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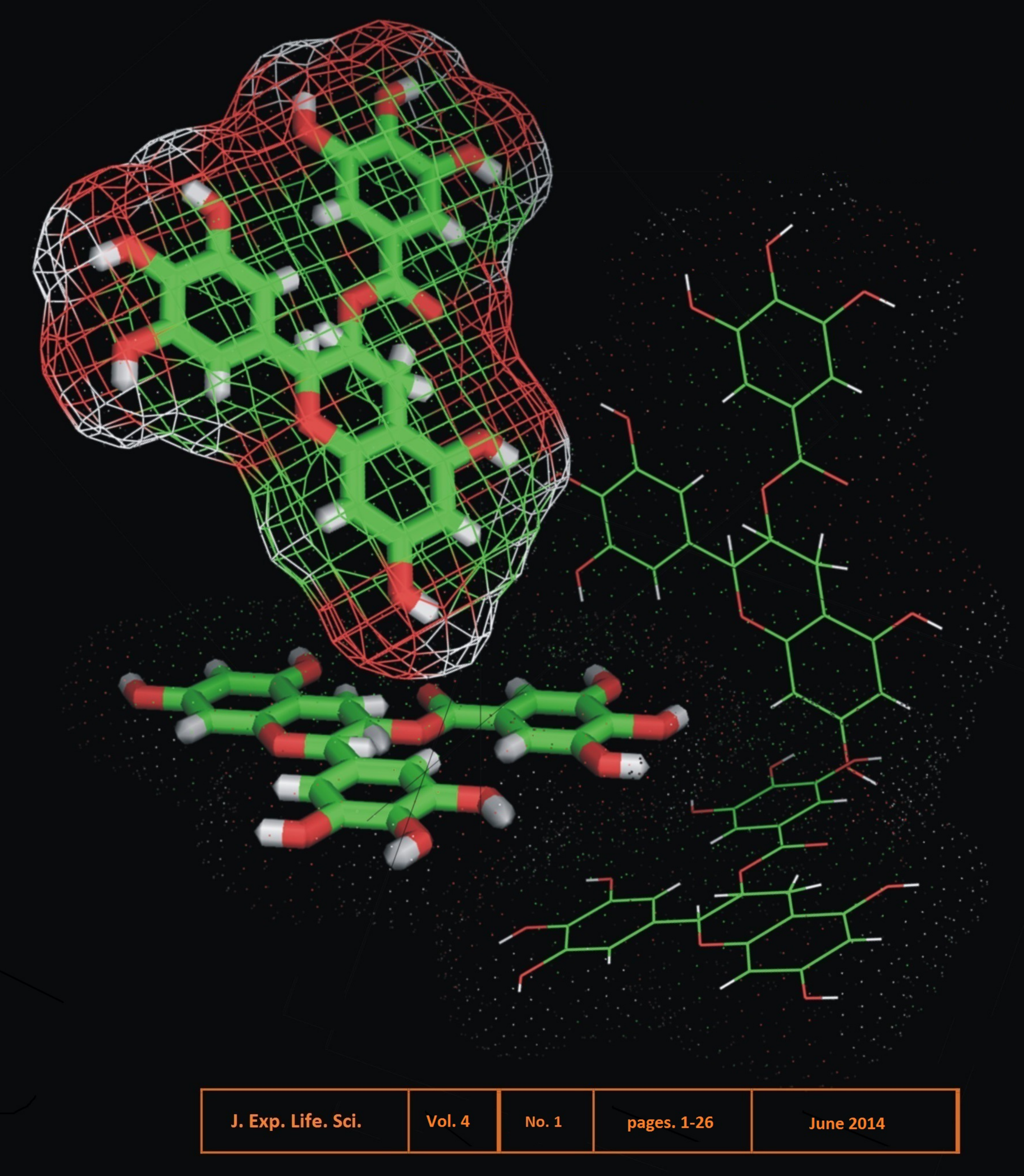

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Aspek Biologi Sel T Regulator CD4+ CD25+ pada Transplantasi Sumsum Tulang

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Abstrak

Graft-versus-host disease (GVHD) dan rejeksi (penolakan) pada transplantasi alogenik merupakan kasus terpenting pada masalah transplantasi jaringan. Di lain pihak ketersediaan donor dengan kriteria MHC-matching sangat terbatas. Fakta bahwa kebanyakan organisme hidup sehat tanpa terjangkit penyakit autoimun menunjukkan adanya mekanisme regulasi yang mampu mencegah teraktivasinya sel-sel autoreaktif. Organisme mempunyai mekanisme yang sangat sempurna untuk menghindari respon mematikan terhadap self-antigen. Mekanisme terpenting pada toleransi imunologi ini adalah yang diperankan oleh sel T regulator. Mekanisme toleransi yang dilakukan oleh sel T regulator mencegah teraktivasinya sel-sel autoreaktif ini nampaknya juga terjadi pada transplantasi, baik transplantasi sumsum tulang maupun organ. Pada review ini kami akan membahas aplikasi sel T regulator $CD4^{+}CD25^{+}$ pada transplantasi alogenik terutama pada transplantasi sumsum tulang (*bone marrow transplantation,* BMT).

Kata kunci: sel T regulator, transplantasi sumsum tulang, toleransi imunologi

Abstract

Graft-versus-host disease (GVHD) and rejection on alogenic transplant is the most important case in tissues transplant. On the other hand, the availability of donor with MHC-matching criteria was limited. The fact that most organisms live healthy without autoimmune disease indicate a regulatory mechanism that prevents activation of autoreactive cells. Organisms have perfect mechanisms for avoiding the deadly response to the self-antigen. The most important mechanisms of immunological tolerance are played by regulatory T cells. Mechanisms of tolerance by regulatory T cells prevents activation of these autoreactive cells were also appears to occur in transplant, either bone marrow or organ transplant. This review discusses the application of regulatory T cells in $CD4^{+}$ $CD25^{+}$ allogenic transplantation, especially in bone marrow transplant (BMT).

Keywords: T-cell regulator, bone marrow tranplant, immunological tolerance

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PENDAHULUAN

Seiring dengan perkembangan ilmu di bidang biologi-kedokteran, saat ini transplantasi sumsum tulang (*bone marrow transplant*-BMT) telah menjadi salah satu strategi untuk mengatasi penyakit turunan maupun penyakit yang timbul akibat pengaruh lingkungan. Di antara penyakit turunan yang telah diatasi dengan transplantasi sumsum tulang misalnya imunodefisiensi dan hemoglobinofati. BMT juga telah dimanfaatkan untuk mengatasi malignansi misalnya leukaemia, pasien yang menderita anemia berat ataupun penyakit infeksi berat akibat aplasia medullaris. Pada pelaksanaan BMT sangat sulit diperoleh donor yang mempunyai kesamaan MHC secara penuh, sehingga penerima transplantasi (*host*) akan hidup dengan dua tipe sel yang bersifat aloreaktif pada tubuhnya [1;2;3]. Dalam kondisi seperti ini *Graft-versus-host disease*-GVHD merupakan masalah paling besar yang dapat menyebabkan kematian. Penyebab utama GVHD adalah adanya sel T *mature* yang ada pada sumsum tulang donor. Penyebab kematian yang lain adalah terjadinya rejeksi (penolakan) donor oleh sistem imun *host*, sehingga transplantasi gagal sejak awal pelaksanaan. Untuk mengatasi penolakan oleh *host* umumnya dilakukan kemoterapi dan radiasi total pada *host*. Kemoterapi yang dilakukan pada kasus ini bertujuan untuk menekan sistem imun *host* yang umumnya menggunakan *cyclosporine* atau *methotrexate*. Pemberian obat-obat imunosupresor meskipun sangat efektif mengontrol rejeksi dan GVHD namun pada akhirnya tidak dapat mengatasi respon imun yang terjadi secara kronik.

Respon imun kronik ini diketahui disebabkan oleh MHC minor [4;5;6;7]. Oleh karena itu meskipun diperoleh donor yang mempunyai keselarasan MHC terhadap *host*

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(*MHC-matching*) tetap tidak ada jaminan bahwa transplan akan diterima selamanya. Selama proses persiapan dan setelah pelaksanaan BMT akan terjadi efek samping yaitu penurunan kualitas hidup bagi pasien. Defektif perkembangan sel-sel hematopoietik (aplasia) akan terjadi setelah pelaksanaan kemoterapi. Cacat perkembangan sel-sel hematopoietik ini akan menyebabkan terjadinya infeksi yang parah di samping munculnya neoplasma. Neoplasma ini merupakan perkembangan sel yang tidak terkontrol dapat bersifat jinak ataupun malignant. Obat-obat imunosupresan tidak saja menarget sel T yang dimaksudkan namun juga mengenai sel-sel dan organ lain sehingga mengarah pada kerusakan organ lain [8;9;10;11;12]. Di samping adanya sifat toksisitas yang telah disebutkan ini, obat-obat imunosupresan juga meningkatkan terjadinya resiko leukemia. Mengingat besarnya efek obat-obat imunosupresan ini, maka sangat penting dikembangkan strategi yang dapat mengatasi rejeksi dan GVHD namun tidak menghambat kerja sel-sel imunokompeten. Semenjak penemuan Bilingham *et al.* yang berhasil menginduksi sitem *tolerance* pada mencit neonatal, maka peneliti-peneliti lain disibukkan untuk mencari tahu mekanisme terjadinya *tolerance* pada sistem alogenik [2]. Semua karya penelitian tersebut sesungguhnya dimaksudkan mencapai satu tujuan yaitu keberhasilan pada transplantasi alogenik.

Telah diketahui bahwa terjadinya toleransi terhadap sel T di dalam tubuh individu telah terjadi sejak awal maturasi sel T pada timus. Teori *clonal deletion* hanya memberikan kesempatan hidup bagi sel T yang memenuhi syarat tertentu dan sisanya akan dieliminasi melalui mekanisme apoptosis. Meskipun seleksi pada timus cukup ketat nyatanya masih ada sel T autoreaktif yang lolos dari seleksi negatif sehingga sel-sel tersebut harus dikontrol dengan ketat agar tidak menimbulkan masalah autoimun [13;14;15;16;17]. Sistem toleran dan *anergy* pada periferal diyakini sebagai hasil kerja yang diperankan oleh sel T regulator (T_{reg}) yang sekaligus juga berperan sebagai supresor. Pada manusia maupun mencit kegagalan sistem toleran akan berakibat munculnya penyakit autoimun. Penyakit ini selanjutnya diketahui terkait dengan mutasi gen penyandi FOXP3 (*forkhead/winged helix transcription factor*). Mutasi pada gen ini akan menimbulkan IPEX (*Immune dysregulation*, *polyendocrinopathy*, *enteropathy, X-linked*) pada manusia. Mencit *scurfy* merupakan salah satu contoh hewan percobaan yang mengalami mutasi pada gen FOXP3. Mencit ini akan segera mati akibat penyakit autoimun. FOXP3 selanjutnya diketahui berperan penting pada perkembangan sel T regulator, $CD4^+CD25^+$. Mencit IL-2 $R\beta^{-/-}$ juga merupakan model mencit eksperimen yang menderita autoimun. Pada mencit jenis terakhir ini meskipun gen FOXP3 tidak cacat namun mencit ini juga tidak mempunyai sel T regulator, $CD4^+CD25^+$. Mencit *scurfy* maupun IL-2 $R\beta^{-/-}$ yang menerima infus sel T regulator akan bidup normal dan bebas dari penyakit autoimun. Dengan demikian jelaslah bahwa komponen utama yang mendukung terselenggaranya homeostasis normal adalah sel T regulator $CD4^+CD25^+$ [18;19;20;21].

