Research Article

In Vitro Generation of Cytotoxic CD4 Lymphocyte Response against Autologous Acute Myeloid Leukemia

Kok Chong Yap¹, Liam Pock Ho¹,², Pui San Yit¹, and Yeow Tee Goh¹

¹Department of Hematology, SGH, Singapore
²Department of Pathology, SGH, Singapore

Corresponding Author: Bernard Yap; email: bernard_y@hotmail.com

Received 21 August 2014; Accepted 28 September 2014

Academic Editors: Luiz Euribel Prestes Carneiro, Ramazan Güneşşar, and Giuseppe Murdaca

Abstract. Relapsed and refractory disease shortens the survival of acute myeloid leukemia (AML) patients. Therefore, additional therapy apart from conventional chemotherapy and stem cell transplantation is urgently needed, especially in eradicating residual leukemic stem cells. The identification of leukemia associated antigens, and the observation that administration of allogeneic T cells may mediate graft versus leukemia effect paved the way to develop various immunotherapy strategies. Effective provision of both tumor antigen and co-stimulation are essential for strategies aimed at enrolling cytotoxic T-lymphocytes to eradicate leukemic cells. In this study, we attempted to up-regulate co-stimulators on leukemic cells (termed “dendritisation” to mimic dendritic cells in providing strong co-stimulation) and couple with inherent leukemic antigens to generate specific antileukemic T-lymphocytes against autologous blast cells. Our study shows evidence of distinct cytotoxicity of CD4 positive T-lymphocytes against autologous blast cells. Dendritised AML blasts were able to function with antigen presenting capability, while still phenotypically existing as blast cells. This was also achievable for a wide range of subtypes of AML, making it a potential immunotherapy against AML.

Keywords: AML, immunotherapy, cytotoxic, CD 4 T-lymphocytes

1. Introduction

Acute myeloid leukemia (AML) is a myeloid neoplasm characterised by the expansion of malignant non-lymphoid hematopoietic progenitor cells resulting in failure of normal hematopoiesis [1]. It is one of the most common acute leukemia affecting adults, and incidence rates are higher with increasing age [2, 3]. It is apparent that after standard therapy, the survival rates of patients are reduced for relapsed or refractory cases. It has been documented that 70% of successfully treated patients by chemotherapy relapse soon after receiving treatment [4]. This high relapse rate occurs despite recent advances in therapeutic approach such as intensive chemotherapy, targeted mechanism therapy as well as stem cell transplantation. Therefore, in order to prolong remission and survival rates of AML patients, there is a continuous need for the development of novel therapy.
Relapsed and refractory AML has often been associated with drug resistant residual leukemic stem cells which are not eradicated completely by intensive chemotherapy. Even among patients who manage to achieve complete remission after therapy, many may experience relapse [5–8]. Relapse of disease in patients who have attained complete remission may be attributed to the presence of residual leukemic stem cells after treatment. Hence, we believe that the eradication of leukemic stem cells is the most effective way to improve the survival rates of AML patients.

There is a higher rate of relapse after syngeneic bone marrow transplantation than allogeneic bone marrow transplantation [9]. The reasons attributing to the better efficacy of allogeneic transplantation in AML treatment may be partly due to the allogeneic immune mechanism, also known as the graft-versus-leukemia effect [10]. In a separate study, it was also found that T cell depletion of the graft was associated with a higher rate of relapse when compared with donor lymphocyte infusion (DLI). DLI was able to re-induce a stable hematological remission in one-fourth of AML patients with relapse [11]. Despite much promises in harnessing DLI against relapsed AML, the increase in malignant cells in aggressive relapsing AML may at times outstrip the clinical effect of the DLI, thereby resulting in a failure to sustain an anti-leukemic response [12]. Another shortcoming of DLI is that patients often develop graft-versus-host disease (GVHD) as a result of the infused allogeneic T cells. Therefore, it would be ideal if the infused cytotoxic T cells have more potent and specific cytotoxic effect against the leukemic cells (Graft versus Leukemia effect) but negligible or no GVHD effect.

