



University of Torino, School of Biotechnology; Fondazione DeBenedetti Cherasco 1547 Onlus; Weizmann Institute of Science, Department of Immunology

Identification of new molecular targets to treat inflammatory diseases.

Synopsis:

Progress of science requires free exchange of knowledge and skills. The success of scientific research in western countries indeed relies on granting and promoting these essential rights.

Despite of ongoing efforts, scientific exchange needs support and continuous fostering. Involvement of private charities is, at least in Italy, essential to promote these activities which might also cover aspects supported by more institutional programs. For example, although often seen as a by-product, links, established through collaborating scientists might crucially help to bring together students from different cultural backgrounds and develop peaceful and respectful relationships between countries. Strengthening the scientific exchange between leader research centres in Piemonte and the Weizmann Institute in Israel will clearly give new opportunities for both scientific advancement and relationship development, breaking prejudice and improving the mutual perspective of each other.

To reach this multifaceted goal, it appears very important to generate a background of collaboration between the two sides so that concepts of scientific exchange and spiritual development might go along in concert. The program should thus be organized in a multi-step process Starting from funding of collaborative projects might end up in long term transfer of competence and people to and from both countries. Initially, proposals from joint bipartite collaborations in leading scientific subjects, where both sides show peaks of excellence, should be supported. The establishment of common scientific interests in a collaborative frame with equivalent support will be the best way to trigger interactions and exchanges of people. Thus, within these collaborative projects, brilliant young scientists should be encouraged to spend a period of time abroad, especially supporting the transfer of research fellows to the Weizmann Institute, where, in contrast to Italy, post-doctoral training is already well established. These projects will thus promote the development of common grounds, excellent training and fostering reciprocal appreciation.

To implement such plan, research in the field of life sciences might be a favourite choice. Biology is currently the most rapidly expanding branch of science and major breakthroughs in this area are expected to have great impact on human health and life quality around the world.

Within high-developed countries, like Italy and Israel, emerging life threatening diseases that have recently driven massive attention from the scientific community comprise chronic dysfunctions with inflammatory origin, like allergy, asthma, arthritis, and inflammatory bowel disease. Interestingly, pathologic inflammatory processes are also known to cause atherosclerosis and to trigger cardiovascular diseases like infarction, stroke and cardiac failure. Overall, current treatments for these dysfunctions of increasing incidence are often insufficient and prone to side effects, frequently leaving patients with a longer life span but a dramatically reduced life quality.

The efforts of research on the molecular mechanisms of inflammation and cardiovascular disease likely will help to define innovative treatments of improved efficacy and reduced side effects. For these reasons, funding of basic and applied studies in this field is critically required. Excellent

research teams both in Piemonte and at the Weizmann Institute are leaders in these areas. Thus the core purpose of the Weizmann Committee of the “Fondazione De Benedetti – Cherasco 1547 Onlus” is to promote the implementation of this challenging project, by supporting bilateral interactions between scientists in Piemonte and at the Weizmann Institute focusing on inflammation and cardiovascular diseases.

Aim and scope of the work:

The aim of this work will be to define the molecular mechanisms of inflammation through the study of genes that control cell migration and leukocyte recruitment. These genes, by controlling inflammatory reactions, might impact on the general outcome of multiple inflammatory diseases including cardiovascular dysfunctions like atherosclerosis and infarction. The Italian and Israeli partners involved in this project will focus on this common aim providing and sharing specific expertises and tools. The two groups are world leaders in complementary areas: the Alon group established a state of the art technology to study migration of inflammatory cells *ex vivo* in the presence of shear forces; the Hirsch group has all the know-how and machinery to generate genetically altered mouse lines and to characterize *in vivo* phenotypes with particular experience in studying inflammation and cardiovascular diseases.

