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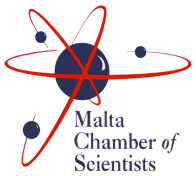

Research Article

The re-emergence of the B1 cell compartment: Is this a pre-lymphoma stage?

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Abstract. Chronic Lymphocytic Leukemia (CLL) are in some cases stereotyped for immunoglobulin variants in different populations, suggesting emergence of B cell subsets following presentation of the same antigen. CLL cells may originate from $CD5^+$ naïve cells and from CD5 memory cells. Gene expression studies characterized a common cell of origin of the two clinical categories of CLL; the unmutated aggressive type and the mutated indolent type. The aim of this study was to investigate the presence of CD5 positive B cells in the elderly and their potential stimulation with exosomes derived from tumor cells. The findings from this study is aimed to create a model to identify instigating carcinomatous factors that may stimulate B1 cells to transform into a CLL-like model.

In this study we show that $CD19^+$ cells (B cells) in cord blood have a high expression of CD5. CD19/CD5 staining of blood samples from senior citizens showed the presence of B cells which also express the CD5 marker, though at a lower expression when compared to CLL cells ($CD19^+/CD5^{dim}$ B cells). Measurement of clonality using λ/κ flow cytometry staining show a monoclonal origin of the human $CD19^+/CD5^{dim}$B cells. Monoclonal B cell Lymphocytosis in the elderly is a potential cell compartment that represents the origin of B cell proliferative disorders. The origin of the B cell proliferative disease requires antigen stimulation. A preliminary experiment showed that sorted lymphocytes can be stimulated by exosomes isolated from 2 cancer cells lines, A549 (lung epithelial) and PC3 (prostate cell line). In comparison with phytohaemagglutinin (PHA) and phorbolmyristate acetate (PMA), known lymphocyte stimulators, the exosomes stimulated the proliferation of monocytic-like cells. Further characterization is required to know the origin of these cells. The result shows that one can speculate that exosomes present cancer-derived antigens and stimulate cell proliferation. Further studies are required to evaluate the potential transformation capacity of cancer-derived exosomes. In addition, various cytokines were measured in the sera of senior citizens to investigate a differential release of cytokines in the presence or absence of the $CD19^+/CD5^{dim}$ B cells. Cytokines examined were not significantly different between the 2 groups and further evaluation of cytokine levels is required.

Keywords: $CD5^+$ B cells, exosomes, CLL, lymphocytosis

1 Introduction

Development of lymphoproliferative disorders have been studied extensively, with a number of observations that elicit interesting mechanisms that are still not fully understood, such as the origin of subpopulations of B cells expressing the CD5 marker. Lymphomas are lineage specific and proliferate and transform into the specific sub-types reflecting the cell of origin (Caligaris-Cappio & Hamblin, 1999; Stevenson & Caligaris-Cappio, 2004). Viral infections are associated with transformation events in lymphomas, such as Epstein-Barr virus associated lymphoproliferative disorders (Rezk & Weiss, 2007) and some strains of *Helicobacter pylori* causing stomach maltomas and lymphomas. Treatment targeting the infection result in complete remission in most of the cases, suggesting that the lymphoproliferative disorder is driven by antigen presentation originating from the infective mechanism (Morgner et al., 2001). Activation of B cells using supernatant of mast cells, identified the role of exosomes as peptide presenting cells, resulting in blast formation,

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proliferation and IgM production, independent of cell-to-cell interactions (Skokos et al., 2001). This supports the role of antigens to stimulate, activate and transform B cells, and prompted us to use exosomes as vehicles for tumour derived antigen presentation in this study.

Of interest, CLL and mantle cell lymphomas are characterised by a subset of B cells that express the CD5 marker (Dono, Cerruti & Zupo, 2004). CD5 positive B cells constitute 80% of B cells in the human newborn decreasing to 10% of B cells in the adult human peripheral blood (Dalloul, 2009). The CD5 molecule is mainly associated with a repressive signal (Soldevila, Raman & Lozano, 2011), indicating that CD5 positive B cells are under tight proliferative control.