The identification of leukemia associated antigens (LAAs) and the observation of anti-leukemic effect of cytotoxic allogeneic T cells in AML patients have spurred the development of various immunotherapy strategies against leukemia. For example, different groups have generated T cells specific for LAAs, by isolating LAAs from leukemic cells of AML patients and subsequently loading them into antigen presenting cells such as the dendritic cells [13–15]. However, this method has its disadvantages especially in the isolation of specific LAAs that varies among individuals. Therefore, this technique requires the identification of specific LAAs that are highly presented in individual patients to generate T cells with anti-leukemic activity. We believe that the success of the cytotoxic T cells hinges on the correct and patient-specific LAAs being presented by the APC to T cells, and hence the success rate of this technique may be modest.

AML is characterised by the accumulation of clonal myeloid progenitor cells [16]. The graft-versus-leukemia effect after allogeneic stem cell transplantation exhibits the potential of immunotherapy against residual leukemic cells [17]. To add on, there have been previous studies that detected specific T lymphocytes that are reactive against tumor-associated antigens such as Wilm’s tumor gene product WT1 and proteinase 3 [18, 19]. Hence, it would be of interest and clinical benefit should there be a suitable adjuvant immunotherapy for treating AML by harnessing cytotoxic T lymphocytes from patients to eradicate autologous leukemic blast cells [16, 20].

One of the major reasons for non-efficient recognition of AML blasts by T lymphocytes in vivo is that 70% of the blasts do not express costimulatory molecules which are necessary for efficient T cell activation [21]. Dendritic cells are the most potent antigen presenting cells present in the human body capable of expressing costimulatory molecules. Hence, these dendritic cells are vital for the initiation of primary specific immune responses and are important means for the induction of anticancer immunity [22]. Therefore, in this study, we exploited the full capacity of dendritic cells to present leukemic antigens to T cells, thereby converting the latter to cytotoxic T cells. Addressing the issue of isolation of correct and specific LAAs, our technique utilizes a protocol that enhances dendritic cells’ antigen presenting capabilities to leukemic blast cells, but retaining the phenotype of the leukemic blast cells - a process we termed as “dendritisation”. By “dendritising” the leukemic blast cells, these malignant cells are able to co-express inherent leukemic antigens and upregulate co-stimulators to generate specific anti-leukemic effect. Unlike another technique that is published which fully converts leukemic blast cells into mature dendritic cells [21], our approach ensures that the leukemic blast cells retain its LAAs that would be presented by its acquired antigen presenting capabilities of the dendritic cells. In addition, since the leukemic blast cells are derived from the patient, the generation of the autologous T cells would therefore reduce the possibility of GVHD. Another advantage for dendritisation is that no effort is required to determine any known LAAs since the dendritised leukemic blast cells would present all known and unknown LAAs. In this study, we attempted to dendritise leukemic blast cells and subsequently generate autologous cytotoxic T cells in vitro from different AML subtypes.

2. Material and Methods

2.1. Patient Samples. Bone marrow and peripheral blood mononuclear cells were collected from AML patients after written informed consent was obtained. Patient consented by signing on the informed consent. This informed consent form was approved by Singhealth Centralised IRB with the approval number: 2008/060/B, for the purpose of laboratory research/study that was performed using the collected bone marrow and peripheral blood mononuclear cells. Bone marrow and peripheral blood mononuclear cells were isolated by density- gradient centrifugation (Ficoll-Paque Plus, GE Healthcare, Sweden) and subsequently washed with Hank’s Balanced Salt Solution (HBSS). Mononuclear cells (MNC) were suspended in complete medium (CM): Sterile water (B.Braun, Bethlehem; USA), powder RPMI 1640 (Sigma Aldrich, St. Louis; USA), 10% fetal bovine serum (Research
Instruments/ Hyclone, South America), 1% L-Glutamine (PAA, Australia), 1% Pen-Strap-Neomycin solution (PAA, Australia), Herpes (Sigma Aldrich, St. Louis; USA), sodium bicarbonate (B.Braun, Melsungen; Germany), and cryopreserved for storage.

