Università degli Studi del Piemonte Orientale "Amedeo Avogadro"
Dottorato di Ricerca in Medicina Molecolare

Research activity report
Dr. Alfredo Amoroso

1. Scientific activity

1.1 - 1st Year

The scientific activity of the first year has been developed at the Department of Bio-medical Sciences, University of Catania, in collaboration with Department of Internal Medicine of P.O. "G. Rodolico", Azienda Ospedaliero-Universitaria "Policlinico-Vittorio Emanuele", Catania, Italy.
The aim of the study was to investigate on the role of CD4+CD25+ regulatory T cells in HCV-related liver disease. The study has been eventually published on "International Journal of Molecular Medicine".

Evaluation of circulating $CD4^+CD25^+$ and liver-infiltrating $Foxp3^+$ cells in HCV-related hepatitis

HCV-related chronic hepatitis is a chronic liver inflammation where the immune system is unable to efficiently clear the viral infection. Although viral factors are certainly involved, several pathophisiological alterations have also been claimed to be involved in the complex pathogenesis of this disease. One possibility is that the naturally tolerogenic microenvironment of the liver favours development of lymphocyte subsets not exerting the appropriate effector functions needed for virus clearance (Mengshol et al., 2007; Racanelli and Manigold, 2007).
Recent studies have raised the possibility that the chronic evolution of the HCV infection may involve *regulatory T cells* (Tregs) (Cabrera et al., 2004), exerting suppressive function on the anti-viral effector immune cells. Tregs have been shown to exhibit a relevant heterogeneity in their differentiation patterns, mechanisms of action, tissue distribution as well as phenotype presentation (Shevach, 2006; Taylor et al., 2006; Wilczynski et al., 2008). However, the main Treg subset is believed to be comprised in the $CD4^+$ T cell subset expressing high levels of CD25 ($CD25^{hi}$) and the transcription factor Foxp3 (*natural* Tregs). HCV-specific $CD4^+$ cells displaying this Treg phenotype have been identified in HCV-related chronic hepatitis (Ebinuma et al., 2008) and the proportions of Tregs have been correlated to the viral load in the PB. Therefore, these Tregs have been suggested to have a deleterious effect on the anti-viral immune response impairing the ability to clear the infection and favouring the development of chronic hepatitis. In line with this possibility, HCV-specific Tregs have been shown to develop during the infection and to suppress the anti-viral cytotoxic $CD8^+$ cell response. However, the real impact of these cells on the disease's out-come is still debated (Dolganiuc and Szabo, 2008; Manigold and Racanelli, 2007).
Aim of this study was to analyse Treg and activated/effector cells in the peripheral blood (PB), and Treg cells in liver biopsies of patients with HCV-related chronic hepatitis and correlate these data with key disease parameters such as viremia, transaminase levels and histological activity, in order to provide further information about the role of these cells in this infection. Results showed that Tregs infiltrating the liver correlate with high levels of activated/effector cells in the PB and lower serum transaminase level, suggesting that these cells play an effective

role in modulating the immune response to HCV while limiting damage to the liver.
Our results show higher proportions of $CD4^+CD25^{low}$ and IFN-γ^+ cells in the patients. By contrast, the proportions of peripheral $CD4^+CD25^{hi}$ cells did not significantly differ. The eleven patients displaying $Foxp3^+$ cells in the liver infiltrates showed significantly higher proportions of peripheral $CD4^+CD25^{low}$ cells. Moreover, we found lower serum transaminase levels in the patients with $Foxp3^+$ immunohistochemistry, although only for ALT such difference resulted statistically significant. In conclusion, these data suggest that the presence of Tregs infiltrating the liver is associated with high levels of activated/effector T cells in the peripheral blood and lower activity of hepatitis. Therefore, liver infiltrating Tregs might play a role in limiting tissue damage and support an effective immune response against HCV.

1.2 - 2nd Year

The scientific activity of the second year can be differentiated into four fields of interest: manuscript revision, analysis of hospital activity data in the field of transfusion medicine (both diagnostic laboratory and blood donation activities), research laboratory (2 activities).