Various studies indicate that B cell stereotypic subsets (Damle et al., 2002; Stamatopoulos et al., 2007) might emerge from the presentation of antigens. Our hypothesis is that these stereotypic subsets might emerge from co-occurrence with solid tumours and the antigenic repertoire presented by apoptotic cancer cells. This is supported by the fact that antibodies produced by CLL cells can bind to cancer neoantigens from epidermoid cells, melanoma and liver cancer antigens (Chu et al., 2008, 2010) and that apoptotic cancer cells secrete exosomes (Fevrier & Raposo, 2004) into the microenvironment eliciting an immune response (Delcayre, Shu & Le Pecq, 2005). Immunogenic tumour-derived exosomes potentially stimulate an indolent subset of immune cells, resulting in transformation and clonal expansion as seen in CLL. In addition studies have shown that CLL mAbs recognize and bind the nonmuscle myosin heavy chain IIA (MYHIIA) exposed on the cell surface of a subgroup of apoptotic cells (MEAC) (Chu et al., 2008, 2010). Interestingly, 94% of CLL mAbs binding to MEACs expressed unmutated immunoglobulins, of which 88% belonged to a stereotyped subset.

In this study we identified a subset of B cells expressing low levels of CD5 and utilised cancer cell derived exosomes to study the potential stimulation effect. Further characterisation of these cells is required to allow proper proliferation and expansion. The ultimate goal of this study is to identify instigating carcinomatous factors that may stimulate B1 cells to transform into a CLL-like model.

2 Methods

2.1 Collection of material

50 adults and 20 neonatal blood samples were consented as per ethical approved project (University Research ethics committee, University of Malta). The criteria for selection of the senior citizens included the absence of dementia and cancer. The serum samples were collected, centrifuged and stored in the Malta Biobank together with the consent forms. Mononuclear cells were isolated from whole blood and stained accordingly for the flow cytometry or the magnetic cell sorting as detailed below.

2.2 Flow Cytometry

A cohort of individuals ($n = 50$) over 65 years of age were collected from Karen Grech Geriatric Wards. Following mononuclear cell isolation, the cells were incubated with antibody mixes (from BD Biosciences) consisting of fluorescein isothiocyanate (FITC) conjugated anti-CD5, PerCP-Cy5.5–conjugated anti-CD19, FITC–anti-κ and phycoerythrin (PE)–anti-λ light chain. At least 200,000 events were acquired on a FACS Calibur equipped with a 488 argon ion laser and 635 red diode laser (Becton Dickinson) and analysed with the CellQuest software system (Becton Dickinson). The ratio of κ^+ and λ^+ events was evaluated following gating of $CD19^+$ subsets. The κ/λ ratio was considered abnormal when it was more than 3:1 or less than 1:3, providing information on the monoclonal proliferation of $CD19^+$ B lymphocytes.

2.3 Magnetic cell sorting (MACS)

For stimulation experiments, $CD19^+$ cells from neonatal whole blood were sorted using the EasySep Human CD19 positive selection kit from Stem Cell Technologies, as per manufacturer's instructions. In summary, mononuclear cells were isolated using Ficoll Histopaque (SIGMA), followed by addition of a positive selection antibody cocktail. After mixing and incubation, the magnetic nanoparticles were added. The cell suspension was placed into the magnet for 5 minutes and the tube was inverted to remove the unbound cells. These cells were labelled as $CD19^-$. Following a number of washes, cells were incubated overnight for subsequent experiments.

2.4 Cytokine level measurements

The level of cytokines in serum were measured by a multiplexed method using a Procarta immunoassay (Affymetrix) on a Luminex platform. The serum and antigen standards were prepared according to the protocol. The captured protein was incubated with antibody conjugated beads for 60 minutes. To detect the captured antibody conjugated beads these were incubated with biotinylated detection antibody for 30 minutes. The beads were then incubated with streptavidin-SE (sape) for 30 minutes and the signal from the beads was read on the Luminex instrument.