2.2. Generation of “dendritised” Blast Cells - “dendritisation” Phase. Cryopreserved MNCs were thawed in 37°C water bath and washed in CM twice. Washed MNCs pellets were subsequently re-suspended in CM. The suspension of MNCs in CM were supplemented with a concertation of human recombinant cytokines [40ng of IL-4 (Peprotech, NJ; USA) per µl of CM, 20ng of GM-CSF (Peprotech, NJ; USA) per µl of CM and 1ng TNF-α (Peprotech, NJ; USA) per µl of CM and incubated at 37 °C and 5% CO2. The blast cells (present in the suspension of MNCs) were dendritised for seven days with one third of the CM and cytokines refreshed on Day 4 during the dendritisation phase. On Day 7 of the dendritisation phase, a complete wash of the blast cells was performed via centrifugation and subsequently re-suspended in fresh CM in preparation for lymphocyte expansion (lymphoexpansion) phase. See Table 1 for illustration of the dendritisation protocol.

2.3. Expansion of lymphocytes - “Lymphoexpansion” Phase. Inherent autologous T-lymphocytes were cultured and expanded in the presence of dendritised blast cells in CM. 100 units of INF-γ (Peprotech, NJ; USA) was added per µl of CM of culture on Day 1 (lymphoexpansion phase)/Day 7 (dendritisation phase). Also, 1µl of IL-2 (300 units/ml) was added to every 1ml of CM of culture on Day 2 (lymphoexpansion phase). No muromonab- CD3 (OKT3) was added into the medium. A separate vial of cryopreserved MNCs were thawed in 37°C water bath, washed in CM twice and subsequently resuspended in CM with IL-2 and INF-γ supplements; this served as the control. One third of the CM and cytokines (IL-2 and INF-γ) were refreshed every four days for both non-control and control flasks. See Table 1 for illustration of the “lymphoexpansion” protocol.

2.4. Immunophenotyping – “Dendritisation” Phase. Cultured cells were harvested at several time points (Days 4–10), washed and stained with mouse anti-human monoclonal antibodies purchased from Beckman Coulter, Ancell Corporation and LifeSpan Biosciences [Beckman Coulter, Marseille Cedex; France: PC5-conjugated anti- CD45, PE-conjugated anti-CD80 (IgG1, clone MAB104), PE- conjugated anti-CD86 (IgG2b, clone HA5.2B7), FITC-conjugated anti-CD34 (IgG1, clone 581), FITC-conjugated anti-CD33 (IgG1, clone D3HL60.251) and FITC-conjugated anti-CD13 (IgG1, clone SJ1D1). Ancell Corporation, Bayport MN; USA: PE-conjugated anti- MHC class II (HLA- DP, DQ and DR, IgG1, clone TDR31.1). LifeSpan Biosciences, USA: FITC-conjugated anti-CD117 (IgG1)].

2.5. Immunophenotyping- “Lymphoexpansion” Phase. Cultured cells were harvested after seven days, washed and stained with mouse anti- human monoclonal antibodies purchased from Beckman Coulter, Marseille Cedex; France: PC5-conjugated anti-CD45 (IgG1, clone J33), PE-conjugated anti-CD4 (IgG1, clone 13B8.2), FITC-conjugated anti-CD8 (IgG1, clone B9.11) and 7AAD antibody (BD Bioscience, USA; Material number 559925).

2.6. Cytotoxicity Assay. Cultured cells were first stained with Beckman Coulter FITC-conjugated-CD8 antibody, blast markers and myeloid markers (FITC-conjugated anti-CD33, FITC-conjugated anti-CD34, FITC-conjugated anti-CD117, FITC-conjugated anti-CD13). CD4 T lymphocytes were obtained via negative selection using anti-FITC conjugated beads (Miltenyi Biotec, Bergisch Gladbach; Germany:130-048-701). CD4 T lymphocytes from both dendritised and control flasks (Effector Cells) were washed and cocultured with freshly thawed autologous blast cells (Target Cells) for 4 h at various effector : target ratio (Effectors: Target ratios of 0:1, 2.5:1, 1:1, 10:1,40:1). After 4 h, cells were washed and stained with mouse anti-human PC5-conjugated anti-CD45(Beckman Coulter, Marseille Cedex; France), respective blast markers, 7AAD (BD Bioscience, USA; material number 559925) and annexin V(BD Bioscience, USA; material number 56421) for flow analysis.