Scientific background:

Increasing evidence indicates that in the signalling transduction events that activate the inflammatory response, phosphoinositide 3-kinases (PI3Ks) play a crucial role. PI3Ks are enzymes able to selectively phosphorylate the 3'-OH residue of phosphatidylinositol(4,5)bisphosphate (PtdIns4,5P₂) and to produce a second messenger (PtdIns3,4,5P₃), which, by recruiting and interacting with multiple proteins, can trigger different intracellular signaling events that control processes such as proliferation and survival, cytoskeletal remodeling and membrane trafficking (Vanhaesebroeck et al., 2001). Class I PI3Ks can be divided in two major subfamilies named Class IA and IB, respectively. Class IA PI3Ks are heterodimers composed of a catalytic subunit tightly coupled to an adapter protein. Mammals possess three catalytic subunits (alpha, beta and delta) that can bind three adaptor proteins (p85alpha, p85beta and p55). PI3Kalpha and beta are ubiquitous but PI3Kdelta is particularly enriched in leukocytes. While these enzymes are mainly activated by tyrosine kinases, the only known Class IB PI3K, PI3Kgamma, is specifically activated by the beta/gamma subunits of trimeric G proteins (Vanhaesebroeck et al., 2001). PI3Kgamma binds to a specific adaptor called p101 which modulates its catalytic activity in response to heterotrimeric G protein activation. Although PI3Kgamma is preferentially expressed in white blood cells, its presence has also been reported in tissues of the cardiovascular system (Prasad et al., 2003).

Studies in genetically engineered mice have recently indicated that class I PI3Ks possess specific and non redundant roles. We and others indeed reported the generation of a PI3Kgamma-deficient mouse strain (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000) and showed that these mice were viable and fertile, and could be maintained in standard animal facilities. However, mutant bone marrow neutrophils did not produce PtdIns(3,4,5)P₃ after stimulation with G protein-coupled seven transmembrane receptor agonists. Similarly, these stimuli did not activate the PI3K-downstream effector Akt/PKB in PI3Kgamma-deficient leukocytes, indicating that PI3Kgamma is the crucial PI3K isoform required for G protein induced PtdIns(3,4,5)P₃ production. In primed PI3Kgamma-null neutrophils, this defect lead to an impaired respiratory burst response following fMLP stimulation. In addition, mutant granulocytes exhibited impaired *in vitro* and *in vivo* chemotaxis towards multiple chemoattractants binding to Galpha_i-coupled receptors like chemokines. In particular, peritoneal neutrophils and macrophages showed a dramatic defective recruitment after induction of septic peritonitis. These results indicate that PI3Kgamma has a crucial

role in controlling leukocyte recruitment in response to inflammatory stimuli (Wymann et al., 2000). A key class of cytoskeleton remodelling enzymes which translate chemokine and cytokine signals to adhesion, polarization and motility are the small Rho GTPases (Vicente-Manzanares and Sanchez-Madrid, 2004) Chemokine signals trigger specific Rho GTPases via both PI3Kgamma dependent and independent pathways. One such pathway involves adaptors of the DOCK family and works conducted by Weizmann scientists suggest a complex crosstalk between PI3K and a member of this family, DOCK-2 (Schulman et al. and Alon, in press). In addition to its key role in cell motility, PI3Kgamma can translate signals from chemokine receptors to various cytoplasmic effectors which are recruited to the surface membrane of lymphocytes, neutrophils and monocytes and activate the adhesiveness of integrins on these immune cells to ligands elevated on blood vessel walls surrounding specific sites of inflammation (Laudanna and Alon, 2006). Without PI3K activation, subsets of these cells arrest on inflamed blood vessels but fail to organize their cytoskeletal machineries to remain adherent on the vessels for minutes thereafter. Without such adhesive capacity maintained for minutes after initial recruitment, the immune cells fail to coordinate their cytoskeletal machineries to successfully cross through the endothelial cells which comprise the blood vessel wall.

