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Conformal Nanoencapsulation of Allogeneic T Cells Mitigates Graft-versus-Host Disease and Retains Graft-versus-Leukemia Activity

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Abstract

Allogeneic transplantation of hematopoietic stem cells (HSC) in combination with T cells has a curative potential for hematopoietic malignancies through graft-*versus*-leukemia (GVL) effects, but is often compromised by the notorious side effect of graft-*versus*-host disease (GVHD) resulting from alloreactivity of the donor T cells. Here, we tested if temporary immunoisolation achieved by conformally encapsulating the donor T cells within a biocompatible and biodegradable porous film (~450 nm in thickness) of chitosan and alginate could attenuate GVHD

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Author Contributions

S.Z. and L.Z. contributed equally to this work.

Notes

The authors declare the following competing financial interest(s): SZ and XH disclosed the technology of conformal nanoencapsulation of cells and tissues to the Technology and Commercialization Office at The Ohio State University, Columbus, Ohio, USA.

without compromising GVL. The nanoencapsulation was found not to affect the phenotype of T cells *in vitro* in terms of size, viability, proliferation, cytokine secretion, and cytotoxicity against tumor cells. Moreover, the porous nature of the nanoscale film allowed the encapsulated T cells to communicate with their environment, as evidenced by their intact capability of binding to antibodies. Lethally irradiated mice transplanted with bone marrow cells (BMCs) and the conformally encapsulated allogeneic T cells exhibited significantly improved survival and reduced GVHD together with minimal liver damage and enhanced engraftment of donor BMCs, compared to the transplantation of BMCs and non-encapsulated allogeneic T cells. Moreover, the conformal nanoencapsulation did not compromise the GVL effect of the donor T cells. These data show that conformal nanoencapsulation of T cells within biocompatible and biodegradable nanoscale porous materials is a potentially safe and effective approach to improve allogeneic HSC transplantation for treating hematological malignancies and possibly other diseases.

Graphical abstract

Keywords

nanomaterials; T cells; chitosan; alginate; graft-versus-host disease; immunoisolation; graft-versus-leukemia

Due to the graft-*versus*-leukemia (GVL) effect, transplantation of allogeneic hematopoietic stem cells in combination with allogeneic T cells is an effective adoptive immunotherapy with curative potential for hematopoietic malignancies. However, the GVL is typically accompanied by graft-*versus*-host disease (GVHD), a major complication of allogeneic hematopoietic stem cell transplantation (HSCT) that can cause mortality.^{1,2} Acute GVHD (aGVHD) generally affects skin, liver, and the gastrointestinal tract.³ Activation of donor T cells and recipient antigen-presenting cells (APCs) plays critical roles in aGVHD initiation. Activation of APCs occurs in the first phase of pathophysiology of aGVHD, in which tissue damage is induced by chemotherapy preceding HSCT.⁴ Recent studies indicate that prior to HSCT, irradiation, and/or chemotherapy can damage the epithelia that line the mucosal barriers of skin and the gastrointestinal tract.^{3,5} Disrupted barrier function leads to translocation of the commensal microbiota present on skin and in the gastrointestinal tract, which fuels continuous activation of innate immune cells, creating and maintaining an inflammatory milieu permissive for subsequent activation of deleterious adaptive immunity. This allows microbial products, such as lipopolysaccharide, to translocate to the circulation, stimulating the secretion of pro-inflammatory cytokines including interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF- α). These pro-inflammatory cytokines stimulate the activation of host APCs that present antigens via MHC class II to CD4⁺ T cells, leading to

the activation, proliferation, and differentiation of donor T cells.⁶ Furthermore, donor CD8+ T cells are also potent in inducing aGVHD. These cells preferentially damage the medullary thymic epithelial cells of recipients and impair negative selection, leading to the production of autoreactive CD4+ T cells that perpetuate the thymic damage and aggravate aGVHD.⁷ Therefore, the blockade of donor T cell activation by systemic immunosuppression is a common approach to combat aGVHD, which however could induce severe complications to patients.^{8,9}