1.2.1 Manuscript revision

A partial revision of the data of the manuscript *Evaluation of circulating CD4+CD25+ and liver-infiltrating Foxp3+ cells in HCV-associated liver disease*, has been necessary prior to publication. The manuscript has been eventually published on *International Journal of Molecular Medicine* (see publication list below).

1.2.2 Transfusion medicine and other clinical activity

My activity at the Immunohematology and Transfusion Medicine Service at Ulss5, in Vicenza, has led to two significant reports, among which one has been published as an abstract to the SIMTI's *40° Convegno Nazionale di Studi di Medicina Trasfusionale*, and reports on a Neonatal Haemolytic Disease case from the rare form of AB0 incompatibility in an African newborn; the second report is still under compilation and deals with the barriers that prevent or discourage extra-European immigrants from blood donation.
Another report has been published about an atypical case od cryoglobulinemia, in collaboration with the rheumatology unit of Ospedale Cannizzaro, Catania.

1.2.3 Research laboratory at the University of Catania

The activity at the University of Catania has been focused on the role of the molecule CD155 (Nectin-like 5 or Polio Virus Rreceptor [PVR]) in the skin microenvironment. For this purpose, different biological materials have been analyzed, such as stabilized melanoma cell lines, stabilized cheratinocytes, and different kinds of skin biopsies like melanoma biopsies or nevi biopsies. Analysis performed has been flow cytometry on the cell lines, immunohistochemistry, Reverse Transcriptase PCR and eventually Micro-Array in all the samples. The results have been published on *Oncotarget* journal.

Nectin like -5 overexpression correlates with the malignant phenotype in cutaneous melanoma

NECL-5 is involved in regulating cell–cell junctions, in cooperation with cadherins, integrins and platelet-derived growth factor receptor, that are essential for intercellular communication. Its role in malignant transformation was previously described. It has been reported that transformation of melanocytes is associated with altered expression of adhesion molecules suggesting the potential involment of NECL-5 in melanoma development and prognosis. To shed light on this issue, the expression and the role of NECL-5 in melanoma tissues was investigated by bioinformatic and molecular approaches. NECL-5 was up-regulated both at the mRNA and the protein levels in WM35, M14 and A375 cell lines compared with normal melanocytes. A subsequent analysis in primary and metastatic melanoma specimens confirmed “in vitro” findings. NECL-5 overexpression was observed in 53 of 59 (89.8%) and 12 of 12 (100%), primary melanoma and melanoma metastasis, respectively; while, low expression of NECL-5 was detected in 12 of 20 (60%) benign nevi. A significant correlation of NECL-5 overexpression was observed with most of known negative melanoma prognostic factors, including lymph-node involvement ($P = 0.009$) and thickness ($P = 0.004$). Intriguingly, by analyzing the large series of melanoma samples in the Xu dataset, we identified the transcription factor YY1 among genes positively correlated with NECL-5 ($r = 0.5$). The concordant computational and experimental data of the present study indicate that the extent of NECL-5 expression correlates with melanoma progression.

1.4 Research laboratory at the Laboratory of Advanced Cellular Therapies (LTCA), Vicenza

At the end of the second year, I started a new collaboration with the Laboratory of Advanced Cellular Therapies (LTCA), Vicenza. *Laboratorio di Terapie Cellulari Avanzate* (LTCA), part of the haematology department of San Bortolo Hospital, in Vicenza, is a new facility on its way to obtain the accreditation for the advanced cellular manipulation and for the production of Advanced Therapy Medicinal Products (ATMP) under GMP quality standards. Presently, LTCA is already working at GLP quality level and is carrying on different research progects in the field of immunology and hematology.
My progect at LTCA has been developed during the third year, mainly focusing on the preclinical development of adoptive T cell-based immunotherapeutic protocols for the prevention and the early treatment of tumoral relapses and opportunistic infections in onco-hematological patients after HSCT.