SEL T REGULATOR $CD4^+$ $CD25^+$ $FOXP3^+$

Pada saat ini para ilmuwan meyakini bahwa sitem toleran pada individu sehat diperankan oleh banyak sel T regulator yang berasal dari populasi sel T. Di antara sel T regulator tersebut yang paling penting adalah $CD4^+$ $CD25^+$ $FOXP3^+$. Sel T regulator $CD4^+$ $CD25^+$ $FOXP3^+$ diketahui berkembang dan terseleksi pada organ timus. Banyak laporan yang berbeda mengenai perkembangan sel T regulator $CD4^+CD25^+FOXP3^+$. Satu grup melaporkan bahwa prekursor sel T regulator $CD4^+$ $CD25^+$ $FOXP3^+$ dapat mengalami seleksi negatif oleh APC, namun grup peneliti lain melaporkan bahwa prekursor sel T regulator $CD4^+$ $CD25^+$ $FOXP3^+$ resisten terhadap mekanisme *clonal deletion* (*negative selection*) pada epitel timus [22;23;24;25;26].

Sampai saat ini perkembangan sel T regulator ini masih merupakan misteri, namun para ilmuwan sudah menemukan persamaan pandang bahwa sel ini mempunyai afinitas ikatan yang tinggi dengan MHC kelas I dan hampir menjadi target seleksi negatif. Mengingat seleksi klon T_{reg} berada pada afinitas MHC kelas I yang tinggi, T_{reg} mempunyai kecenderungan menjadi *self-reactive*. Sifat ini sangat penting karena salah satu mekanisme kerja T_{reg} adalah menarget dan mengontrol sel autoreaktif dengan cara kontak antar sel [27;28;29;30].

T_{reg} pada mencit muncul setelah hari ketiga. Timektomi sebelum hari ketiga berakibat munculnya penyakit autoimun pada mencit model tersebut, sebaliknya jika dilakukan setelah hari ketiga mencit tetap hidup normal. Setelah hari ketiga sel T_{reg} telah berada pada organ limfoid periferal dan T_{reg} tersebut akan terus berproliferasi secara homeostasis untuk

memenuhi kebutuhan fisiologi individu yang bersangkutan. Mencit yang mengalami gangguan autoimun juga akan sembuh jika ditransfer dengan sel T regulator $CD4^+CD25^+$ atau ditransfer dengan total limfosit dari mencit yang umurnya lebih dari tiga hari. T_{reg} berproliferasi dengan memanfaatkan interleukin-2 (IL-2). T_{reg} yang teraktivasi akan melakukan kerjanya yaitu menekan reaktivitas sel-sel autoregresif. T_{reg} akan teraktivasi jika terjadi ikatan CTLA-4 yang ada pada permukaan T_{reg} dengan molekul CD80/CD86 yang berada pada permukaan APC. Ikatan CTLA-4:CD80/CD86 akan meningkatkan ekspresi IDO (*Indoleamine* 2, 3-*dioxygenase*) pada APC. Peningkatan IDO akan menghabiskan ketersediaan triptofan pada daerah di sekitar APC tersebut. Keadaan ini mengakibatkan terhentinya proliferasi sel T efektor maupun sel-sel autoreaktif yang lain. Beberapa fakta yang lain menunjukkan bahwa kurangnya triptofan akan menyebabkan sel-sel efektor akan melakukan apoptosis [31;32;33].

T_{reg} tidak saja mencegah munculnya penyakit autoimun, namun dapat mengontrol sel T reaktif pada IBD sehingga inflamasi dapat dihindari. Dari perspektif ini jelaslah bahwa T_{reg} tidak saja berguna sebagai agen preventif namun juga kuratif. Terjadinya supresi oleh T_{reg} diperantarai oleh molekul efektor IL-10 dan TGF-β yang dihasilkan oleh T_{reg}. Prasyarat lain untuk terlaksananya supresi bahwa target harus peka terhadap TGF-β. Dalam kaidah ini jelaslah bahwa T_{reg} tidak akan efektif terhadap sel-sel autoreaktif yang berasal dari mencit $TGF\text{-}\beta^{-/-}$. Telah diketahui T_{reg} mempunyai peran yang sangat penting pada inplantasi vetus pada rahim induknya. Diketahui pula T_{reg} juga menghambat sel-sel efektor yang mestinya bekerja mengatasi virus, parasit, bakteri, dan fungi. Efek samping kerja T_{reg} ini menjadi masalah penting pada terapi menggunakan T_{reg}, karena T_{reg} juga menekan sel-sel anti-tumor baik dari populasi sel T sitotoksik, NK maupun sel-sel imun lain tanpa spesifitas. T_{reg} yang telah teraktivasi oleh suatu antigen tidak saja melakukan supresi terhadap sel pembawa antigen tersebut namun T_{reg} akan melakukan kerja nonspesifik pada seluruh sel-sel efektor, sehingga penggunaan T_{reg} pada terapi harus memperhitungkan hal-hal yang terkait dengan pentingnya fungsi sel efektor [34;35;36;37;38].

Ekspresi molekul CD25 pada limfosit T tidak serta merta mewujudkan sel T regulator. Hal ini terkait fakta bahwa sel yang teraktivasi akan memunculkan molekul ini sebagai konsekuensi kepentingan proliferasi dimana pada saat itu sel membutuhkan IL-2 sedangkan CD25 merupakan IL-2Rα. Sebaliknya sel T yang tidak mengekspresikan molekul CD25 pun dapat berfungsi sebagai sel T regulator apabila sel tersebut mengekspresikan FOXP3. Dalam tinjauan ini keberadaan FOXP3 lebih penting untuk menjadi klaim sebagai sel T regulator, namun demikian *marker* CD25 tetaplah cara termudah untuk memperoleh sel T regulator. Pada berbagai eksperiman telah diketahui $CD4^+CD25^-FOXP3^+$ terbukti mempunyai kapasitas yang sama dengan sel T regulator $CD4^+CD25^+$. Dengan adanya fakta ini peneliti telah melakukan kajian mendalam ternyata pada individu yang relatif muda hampir dipastikan bahwa $CD4^+CD25^+$ merupakan sel T regulator yang mengekspresikan FOXP3. Sehingga untuk memperoleh sel T regulator dari mencit diperlukan mencit berumur di bawah 10 minggu dan cukup mengisolasi $CD4^+CD25^+$ untuk memperoleh sel T regulator [39;40].

Jauh sebelum mekanisme toleran diketahui, grup peneliti Nicole Le Douarin *et al.* telah melakukan xeno-transplantasi dimana epitelium timus dipindahkan ke spesies lain pada saat embrio. Ternyata embrio tersebut pada hidupnya toleran terhadap jaringan yang berasal dari donor yang sebelumnya telah menyumbangkan epitelium itu. Hasil ini menjadi bukti berjalannya mekanisme toleransi melalui manipulasi imunologi. Pada saat Nicole Le Douarin *et al*. memperoleh hasil tersebut belum banyak yang bisa dijelaskan, namun pada saat ini diketahui bahwa mekanisme toleran dimediasi oleh kerja sel T regulator $CD4^+$ $CD25^+$ yang perkembangannya mutlak memerlukan timus [41;42].

GRAFT-VERSUS-HOST DISEASE **(GVHD)**

Pada kasus terjadinya kanker darah pasien diberi terapi dengan kemoterapi maupun radiasi. Terapi ini dimaksudkan untuk menghilangkan baik sel-sel limfosit maupun mieloid. Namun demikian ablasi *in vivo* pada sel limfosit maupun mieloid tidak sepenuhnya bisa menghilangkan sel-sel ini. Sel-sel yang tersisa ini pada akhirnya menjadi salah satu faktor penyebab rejeksi maupun GVHD. Saat ini diketahui bahwa faktor paling penting yang memperburuk transplantasi sumsum tulang adalah adanya kontiminasi sel T *mature* donor. Untuk mengatasi masalah ini, dengan mudah para peneliti melakukan deplesi sel T *mature* donor. Inkubasi sel donor dengan anti-CD3 atau anti-Thy1.2 selama 30 menit diikuti

dengan penambahan komplemen akan menyebabkan semua sel T *mature* mati akibat reaksi komplemen *in vitro*. Namun demikian pada akhir-akhir ini timbul masalah baru, karena terbukti sel-sel T *mature* yang dianggap sebagai sel kontaminan ini ternyata sangat dibutuhkan untuk memusnahkan sel-sel kanker darah yang tersisa, yang selanjutnya dikenal dengan istilah GVL (*graft-versus-leukemia*). Dalam pandangan terakhir ini maka untuk mengatakan bahwa BMT berjalan sukses harus dipenuhi kriteria terjadi toleransi dimana tidak terdapat rejeksi maupun GVHD dan mekanisme GVL tetap berjalan dengan sempurna [43].

Untuk memperoleh toleran ini dapat dilakukan dengan cara melakukan transfusi sumsum tulang yang sebelumnya telah di-*blocking* dengan berbagai antibodi yang spesifik untuk sel T ataupun dengan cara mem-*blocking* ligannya, misalnya dengan melakukan *staining* dengan anti-CD4, CD8, anti-B7, dan anti-CD154 yang dikuti dengan komplemen. Pada percobaan yang dilakukan oleh Johnson dimana mencit diradiasi dengan dosis letal dan diinjeksi dengan sumsum tulang yang sebelumnya telah dilakukan deplesi sel T, mencit tidak mengalami GVHD. Ketika lima minggu setelah BMT dilakukan infuse sel T donor, mencit tetap tidak mengalami GVHD. Sebaliknya jika sebelum menginfusi sel T donor itu mencit diinjeksi *in vivo* dengan antibodi yang mendeplesi sel T maka mencit menderita GVHD yang mematikan. Percobaan ini memberikan informasi bahwa sel T donor telah berkembang menjadi sel T regulator pada timus *host*, sehingga ketika sel T donor pada minggu ke lima ditambahkan tidak akan menimbulkan GVHD karena sel T tersebut dapat diregulasi oleh sel T_{reg} yang telah ada. Sebaliknya jika sebelum menerima sel T donor sel T dideplesi terlebih dahulu, maka semua sel T termasuk sel T_{reg} telah hilang pada mencit tersebut sehingga sel T yang diinjeksikan berikutnya tidak dapat diregulasi dan akan berkembang menjadi efektor yang menyebabkan GVHD. Bentuk percobaan yang pendekatannya menyerupai kejadian GVHD akut pada klinik awalnya dilakukan pada laboratorium Strober, Blazar, dan Salomon. Pada mencit yang diradiasi dengan dosis letal dan diinfusi dengan sel T donor, *host* akan menderita GVHD setelah beberapa minggu. Pada model ini deplesi sel T yang membawa molekul CD25 akan memacu GVHD dan letal lebih cepat [44;45;46]. Sebaliknya penambahan sel T donor $CD4^{+}CD25^{+}$ akan menunda GVHD, terutama jika sel T_{reg} yang ditambahkan cukup besar misalnya 1:1 untuk perbandingan sel T yang diinfusikan atas sel T_{reg} yang ditambahkan [44;45;46;47].

Dalam percobaan yang dilakukan oleh para ilmuwan sejauh ini hasil percobaan menggunakan T_{reg} mempunyai hasil yang bervariasi mulai dari tertundanya GVHD sampai terjadi proteksi penuh sehingga mencit hasil transplantasi hidup sehat setidaknya sampai 3 bulan pasca transplantasi. Sejauh ini belum ada yang bisa menjelaskan namun diduga sangat dipengaruhi oleh jumlah sel T regulator dan jumlah sel efektor. Hasil yang sama juga terjadi pada percobaan GVHD yang diinduksi dengan infusi splenosit yang berbeda pada MHC-minor. Pada sistem ini T_{reg} donor dan *host* sama-sama berperan mencegah terjadinya GVHD [48]. Untuk mengatasi GVHD nampaknya memerlukan sel T_{reg} dalam jumlah yang banyak, yaitu untuk mengimbangi sel T efektor. Namun sayangnya T_{reg} khususnya $CD4^{+}CD25^{+}$ bersifat non-responsif terhadap stimuli melalui jalur TCR (*T-cell reseptor*). Strategi pengembangan T_{reg} *in vitro* ini sangat penting untuk memenuhi kebutuhan T_{reg} pada pelaksanaan terapi. Oleh karena itu harus diketahui apakah T_{reg} hasil ekspansi *in vitro* juga efektif untuk melakukan supresi *in vivo* [44;45;46;47;48].