While the use of encapsulation to mitigate GVHD has not yet been reported, microencapsulation of cells and natural tissues (*e.g.*, islets) in a matrix or shell of a few hundred microns has been explored to isolate the implanted cells or tissues from the host immune system for cell-based medicine.^{10–17} This is because the matrix/shell made of biocompatible biomaterials, such as alginate, chitosan, poly(L-lysine), poly(lactic-co-glycolic acid), and hyaluronic acid, is semipermeable, allowing sufficient transport of oxygen, nutrients, and metabolic wastes while preventing direct contact between the encapsulated cells and host cells including immune cells.^{10–17} In addition, microencapsulation technology has been utilized for miniaturized 3D culture of various cells to better mimic their 3D microenvironment than the conventional 2D culture.^{17–20} However, these microencapsulation technologies with the use of biomaterial systems of a few hundred microns are not suitable for encapsulating donor T cells for HSCT because they are of the same scale as many blood vessels and may obstruct blood flow after systemic injection to cause morbidity and mortality. In other words, conformally coating the donor T cells with a nanoscale biocompatible and biodegradable film without significantly changing their size and surface charge is desired to block the direct contact between the donor T cells and host APCs in the setting of allogeneic transplantation to minimize GVHD.

The objective of this study was to determine if conformally encapsulating T cells within a biocompatible and biodegradable nanoscale porous film of chitosan and alginate could attenuate GVHD without compromising the GVL effect. We successfully encapsulated murine T cells within the nanoscale film of chitosan and alginate by sequentially soaking the cells in isotonic solutions of chitosan and alginate by utilizing the electrostatic interactions between the positively charged chitosan and the negatively charged plasma membrane of cells and alginate. We observed that this nanoscale conformal encapsulation did not compromise the viability, proliferation, and function of the donor T cells. Moreover, the use of encapsulated T cells reduced GVHD severity without compromising the GVL effect. Our data suggest the great potential of applying the nanomaterial-encapsulated T cells to augment allogeneic HSCT for treating hematopoietic and possibly many other diseases.

RESULTS

Conformal Nanoencapsulation of T Cells without Significantly Altering Their Size and Surface Charge

As shown in Figure 1A, we conducted conformal encapsulation of the donor T cells by sequentially soaking them in isotonic solutions of chitosan and alginate. The cationic chitosan was deposited on the negatively charged cell membrane first *via* electrostatic interactions. The surface ζ -potential of the T cells was approximately -19.5 mV before

encapsulation, and after being coated with the first chitosan layer, the surface ζ -potential shifted to -0.3 mV, indicating successful deposition of the cationic chitosan on the cell membrane (Figure 1B). Subsequently, the chitosan-coated T cells were soaked with alginate to deposit the negatively charged alginate over the cells, again based on electrostatic interactions. This deposition of alginate reversed the surface ζ -potential back to negative (approximately -24.6 mV, Figure 1B). Then, the cells were further sequentially coated with chitosan and alginate one more time to complete the conformal encapsulation, which changed the surface ζ -potential to $+27.4$ and -26.9 mV, respectively. The final ζ -potential of the conformally encapsulated T cells was negative and similar to that of the cells without encapsulation. To visualize the conformally coated film on the T cells, alginate labeled with fluorescein isothiocyanate (FITC) was used to form the film using the aforementioned procedure (Figure 1A). A thin film of 442.8 ± 90.5 nm with strong green fluorescence of FITC is clearly observable around the cell surface, while it is absent over the cells without encapsulation (Figure 1C, left panels). Further scanning electron microscopy (SEM) images show that there were many microvilli on the surface of non-encapsulated T cells, while the surface of encapsulated T cells appeared smooth with a porous structure (Figure 1C, right panels). This further indicates successful conformal encapsulation of the T cells within the porous film of chitosan and alginate, where the microvilli are embedded. The encapsulation efficiency was quantified by flow cytometry, which shows at least 85.7% cells (boxed region in Figure 1D) were successfully encapsulated within the nanoscale film.

Capability of Binding to CD3 Antibody and IL2-Induced Proliferation of T Cells Are Not Significantly Affected by Encapsulation

Typical fluorescence images showing the degradation or disassembly of the conformally coated porous film of chitosan and alginate (labeled with FITC) within 72 h *in vitro* are given in Figure 2A. Importantly, the encapsulated T cells retained their capability of binding to the CD3 antibody (Figure 2B), probably due to the porous structure of the conformal film. This figure also shows that the purity of the isolated T cells is $\sim 90\%$. Moreover, the conformal encapsulation did not affect the proliferation of the T cells stimulated by IL-2 (Figure 2C), which was further confirmed by the BrdU assay (Figure 2D).