3. Results

3.1. Patient characteristics. Patients were aged between 18 and 73 years, and had subtypes (M1, M2, M4) of AML according to the French-American-British (FAB) classification. Patients’ demography and main clinical parameters are summarized in Table 2.

3.2. “Dendritisation” Phase- Generation of “Dendritised” Leukemic Blast Cells. There was distinct up-regulation of co-stimulators (CD80, CD86) and MHC II in the dendritised blast cells from Day 1 onwards. The strength of the expression of co-stimulators and MHC II increased to a maximum Delta Mean Fluorescent Index (dMFI) around Day 7 after the start of “dendritisation” (See Figure 1). In addition, cell surface projections akin to dendritic cells were observed in the dendritised blasts of all the seven patients in this study.

3.3. “Lymphoexpansion” Phase- Generation of autologous cytotoxic T cells. After “dendritisation” of leukemic blast cells, the dendritised leukemic blast cells were co- cultured with the inherent T lymphocytes that were present, in the presence of IL-2 and INF-γ (i.e., lymphoexpansion phase). There was a distinctive increase in the proliferation of T lymphocytes (both CD4 and CD8 positive T lymphocytes). However, as the number of days of lymphoexpansion...
increased, there was a predominance of CD4 positive T lymphocytes than CD8 positive T lymphocytes (See Figure 2(a)). Expanded CD4 lymphocytes, both that were co-cultured with dendritised blast cells and controls, exhibited effector memory phenotype (TEM) (CD45RA−, CD45RO+, CD62L−, CCR7−, CD127−, CD25+, and CD27±, CD28±) (See Figure 2(b)). However, despite T lymphocytes from both control and dendritised blast cells having the same TEM phenotype, there was a distinct difference in the expression of intra-cellular cytotoxic granules. It was observed that there was a significantly higher IFN-γ expression in the T lymphocytes that was co-cultured with dendritised blast cells than in the T lymphocytes from the controls. There was also a slight increase in granzyme A and granulysin in the T lymphocytes that was co-cultured with dendritised blast cells than in the T lymphocytes from the controls (See Figure 2(c)).

3.4. Functional Test: Cytotoxicity Assay. Figure 3 shows the comparison of the anti-leukemic cytotoxicity effect between CD4 positive T lymphocytes that were primed with dendritised blast cells and those that were primed with only IL-2 in the control flask for Patient 2. The percentage of live leukemic blast cells (represented by 7AAD and Annexin V double negative cells) showed a distinctive decrease from 83% to 55% (Effectors:Target ratio from 0:1 to 40:1) when co-cultured with CD4 T lymphocytes that were primed with dendritised blast cells (see Figure 3(a)). However, there was no change in the percentage live leukemic blast cells that were co-cultured with the CD4 lymphocytes from the control flask (see Figure 3(a)). Figure 3(b) shows the cumulative average percentage of dead blast cells that were co-cultured with CD4 T lymphocytes and primed with dendritised blast cells and controls. Cytotoxic- capability of CD4 T lymphocytes that were primed with dendritised blast cells were higher that CD4 T lymphocytes from the controls. CD4 T lymphocytes that were primed with dendritised blast cells exhibited increasing cytotoxicity effect against autologous blast cells with increasing amount of CD4 T lymphocytes (Percentage of blast cells killed increased from 16% (0:1) to 21.7% (1:1) to 25.2% (2.5:1) to 40% (10:1) to 45%(40:1), whereby ratio in brackets represents ratio of effector cells : blast cells respectively. On the contrary, CD4 T lymphocytes did not exhibit cytotoxicity effect towards autologous blast cells (blast cells killed: 16.8% (0:1) to 21.3% (1:1) to 15.8% (2.5:1) to 21.7% (10:1) to 17.8% (40:1). At effector:target ratio of 10:1 and 40:1, the CD4 T lymphocytes that were primed from dendritised blast cells exhibited close to a significant 30% cytotoxicity. The spontaneous percentage blast cell death at 0:1 effector:target ratio are approximately similar at 16% in both control and dendritised groups. Results from Figure 3(b) are derived from four patient samples.