Meskipun pada stimuli dengan anti-CD3 T_{reg} bersifat anergi (*nonresponsive*) namun strategi dengan menggunakan stimuli anti-CD3/CD28 ditambah IL-2 ternyata mampu menstimuli proliferasi hingga 10^{4} kali lipat pada kultur minggu ke-6 [49]. Mencit diinfusi dengan sel sumsum tulang yang dicampur dengan sel T dan dimasukkan ke *host* bersama T_{reg} yang dikembangkan *in vitro*. Pada desain penelitian ini kejadian GVHD dapat dihindarkan [50]. Penambahan TGF-beta secara *in vitro* pada kultur T_{reg} dapat meningkatkandaya proteksi T_{reg}. Hasil ini menggambarkan meskipun secara klinik jumlah T_{reg} yang dapat diperoleh dari biopsis pasien, propagasi *in vitro* dapat dilakukan dengan mudah sehingga sel T regulator untuk terapi GVHD semakin menjanjikan dan mudah dikerjakan. Ke depan diyakini bahwa pemenuhan kebutuhanT_{reg} untuk terapi akan menggunakan sistem *in vitro*. T_{reg} pihak donor yang dikembangkan *in vitro* bersama sel host mempunyai efektivitas yang lebih baik untuk mencegah terjadinya GVHD. T_{reg} yang dikembangkan dengan cara menstimulasi dengan anti-CD3 tidak efisien untuk mengatasi GVHD, sebaliknya yang dikembangkan dengan cara menstimulasi dengan APC tipe host akan sangat efisien menghambat terjadinya GVHD [51].

Telah diketahui bahwa T_{reg} yang spesifik pada *antigen host* akan lebih survival jika diinfusikan pada *host*. Untuk tujuan terapi, sel T_{reg} akan distimuli dengan APC tipe *host* agar diperoleh fungsi optimum dalam melakukan regulasi. Sel T_{reg} dari *host* juga mempunyai kontribusi mencegah terjadinya GVHD. Telah diketahui bahwa sel T regulator bekerja secara non-spesifik. Artinya ketika T_{reg} telah menjadi efektor, sitokin yang dihasilkan dapat bekerja pada semua sel yang teraktivasi tidak terkait dengan antigen yang menyebabkan aktivasi itu. Trenado *et al.*, melaporkan bahwa T_{reg} yang distimuli dengan APC *host* jauh lebih efisien daripada jika APC berasal dari spesies lain yang tidak dikenal. Hal ini diduga T_{reg} yang teraktivasi *in vitro* tersebut langsung bekerja dan bisa mengenali targetnya ketika berada pada sirkulasi *host*. Pada studi menggunakan T_{reg} dari mencit transgenik, GVHD dapat dihambat jika T_{reg} mendapat stimuli antigen yang sesuai (2-4,44).

Dalam hal ini ketika mencit transgenik tersebut spesifik untuk OVA, maka GVHD hanya dapat dihambat jika *host* diberi stimuli OVA untuk mengaktifkan T_{reg} yang diinjeksikan. Hasil penelitian yang telah dilaporkan oleh banyak ilmuwan hampir dapat disimpulkan bahwa T_{reg} memerlukan antigen spesifik untuk terjadinya aktivasi dan berubah menjadi efektor, namun kerja selanjutnya tidak terkait dengan antigen yang menstimuli itu. Dalam penelitian lain diterangkan bahwa sel T $CD4^+$ yang ditransduksi dengan FOXP3 dapat menghambat GVHD dengan sangat efisien jika T_{reg} yang dimaksud mengenal salah satu *antigen host*. Pada keterangan terakhir ini menjadi gambaran bahwa T_{reg} bekerja tidak selalu menggunakan antigen spesifik secara absulut, namun yang dibutuhkan adalah adanya antigen yang bisa dikenali sehingga tercapai fase efektor. T_{reg} yang bekerja secara aktif ternyata mengekspresikan molekul CD62L pada level yang tinggi. CD62L merupakan molekul adhesi untuk homing, dengan demikian diperkirakan bahwa T_{reg} bekerja dengan efisien pada limfoid sekunder untuk melakukan fungsi sebagai sel T regulator [49; 51].

T_{reg} bekerja dengan menekan proliferasi dan fungsi efektor targetnya. Untuk mengatasi GVHD T_{reg} juga bekerja pada berbagai macam jaringan seperti kulit, paru, liver, dan saluran pencernakan. Molekul CCR5 diperlukan oleh T_{reg} untuk homing pada jaringan-jaringan target [52]. Pada eksperimen model colitis sitokin yang diketahui berperan sebagai imunosupresor adalah IL-10 dan TGF-beta. Pada eksperimen model GVHD sampai saat ini hanya IL-10 yang dilaporkan terlibat sebagai molekul supresor [4;5;6].

Pada percobaan menggunakan T_{reg} yang langsung diisolasi dari mencit normal tipe donor ternyata mampu mencegah GVHD dan bebas dari kematian karena GVHD. Sebaliknya injeksi T_{reg} yang diisolasi dari IL-$10^{-/-}$ hanya menunda efek letal GVHD dan hanya 40% yang hidup setelah 3 bulan transplantasi. Mencit yang tersisa inipun menunjukkan GVHD dengan sangat jelas. Dengan demikian dapat dikatakan bahwa IL-10 mempunyai peranan yang sangat penting sebagai molekul supresor bagi T_{reg} namun bukan satu-satunya faktor yang digunakan untuk mencegah terjadinya GVHD. CTLA-4 dan TGF-beta berperan pada sebagian mekanisme perlindungan terhadap GVHD pada tercobaan menggunakan T_{reg} yang diisolasi dari mencit IL-$10^{-/-}$. T_{reg} dapat menggunakan berbagai macam mekanisme efektor untuk dapat bekerja. Studi GVHD menggunakan mencit umumnya dilakukan selama tiga bulan dari awal transplantasi, hal ini sangat berhubungan dengan masa hidup mencit. Namun manusia mempunyai masa hidup yang lebih panjang daripada mencit, sehingga efek kronis harus terus diikuti. Pada percobaan BMT alogenik yang pernah dilakukan oleh Taylor et al., yaitu analisis 7 bulan setelah transplantasi ditemukan beberapa informasi antara lain terjadi GVHD yang serius pada ognan paru, kulit, usus, liver, dan limpa [43].

Dengan demikian meskipun nampaknya T_{reg} dapat melindungi host dari GVHD dan tanda klinik lainnya seperti berkurangnya bobot badan, tidak ada gangguan, namun ternyata tidak sepenuhnya dapat melindungi GVHD selama hidup. Hal ini dimungkinkan karena adanya MHC-minor yang selanjutnya menstimuli sel T sehingga berkembang menjadi sel-sel efektor yang dapat melakukan infiltrasi pada berbagai organ secara kronis. T_{reg} yang diinjeksikan tidak akan hidup dalam waktu yang sangat panjang, sehingga aplikasi T_{reg} perlu diulang sesuai dengan kebutuhan. Namun demikian ada dugaan bahwa T_{reg} yang diinjeksikan akan terus berkembang sesuai dengan homeostasis dan sel tersebut secara "cerdas" akan berproliferasi sesuai dengan kebutuhan fisiologi. Gejala GVHD yang muncul dalam waktu yang relatif lama setelah pelaksanaan transplantasi kemungkinan juga sebagai akibat reaksi *de novo* yaitu aktivitas dari sel-sel autoreaktif yang lolos dari proses seleksi negatif. Pada transplantasi alogenik dalam waktu yang panjang memungkinkan terjadinya

gangguan seleksi negatif sehingga sel-sel autoreaktif tetap hidup. Gangguan mekanisme seleksi negatif (*clonal deletion*) ini disebabkan oleh MHC yang tidak selaras (*MHC mismatching*) antara epitelium timus *host* dengan APC yang berkembang dari donor. Pada pelaksanaan BMT, kemoterapi maupun radiasi tidak dapat menghilangkan sel-sel resisten baik dari populasi sel T maupun APC *host*. Sisa-sisa sel tersebut pada akhirnya akan teraktivasi dan menjadi penyebab timbulnya GVHD [51;52].

Menurut pemahaman sejauh ini, bahwa sel sumsum tulang yang ditransplantasi akan berkembang menjadi sel-sel hematopoietik dan khususnya sel T akan mengalami seleksi pada timus *host*. Dengan demikian hanya sel-sel toleran yang akan lulus hidup. Jika sel sumsum tulang yang ditransplantasikan terkontaminasi sel T *mature* akan terjadi aloreaktif. Oleh karena itu inhibisi GVHD oleh T_{reg} hakekatnya sama dengan melakukan deplesi sel *mature* sebelum transplantasi, keduanya dimaksudkan untuk memperoleh toleran sehingga tidak terjadi serangan sistem imun pada *host* atau sebaliknya. Edinger *et al.*, Rifa'i *et al.*, dan Trenado *et al.*, menunjukkan bahwa T_{reg} secara signifikan menghambat terjadinya GVHD pada sistem alogenik khususnya BALB/c yang ditransfusi dengan C57BL/6 [8;10;12;44;53]. Ketika mencit ini selanjutnya diinjeksi dengan sel kanker A20, sel kanker akan dibunuh oleh sel dari C57BL/6 dan GVHD tetap saja terhambat. Dalam kaidah ini nampaknya sel T_{reg} merupakan sel cerdas yang dapat membedakan antara GVHD dan GVL, dalam kaitan ini T_{reg} hanya menghambat GVHD sehingga keberadaannya tidak merugikan dan GVL tetap terjadinya. Sampai saat ini tidak diketahui bagaimana T_{reg} membedakan GVHD dan GVL. Meskipun disebut-sebut sebelumnya bahwa T_{reg} bekerja nonspesifik namun ada kecenderungan bahwa T_{reg} lebih efisien menghambat aktivasi CD4 daripada CD8. Berbeda dari penelitian sebelumnya Cohen *et al.* menyatakan bahwa T_{reg} tidak dapat membedakan antara GVHD dari GVL. Akhir-akhir ini ada pertimbangan untuk tidak menghilangkan sel T yang mengkontaminasi sumsum tulang sebelum pelaksanaan BMT. Sel T yang terikut pada sumsum tulang tersebut diduga meningkatkan *engraftment* sel mieloid dan limfosit [44].

PENOLAKAN TRANSPLAN OLEH *HOST*

Transplantasi sumsum tulang tidak saja terhambat oleh masalah GVHD tetapi juga masalah rejeksi oleh *host*. Tata pelaksanaan BMT dengan obat-obat imunosupresif dapat menimbulkan efek samping yang sangat berat. Radiasi dan kemoterapi dapat menyebabkan kerusakan jaringan yang sangat parah terutama pada jaringan yang tersusun oleh sel-sel yang normalnya berproliferasi sangat cepat misal paru-paru dan intestinum. Pasca transplantasi memicu terjadinya infeksi dan keracunan hepar, hal ini banyak disebabkan karena sebelum pelaksanaan transplantasi menggunakan obat-obat imunosupresan. Dalam percobaan yang dilakukan oleh Rifa'i *et al.*, sel sumsum tulang mencit BABL/c yang ditransfusikan ke mencit IL-2Rβ $^{-/-}$ dengan *background* C57BL/6 ternyata terjadi kimera pada sel-sel hematopoietik (data tidak dipublikasi). Dalam hal ini terjadi toleransi pada sistem alogenik ini dan dua sel alogenik berkembang tanpa menunjukkan aloreaktif. Toleransi ini dapat terjadi jika transplantasi dilakukan sedini mungkin misal mencit belum berumur lebih dari tiga hari. Untuk mencit yang telah dewasa penolakan akan terjadi sangat cepat dan tidak ada sel donor yang dapat dideteksi setelah 7 hari transfusi. Untuk mencit dewasa, dengan radiasi dosis letal memungkinkan sel-sel sumsum tulang donor alogenik diterima oleh host dan terjadi toleransi. Demikian juga apabila infusi sumsum tulang (*bone marrow*, BM) berasal dari campuran (50%:50%) tipe donor dan *host*, radiasi letal dosis memediasi terjadinya toleransi dan sel berkembang saling berdampingan dan terjadi toleransi. Pada kasus yang terakhir ini diduga sel-sel T donor maupun *host* telah melalui seleksi pada timus *host* sehingga hanya sel-sel toleran yang dapat hidup sedang yang lain akan dieliminasi dengan mekanisme apoptosis. Pada percobaan yang dilakukan oleh Waldmann *et al.*, tingkat keberhasilan BMT alogenik semakin tinggi apabila host diinjeksi dengan antibodi yang dapat mengeliminasi sel T [45].