Encapsulated T Cells Retain Cytotoxicity and Cytokine Secretory Functions

To examine the cytotoxicity of encapsulated T cells against tumor cells, the encapsulated T cells and control (non-encapsulated) cells were stimulated with CD3–CD28 beads and then incubated with the A20 and P815 tumor cells for 6 h. We observed that the encapsulated T cells were able to lyse both the A20 and P815 tumor cells, and no significant difference was observed between the encapsulated and non-encapsulated T cells (Figure 3A,B). Although IL-2 secretion was moderately decreased at 48 h after stimulation with CD3–CD28 beads, no significant difference was observed at 96 h (Figure 3C). At both time points, no significant difference in TNF- α and IFN- γ secretion was observed (Figure 3D,E).

Encapsulated T Cells Reduce GVHD Severity and Enhance Survival of Mice with GVHD

Compared to mice transplanted with allogeneic encapsulated T cells, the mice transplanted with control (non-encapsulated) cells developed more severe GVHD symptoms including ruffled fur, fur loss, and hunching (Figure 4A) together with weight loss, reduced activity,

and diarrhea. From day 21 to day 50, the clinical GVHD scores (calculated based on the aforementioned six symptoms)²¹ for mice transplanted with encapsulated T cells were significantly lower than those of mice transplanted with the non-encapsulated T cells (Figure 4B). Mice in the non-encapsulated T cell group started to die on day 27, and all died of GVHD within 60 days post-transplantation. In contrast, transplantation with encapsulated T cell significantly prolonged animal survival compared to transplantation with non-encapsulated T cells ($p < 0.05$; 50% survival at day 60) (Figure 4C).

Pathologic analysis of liver sections confirmed the aforementioned clinical score data. Portal inflammation, bile duct damage, lobular inflammation (H&E stain), and fibrosis (Trichrome stain) were detected in the mice transplanted with non-encapsulated T cells, and 100% of portal tracts were affected by inflammation, while these pathological symptoms were less severe in the mice transplanted with encapsulated T cells and the differences between the two groups were statistically significant (Figure 4D–F).

Encapsulation of T Cells Increases Donor Bone Marrow-Derived Cells and Decreases Total Engrafted Donor T Cells and CD8+ T Cells

In our transplantation model, grafts contained a mixture of donor bone marrow cells (BMCs) and donor T cells (encapsulated or non-encapsulated). To measure the effects of encapsulation on the engraftment of these cells separately, we used BMCs from Ly5.1 C57BL/6 mice and T cells from Ly5.2 C57BL/6 mice, both of which carry the H2Kb MHC class I background to distinguish from H2Kd MHC class I of recipient BALB/c mice. In this transplantation model, we measured donor bone marrow (BM)-derived cells and engrafted donor T cells in peripheral blood by flow cytometry on days 19, 33, and 50 post-transplantation. As shown in Figure 5A, the number of donor BM-derived cells in mice transplanted with encapsulated T cells was higher than that in mice transplanted with non-encapsulated T cells. However, the donor CD3+ (total) T cells, CD3+CD8+ T cells, and CD3+CD4+ T cells in the encapsulated were significantly less than those in the non-encapsulated group (Figure 5B–D). The number of donor NKT cells was not significantly different between the two groups (Figure 5E). When mice were moribund, they were sacrificed and splenocytes were harvested to analyze BM-derived donor cells, CD3+ T cells, CD4+ T cells, and CD8+ T cells, all of which showed no significant difference between the two groups (Figure 5F).

Encapsulated T Cells Retain Their GVL Activity

A lymphoma A20 model was used to compare GVL effects of encapsulated and non-encapsulated T cells. Obvious tumor growth was observed in the mice engrafted with BMCs alone at 2 weeks post-transplantation, while no tumor cells were observable in the mice engrafted both BMCs and T cells (either encapsulated or non-encapsulated) throughout the 70 day observation period, demonstrating that the encapsulated T cells retained their GVL effect (Figure 6A). In this model with both GVL and GVHD, transplantation with encapsulated T cells significantly improved animal survival (81% for encapsulated T cells *versus* less than ~20% for non-encapsulated T cells and BMCs alone at day 83, $p < 0.05$) (Figure 6B). Consistent with these data, flow cytometry analyses indicated that there was no evidence of lymphoma in the spleen tissue in mice engrafted with encapsulated T cells or

non-encapsulated T cells together with BMCs, but a tumor population was detected in mice transplanted with BMCs alone (Figure 6C).