Based on recent studies, PI3Kgamma may contribute differently to distinct set of interactions between different immune cells and inflamed vascular sites, since the composition of adhesive and cytokine signals varies with the immune and endothelial cell type, the duration and the magnitude of the inflammatory stimulus. In addition to its key role in cell motility, PI3Kgamma, also controls other important aspects of inflammation. In fact, mast cells lacking PI3Kgamma show a significantly reduced response after stimulation with multiple agonists, including the IgE/antigen complex. In the absence of PI3Kgamma, mice are protected in an in vivo model of anaphylactic shock (Laffargue et al., 2002). These observations, together with the finding that PI3Kgamma-deficient mice are viable in normal conditions, open the possibility that a specific inhibitor for PI3Kgamma might have a great therapeutic potential in inflammatory and diseases and recently experimental PI3K γ inhibitors were reported to be efficacious to treat rheumatoid arthritis (Camps et al., 2005). However, PI3K γ appears to function also in the cardiovascular system as well. As we previously reported, PI3K γ controls heart muscle contractility (Crackower et al., 2002). Interestingly, this effect is independent on its enzymatic activity and involves a complex formation with the phosphodiesterase 3B which controls the level of intracellular cAMP (Patrucco et al., 2004). On the other hand, the kinase activity of PI3K γ is required for vascular responses to angiotensin II, suggesting that inhibiting PI3K γ could represent a novel way to treat hypertension (Vecchione et al., 2005).

How these different biochemical functions of PI3K γ are accomplished inside a cell is still a matter of intense study. The scenario depicted by the literature on PI3K γ as well as other isoforms suggests that different signaling components are differently assembled in distinct cell types and each assembly may give rise to multiple responses depending on the stimulus and the cellular environment. The challenge for the future is thus to define at a more refined molecular level these different signaling complexes, in order to find new targets for more precise and efficacious therapy in inflammatory diseases.

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Wymann, M. P., Sozzani, S., Altruda, F., Mantovani, A., and Hirsch, E. (2000). Lipids on the move: phosphoinositide 3-kinases in leukocyte function. *Immunol Today* 21, 260-264.

Experimental plan:

The project will be divided in three workpackages to be developed partly in Torino and partly at the Weizmann Institute.

1) Identification of the mechanisms of chemokine-induced cell migration

1.1) Study of the in vivo and in vitro effects of the expression of a PI3K γ mutant with a constitutive enzymatic activity (Torino).

1.2) Study of the role of PI3K γ mutations in the ability of different types of leukocytes to translate G-protein stimulatory signals into the induction of firm integrin-mediated adhesion to the endothelial wall (Weizmann).

2) Definition of the kinase independent effects of PI3K γ .

2.1) Analysis of the PI3K γ containing signalosome in leukocytes and cardiomyocytes by proteomic analysis (Torino).

2.2) Genetic and bio-informatic analysis of the PI3K γ interactome (Torino-Weizmann).

2.3) Novel functions of PI3K γ and DOCK2 in chemoattractant triggered vascular adhesion, motility and inflammatory responses (Weizmann).

3) Definition of the role of PI3K γ in cardiovascular disease models

3.1) Study of the influx of the inflammatory reaction in cardiac hypertrophy (Torino).

3.2) Study of the effects of the absence of PI3K γ in acute myocardial infarction and organ ischemia/reperfusion (Torino).

3.3) Study of the effects of PI3K γ mutation in angiogenesis and neovascularization (Torino-Weizman).

Coordination and Management

For the management of this project, a special effort will be made to optimize coordination. Activities will be periodically evaluated in meetings of group leaders, students and staff scientists directly involved in the experimental work that will take place every six months. At each meeting, the overall state of the project will be evaluated and advancements will be assessed. Discussion of eventual bottlenecks or unexpected difficulties will help to keep the development of the project within the proposed tracks. At the same time, results from the two units will be integrated and working hypotheses as well as mechanistic models conjunctly elaborated. The two leaders of the partner groups, although not sharing a recent history of collaboration, know each other since long and this will further assure an open and trustworthy discussion.