Pendekatan yang terbaik untuk memperoleh toleransi imunologi pada sistem alogenik adalah dengan menginjeksikan *professional regulatory T cells,* baik pada transplantasi sumsum tulang maupun transplantasi organ. Dalam kajian keseluruhan dan informasi yang ada pada awalnya diduga bahwa sel T regulator yang baru diisolasi dari donor akan lebih efektif dibandingakan dengan T_{reg} yang dikembangkan *in vitro*. Pada percobaan sistem alogenik dimana host diradiasi dengan dosis letal dan ditransfusi dengan BM singenik dan BM alogenik kemudian ditambahkan sel T dari *host*, ternyata terjadi penolakan sel donor

dengan sangat cepat. Dalam hal ini mudah dipahami bahwa sel *mature* tipe *host* akan mengeliminasi donor. Pada percobaan yang sama ketika T_{reg} $CD4^+CD25^+$ ditambahkan ternyata tidak mampu membantu penerimaan donor oleh *host*. Pada kasus ini rejeksi yang dilakukan oleh sel-sel *mature* tersebut tidak mampu dihentikan oleh sel regulator yang ditambahkan. Hal yang sangat menarik ketika dilakukan injeksi dengan T_{reg} yang sebelumnya distimuli dengan APC tipe donor secara *in vitro* ternyata dapat mempertahankan donor dari eliminasi sistem imun *host*. Hasil yang diutarakan terakhir ini menjadi hal yang sangat menarik karena T_{reg} yang dikembangkan *in vitro* sangat efisien dan stimuli *in vitro* dengan APC tipe donor dan penambahan IL-2 menjadi kunci penting yang harus diteliti lebih lanjut [45].

KESIMPULAN

$CD4^+CD25^+FOXP3^+$ merupakan *marker* yang paling tepat untuk menunjuk sel T regulator. Sel T regulator baik yang berasal dari isolasi langsung ataupun yang dikembangkan *in vitro* mempunyai daya regulasi yang sangat baik. Sel T regulator dapat membantu enggrafment donor pada *host* dan mengatasi atau setidaknya menunda GVHD. Sel T regulator tidak saja menekan aktivitas sel-sel aloreaktif namun juga menekan semua sel-sel efektor yang normalnya berguna untuk mengatasi infeksi. Jumlah sel T regulator yang diaplikasi untuk terapi merupakan faktor penting agar GVHD maupun rejeksi dapat teratasi namun sistem imun tetap berjalan dengan baik.

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Immunomodulator Testing on Ethanol Extract of *Gynura procumbens* Leaves to *Mus musculus* Adaptive Immune System: in Vitro Study

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Abstract

Immunomodulator is a substance that has an ability to modulate the activity and function of immune system. *Gynura procumbens* supposed to has benefit as an immunomodulator because of it afficacy to cure many diseases. The aim of this study is to determine the effect and dose variations of *G. procumbens* extract on biological aspect of $CD4^+CD62L^-$, $CD4^+CD62L^+$, $CD8^+CD62L^-$ and $CD8^+CD62L^+$ T cells. *G. procumbens* extract concentrations that used in this experiment were 0 µg /ml, 0.1 µg/ml, 1 µg/ml, and 10 µg/ml. Spleen cells were cultured for 4 days in 5% CO_2 incubator at a temperature of 37˚C. Cultured cells were harvested and analyzed by flowcytometry to asses cell surface molecule expression. The resulting data were tabulated and analyzed using ANOVA analysis with a significance of 0.05% on SPSS version 16. Results showed that the extract of *G. procumbens* can increase the proliferation of $CD4^+CD62L^-$, $CD4^+CD62L^+$, $CD8^+CD62L^-$ and $CD8^+CD62L^+$ T cells compared to the control. Dose of 1 µg/ml showed the highest effect to promote cell activation compared with the dose of 0.1 µg/ml and 10 µg/ml. Dose of 10 µg/ml could suppress $CD4^+CD62L^-$, $CD4^+CD62L^+$, $CD8^+CD62L^-$ and $CD8^+CD62L^+$ T cells development. This study suggests that the ethanol extract of *G. procumbens* has benefit as an immunomodulator and involved in the immune system.

Keywords: *Gynura procumbens*, immunity, immunomodulator, in vitro, lymphocytes.

INTRODUCTION

Immunomodulator is a substance that has an ability to modulate the activity and function of the immune system. Immunomodulator has ability to influence the number of cells that play role in the adaptive immune system such as $CD4^+$ and $CD8^+$ T cells. Research on immunomodulator that has been done used the recombinant cytokines. Recombinant cytokines that were used were interferon-γ (IFN-γ) in combination with vaccines [1,2]. The weakness of this substance are unstable, easily degraded, and have negative effects such as neutrophilia or defective neutrophil function, lymphophenia and monocytophenia [3]. Based on that case, we need another alternative such as active compound in a plant that has immunomodulator activity. One of them is *Gynura procumbens* or *Sambung Nyawa*.

G. procumbens has been long used by people in Java as a traditional medicine. Based on recent studies, leaves of *G. procumbens* have a lot functions such as anti-cancer, anti-inflammation of the kidneys and as anti-diabetic [4]. The immunological side *G. procumbens* such as immunomodulator activity has not been studied yet. In fact, with so many functions of *G. procumbens*, it is possible that these plants have immunomodulator capabilities that can affect the activity of immunocompetent cells such as $CD4^+CD62L^-$, $CD4^+CD62L^+$, $CD8^+CD62L^-$ and $CD8^+CD62L^+$ T cells. This study aimed to determine the effect of *G. procumbens* and dose variations of *G. procumbens* extract on the number of $CD4^+CD62L^-$, $CD4^+CD62L^+$, $CD8^+CD62L^-$ and $CD8^+CD62L^+$ T cells.

MATERIALS AND METHODS

Medium Preparation

Culture medium that was used in this study was RPMI medium with antibiotics penicillin and streptomycin. The medium were put in 2 propylene tubes, each as much as 5 ml and used as control medium. *G. procumbens* extract powder weighed 0.2 gr and diluted with 200 ml of sterile water and then homogenized. Then, a stock solution with a concentration of 1mg/ml. 100 µl was diluted with 9900 µl control medium to obtain a dose of 3 medium with a concentration 10 µg/ml. 500 µl dose in 3 medium was diluted with of 4500 µl control medium to obtain a dose 2 medium with a concentration 1 µg/ml. 500 µl dose 2 medium was diluted with of 4500 µl control medium to obtain a dose 1

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medium with a concentration 0.1 µg/ml. Dose 1, 2, and 3 medium filtered with cell strainer (Millipore membrane) and transferred into a new propylene tube. After filtered, 4 types of medium were added with 1 µl of 2-mercaptoethanol, 10% Fetal Bovine Serum (FBS), and 1% α-CD3.

Cell Isolation and Counting

Spleen organ was isolated from mice. Spleen was washed in petri dish that contained PBS. The spleen was transferred to another petri dish which also contained PBS, and then crushed. The suspension was filtered and transferred into propylene tube, then added with PBS until 12 ml suspension. Suspension of cell was centrifuged with a speed of 2500 rpm for 5 minutes at 4˚C. Pellet resuspended in 1 ml of control medium. This suspension taken 20 µl and added with 80 µl evans blue and then homogenized by pipetting. The number of cells was counted with haemocytometer.

Cell culture

Each medium, i.e. control, dose 10 µg/ml, 1 µg/ml, and 0.1 µg/ml were added with ± 122 µl of cell suspension and mixed gentle. Each of these medium which contain cell was inserted into the well in plate. The cells were incubated in a 5% CO_2 incubator at 37˚C for 4 days. After 4 days, the cells were harvested and then centrifuge with a speed of 2500 rpm for 5 min at 4˚C. Pellet was resuspended in 1 ml PBS. Each sample was taken 20 µl and added with 80 µl evans blue after that was homogenized by pipetting to count the number of cells. Each sample was also taken 300 µl and transferred into micro tube containing 500 µl PBS. A sample in 3 micro tubes was centrifuged with a speed of 2500 rpm for 5 minutes at 4˚C. Pellet stained with antibodies with extracellular staining.

Flowcytometry Test

Pellet in micro tube was added with 50 µl antibody. Antibodies that were used were FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse CD8, and PE/Cy5-conjugated rat anti-mouse CD62L. After that, the samples were homogenized by pipetting and incubated in the ice box for 20 minutes. Each sample in a micro tube was transferred to the flowcytometry cuvettes and added with 300 µl PBS. Next, the samples were ready for running with flowcytometer.

Experimental Design and Data Analysis

The design of this study was experimental research with a completely randomized design consist of 4 treatments (control, dose 10 µg/ml, 1 µg/ml, and 0.1 µg/ml). Flowcytometry results were visualized using BD CellQuest PRO™ software then the resulting data were tabulated and analyzed using ANOVA analysis with a significance of 0.05% on SPSS version 16.

RESULT AND DISCUSSION

Population of $CD4^+CD62L^-$ and $CD4^+CD62L^+$ T Cells

Extract of *G. procumbens* with 4 different doses gave significant different results on the activation of $CD4^+$ T lymphocytes (Fig. 1). Control treatment 0 µg/ml indicated the relative numbers of $CD4^+CD62L^-$ T cells were 23.91%, and the absolute numbers of cells were $5376x10^3$ cells. Dose of 0.1 µg/ml in cell culture showed greater cell numbers compared with control with relative number 29.54% as many $17015.1x10^3$ cells. Dose of 1 µg/ml showed the highest absolute number of 4 treatments given with the absolute number $30272.5x10^3$ cells (39.41%). The absolute numbers of cells in a 10 µg/ml dose were $9301x10^3$ cells (37.67%). This number was decreased compared to dose 0.1 µg/ml and 1 µg/ml, but still higher than the control.

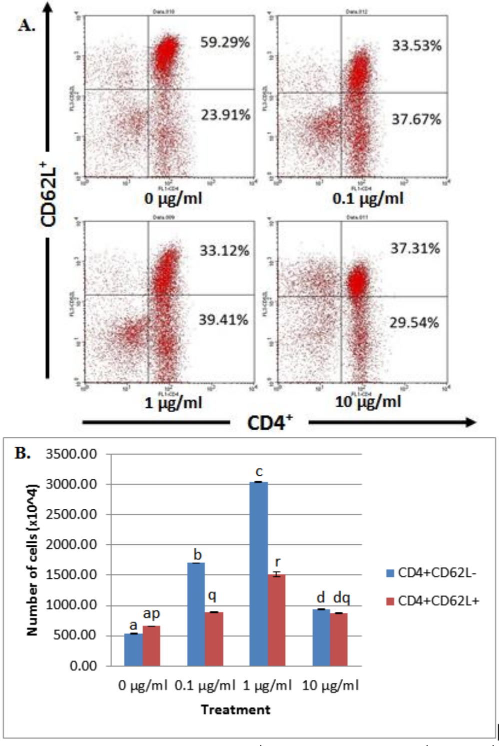

Figure 1. Population of $CD4^+CD62L^-$ and $CD4^+CD62L^+$ T Cells: A. Relative number; B. Absolute number.

Ethanol extract of *G. procumbens* was also affected the proliferation of $CD4^+CD62L^+$ T cells. The relative numbers of the cells in the control treatment were 52.29% and the absolute numbers were $6551.2x10^3$ cells. Relative numbers of $CD4^+CD62L^+$ T cells in the 0.1 µg/ml dose of the extract were 37.31% and the absolute numbers were $8822.5x10^3$ cells. The relative numbers of cells in the 1 µg/ml dose treatment were 33.12% with the absolute numbers $15164.6x10^3$ cells. Treatment dose of 10 µg/ml showed the relative number of 33.53% with the absolute number $8733.7x10^3$ cells.