Encapsulation of Donor T Cells Mitigates GVHD in Allogeneic Transplantation Tumor Model

The clinical symptom scores of mice in the encapsulated T cell group were lower than those of mice in the non-encapsulated T cell group on day 13 and onward post-transplantation (Figure 7A). Compared with the non-encapsulated T cell group, the body weights of mice in the encapsulated T cell group were significantly higher, which started at day 42 until the end of the experiment (Figure 7B). In this allogeneic transplantation tumor model, the donor BM-derived cells, donor CD3+ T cells, and CD4+ T cells were significantly greater in the encapsulated than non-encapsulated T cell group, while the number of CD8+ T cells and NKT cells were not significantly different between the two groups (Figure 7C). To determine whether reduction of GVHD severity by encapsulated T cells is due to less responsiveness to antigens presented by APCs, we isolated allogeneic macrophages and cocultured them with encapsulated or non-encapsulated T cells. The results showed that encapsulated T cells are less responsive to cocultured macrophages in terms of the T cell proliferation (Figure 7D).

DISCUSSION

Allogeneic HSCT has the potential to cure hematologic malignancies. In both experimental and clinical bone marrow transplantation, however, donor immune responses to allogeneic antigens often trigger acute and chronic GVHD.^{22,23} Currently available therapies for GVHD, including steroid and immunosuppressive drugs (*e.g.*, mycophenolate mofetil and methotrexate), are effective, but they are often associated with severe side effects including infection and secondary cancer, and some patients develop drug resistance.^{8,9} Therefore, development of effective treatment strategies with less adverse effects is needed. Both GVL and GVHD reactivity are largely systemic responses that are primarily mediated by donor T cells.^{24,25} Donor T cells play a crucial role in GVHD-associated pathologic damage. Selective depletion of T cells that cause GVHD from allogeneic stem cell grafts and preservation of T cells specific for pathogens may improve the outcomes of HSCT.^{26,27} After HSCT, donor T cells are activated first by host APCs through direct cell contact. Chen and colleagues reported that IMMU-114, a humanized anti-human leukocyte antigen-DR (HLA-DR) mAb, selectively inhibited the proliferation of HLA-DR⁺ T cells and reduced CD25 alloreactive T cells in allogeneic mixed leukocyte reactions (MLRs) by depleting human PBMCs of all APCs, including B cells, monocytes, myeloid DC type-1 (mDC1), mDC2, and plasmacytoid DCs (pDCs), which could efficiently suppress GVHD.^{28,29} Therefore, we hypothesized that temporarily encapsulating T cells within nanoscale biomaterials could attenuate GVHD by reducing the direct contact between donor T cells and host APCs.

In recent years, a variety of natural and synthetic biomaterials with diverse chemical or physical properties have been used to engineer cells or tissues for therapeutic applications. In particular, encapsulation of cells in microcapsules of a few hundred microns using these

materials by electrospray or microfluidics for implantation has attracted a great deal of attention for modern cell-based medicine because it protects the implanted cells from the host immune cells and enables sustained release of a therapeutic compound without the need of frequent injection.^{10–17} However, these approaches are not applicable for encapsulating cells that circulate with blood because of their large size that can cause blockage of blood flow. We overcome this issue by conformally encapsulating single T cells within a nanoscale semipermeable film of chitosan and alginate, which does not significantly change their size and surface charge. Chitosan and alginate are used because they are naturally derived biodegradable polymers (polysaccharides) with great biocompatibility and opposite charge.^{11,30–32} The product from degradation of chitosan is glucosamine (amino sugar), while the degradation product of alginate is oligosaccharide, both of which are not cytotoxic. We found that the conformal encapsulation does not affect the proliferation of T cells or their secretion of IFN- γ , TNF- α , and IL-2. This is probably because the conformal film of alginate and chitosan is thin (~450 nm), biodegradable, biocompatible, and possibly soft. Moreover, the film is porous (Figure 1C), which may allow sufficient transport of nutrients to and substances produced by the cells (including metabolic wastes and secreted cytokines) away from the encapsulated T cells. Furthermore, the porous nature of the film may allow the encapsulated T cells to communicate freely with their microenvironment either *in vitro* or *in vivo* in the host. The latter may further lead to their adaptation to the host microenvironment, resulting in reduced alloreactivity and a decrease in GVHD severity and incidence. Indeed, our data show that the conformally nanoencapsulated donor T cells could bind to antibodies, maintain their capability of cytokine secretion, and were able to reduce GVHD severity and extend the lifetime of transplants in our animal model.