1.3 - 3rd Year

The scientific and research activity of the third year has been entirely developed at the Laboratory of Advanced Cellular Therapies (LTCA), Vicenza.
The *Laboratorio di Terapie Cellulari Avanzate* (LTCA), part of the haematology department of San Bortolo Hospital, in Vicenza, is a new facility on its way to obtain the accreditation for the advanced cellular manipulation and for the production of Advanced Therapy Medicinal Products (ATMP) under GMP quality standards.. Presently, LTCA is already working at GLP quality level and is carrying on different research progects in the field of immunology and hematology.
My activity at LTCA focused on the optimization and preclinical development of adoptive cell-based immunotherapeutic protocols for the prevention and the early treatment of tumoral relapses and opportunistic infections in onco-hematological patients after HSCT. Results will be discussed in the final thesis.

1.3.1 Introduction

Adoptive transfer of lymphocytes with anticancer properties has the potential to break the tolerance to tumour antigens and to generate high avidity effector T-cells. Cytokine-induced killer cells (CIK) are obtained ex-vivo by culturing for 21 days peripheral blood mononuclear cells (PBMC) obtained by leucapheresis or by density gradient separation from whole blood, in a interferon gamma (IFNgamma), interleukin-2 (IL-2) and anti-CD3 monoclonal-antibody (mAb) enriched medium, as firstly described by Negrin (2001).
CIK have demonstrated an effective antitumor potential both in vitro and in vivo against solid tumours and haematological malignancies. Bulk CIK cells can be described as an heterogeneous population constituted of a majority of cells with a CD3+CD56+ phenotype (NKT) and a minor fraction of NK (CD3-CD56+) and T lymphocytes (CD3+CD56-). Compared to the so called "lymphokine-activated killer cells", the use of CIK offers advantages because of their better in vivo activity without the need of exogenous administration of IL-2.
Among the bulk of CIK cell population, significante functional other than phenotypical differences can be observed. CD3+CD56- cells display a limited cytotoxic activity if compared to that of CD3+ CD56+ and CD3-CD56+ cells. On the other side, these last two populations show a low proliferative potential, thus making their isolated use unfeasible, as theu would not grant a sufficient long term immunity. However, it is known that CD3+CD56+ cells in the CIK bulk derive from the CD3+CD56-CD8+ cytotoxic T cells (CTL), acquiring N-CAM (CD56) surface molecule during the culture. This polulation, though displaying limited cytotoxic activity when compared to the other mentioned populations, show great proliferative potential. Therefore, they could represent the population granting the long term immunity once infused to patients.
The main limitation preventing the successful clinical translation of several adoptive immunotherapy strategies including CIK is the obtainment of sufficient numbers of anti-tumor immune effectors cells and their in-vivo persistence. The antitumor activity of CIK seems to be associated with the CD3+CD56+ subset which has an "in vitro" fold expansion that varies from few to more than 1000 folds. The reason of this variability is unclear and additional strategies are currently under investigation to improve expansion rates especially in "poor expander" patients. The percentage of CIK CD3+CD56+ cells at the end of expansion is critical in predicting the efficacy of the therapy "in vivo", and constitutes an essential criteria for batch release. Recent data show that NKT (CD3+CD56+) cells reach the highest concentration in culture from day 17 to day 21, whereas a delay from one to a few days in cell harvesting causes a dramatic drop of their concentration. Understanding the CIK growth dynamics could provide significant insight into the limiting steps involved in cell expansion and differentiation. Recent studies of multivariate analysis have shown as it is possible to identify critical "check-points" during the in-vitro expansion, useful to predict the final outcome of the CIK culture. Further efforts seem therefore necessary to optimize the expansion procedures.
Interesting studies have focused on the other populations copresent in the initial PBMC culture, like neutrophils and monocytes. Activated neutrophils have been shown to be able to increase cytotoxic activity of both NK and T cells. The role of monocytes is, on the other hand, still controversial.
Other studies point out how diffeneces in the isolation procedures might be responsible for significant functional differences in the final cellular product.
Present issues are then whether these population, being incidentally present in the initial PBMC culture as residual contaminants, can influence the final outcome of CIK expansion, and whether different qualities of the reagents used for gradient separation can make a significant difference.
The aim of my study was to evaluate the impact and relevance with regard to cell recovery, expansion capacity, phenotypical and functional features of CIK cells cultured from PBMC obtained from different density gradient separation reagents.