The pattern of $CD4^+CD62L^-$ T cell proliferation showed the same trend with the proliferation of $CD4^+CD62L^+$T cells. The number of cells increased at treatment doses 0.1 µg/ml, 1 µg/ml and 10 µg/ml compared to the control treatment. Dose 1 µg/ml showed the highest cell number compared to other treatments. The number of cells decreased in a dose of 10 µg/ml when compared to dose 0.1 µg/ml and 1 µg/ml. Comparison of the number of $CD4^+CD62L^-$ T cells and $CD4^+CD62L^+$ T cell can be seen clearly that in the control treatment $CD4^+CD62L^+$ T cell count more than $CD4^+CD62L^-$ T cells, while cells after treatment with the *G. procumbens* extract given, the number of $CD4^+CD62L^+$ T cells tend to decrease compared to $CD4^+CD62L^-$ T cells. CD62L was a molecule that is owned by the naive cells [5]. It showed that extracts of *G. procumbens* has the ability to activate $CD4^+$ T cells so $CD4^+CD62L^+$ T cells lose CD62L molecule into $CD4^+CD62L^-$ T cells.

The increase of activated $CD4^+$ T cells number after the administration of extracts *G. procumbens* supposedly because the content of flavonoids and saponin in *G. procumbens* as an immunostimulant. According to Middleton *et al.* [6], flavonoids have the ability to trigger the activity of MAP Kinase. Mitogen can stimulate the increase of IL-2. IL-2 is a proliferation and differentiation factor of immunocompetent cells. Concentration of cyclin D2 and cyclin E would have increased when the cells were exposed to IL-2. IL-2 was also served to inhibit the p27 concentration. Under these conditions, IL-2 was able to induce cell cycle continuation of the G1 phase to the S phase of the cell cycle so it proliferated actively [7]. Saponin has the ability to increase cytokine IFNγ [8]. Lee *et al*. [9] and Shi *et al*. [10] mentioned that IFNγ can stimulate the up-regulation of MHC-II expression so that more T cells differentiate into $CD4^+$ T cells.

The decrease of the number of cells in the dose 3 treatment showed that *G. procumbens* at a dose of 10 µg/ml have immunosuppressive effects on $CD4^+$ T cells. This immunosuppressive effect was also obtained from the ethanol extract of leaf flavonoids in *G. procumbens*. According to Schroeter *et al.* [11], the actions of flavonoids were very complex, sometimes synergistic and antagonistic at times depend on the specific components, cell type, concentration, and experimental design. Based on this statement, it can be seen that the concentration of flavonoids in the given doses influenced the cells. Immunosuppressant tends to inhibit the transcription of cytokines so the numbers of cytokines such as IL-2 and IFNγ that play role in cell activation were decreasing [5].

Population of $CD8^+CD62L^-$ and $CD8^+CD62L^+$ T Cells

Extract of *G. procumbens* in cell cultures influence the activation of $CD8^+$ T lymphocytes (Fig. 2). The relative numbers of $CD8^+CD62L^-$ T cells in a control treatment were 4.04% and absolute number of cells $2421.1x10^3$ cells. Treatment with the *G. procumbens* ethanol extract showed the higher proliferation compared with control treatment. The numbers of cells in dose 1 treatment were $4584.6x10^3$ cells (4.70%), dose 2 were $7767.5x10^3$ cells (3.38%), and dose 3 were $3518.2x10^3$ cells (2.93%). Same with $CD4^+$ T cells, a dose 2 also showed the highest proliferation ability of the $CD8^+$ T cells.

Extract of G. procumbens cell culture also affect proliferation of $CD8^+CD62L^+$ T cell. The relative number of $CD8^+CD62L^+$ T cells in the control treatment were 2.07% and the absolute number were $404x10^3$ cells. The dose 0.1 µg/ml treatment showed the relative number of cells 3.31% and the absolute number were $615.5x10^3$ cells. At a dose 1 µg/ml, the relative number of cells 2.63% was as many $1029.1x10^3$ cells. While at a dose 10 µg/ml, the relative number of cells 1.95% was as many $292.8x10^3$ cells. These results indicated that proliferation of $CD8^+CD62L^+$ T cell was higher in the treatment with the *G. procumbens* extract dose 0.1 µg/ml and dose 1 µg/ml, while the dose 10 µg/ml cell number decreased compared to the control.

The increase in $CD8^+$ T cells was affected by the increase in cytokines IL-2 and IFNγ that

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induced flavonoid and saponin in *G. procumbens* extracts as described previously. The increase of CD8$^+$ T cells were also influenced by CD4$^+$ T cells that activated because CD4$^+$T cells that were activated would be differentiated into Th1 that producing IL 2 and IFN-γ [12]. Dose 10 μg/ml provides suppressive effect on CD8$^+$ T cells because this dose also has suppressive effects on CD4$^+$ T cells. IL-2 that produced by CD4$^+$T cells as addition used for the up-regulation itself and itwas also used by CD8$^+$T cells as a stimulant for proliferation. It is explained further that the CD8$^+$ T cells have a higher affinity than the affinity of CD4$^+$T cells in the use of IL-2 [12].

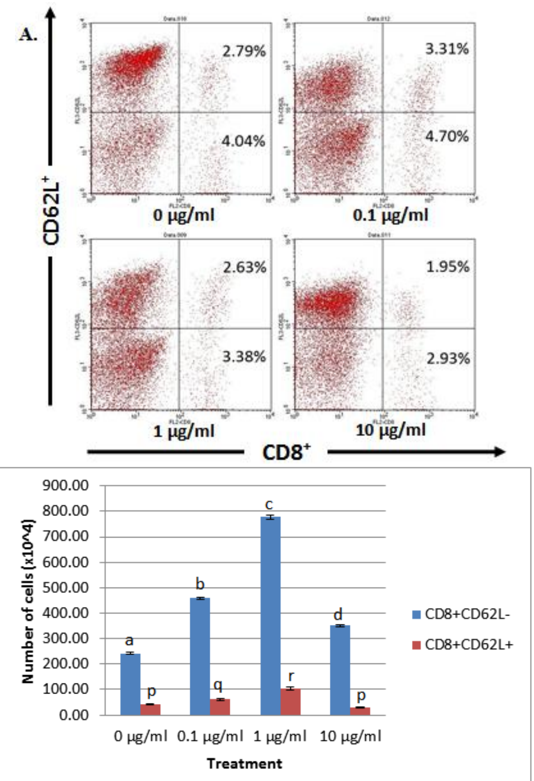

Figure 2. Population of CD8+CD62L- and CD8+CD62L+ T Cells: A. Relative number; B. Absolute number.

CONCLUSION

We concluded that the extract of *G. procumbens* can increase the proliferation of CD4$^+$CD62L$^-$, CD4$^+$CD62L$^+$, CD8$^+$CD62L$^-$, and CD8$^+$CD62L$^+$ T cells. Dose of 1 μg/ml showed the highest cell activation capability compared to the dose of 0.1 μg/ml and 10μg/ml. Dose of 10 μg/ml was given suppressants effect to CD4$^+$CD62L$^-$, CD4$^+$CD62L$^+$, CD8$^+$CD62L$^-$, and CD8$^+$CD62L$^+$ T cells because at that dose the number of cells was decreasing.

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In Vitro Immunomodulatory Activity of Aqueous Extract of *Moringa oleifera* Lam. Leaf to the CD4 +, CD8$^+$ and B220$^+$ Cells in *Mus musculus*

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Abstract

Immune system is a system of biological structures and processes within an organism that protects against disease. It can be promoted by substance referred as immunomodulator. Usually people use synthetic drug or synthetic immunomodulator to get quick response against the disease. This habit lead to arising side effects such as nausea, bone marrow degradation, thrombocytopenic purpura and agranulocytosis. As alternative, natural immunomodulator derived from active compound in plant. The objectives of this study are to determine the effect of aqueous extract of *Moringa oleifera* Lam. leaf to the population of CD4$^+$ and CD8$^+$ T cells, and also B220$^+$ cell on *Mus musculus* through in vitro study and to analyze the difference of immune response in treatment and non-treatment group (control). In this experiment we used spleen from Balb/C mice. Cells were grown in RPMI medium with three doses (10 µg/ml, 1 µg/ml, 0,1 µg/ml) of *M.oleifera* extract. The cells were grown for four days culture in the CO_2 incubator at 37°C with 5 % CO_2. The cells number and expression were analyzed by flowcytometry. Data was analyzed using one-way ANOVA with α=0,05 by SPSS 16.0 for windows with complete randomized design. The result shows that the extract has immunostimulant activity and the low dose (0,1 µg/ml) can increase the cell number of CD4$^+$ and CD8$^+$, while high dose (10 µg/ml) significantly increase B220$^+$ cells compared to the control. This result strenghten that *M.oleifera* has immunomodulator activity to immunity system and worth to be developed into medicinal drug.

Keywords: immunomodulator, in vitro, *Moringa oleifera*, T cell

INTRODUCTION

Human health can not be separated from the surrounding environtment. The number of infectious pathogens such as viruses, bacteria and fungi can easily attack if the body is in an unfit state due to weak immune system (deficiency) and will lead to arising many diseases. People often deal the emerging diseases with antibiotics or other synthetic drug treatment to get quick healing effect. This habit will lead to another problem such as antibiotic and syntetic drug resistance. Besides that, certain drugs can produce side effects such as nausea, bone marrow damage, thrombocytopenic purpura, and agranulocytosis that can lead to other diseases. Herbs are safe to use with no side effects and in some cases is the most effective choice [1]. Because of that case, many species of medicinal plants are widely used in herbal medicine [2].

Prevention of diseases and free radicals which attack the body can be done by modulating the immune system. Immunomodulator is a substance which able to modulate the function and activity of the immune system. *Moringa oleifera* Lam. is one of the species which known for having the immunomodulatory activity to the immune system [3].

Moringa oleifera Lam. is Family *Moringaceae* that most known and widely distributed in the world. This plant has a high value because almost all parts of the plant (leaf, roots, stems, flowers, fruit peel and seeds) can be used as highly nutritious food and also has been reported to have antimicrobial compound [4]. This plant also serves as an immune system builder and is used in some countries to overcome malnutrition and malaria [5]. This herb was choosen due to its rich phytochemical compound, which including saponins, carotenoids, phenolic compounds and flavonoids. Saponin and flavonoid serve as natural immunomodulator that expected to enhance lymphocytes cells development which is very important in immunity system [6].

Based on research conducted by Hefni [7], aqueous extract of *M. oleifera* that was given orally to mice infected with *Salmonella typhi*

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showed immunomodulatory effects because it was able to increase the number of the cell population of HSC ($CD34^+$), B lymphocytes ($B220^+$), precursors of erythrocytes ($TER119^+VLA\text{-}4^+$), mature erythrocytes ($TER119^+$), expression of naive T cells ($CD62L^+$) and proinflammatory cytokines IFN-γ. Therefore, this research aims to assess the effect of aqueous extract of *M. oleifera* Lam. leaf to the population of T cell $CD4^+$, $CD8^+$ and $B220^+$ on *Mus musculus* through in vitro study and to analyze the difference of immune response in treatment and non-treatment group (control).

MATERIALS AND METHODS

This research was conducted on September 2013 to March 2014 at Laboratory of Animal Physiology, Department of Biology and Biomedical Laboratory, Faculty of Medicine, University of Brawijaya.

Material and Equipments

The materials which is used are aqueous extract of *M. oleifera* leaf, *Mus musculus* Balb/C, RPMI 1640 medium, sterile water, α-CD3, FBS (fetal bovine serum), 2-mercaptoethanol, antibiotics (penicillin - streptomycin), PBS, 70 % alcohol, and monoclonal antibodies (FITC conjugated rat anti-mouse CD4, PE conjugated rat anti - mouse CD8, PE/Cy5 conjugated rat anti - mouse B220.

The equipments used for this research are erlenmeyer glass, medium bottles (Schott), CO_2 incubator, freeze drying machine, autoclave, microscope, microtube, milliphore membrane, micropipette, syringe, propylene tube, centrifuge, BD FACS Calibur ™ flow cytometer, petri dish, wire, surgical scissor, cuvettes, LAF (laminar air flow), haemocytometer and 48 wells culture dish.

