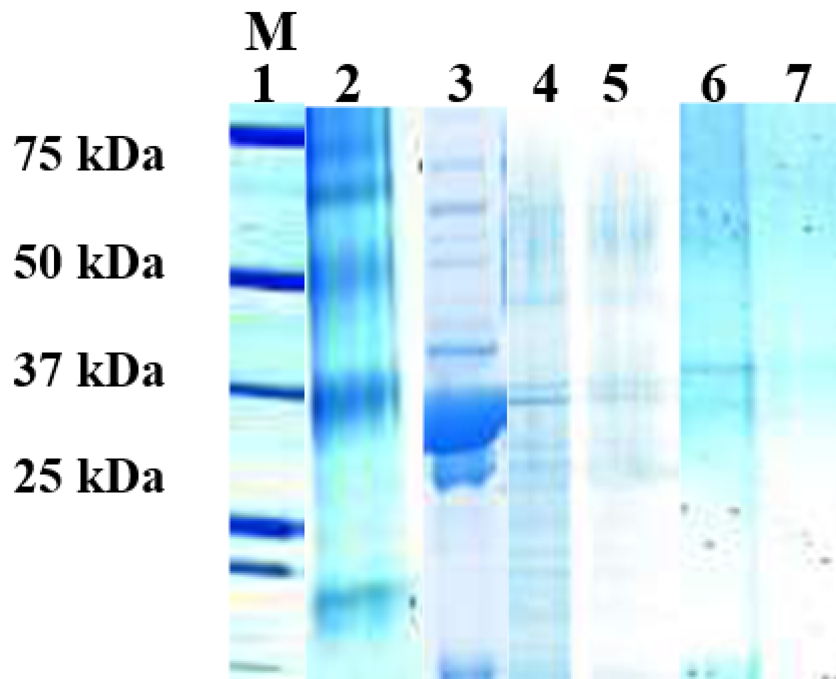

Figure 4

SDS-PAGE of mammalian seminal plasma (lane 2), as well as control protein lysates from synovial fluid (lane 3), but also from control mammalian brain and pancreas (lanes 4 & 5); as well as extracts from mammalian brain and pancreas with added extract of malignant cells to each one of them (lanes 6 & 7); M – marker (lane 1), Coomasie-blue staining

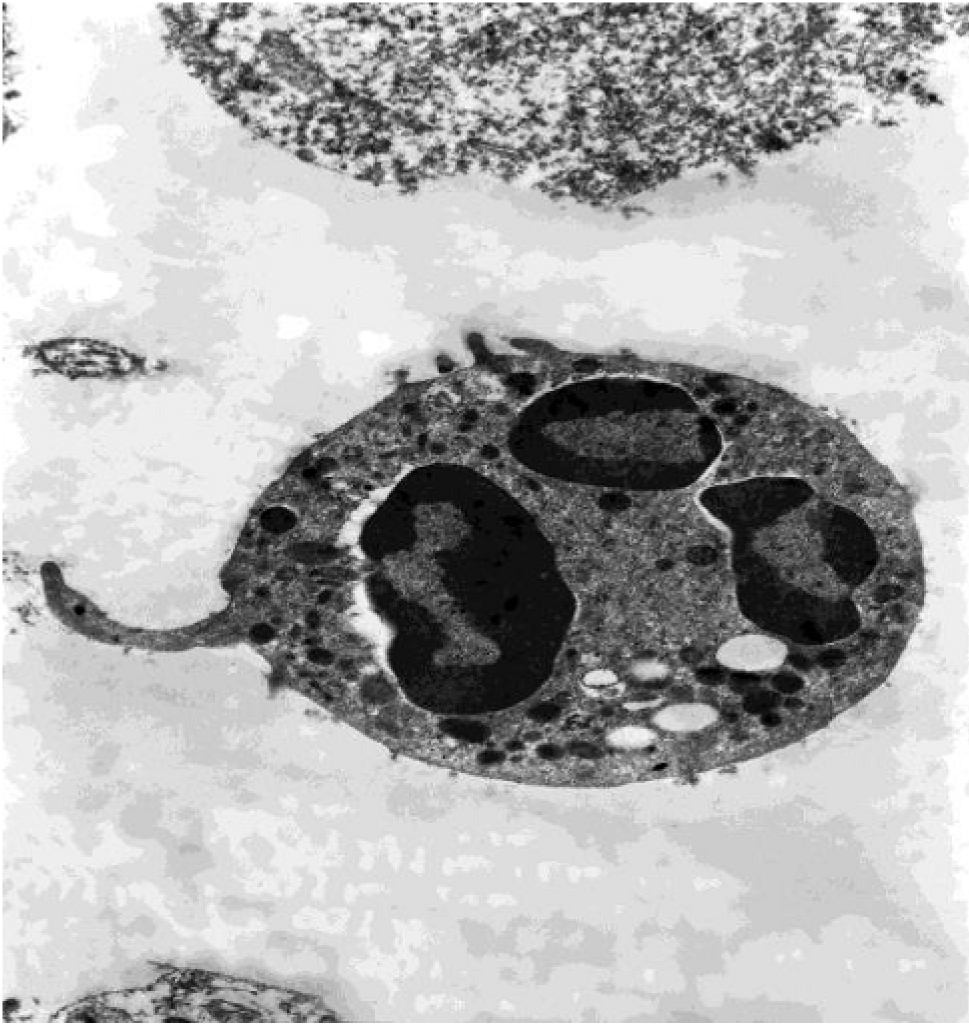

Figure 5

Electronogram of a leukocyte from an ejaculate of a patient with prostatitis, TEM, x10 000