1.3.2 Materials and Methods

Sample collection
Samples have been obtained by buffy-coats of whole blood donations from healthy blood donors.
Isolation of PBMC
PBMC have bee obtained by density gradient centrifugation of buffy-coats by using four different reagents: GE-Healthcare Ficoll-Paque PLUS, Sigma-Aldrich Histopaque®-1077 Hybri-Max™, Biochrom Biocoll, Axis-Shield Lymphoprep.
Cell culture
CIK expansion has been obtained by using Negrin's protocol (see references).
Cell count
Cell counts have been performed by optical microscope count on Bürker chamber by using trypan blue staining at days 3, 7, 10, 14, 17, 21.
Phenotypical analysis
Phenotypical analyisis have been performed by flow cytometry, by using the following antibodies: CD3, CD4, CD8, CD56, CD19, CD14, CD33, CD45. Analysis were performed at days 3, 7, 10, 14, 17, 21.
Cytotoxicity assay
Cytotoxicity assays have been performed by using calcein-release assay, at days 7, 14, 21.
Statistical analysis
Statistical analysis have been performed by using ANOVA test by Prism 5 software (Graphpad, San Diego, CA), and by Turkey's post-test for multiple couple comparisons. $p<0,05$ was considered significant.

1.3.3 References

- **Biol Blood Marrow Transplant**. 2001;7(4):216-22. *Expansion of cytotoxic CD3+ CD56+ cells from peripheral blood progenitor cells of patients undergoing autologous hematopoietic cell transplantation*. Alvarnas JC, Linn YC, Hope EG, Negrin R.
- **Expert Opin Biol Ther**. 2009 Jul;9(7):831-40. *Cytokine induced killer cells as adoptive immunotherapy strategy to augment graft versus tumor after hematopoietic cell transplantation*. Sangiolo D, Mesiano G, Carnevale-Schianca F, Piacibello W, Aglietta M, Cignetti A.
- **J Hematother Stem Cell Res**. 2002 Apr;11(2):265-76. *Donor lymphocyte infusion: the use of alloreactive and tumor-reactive lymphocytes for immunotherapy of malignant and nonmalignant diseases in conjunction with allogeneic stem cell transplantation*. Slavin S, Morecki S, Weiss L, Or R.
- **Nat Rev Immunol**. 2011 Jul 25;11(8):519-31. doi: 10.1038/nri3024. *Neutrophils in the activation and regulation of innate and adaptive immunity*. Mantovani A, Cassatella MA, Costantini C, Jaillon S.
- **J Exp Med**. 2012 Mar 12;209(3):565-80. doi: 10.1084/jem.20111908. Epub 2012 Mar 5. *Neutrophil depletion impairs natural killer cell maturation, function, and homeostasis*. Jaeger BN, Donadieu J, Cognet C, Bernat C, Ordoñez-Rueda D, Barlogis V, Mahlaoui N, Fenis A, Narni-Mancinelli E, Beaupain B, Bellanné-Chantelot C, Bajénoff M, Malissen B, Malissen M, Vivier E, Ugolini S.
- **Blood**. 2011 Feb 3;117(5):1677-86. doi: 10.1182/blood-2010-06-287243. Epub 2010 Nov 22. *Human neutrophils interact with both 6-sulfo LacNAc+ DC and NK cells to amplify NK-derived IFN{gamma}: role of CD18, ICAM-1, and ICAM-3*. Costantini C, Calzetti F, Perbellini O, Micheletti A, Scarponi C, Lonardi S, Pelletier M, Schakel K, Pizzolo G, Facchetti F, Vermi W, Albanesi C, Cassatella MA.
- **Haematologica**. 2011 Oct;96(10):1543-7. doi: 10.3324/haematol.2011.044578. Epub 2011 Jun 28. *On the potential involvement of CD11d in co-stimulating the production of*

interferon-γ by natural killer cells upon interaction with neutrophils via intercellular adhesion molecule-3. Costantini C, Micheletti A, Calzetti F, Perbellini O, Tamassia N, Albanesi C, Vermi W, Cassatella MA.