Moringa oleifera extraction

Moringa oleifera leaf was obtained from Tirtomarto Village, District Ampelgading, Malang in August 2013. This method was performed in the Laboratory of Biochemistry, Department of Chemistry, Brawijaya University. *Moringa oleifera* leaf was successively extracted with water by maceration method. Extraction process was done by taking the mature leaf, washed and air-dried, then crushed into powder to obtain the simplisia form. The result of this extraction process was in gel form. To obtain the dry extract, it was freeze dried for ± 24 hours.

Cell Isolation

Mus musculus used in this study were male mice, Balb/C strain. It was killed by neck dislocation. After dislocation, the mice were dissected using surgical scissor on a surgical board in the dorsal part, and then the spleen was taken and washed with PBS.

Spleen was placed in a petri dish containing PBS ± 2ml. The organ then crushed with a syringe base, clockwise until the organ was destroyed. The homogenat was put in a 15 ml propylene tube filtered with wire to take out the debris. PBS was added to the tube untill 12 ml. After that, cell was centrifuged at 2500 rpm for 5 min and 4^oC. The supernatant was discarded and the pellet was taken. Pellet was resuspended by micropippete in 1 ml of RPMI media. Then it was taken 20 µl into a microtube, homogenized with 80 µl evans blue dye, and placed in a haemocytometer to count the number of living cells by microscope. Living cells were characterized by cells that were not stained blue. The number of cells needed for culture was $3x10^6$ cells for each well.

Cell Culture and Treatment

The cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), antibiotic (penicillin & streptomycin), 1% α-CD3, 1 µl 2-mercaptoethanol, respectively. The media was mixed with three doses of *M.oleifera* extract (0,1 µg/ml, 1 µg/ml, 10 µg/ml) and 0 µg/ml (control), and seeded into 48 wells culture dish. Each well contains approximately 1 ml of medium and cells suspension. All process were done in Laminar Air Flow (LAF) with sterile room and aseptic methods to avoid contamination. The equipments for cell culture were under sterile condition by autoclaving in 121° C. Total of 70% alcohol was used for sterilized the LAF surface. Furthermore, the dish was kept at CO_2 incubator with temperature 37^oC, 5% CO_2 for four days.

Cell Harvesting

Cell harvesting was conducted after four days of cell culture by slowly pipetting the medium, then put it in 15 ml propylene tube. Furthermore, the yields was centrifuged at 2,500 rpm for 5 min and 4^oC. Supernatant was discarded and the pellet was taken, resuspended with 1 ml PBS. Result was divided into two microtube for two groups of antibody staining. Total 500 mL of PBS was added to each micro tube, and then centrifuged again. The supernatant was

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discarded and the pellet was taken. Pellet ready to be labelled with specific antibody.

Antibody staining

The extracellular staining was conducted by antibody and divided into two groups of staining, group A ($CD4^+$, $CD25^+$, $B220^+$) and group B ($CD4^+$, $CD8^+$, $CD62L^+$). It was done with added 50 mL specific antibody to the pellet of second centrifugation, pipetting, incubated in an ice box, added 300 mL PBS and transferred to flow cytometry cuvet.

Flow Cytometry Analysis

Flowcytometric analysis was to determine the cell number of $CD4^+$, $CD8^+$ and $B220^+$. After transfer into the flow cytometry cuvet, samples were ready for analysis according to the parameters that had been set in the flow cytometry FACS Calibur machine. Obtained result analyzed by Cell Quest Pro^{TM} software and data was analyzed using one-way ANOVA with α=0,05 by SPSS 16.0 for windows with complete randomized design.

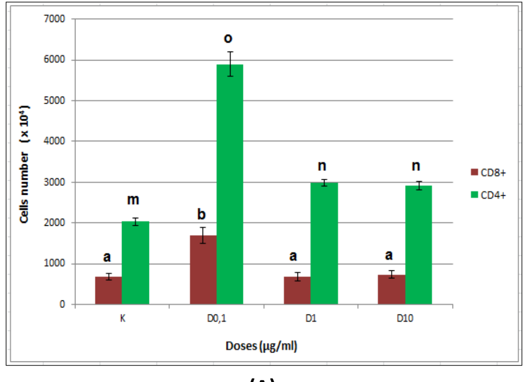
RESULT AND DISCUSSION

Absolute Cells Number of $CD4^+$, $CD8^+$ and $B220^+$ in the Spleen of *Mus musculus*

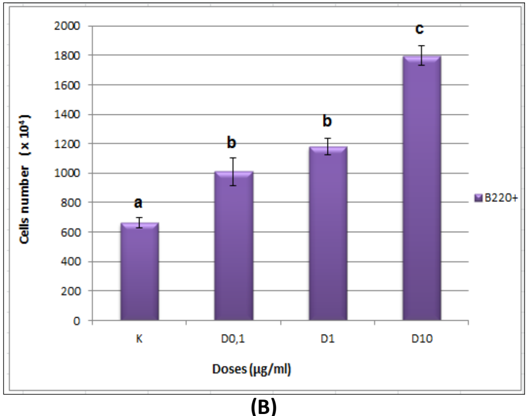
The aqueous extract of *M.oleifera* leaf that was given in lymphocytes of Balb/C mice showed immunomodulatory activity as immunostimulant. This activity can be seen through the increase in the cells number of $CD4^+$ (T helper cells), $CD8^+$ (T cytotoxic cells) and $B220^+$ cells in different doses. CD4+ and CD8+ are surface molecules which present in the activated-T cells. T cell activation can be caused by antigen infection.

In normal cells, the $CD4^+$ and $CD8^+$ molecule should not be excessive expressed. Excessive $CD4^+$ and $CD8^+$ molecules expression in normal cells indicated reactivity is present and that should be under apoptotic to mantain the homeostatic condition in the body.

The effective dose to stimulate highest cell proliferation in $CD4^+$ and $CD8^+$ cells is in the lowest dose (0,1 μg/mL), but the effective dose in $B220^+$ is in the highest dose (10 μg/ml) instead. The number of $CD4^+$ cells in control is $20x10^6$ cells, in 0,1 μg/ml, 1 μg/ml and 10 μg/ml is $59x10^6$, $30x10^6$ and $29x10^6$ cells, respectively. The number of $CD8^+$ cells in control is $6,82x10^6$ cells, in 0,1 μg/ml, 1 μg/ml and 10 μg/ml is $17x10^6$, $6,8x10^6$ and $7,3x10^6$ cells, respectively. While the number of $B220^+$ cells in control is $6,6x10^6$ cells, in 0,1 μg/ml,1 μg/ml and 10 μg/ml is $10x10^6$, $12x10^6$ and $18x10^6$ cells.



(A)



(B)

Figure 1. Effect of aqueous extract of *M. oleifera* leaf to the absolute cells number of A ($CD4^+$ & $CD8^+$) and B ($B220^+$) after four days culture with dose 0 μg/ml (control), 0,1 μg/ml, 1 μg/ml, 10 μg/ml. Cells were isolated from the spleen of *M. musculus*. Number of cells were analyzed by flowcytometry.

Based on ANOVA, the absolute cell number of $CD4^+$ T cell in all dose treatment shows significant difference compared to the control ($p<0,05$). However, dose 1 μg/ml and 10 μg/ml have no significant difference ($p>0,05$). The lowest dose (0,1μg/ml) stimulate the highest cell proliferation and in higher doses, the number of cell is decrease. It indicated that absolute cells number of $CD4^+$ is affected by the difference of treatment (doses).

Figure 1 shows that $CD4^+$ cells has the highest proliferation number than $CD8^+$. It can be assumed because $CD4^+$ plays crucial role to secrete many kinds of cytokine after differentiated into Th1 and Th2. Increasing number of $CD4^+$ will affect the number of $CD4^+$ cell itself and another T cells such as $CD8^+$ or

CD25$^+$ through the cytokine secretion. It also stimulates B cell to secrete antibody [8].

One of the cytokines produced by CD4$^+$ is interleukin-2 (IL-2), which is very essential in increasing proliferation of T cells and B cells [9]. IL2 will regulate cell communication to activate the other cells. The cytokine synthesis is initiated by gene transcription and occurs due to the presence of a stimulus, in this case the stimulus derived from active compounds in the extract of *M. oleifera*. IL-2 is one of the earliest immune response of T helper cells after activated by a stimulus. These cells will also bind to the APC, which generally are macrophages after doing phagocytosis. It rise the immune response in the body [10].

The immunomodulatory activity of *M.oleifera* also present in CD8$^+$ cell. The lowest dose (0.1 μg/ml) can stimulate the highest cells proliferation. The high number of CD8$^+$ cells also affected by CD4$^+$ because it secrete IL2 and IFN-γ to induce the proliferation of CD8$^+$ [11]. Figure 1 also tells us that the significant difference only present in 0,1 μg/ml compared to the control, therefore the other doses (1 μg/ml & 10 μg/ml) have no significant difference with control. It means that the cells are not affected by these two doses treatment.

Compared to the CD4$^+$ cell, the number of CD8$^+$ is lower. This can be assumed because in this experiment, we used normal cell (without antigen infection), so the cell doesn't require much CD8$^+$. CD8$^+$ is the T cell that already activated and has the ability to killing a target identified as cytoplasmic antigen and will recognize and killed infected cells, so it is called cytotoxic T cells [12]. When these cells destruct the targets – which considered as foreign antigens, will cause systemic inflammation [9].

Cytotoxic T cells directly attack cells that contain foreign antigens or abnormal molecules on the surface. Cytotoxic T cells are particularly useful for attacking viruses because viruses often hiding from the immune system as it grow within infected cells. These cells recognize small fragments of the virus which is visible from the cell membrane and then kill the infected cells [13]. CD8+ has many subset with diverse function, e.g. subset CD8+CD122+ is a regulatory T cells which are naturally occurring [14]. This cell is indispensably important in the maintenance of immune system homeostasis and expressed in mice, but not identified in human. Human showed CD8$^+$CXCR3$^+$ instead, with same function with CD8+CD122+ [15].

Aqueous extract of *M.oleifera* leaf also shows immunostimulant activity in B cells (B220$^+$). All of the three doses can increase the number of B220$^+$ cells compared to the control. The number of B220$^+$ cells increased significantly (p<0,05) due to higher dose given and the number of cells is the highest at the highest dose treatment (10 ug/ml). B220$^+$ (CD45R) are a cell surface marker on B cells and expressed mostly in all cell B lineages. CD45 is a tyrosine phosphatase that participates in modulating the immune response in both B cells and T cells [16].

One of the factors that influence the activation of B cells is IL-2 produced by CD4$^+$. When cells expressed high number of CD4$^+$, which is in dose 0,1 mg/ml, it showed the lowest number of B220$^+$ expression. Otherwise, when the number of CD4$^+$ is low (doses 1 and 10 mg/ml), then the number of B220$^+$ cell is high. This is supposed because only B cells in the late stages of development, which is B cell that already differentiate into plasma cell, it has decreased expression of B220$^+$ (downregulated) [17]. In the other word, B220$^+$ is a marker for naive B cells. Naive B cells are the B cells that have not been activated by the presence of antigen. IL-2 secreted by CD4$^+$ help the B cell activation, resulting in the higher secretion of IL-2, then the cell surface expression of B220$^+$ will decrease. This is because the activated B cell will have decrease expression of B220$^+$.

Immunostimulatory activity found in the increasing number of CD4$^+$, CD8$^+$ and B220$^+$ due to the active substances such as saponins and flavonoids in the aqueous extract of *M. oleifera* leaf [18]. Cell proliferation induced by lymphocyte respond can be caused by exogenous stimuli in the form of the active compounds of plants which act as immunostimulator [11,19]. The active compounds in *M.oleifera* act as MAPK (mitogen activated protein kinase), which will stimulate T and B cell proliferation through the expression of IL-2 [7].