The filter plate was prepared, including standards, samples and a blank. Antibody polystyrene beads were added with antigen standards and serum samples. Following incubation at room temperature the plate was mixed for 60 minutes at 700 rpm, washed and incubated with the detection antbodies. Following addition of SAPE and washes the fluorescence was read using the

Luminex Analyser.

2.5 Exosome isolation

The prostate cancer cell line, PC3 and the epithelial lung cancer cell line, A549 were cultured and expanded to have at least 1×10^8 cells. The cells were serum deprived for 24 hours and 100 ml of supernatant was harvested from each cell line.

Exosomes were isolated using an optimized protocol kindly provided by Professor F. Cappello (University of Palermo) (Merendino et al., 2010). The supernatant was centrifuged at 2000 revs for 5 minutes to remove the cells and cell debris, and transferred to ultracentrifuge tubes. Following a 2 hour spin at 110,000 g in a ultracentrifuge (RotorT25) the resulting exosome pellet was collected and suspended in 150 µl of PBS. The exosome suspension was stored at 4 °C until use. Figure 1 shows the resulting electron micrograph of the exosomes isolated from the A549 cell line.

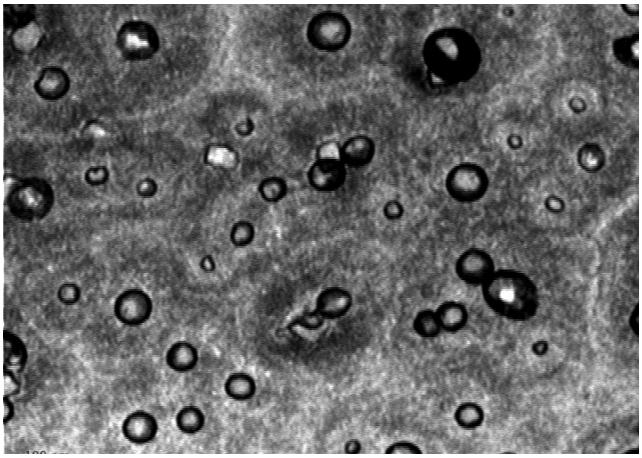

Figure 1: Electron micrograph showing exosomes isolated from A549 cell line. The image was taken at Dipartimento di Biomedicina Sperimentale e Neuroscienze Cliniche, Sez. Anatomia Umana, Università degli Studi di Palermo [Prof. Francesco Cappello].

2.6 Stimulation experiments

The cells from the magnetic sorting preparation were used for stimulation experiments. Both the $CD19^+$ cells and the $CD19^-$ cells were used in this experiment. Stimulation was done using known lymphocyte stimulators, Phytohemagglutinin (PHA) and Phrobolmyristate acetate (PMA) or with exosome suspension isolated from cancer cell lines. To remove macrophages, the cells were incubated overnight and adherent cells were removed by transferring suspension cells to a new culture flask. The cells were divided into separate wells and treated with a final concentration of 50 µg ml^{-1} PHA, 80 nM PMA, and with exosome suspension derived from 2 cancer cell lines (PC3: prostate cell lines; A549: lung cancer cell lines), or left untreated. PHA was added to cells every third day and exosomes once on day 1.

3 Results

3.1 Characterisation of a $CD19^+$ $CD5^{dim}$ B cells subset

The $CD19^+$ fraction of the mononuclear cells derived from neonate cord blood, was positive for CD5 (ROI 1; Figure 2A). Of interest, the cells lacked a CD5 positive ($CD19^-$) T cell fraction. From a cohort of 50 senior citizens, 25 samples were selected on the basis of the number of $CD19^+$ events (> 100 events). In addition to the $CD19^+$ B cells and the $CD5^+$ T cells, immunophenotyping identified a $CD19^+$ $CD5^{dim}$ fraction (Figure 2B). The percentage of B cell subtypes in the cohort are summarised in Table 1. In the age group between 65 and 75 years, less than 20% of total B cells ($CD19^+$) stained for CD5 ($CD19^+$ CD5dim), while other age groups ($n = 22$) showed samples with higher percentages (Figure 3). Of interest, one of the senior citizens samples showed that more than half (57.12%) of the B cells were typed as