- **Immunol**. 2012 Apr 1;188(7):3150-9. doi: 10.4049/jimmunol.1103414. Epub 2012 Feb 20. *T lymphocyte priming by neutrophil extracellular traps links innate and adaptive immune responses*. Tillack K, Breiden P, Martin R, Sospedra M.
- **Blood**. 1992 Nov 1;80(9):2221-9. *Role of monocytes in the expansion of human activated natural killer cells*. Miller JS, Oelkers S, Verfaillie C, McGlave P.
- **J Immunother**. 1998 Nov;21(6):409-17. *IL-2 expansion of T and NK cells from growth factor-mobilized peripheral blood stem cell products: monocyte inhibition*. Ageitos AG, Singh RK, Ino K, Ozerol I, Tarantolo S, Reed EK, Talmadge JE.
- **Eur Heart J**. 2007 Mar;28(6):766-72. Epub 2007 Feb 13. *Cell isolation procedures matter: a comparison of different isolation protocols of bone marrow mononuclear cells used for cell therapy in patients with acute myocardial infarction*. Seeger FH, Tonn T, Krzossok N, Zeiher AM, Dimmeler S.
- **Cytometry B Clin Cytom**. 2013 Sep 3. doi: 10.1002/cyto.b.21124. *Multivariate statistical data analysis as a tool to analyze ex vivo expansion dynamics of cytokine-induced killer cells*. Zanon C, Stocchero M, Albiero E, Castegnaro S, Chieregato K, Madeo D, Rodeghiero F, Astori G.

2. Publications

- Amoroso A, D'Amico F, Consolo M, Skarmoutsou E, Neri S, Dianzani U, Spandidos DA, Mazzarino MC. *Evaluation of circulating CD4+CD25+ and liver-infiltrating Foxp3+ cells in HCV-associated liver disease*. **Int J Mol Med**. 2012 Jun;29(6):983-8. doi: 10.3892/ijmm.2012.947. Epub 2012 Mar 22.
- Bevelacqua V, Bevelacqua Y, Candido S, Skarmoutsou E, Amoroso A, Guarneri C, Strazzanti A, Gangemi P, Mazzarino MC, D'Amico F, McCubrey JA, Libra M, Malaponte G. *Nectin like-5 overexpression correlates with the malignant phenotype in cutaneous melanoma*. **Oncotarget**. 2012 Aug;3(8):882-92.
- Consolo M, Amoroso A, D'Amico G, La Rosa L, Vinci M. *Atypical onset cryoglobulinemia: case report*. **Clin Ter**. 2012 May;163(3):223-225.
- La Raja M, Amoroso A, Micciolo R, Monastero A, Dalla Valle C, Schieven E. *MEN grave da incompatibilità AB0 in neonato di origini africane*. 40° Convegno Nazionale di Studi di Medicina Trasfusionale; 2012.

3. Conferences and seminars

- ***GIC**: Scuola Nazionale di Citometria – Corso di Immunologia*. Urbino, September, 29th – October, 2nd, 2010.
- *New Aspects on Autoimmunity and Autoinflammation*. Prof. Klaus Bendtzen, Institute for Inflammation Research, Rigshospitalet National University Hospital, Copenhagen, Denmark. Catania, November, 16th, 2010.
- *Modulation of the Immune System: Treatment Options and new Developments*. Dr. Chris Rundfeldt, Consulting service for preclinical development / Translational Medicine, Magdeburg, Germany. Catania, April, 6th, 2011.
- **SICiCS**: *La citofluorimetria nel percorso decisionale clinico della medicina di laboratorio*. Parma, November, 15th, 2011.
- *Citometria di 2° livello: applicazioni avanzate di diagnostica e ricerca* – Corso di perfezionamento universitario. **Università degli Studi di Milano**; 19-23 marzo 2012.
- **Myltenyi Biotec** webinar: *Flow cytometric quality control of CliniMACS® TCRα/β/CD19 depleted cell products*. November 16th, 2012.
- **TerumoBCT**: *Corso addestramento Trima Accel SW 5.1*. Montecchio Maggiore (VI), April 9th, 2013.
- **SIMTI**: *III Conferenza Nazionale dei Servizi Trasfusionali*. Genova, May 16th-18th, 2013.