IL-2 is a major autocrine growth factor for T cells and responsible for the progression from GI to S phase in the cell cycle. It also acts as a growth factor for B cells and as a stimulus for antibody synthesis [20]. Flavonoid exert an antioxidant effect which stimulates immune system through affecting the enzyme involved in inflammatory reaction [21,22]. While saponin increase the production of many kind of immune mediators and can stimulate the viability of cells, which plays role in immune system. The activity of saponin may be mediated by upregulation of

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secretory molecules in macrophages and neutrophils and this compound also plays role in activation of these cells [23].

Cells Expression Profile of CD4+, CD8+ and B220+ by Flow Cytometry Analysis

The profiles showed the expression of relative cell number of CD4+, CD8+ and B220+ (Fig. 2 and 3). The expression of CD4+ on Fig. 2 shows that the lower dose (0,1 μg/ml) can stimulate CD4+ expression (54,09%), but the effective dose which can stimulate the highest cell number is dose 1 μg/ml (58, 71 %).

The lowest expression is in the dose 10 μg/ml (51,50%), while in control is 50,20%. Therefore, in CD8+, the dose with highest cells expression is in the lower dose (15, 57%), compared with dose 1 μg/ml (13,43%) and 10 μg/ml (12, 86 %), while in control is 16,86%.

Expression of B220+ shows that the high dose of *M. oleifera* extract is effective to increase the B220+ expression (Fig. 3). The highest cell expression number is in the highest dose (10 μg/ml) (28,96%), while in dose 0,1 μg/ml is 10,82%, in 0,1 μg/ml is 9, 89 % and control is 11,30%. From those data, we can see the immunostimulant activity in the CD4+, CD8+ and B220+ cells.

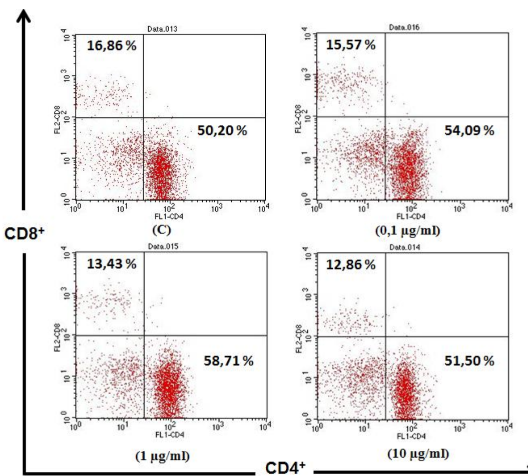

Figure 2. Cells expression profiles of CD4+ and CD8+. This result obtained through flow cytometry and analyzed by Cell Quest Pro™ software.

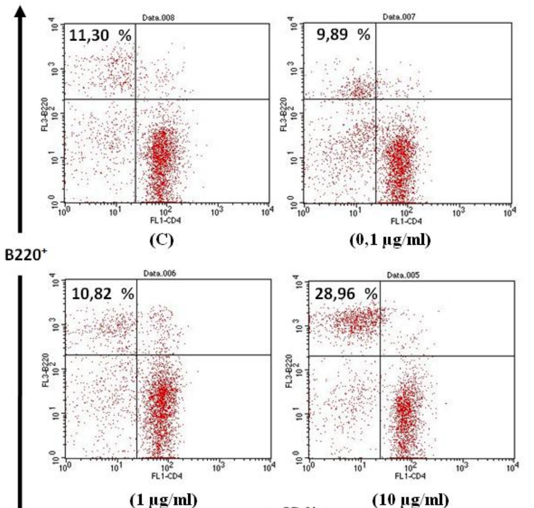

Figure 3. Expression profile of B220+ cells. This result obtained through flow cytometry and analyzed by Cell Quest Pro™ software.

CONCLUSION

The aqueous extract of *M. oleifera* leaf has activity as immunomodulator through it's active compound, such as saponin and flavonoid, which act as an immunostimulant on CD4+ (T helper cell) and CD4+ (T cytotoxic cell), as well as B220+. The low dose (0, 1 μg/ml) is effective to increase the number of CD4+ and CD8+ cells, while high dose (10 μg/ml) is effective to increase the number of B220+ cells.

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Identification of Coral Reefs in Mamburit Waters, Sumenep Regency

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Abstract

This research was conducted in September to October 2013 in Mamburit Waters, Sumenep Regency. This study was aimed to assess the percentage of coral reefs and acknowkedge the type of the coral reefs. Coral reefs was observed with the *Line Intercept* (LIT) method laid parallel to the coastline in the depth of 3 m and 10 m in *windward* and *leeward* area. Total of 59.88% coral reefs lived in *leeward* area in 3 m depth includes in good category and the percentage of dead coral reefs and other fauna for 40.12%. In the 10 m depth, 69.19% live coral reefs include as medium category with percentage of dead coral reefs and other fauna for 30.81%. *Winward* location of 3 m depth has 68.38% live coral reefs in good category and percentage of dead coral reefs and other fauna for 31.62%. Whereas for 10 m depth, 40.86% of live coral reefs include as medium category with percentage of dead coral reefs and other fauna for 59.14 %.

Keywords: coral reefs, *leeward*, *windward*, Mamburit

INTRODUCTION

Kangean is an island in East Java Province with 2 districts: Kangayan and Arjasa that has coral reefs cover area of 2,900.5 ha and 3,536.2 ha consecutively. It is located in eastern of Madura Island and northern of Bali Island [1]. Coral reefs also function as habitat for biota feeding ground, spawning, nursery ground, reproduction area. Diverse sea organism of various tropic levels such as fish, crustacea, mollusc, echinodermata, polychaeta, and other biota living surround the coral reefs. Coral reefs provide some habitat for these living organisms [2], each component in this community has tight dependance of each other [3].

The Island of Mamburit has very good potencies to be developed into underwater tourism site. It is due to its diverse resources, especially from coastal resources, e.g. sea biota, coral reefs, fish, mollusc, etc. Most people in Mamburit Island are fishing for their occupation. From the general fisheries catches, percentage of living coral reefs needs to be concerned because it affects the public economy in fisheries. The less percentage of coral reefs, the less sea biota evolves [4].

Moreover, fishermen in Mamburit Island exploit coral reefs for building materials. Fishermen also fishing with small boats when low tide thus the coral reefs get massive damages. The accurate data of coral reefs in Mamburit Island is absence thus it needs direct review from authority so that fisheries become more environment-friendly. It also to prevent the exploitation of coral reefs for building materials, thus it is important to identify the coral reefs, to determine the percentage of living or dead coral reefs. This study is aim to assess the percentage and the type of the coral reefs. Result of this study is expected to provide information especially to the local government and researchers to be referenced.

MATERIALS AND METHODS

This research is conducted in September to October 2013 in waters area of Mamburit, Sumenep Regency (Figure 1). This study was initiated by the determination of study sites by using Line Intercept (LIT) method parallel to coast line. This research conducted in four stations (Table 1) and positioned by Global Positioning System (GPS). Each windward and leeward area divided into four observation plot. Coral reefs along the roll meter were identified and the growth was observed.

Table 1. Study Site Potition

Location	Direction	Depth (m)	Coordinates
I	winward	3	6°50'48.39" S 115°13'5.84" E
II	winward	10	6°50'30.13"S 115°13'19.54" E
III	leeward	3	6°50'29.24" S 115°12'56.97" E
IV	leeward	10	6°50'13.88" S 115°13'9.54" E

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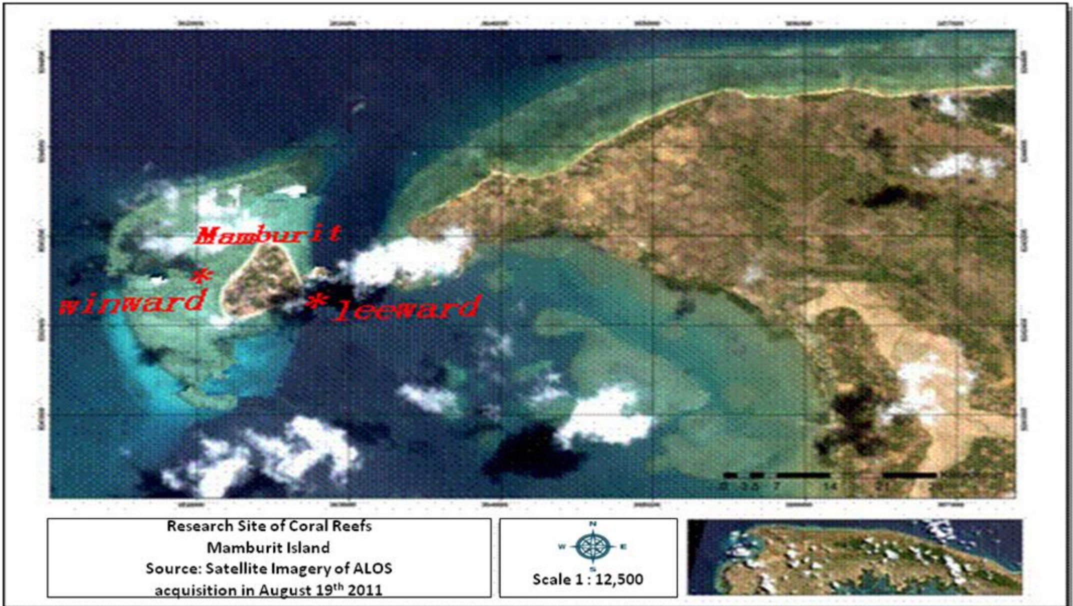

Figure 1. Observation Site of Coral Reefs in Mamburit Island (ALOS Imagery).

Measurement of Environment Parameter

Environment parameters were measured on each station consisted of depth, temperature, salinity, surface currents, water transparency, and DO. Methods of measurement refer to Sari and Usman [5].

Depth (Meter)

Depth measured in each station along with the observation on coral reefs coverage. The depth of waters' bottom (coral reef) was acknow-ledged by deep gauge on the hose regulator of diving equipment.

Temperature (°C)

Temperature measured in each determined station with thermometer. Thermometer dipped in the water and measured number recorded.

Salinity (‰)

Salinity was measured by refractometer in each station. Refractometer dipped in the water and the scale showed the salinity value.

Surface current (ms^{-1})

Current was measured with floating ball with string length of 5 m. Measurement conducted by releasing the floating ball along with stop watch and then the result recorded.

Water Transparency (meter)

Trancparency of water measured with secchi disc. Secchi disc entered into the water until the white part of the disc was not seen. The string length showed the transparency of the water.

DO (mgm^{-3})

The tool was calibrated on zero scale and then probe was dipped in the water for a while until the tool showed the numbers of DO.

Data Analysis

Percentage of coral reefs coverage includes the coverage of dead coral, live coral, and other lifeform. It counted with formula of English *et al.* [6]:

$$C = \frac{a}{A} x100\%$$

Description: C = percentage coverage of lifeform i
a = transect length of lifeform i
A = total length of transect

Data analysis to calculate the percentage of coral reefs coverage refers to formula of UNEP [7] and living coral reefs categorized based on Ofri [8] in Table 2.

$$\%\ Coverage = \frac{Total\ Length}{Transect\ Length} \times 100$$

Table 2. Category of living coral reefs coverage

Percentage (%)	Category
00.0 – 24.9	bad
25.0 – 49.9	medium
50.0 – 74.9	good
75.0 – 100.0	very good

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RESULT AND DISCUSSION

Parameter of Mamburit Waters

The highest salinity is in the 10 m depth of Leeward, followed by 10 m and 3 m depth of winward, and 3 m depth of Leeward. According to Eliza [9], coral organism is living in average salinity of 35‰. The highest salinity is 3635‰ in Leeward area, however the average salinity in Mamburit is 35‰; the salinity that appropriate for the growth of coral reefs. The increase of salinity leads to mortality for the coral reefs.