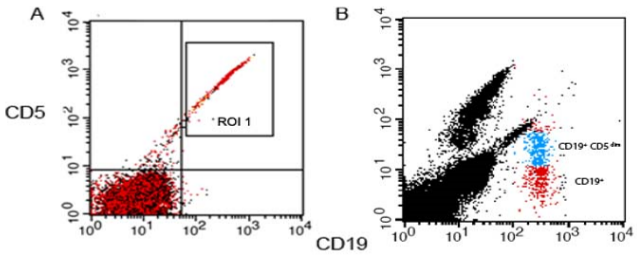

Figure 2: Flow cytometry of stained (CD5 and CD19 antibodies) mononuclear cells derived from (A) neonates and (B) adult (age of 79). The quadrants in (A) were set using isotype controls. [ROI 1 = Region of Interest 1].

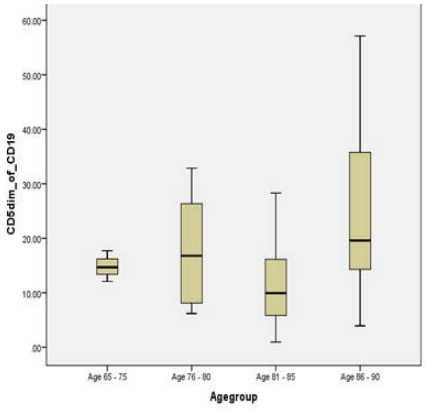

Figure 3: Box plot showing distribution of $CD19^+$ $CD5^{dim}$ percentage across age groups. Age group 65–75 ($n = 3$); age group 76–80 ($n = 8$); age group 81–85 ($n = 7$) and age group 86–90 ($n = 7$).

Table 1: Percentage of B cell subtypes in the senior citizen cohort.

Sample	Age	$CD5^{dim}$ [$CD19^+$ gated]		$CD5^{neg}$ [$CD19^+$ gated]	
		Events	% of $CD19^+$	Events	% of $CD19^+$
A8	87	381	57.12	285	42.73
A16	86	68	40.48	107	63.69
A22	77	68	32.85	168	81.16
A13	90	68	31.05	168	76.71
A21	76	41	30.15	88	64.71
A9	81	73	28.29	180	69.77
A49	79	141	22.56	362	57.92
A3	84	87	21.59	308	76.43
A32	80	39	20.42	140	73.3
A10	90	141	19.58	527	73.19
A42	73	28	17.72	120	75.95
A45	65	64	14.68	352	80.73
A39	87	20	14.39	118	84.89
A6	90	68	14.23	168	35.15
A11	76	50	13.16	289	76.05
A27	65	33	12.09	237	86.81
A14	84	11	10.68	80	77.67
A1	85	30	9.93	271	89.74
A38	85	11	9.91	90	81.08
A28	79	24	8.25	257	88.32
A50	78	39	7.93	377	76.63
A23	77	14	6.19	210	92.92
A44	89	9	3.93	210	91.7
A51	83	3	1.76	156	91.76
A19	83	3	0.95	313	98.74

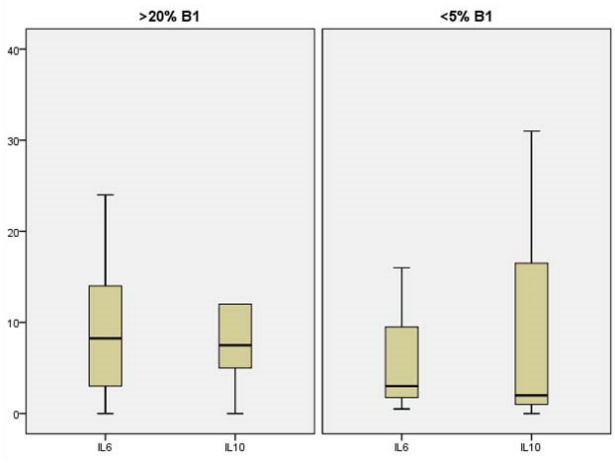

Figure 4: Distribution of IL6 and IL10 levels. The left panel shows the results obtained for individuals which have a $CD19^+/CD5^{dim}$: total $CD19^+$ ratio higher than 0.2 (> 20% B1), while the right panel individuals with a $CD19^+/CD5^{dim}$: total $CD19^+$ ratio lower than 0.05 (< 5% B1). [B1 = $CD19^+/CD5^{dim}$ cells].