4. Discussion

In this study, we report the successful generation of dendritised leukemic blast cells in the presence of GM-CSF, IL-4 and TNF-α based on our “dendritisation” approach. The patients that were reported in this study were newly diagnosed with AML of different subtypes (M1, M2 and M4) and blast percentages (ranging from 3%–91%). Generation of dendritised blast cells was successful in all patient samples with dendritised blast cells expressing co-stimulatory molecules (CD80 and CD86) and MHC II while retaining its blast cell phenotype (CD117+ and CD34+). Our protocol
Figure 1: Flow-cytometry analysis of co-stimulatory molecules (CD80 and CD86). MHC II and “dendritised” leukemic blast cells.

(a) Distinctive increase in the co-stimulatory molecules (CD80 and CD86) and MHC II when compared between non-dendritised (Day 0) leukemic blast cells and dendritised blast cells at Day 7. Diagram shown is representative for Patient 1 and consistent with all 7 patients. Leukemic blast cells from 7 patients were co-cultured in a concortion of IL-4, GM-CSF and TNF-α over a period of 10 days. The dMFI of CD80, CD86 and MHC II peaked at approximately Day 7. (b) Flow cytometry analysis shows the progressive increase in the number of leukemic blast cells which are positive for stem cell marker CD34, expressing CD80, CD86 and MHC II as the dendritisation days increases from Day 0 to Day 7.

is better than other previous studies which were only 70% successful in differentiating leukemic blast cells to mature dendritic cells [23–25]. Indeed, there have been existing studies which documented the generation of “leukemic dendritic cells” [26] and “immunocompetent dendritic cells” [21] that were derived from blast cells. However, a major difference between our dendritised blast cells and the dendritic cells that were generated in other studies [21, 26] was that our dendritised blast cells remained as blast cells (albeit possessing antigen presenting capabilities) whereas the dendritic cells from other studies fully differentiated to dendritic cells. We believe that our dendritised blast cells still possessed inherent leukemic antigens. However, it is uncertain if there is a reduction in the leukemic antigens present in the dendritic cells that were generated from other studies.

This study shows that apart from the expression of high level of co-stimulatory molecules, our dendritised blast cells also possess up-regulated MHC II which was not reported in dendritic cells generated from other studies. Contrary to the fact that MHC II presents exogeneous antigens, there have been also multiple pathways that have shown to contribute to the MHC class II mediated presentation of cytoplasmic and nuclear viral, tumor and self antigen [27]. These pathways include chaperone mediated autophagy (CMA), macroautophagy, a TAP-dependent pathway and intracellular antigen transfer which eventually present the antigens to CD4 positive T lymphocytes [27]. In our study, it
Figure 2: Flow cytometry analysis of expanded T-lymphocytes. (a) Flow cytometry diagram illustrating significant increase in both CD8 and CD4 T lymphocytes from both control and dendritised culture flask. Beyond Day 28 of lymphexpansion, there was a greater increase in CD4 T lymphocytes in comparison to CD8 T lymphocytes for both control and dendritised culture flask. (b) Both CD4 positive T lymphocytes from control and dendritised flasks exhibit effector memory (TEM) phenotype (CD45RA−, CD45RO+, CD62L−, CCR7−, CD127−, CD25+, and CD27±, CD28±). (c) There was a significantly higher expression of INF-γ in CD4 positive T lymphocytes that was co-cultured with dendritised leukemic blast cells in comparison to the control CD4 positive T lymphocytes. There was also a slightly higher amount of granzyme A and granulysin in T lymphocytes that was co-cultured with “dendritised” leukemic blast cells than the control CD4 positive T lymphocytes. Unfilled Histogram = Control CD 4 T- Lymphocytes Filled Histogram = CD 4 T- Lymphocytes Co-Cultured with “dendritised” Blast Cells.
was observed that the MHC II and co-stimulatory molecules (CD80 and CD86) attained a maximum level around Day 7. Hence, we believe that the antigen presenting capability of our dendritised blast cells peak at Day 7 of dendritisation phase. This would mean that our dendritised blast cells would be able to efficiently utilize the up-regulated MHC II to present inherent leukemic antigen to surrounding CD4 positive T lymphocytes, in the presence of up-regulated CD80 and CD86.