In addition to these periodical meetings, the two groups will be in constant communication by traditional means. A simple user restricted web site will be organized to facilitate exchange of results and techniques between the involved researchers.

Particular attention will be dedicated to promote exchange of students between the two labs, and with greater emphasis to promote training programs of Italian young researchers in the Weizman Institute.. In specific circumstances, selected issues will thus be addressed by transfer of technology and expertise between the two groups. For example, the Hirsch group will help to establish a colony of PI3K γ mutant mice in Israel by providing all the genotyping techniques required. A new research center focused on Inflammation Research: In Health, Disease, and Therapy has been recently set up in the Weizmann Institute. The Alon lab will introduce these mice to other groups in his department as well as to scientist members of this new center.

Special attention will be paid to the compilation of the common publications: after the definition of initial drafts, the finalization will be carried out in small group meetings specifically aimed at stimulating the integration of results.

The intellectual and industrial property rights resulting from this joint research program will be the co-ownership of WIS (45%), Genetics, Biology and Biochemistry of Torino University (45%) and Fondazione De Benedetti (10%). Each party shall be solely responsible for distributing to its respective investors any share of net revenues in accordance with its respective patent policy. In case of co-owned patent neither party will act as regards exploitation or grant of licenses without the consent of the other party, nor will act in a way able of endangering the validity of the co-owned patents.

Budget

Each core group requests a PhD student and a Post-Doc with a salary adequate to local custom.

A total of 125.000 Euros/year for both groups is requested to cover research costs such as consumables, animal care, laboratory maintenance. This money will be equally shared by the two groups.

Funding of the Israeli partner will consist of the following: About 60% (37.500 Euros/year) of the costs will be funded by Weizmann internal sources; 40% of the costs (25.000 Euros/year) will be funded through the "Fondazione De Benedetti – Cherasco 1547 Onlus".

Funding of the Torino partner will come from local institutions and banks.

A total of 25.000 Euros/year is requested for young students from Torino to the Weizman Institute. Together with this, the Weizman internal funds will have to cover 50% of the total cost.

Unit Ronen Alon:

As a start up for the program, an initial sum of 25.000 Euros/year for 3 years will be provided by the “Fondazione De Benedetti – Cherasco 1547 Onlus”. This will cover the following items to be of essential use within the project:

Consumables:	
General and cell culture material: <i>plastic, glass ware, chemicals, pipettes & tips, sterile plastic for cell culture & misc. materials.</i>	10.000
Antibodies: <i>1st, 2nd, labelled antibodies for facs analysis, antibodies anti phosphoproteins, other antibodies.</i>	8000
Cell culture: <i>cell culture media, FCS.</i>	5000
Reagents for molecular biology Genotyping, PCR, protein analysis): <i>DNA purification and manipulation (e.g cartridges or Maxi preps), restriction enzymes, DNA modification enzymes, DNA labeling kits, HM Agarose, Taq-polymerase, PAGE, membranes, dNTPs, Mw standards (DNA and protein), DNA sequencing kit.</i>	7000
Radioactive tracers: <i>³²PαATP for Nucleic acid labeling and kinase assays</i>	4200
Oligonucleotides: <i>PCR, mutagenesis, PCR screening of mice</i>	2500
Growth factors and agonists	1500
Chemicals: <i>ficoll, percoll, drugs.</i>	2000
Glassware: <i>general equipment</i>	800
Equipment:	
Computers	2500
Microscopy meterial	6000
Lab instrumentation to be specified	18000
Others:	
Publication costs:	800
Meeting fees and travel expenses:	2500
Ordinary maintenance of equipment:	4200
Sum of 3-year expenses:	75000

Unit Hirsch:

As a start up for the program, an initial sum of 25000 Euros/year for 3 years will be provided. This will cover the following items to be of essential use within the project:

Consumables:	
Ordinary maintenance of the animal facility: <i>food, straw, cages, lids, bottles.</i>	10000
General and cell culture material: <i>plastic, glass ware, chemicals, pipettes & tips, sterile plastic for cell culture & misc. materials.</i>	7500
Antibodies: <i>1st, 2nd, labelled antibodies for facs analysis, antibodies anti phosphoproteins, other antibodies.</i>	5000
Cell culture, embryo culture and microinjection: <i>cell culture media, FCS, LIF.</i>	2500
Reagents for molecular biology Genotyping, PCR, protein analysis): <i>DNA purification and manipulation (e.g cartridges or Maxi preps), restriction enzymes, DNA modification enzymes, DNA labeling kits, HM Agarose, Taq-polymerase, PAGE, membranes, dNTPs, Mw standards (DNA and protein), DNA sequencing kit.</i>	7000
Cell proliferation and apoptosis: <i>kits.</i>	1700
Radioactive tracers: <i>³²PαATP for Nucleic acid labeling and kinase assays</i>	4200
Reagents for histology: <i>Solvents, plasticware, blades, staining solutions</i>	2500
Oligonucleotides: <i>PCR, mutagenesis, PCR screening of mice</i>	2500
Growth factors and agonists	800
Chemicals: <i>PtdIns(4,5)P2, others</i>	800
Glassware: <i>general equipment</i>	800
Equipment:	
Computers	2500
Microfuge	1700
Microspectrofotometer for molecular biology	10000
Lab instrumentation to be specified	8000
Others:	
Publication costs:	800
Meeting fees and travel expenses:	2500
Ordinary maintenance of equipment:	4200
Sum of 3-year expenses:	75000

Proponents CV and Selected Publication Record

Emilio Hirsch (Born in Torino, 22nd July, 1965):

A. Education

1988	Turin; Italy	University of Turin	Graduation	Biology
1994	Turin; Italy	University of Turin	Ph.D.	Human Biology: molecular and Cellular Ba
1995-1997	Martinsried (Munich), Germany	Max Planck-Institute for Biochemistry	Post-doctoral training	Cell Adhesion and Migration; Gene Targeting

B. Positions and Honors.

March-June 1989	Abano Terme (Padova), Italy	Fidia	Research Fellow	Recombinant protein product
July-November 1989	Paris, France	Laboratoire de Biochimie Genetique; Hopital Necker	Research Fellow	Transgenic mouse production
1995-2000	Torino, Italy	University of Turin, School of Medicine, Department of Genetics, Biology : Biochemistry	Assistant Professor (Ricercatore Universitario)	Transgenic mice; cell adhesion and migration
1995-1997	Martinsried (Munich), Germany	Max Planck-Institute for Biochemistry	Post-doctoral fellow	Cell Adhesion and Migration;
2000-2005	Torino, Italy	University of Turin, School of Medicine, Department of Genetics, Biology : Biochemistry	Associate Professor (Professore Associato)	Gene Targeting; cell adhesion and migration; GPCR signaling
2002-2005-			Member of the editorial board of Thrombosis and Haemostasis (IF 2003: 4.95)	
	Torino, Italy	University of Turin, School of Medicine, Department of Genetics, Biology : Biochemistry	Full Professor (Professore Straordinario)	Cell migration; GPCR signalling and cardiac physiopathology