$CD19^+$ $CD5^{dim}$. In this sample, flow cytometry using λ/κ staining showed a monoclonal origin of the human $CD19^+/CD5^{dim}$ cells.

3.2 IL6 and IL10 levels are not affected by presence of $CD19^+$ $CD5^{dim}$ cells

Given that the variance between age groups showed an increase of $CD19^+/CD5^{dim}$ cells with older age groups, and that the age group between 65 and 75 were all below 20% of B cells (Figure 3), we used this as a threshold to compare cytokine levels between those individuals with a percentage higher than 20% and individuals in which the $CD19^+/CD5^{dim}$ cells are less than 5% of the total B cells. Both IL6 and IL10 showed a high variance in the different groups and although there is no significant difference, the average of cytokine levels is higher in individuals having a $CD19^+/CD5^{dim}$: total $CD19^+$ ratio higher than 0.2 (> 20% B1; Figure 4).

3.3 Cancer cell line derived Exosomes stimulate mononuclear cell expansion

The cord-blood derived $CD19^+$ fraction, was cultured in the various stimulants or left unstimulated. In ad-

dition, the remaining fraction following separation was also used in the experiment. The $CD19^+$ fraction was kept in culture only for 3 days and hence further experiments were not possible. Hence, further experiments require the isolation of this fraction from a monoclonal lymphocytosis individual characterized in the screening described above. The other fraction was viable for the whole duration of the experiment. The fraction consisted of mononuclear cells with $CD19^+$ cells depletion. Proliferating cells under PHA stimulation were confirmed to be $CD5^+$, while cells under exosome stimulation are not $CD19^+$ or $CD5^+$ (Figure 5A–5C). Infact, electron microscopy show clearly that the proliferating cells are larger and cytospins suggest selection for the monocytic lineage. Of interest, at day 12 (Figure 5D–5F) cells in the presence of exosomes show proliferation of lymphocytes and monocytic cells. Proliferation of lymphocytes was absent at day 12. Hence eventual experiments should be designed to add exosomes at various intervals. All cultures were stopped at day 21.

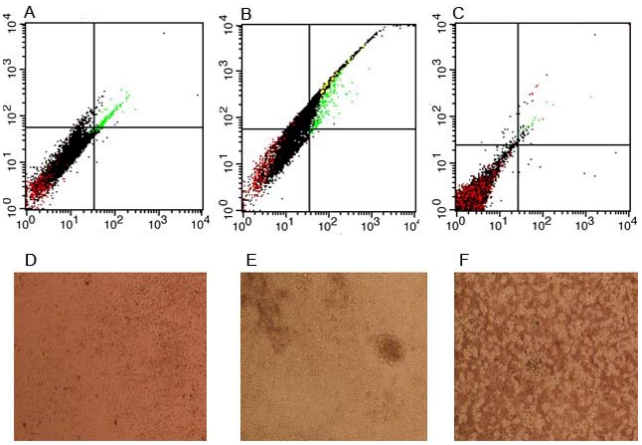

Figure 5: Flow cytometry of CD5/CD19 staining mononuclear cells (A-C). A: Isotype control; B: PHA stimulated cells and C: cells exposed to exosomes derived from PC3. Lower panel (D-F) shows light microscopy images of D: unstimulated cells; E: PHA stimulation at day 12; F: cells exposed to PC3-derived exosomes at day 12.