After the generation of dendritised blast cells, the next phase of our study was to expand autologous T lymphocytes that were presented with the inherent leukemic antigens by the generated dendritised blast cells. We observed an increase in proliferated CD8 positive and CD4 positive T lymphocytes. However, it was apparent that towards the end of the “lymphoexpansion” phase, there was a more significant increase in the CD4 positive T lymphocytes than CD8 positive T lymphocytes. This could possibly be due to the treatment of inflammatory signal such as INF-γ that could induce MHC II expression on the surface of a variety of APC [27]. This observation was expected because there was an up-regulation of MHC II molecules in the generated dendritised blast cells that were responsible for the presentation of inherent leukemic antigens to the CD4 positive T lymphocytes. Similar observation was made for the proliferated T lymphocytes in the control arm.

Both CD4 positive T lymphocytes from control and dendritised flasks exhibited effector memory (TEM) phenotype (CD45RA+, CD45RO+, CD62L−, CCR7−, CD127−, CD25+, and CD27±, CD28±). Human TEM are memory cells that have lost the constitutive expression of CCR7 and CD62L, and display characteristic sets of chemokine receptors.
receptors and adhesion molecules that are required for homing to inflamed tissues [28]. Therefore, the CD4 positive T lymphocytes from both control and dendritised flasks were characterized to have rapid effector function. Nonetheless, access of cytoytic capability of CD4 positive T lymphocytes remained paramount despite knowing the TEM phenotype of the CD4 positive T lymphocytes. In our study, we have shown consistent stimulation of autologous killing of leukemic blast cells through priming with dendritised blast cells via flow cytometry cytotoxicity assay (see Figure 3). At effector:target ratio of 1:1, there was no autologous cytotoxicity for CD4 positive T lymphocytes from both dendritised and control flasks. However, as the effector:target ratio increased to 2.5:1, 10:1 and 40:1, there was a significant increase in the cytotoxicity effect for the CD4 positive T lymphocytes in comparison to the control CD4 positive T lymphocytes. The extent of cytotoxicity towards autologous blast cells reached to about 30% (E:T ratio of 40:1) more for CD4 positive T lymphocytes that was primed from dendritised blast cells as compared to control CD4 positive T lymphocytes.

In conclusion, our study shows evidence of distinct cytotoxicity of CD4 positive T lymphocytes against autologous blast cells. “Dendritised” AML blasts were therefore able to function with antigen presenting capability, while still phenotypically existing as blast cells. This was also achievable for a wide range of FAB subtypes of AML making it a potential immunotherapy against AML. We believe that our study findings support the feasibility of using cultured and “dendritised” AML blasts cells to present leukemic antigens to autologous T cells hence conferring cytotoxicity against autologous blast cells and their possible use in clinical trials in the future.

Funding

This work is supported by: National Medical Research Council [SRG-CG#04/2010] and Cell Pro & Immune Recon from Singapore Cancer Syndicate [SGH-SCS-BMTC].

Acknowledgements

The authors appreciate the support of SingHealth/Duke-NUS Academic Medicine Research Institute and Taara Madhavan (Associate in Clinical Sciences, Duke-NUS Graduate Medical School) in editing this manuscript.

Contribution

Mr. Bernard Yap and Dr. Ho Liam Pock have contributed equally to this manuscript.

Declaration of Conflicts

All authors have nothing to declare.

References