C. Selected Publications

1. Hirsch E, Iglesias A, Potocnik AJ, Hartmann U, Fassler R. 1996. Impaired migration but not differentiation of haematopoietic stem cells in the absence of beta1 integrins. **Nature** 380:171-175.
2. Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, Sozzani S, Mantovani A, Altruda F, Wymann MP. 2000. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. **Science** 287:1049-1053.
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7. Brancaccio M, Fratta L, Notte A, Hirsch E, Poulet R, Guazzone S, De Acetis M, Vecchione C, Marino G, Altruda F, Silengo L, Tarone G, Lembo G. 2003. Melusin, a muscle-specific integrin beta1-interacting protein, is required to prevent cardiac failure in response to chronic pressure overload. **Nature Medicine** 9:68-75.
8. Patrucco E, Notte A, Barberis L, Selvetella G, Maffei A, Brancaccio M, Marengo S, Russo G, Azzolino O, Rybalkin SD, Silengo L, Altruda F, Wetzker R, Wymann MP, Lembo G, Hirsch E. 2004. PI3Kgamma modulates the cardiac response to chronic pressure overload by distinct kinase-dependent and -independent effects. **Cell** 118:375-387.
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Ronen Alon (Born in Tel Aviv, 1961):

A. Education

- 1987 Tel-Aviv University, Tel-Aviv, Israel B.Sc. Chemistry
1988 Tel-Aviv University, Tel-Aviv, Israel M.Sc. Biochemistry
1993 Weizmann Institute of Science, Rehovot, Israel Ph.D. Biophysics
1993-1996 Harvard Medical School, Boston, MA Post. Doc. Pathology

A. Positions and Honors.

Positions and Employment

- 1996 - Senior Investigator, Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel
2003- Associate Professor with tenure, Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel

Other Experience and Professional Memberships

- 2001- Board Member, Israeli Immunological Society
2002-2003 Advisory Board Member, Biokine Therapeutics, Rehovot, Israel
2004-2008 Coordinator of the Weizmann Institute subnetwork of MAIN, an EU funded network of excellence on Migration in Inflammation (R. Pardi, head)
2006-Vice-President of the Israeli Immunological Society

Honors

- 1993 EMBO Long Term Research Fellowship
1995 Dorot Foundation Research Fellowship
1995 Alon Fellowship
1997 Jakubskind-Cymerman Award
1998 Incumbent of the Tauro Career Development Chair in Biomedical Research
2004 Christian Crone Travel Award
2006-The Linda Jacobs Chair in Stem Cells and Immunity

B. Selected Publications.

1. Cinamon, G., Shinder, V. and Alon, R. (2001) Wall shear forces promote lymphocyte migration across inflamed vascular endothelium presenting apical chemokines. **Nature Immunology** 2, 515-522.
2. Dwir, O., Kansas, G.S., and Alon, R. (2001) Cytoplasmic anchorage of L-selectin controls leukocyte capture and rolling by increasing the mechanical stability of the selectin tether. **J. Cell. Biol.** 155, 145-156.
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5. Kinashi, T., Aker, M., Sokolovsky-Eisenberg, M., Grabovsky, V., Tanaka, C., Shamri, R., Feigelson, S., Etzioni, A. and Alon, R. (2004) LAD-III, a leukocyte adhesion deficiency syndrome associated with defective Rap1 activation and impaired stabilization of integrin bonds. **Blood**, 103, 1033-1036.

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8. Alon, R., Feigelson, S., Manevich, E., Rose, D.M., Schmitz, J., Overby, D.R., Winter, E., Grabovsky, V., Shinder, V., Matthews, B.D., Sokolovsky-Eisenberg, M., Ingber, D.E., Benoit, M., and Ginsberg, M.H. (2005) α 4 β 1-dependent adhesion strengthening under mechanical strain is regulated by paxillin association with the α 4 cytoplasmic domain **J. Cell. Biol.** 171, 1073-1084.
9. Luster, A.D., Alon, R. and von-Andrian, U.H. (2005) Immune cell migration in inflammation: Present and future therapeutic targets. **Nature Immunology.** 6, 1182-1190.
10. Shulman, Z. Pasvolsky, R., Woolf, E. , Grabovsky, V. Feigelson, S.W., Erez, N., Fukui, Y., and Alon, R. DOCK2 regulates chemokine-triggered lateral lymphocyte motility but not transendothelial migration. **Blood** (in press).

On behalf of:

University of Torino, School of Biotechnology:
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Signature: Date:

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