Temperature parameter in 10 m depth of Leeward area is 29°C, while 3 m depth of Leeward is 30°C. Otherwise, 10 m and 3 m depth of Windward is similar, i.e. 30°C. The high temperature was due to the measurement that conducted in the day light, and the weather condition at the time is hot. However, the temperature still supports the growth of coral reefs in Mamburit waters. Effendi [10] stated that temperature for coral reefs to grow is ranged between 22-29°C with maximum point of 36°C. The change on sea water's temperature caused Zooxanthellae off of their host, i.e. coral reefs.

pH in the depth of 10 m Leeward is 7.3, while in 3 m depth is 7.4. Otherwise in Windward, pH of 10 m and 3 m are same, i.e. 7.3. Acidity degree (pH) in sea waters is commonly average 7.5 - 8.4. Ministry of Environment determined the ideal pH for sea biota ranged 7-8.5, while Salm [11] suggested normal pH in waters area ranged 8.0-8.3.

DO in Leeward area of 10 m depth is 6.7 $mg.m^{-3}$, while in 3 m is higher, i.e. for 6.9 $mg.m^{-3}$. Otherwise, DO in windward of 10 m depth is 7.9 $mg.m^{-3}$, whereas in 3 m is 7.7 $mg.m^{-3}$. The DO in Mamburit area is sufficient for the growth of coral reefs.

Current speed in Mamburit waters of Leeward area is similar for 10 m and 3 m, i.e. 10.47 $m.s^{-1}$. Meanwhile, windward of 10 m depth has 14.31 $m.s^{-1}$ current speeds, whereas in 3 m depth, the current speed is 13.14 $m.s^{-1}$. Nontji [12] explained that the current speed on the water surface is 0.06 – 0.64 $m.s^{-1}$. Water current is needed to obtain food, e.g. zooplankton, and oxygen, as well as to clean the surface of coral reefs from sediment thus the growth of the coral reefs will not obstructed.

Waters transparency is supporting factor for coral reefs growth. Waters transparency in Mamburit ranged 3-10 m. The transparency was relatively high, showed that the water condition of Mamburit is relatively clean and clear.

Percentage of Coral Reefs in Mamburit

The growth form of coral reefs in windward area of Mamburit found a total of 11 types of coral reefs include: ACB, ACD, ACS, ACT, CF, CHL, CM, CME, CS, CMR, and CB (Table 3). We found 11 types of coral reefs in windward area, plot I at the 3 m depth with 64.82% living coral reefs which categorized as good. The coral reefs were dominated by particular type of CF for 27.02%, with dead coral reefs and other fauna for 35.18%.

A total of 7 types of coral reefs were found in Plot II, with 71.94% living coral reefs categorized as good. Dead coral reefs and other fauna were found as much as 28.06%. Coral reefs in this location were dominated by the type of ACD for 6.96%.

Otherwise, 8 types of coral reefs in the depth of 10 m, Plot III for 41.54% with dead coral and other fauna for 58.46%. It is categorized as medium. This location was dominated by the type of ACB for 25.12%. We found 6 types of coral reefs in Plot IV with 40.18% living coral categorized as medium, while the dead coral and other fauna for 59.82%.

Table 2 and 3 showed the percentage of each type of coral reefs. The higher light intensity supports the growth of coral reefs because the light will affect the abundance of Zooxanthellae [13]. This has to be related to the 3 m and 10 m depth; which the highest percentage of living coral reefs found in the depth of 3 m. It is caused by the light intensity that reached the bottom of the sea, whereas many shatter of coral reefs found in 10 m depth. The shatter was caused by the waves and human activities.

Coral reefs with good category commonly found in the depth of 50 m and 20 m. However, coral reefs that found in the depth of 3 m and 10 m has more species diversity and a better growth compared to the ones in the depth of 50 m and 20 m; affected by the light intensity which enter the waters depth [4,12]. Coral reefs in the depth of 3 m and 10 m of Windward indicate that the Mamburit waters are still clear.

The condition accelerates the growth of coral reefs because the photosynthesis process from solar light towards the coral reefs that penetrate the bottom of the sea. The average percentage of living coral reefs in 3 m depth Windward area is 68.38% (categorized as good), with dead coral and other fauna for 31.62%. Otherwise for 10 m depth, the percentage of living coral is 40.86% (medium category), with dead coral and other fauna for 59.14%.

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Table 3. Coral Reefs in Windward Area of Mamburit

Living coral reefs	3 m depth of windward				10 m of windward			
	Plot I		Plot II		Plot III		Plot IV	
	length (cm)	%	length (cm)	%	length (cm)	%	length (cm)	%
Acropora branching (ACB)	813	16.26	872	17.44	1256	25.12	995	19.9
Digitate(ACD)	348	6.96	1467	29.34	17	0.34	142	2.84
Sub massive(ACS)	358	7.16	353	7.06	46	0.92	75	1.5
Tabulate(ACT)	78	1.56	290	5.8	205	4.1		
Branching(CB)	33	0.66						
Foliose (CF)	1351	27.02			5	0.1	90	1.8
Heliopora (CHL)	26	0.52	75	1.5	80	1.6		
Massive (CM)	196	3.92	540	10.8	432	8.64	677	13.54
Millepora (CME)	30	0.6			36	0.72	30	0.6
Mushroom (CMR)	8	0.16						
Sub massive (CS)								4.14
Total percentage %		**64.82**		**71.94**		**41.54**		**40.18**
average		**6.48**		**11.99**		**5.19**		**6.69**
Dead coral reefs								
Hard coral (DC)	275	5.5	291	5.82	821	16.42	27	0.54
Dead coral with algae (DCA)	235	4.7	367	7.34	118	2.36	70	1.4
Others: anemon, gorgonian (OT)	12	0.24	587	11.74			207	4.14
Rubble (R)	941	18.82	30	0.6	1721	34.42	1875	37.5
Abiotik sand (S)	82	1.64			247	4.94	812	16.24
Soft coral(SC)	18	0.36			16	0.32		
Spongs (SP)	121	2.42	128	2.56				
Turf alga (TA)	75	1.5						
Total percentage %		**35.18**		**28.06**		**58.46**		**59.82**
Average		**4.49**		**33.06**		**11.69**		**11.96**

Table 3. Coral Reefs in Leeward Area of Mamburit

Living of Coral reefs	3 m depth of leeward				10 m depth of leeward			
	Plot I		Plot II		Plot III		Plot IV	
	length (cm)	%	length (cm)	%	length (cm)	%	length (cm)	%
Acropora branching (ACB)	970	19.4	1308	26.16	2147	42.94	1359	27.18
Digitate (ACD)	244	4.88	92	1.84	361	7.22	356	7.12
Sub massive(ACS)	475	9.5	217	4.34	102	2.04	400	8
Tabulate(ACT)	91	1.82	55	1.4	7	0.14	66	1.32
Branching(CB)	151	3.02	68	1.76				
Foliose (CF)	158	3.16			13	0.26	914	18.28
Heliopora (CHL)	37	0.74	30	0.6				
Massive (CM)	825	16.5	1039	24.06	604	12.08	553	11.06
Mushroom (CMR)	228	4.56						
Millepora (CME)					37	0.74		
Total percentage %		**63.58**		**56.18**		**65,42**		**72.96**
Average		**7.06**		**8.02**		**9.34**		**12.16**
Deat coral reef								
Hard coral (DC)	319	6.38	283	5.66	141	2.82	553	11.02
Dead coral with algae (DCA)	206	4.12	187	3.74	113	2.26	551	4.62
Others: anemon, gorgonian (OT)	5	0.1						
Rubble (R)	955	19.1	1029	20.58	114	2.28	332	6.64
Abiotik sand (S)	285	5.7	185	3.7	1342	26.84	238	4.76
Soft coral(SC)	15	0.3	135	2.7				
Spongs (SP)	6	0.12	348	6.96	19	0.38		
Turf alga (TA)	30	0.6						
Halimedae (H)			24	0.48				
Total percentage %		**36.42**		**43.82**		**38.58**		**27.04**
average		**4.55**		**6.26**		**6.91**		**6.76**

Windward area was dominated by Coral Massive (CM) and Acropora Brancing (ACB). It implies that the greater light intensity, the greater growth of the coral reefs [13]. Light that enter the waters is needed by Zooxanthellae which live in the tissues of coral reefs, which affect the growth of coral reefs. Thus the light penetration that depends on the depth of the sea affects the growth of coral reefs [4].

Light intensity in the depth of 10 m is able to penetrate to the bottom of the sea, but the percentage of living coral reefs decreased compare to the coral that lives in the depth of 3 m. There are several issues found in the study site as follows. Shattered coral reefs in the depth of 10 m were caused by the waves, fishing (fish, sea cucumber, etc) which lived in the coral reefs. The fishing executed by diving and crack open the coral reefs thus it destructed and shattered.

Table 3 showed 11 types of coral reefs' growth form in Leeward area of Mamburit water, i.e. ACB, ACD, ACS, ACT, CF, CHL, CM, CME, CS, CMR, and CB. Specifically, in the 3 m depth of Leeward, we found 10 types of coral in Plot I. The living coral reef is 63.58%, categorized as good and dominated by ACM type. Percentage of dead coral is 19.4% and other fauna is 36.42%.

We found 7 types of coral reefs in Plot II, with 56.18% living coral (good category). The coral dominated by ACB coral type for 26.16%, whereas dead coral and other fauna found for 43.82%. Similarly in Plot III of 10 m depth, we found 7 types of coral reefs for 65.42%, which categorized as good. Coral reef of ACB is dominant with percentage of 42.94%, while the dead coral and other fauna for 34.58%.

In Plot IV, we found 6 types of coral reefs for 72.96%, dominated by ACB type (27.18%), with dead coral and other fauna for 27.04%. Average percentage of living coral in 3 m depth is 59.88% (medium category), while dead coral and other fauna for 40.12%. Living coral reefs in the depth of 10 m is higher, i.e. 69.19% includes in good category with dead coral and other fauna for 30.81%.

Coral reefs in Leeward area mostly found shattered because in low tide, fishermen walk on the coral for fishing. Thus many coral reefs were damaged. It is different with the Windward area that faces the upwind. Fishermen are rarely fishing here. Fishing activities mostly conducted in Leeward area, however the coral reefs percentage in both locations was not much different.

CONCLUSION

Percentage of living coral reefs in Leeward area of 3 m depth is 59.88% categorized as good, with dead coral reefs and other fauna for 40.12%. Whereas for 10 m depth, the living coral reefs is 69.19% includes in medium category with dead coral reefs and other fauna 30.81%. Winward area of 3 m depth has living coral reefs for 68.38% in good category with dead coral reefs and other fauna of 31.62. Whereas for 10 m depth, the living coral reefs is 40.86% include in medium category with dead coral reefs and other fauna of 59.14%. Type of coral reefs in Mamburit waters are ACD, ACB, CF, CM, ACT, CB, CHL, CME, CMR, and CS.

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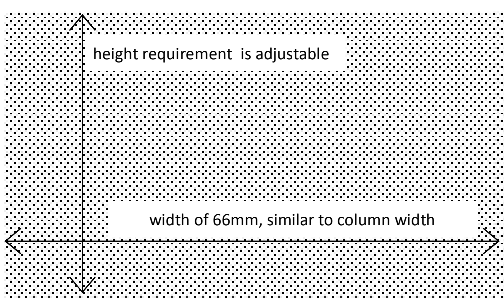

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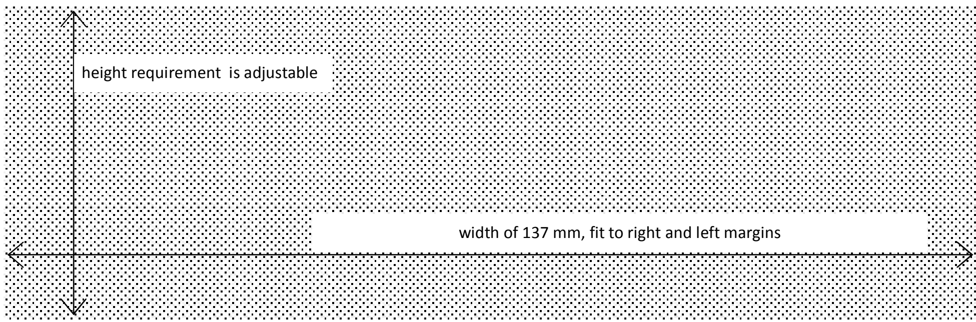

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