4 Discussion

Previous studies show that the presence of CD5 positive B cells increases with age in senior citizens (Geiger et al., 2000); CD5 positive B cells are detected in human malignancies (Wang, Amato, Rabah, Zheng & Fernandes, 2002) and that the presence of CD5 positive B cells in relatives of Chronic lymphocytic leukemia (CLL) patients, increases the risk of acquiring the disease (Rawstron et al., 2002). This suggests that these cells are potential precursors that require a transformation event, to progress into a malignant phenotype. To study transformation to malignant phenotype, a source of physiologically occurring CD5 positive cells is required. CD5 expressing B cells include (1) the life-long pool of B1 cells homing primarily to the peritoneal cavity, and having the capacity of self renewal; (2) the $CD5^+$ B cell subpopulation in neonatal blood (Erkeller-Yuksel et al., 1992); and (3) monoclonal lymphocytosis in the elderly (Ghia et al., 2004). Hence we decided to immunophenotype cord blood and a cohort of senior citizens. Of interest all $CD19^+$ cells in neonatal derived lymphocytes highly express the $CD5^+$ marker. In addition we show the presence of a subset of $CD19^+$ B cells with low expression of CD5, $CD19^+/CD5^{dim}$. The percent of $CD19^+/CD5^{dim}$ to total $CD19^+$ increases with age (Figure 3), with percentages higher than 40% found in 2 individuals over 80 years of age. Weak expression of CD5 in $CD19^+$ B cells were previously described in a patient cohort of CLLs (Wang et al., 2002), showing heterogeneity of CD5 expression levels. The significance of the differential level of CD5 expression in B cells is unknown, and hence understanding their origin and function towards initiation or development of B cell malignancy is imperative.

Survival of CD5 expressing B cells depends on CD5-dependent IL10 production (Gary-Gouy et al., 2002). In addition to B1 cells, a subset of CD5 positive B cells produce IL10 and have a regulatory function (Yanaba et al., 2008). Although the $CD19^+/CD5^{dim}$ cells characterised in this study have a low expression of CD5, we measured the level of IL10 and other cytokines in the sera of senior citizens. As shown in Figure 4, the IL10 level varies considerable, but there is a trend to a higher mean level of IL10 in elderly with higher proportion (percent of total B cells) of $CD19^+/CD5^{dim}$ cells. IL6 was selected for analysis since with increasing age, this cytokine is more abundant and provides an inflammatory response required for the formation of various cell clones and in the transformation of the benign condition, MGUS into Multiple Myeloma (Fayad et al., 2001). Our interest is that IL6 might also be involved in the transformation of MBL into a malignant CLL condition. Further analysis is required to quantify IL10 release in sorted cells. Of interest, the stimulation of B cells with phorbolmyristic acetate (PMA), increases the expression of CD5 (Youinou, Mackenzie, Jouquan, Le Goff & Lydyard, 1987). This suggests that cells can acquire the expression of CD5 through an activation stimulus. In this study we used neonatal-sorted $CD19^+$ $CD5^+$ cells to address the potential activation of CD5 positive B cells using well known activators, such as PMA, and cancer cell- derived exosomes as a vehicle to present cancer antigens. In the stimulation experiment, we used both positively selected $CD19^+$ cells and the mononuclear cells depleted of $CD19^+$ cells, derived from neonatal blood.

The positive selected $CD19^+$ cells lost viability after 3 days, and the stimulation experiment was performed using the cells depleted of $CD19^+$ cells ($CD19^-$ fraction).

In comparison with PHA and PMA (known lymphocyte stimulators), the exosomes isolated from 2 cancer cells lines, A549 (lung epithelial) and PC3 (prostate cell line) stimulated the proliferation of monocytic-like cells. Characterization is required to know the origin of these cells and further studies should utilise $CD19^+/CD5^{dim}$ cells from elderly individuals, ideally from the 2 individuals that have a high percentage of cells expressing the CD5 marker.

5 Conclusion

Creating a cellular model of B cell transformation following antigen presentation will provide a tool to study the origin of CD5 positive cells and to understand the role of tumour derived-exosomes in antigen presentation and activation of B cells. In this paper we show the presence of a specific population of CD5 positive cells that re-emerge in the elderly individuals, and the capacity of tumour derived exosomes to stimulate lymphocytes and monocyte-like cells. Further studies are required to expand the $CD19^+$ $CD5^+$ cells. Understanding stimulation and proliferation of these cells will be useful as a CLL-like model.

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