GVHD is associated with changes in the populations of immune cells, such as increased CD3⁺ T cells, CD4⁺ and CD8⁺ effector memory cells, monocytes, CD86 expression, BAFF/B cell ratio, together with deficiency of Treg, NK cells, pDC, and NKT cells, *etc.*³³ In this study, immune cell subsets were measured at the early (before the median day of GVHD onset) and late stage post-transplantation to determine which subsets are responsible for GVHD mitigation by the encapsulated T cells. In a GVHD model, we found that the number of donor CD8⁺ T cells and CD4⁺ T cells in mice transplanted with non-encapsulated T cell was not significantly different from that in mice transplanted with encapsulated T cells on day 33 after transplantation, indicating that the reduced aGVHD in mice treated with the encapsulated T cells is a result of the temporary immunoisolative effect of the conformal encapsulation. We also found that the number of donor BMCs in the encapsulated T cell group was higher than that of the non-encapsulated group, suggesting that T cell encapsulation may facilitate engraftment of stem cells. This should contribute to the significantly extended lifetime of the mice treated with the nanoencapsulated T cells.

Alloreactive T cells are capable of mediating specific GVL reactivity due to the hematopoiesis-restricted expression of HLA class II.^{34,35} A20 is a rapidly dividing, B-cell-derived lymphoma cell line that typically causes death in nearly all recipients transplanted with BMCs alone.³⁶ In this study, in order to determine if GVHD and GVL reactivity could be effectively dissociated by encapsulating T cells, we further studied the GVL effect of the encapsulated T cells using the allogeneic transplantation model of the A20 tumor cells. We observed that although the A20 tumor cells were observed in mice transplanted with BMCs

alone, there was no evidence of lymphoma in the spleen tissue in mice treated with the BMCs and T cells either with or without conformational encapsulation. Our data suggest that the encapsulated T cells actively participate in an overt GVL response. Consistent with the data collected from the model with GVHD alone, in the aforementioned combined GVL and GVHD model, encapsulated T cells also reduce the severity of GVHD. Additionally, proliferation of the encapsulated T cells incubated with allogeneic macrophages was lower than that of non-encapsulated T cells, indicating that the encapsulated T cells attenuate the severity of GVHD most likely by preventing the donor T cells from being activated by the host APCs.

CONCLUSION

In summary, we successfully developed a conformational nanoencapsulation technology to engineer T cells without significantly changing their size and surface charge for transplantation to treat hematopoietic malignancy. This nanoencapsulation temporarily prevents the direct cell contact between the transplanted T cells and host APCs. As a result, the alloreactivity of donor T cells was minimized, leading to significantly reduced GVHD and enhanced animal survival for allogeneic HSCT for the treatment of hematopoietic malignancy. To our knowledge, this is the first report showing that nanoencapsulated T cells mitigate GVHD without compromising the GVL effect. Our study demonstrates a nanotechnology-based strategy to control GVHD in allogeneic transplantation, which has great potential to be translated into the clinic to benefit patients with hematological malignancies.

MATERIALS AND METHODS

Mice

BALB/c mice (recipient, 8–12 weeks old), Ly5.1⁺, and Ly5.2⁺ C57Bl/6 mice (donor) were either purchased from The Jackson Laboratory or bred in the University Laboratory Animal Resources of The Ohio State University (OSU). All animals were housed in University Laboratory Animal Resources of OSU. All animal experiments were conducted according to a protocol approved by the OSU Institutional Animal Care and Use Committee.

Cell Culture

A20 tumor cells expressing MHC class I H-2d molecules from a BALB/c B-cell lymphoma cell line were used in this study. The A20 cells were further modified by transducing with a luciferase gene (thereafter A20-luc), which allowed for noninvasive visualization of tumor progression. Both modified and unmodified A20 cells were cultured in RPMI 1640 (Life Technologies, Rockville, MD, USA) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT, USA), 100 unit/mL penicillin (Life Technologies), 100 μ g/mL streptomycin (Life Technologies), and 50 μ M 2-mercaptoethanol (Sigma, St. Louis, MO, USA). The P815 cell line was used as target cells for cytotoxic T cell assays and was cultured in RPMI 1640 supplemented with 2 mM glutamine, 10% FBS, 100 unit/mL penicillin, and 100 μ g/mL streptomycin.

Reagents

FITC-anti-Ly5.2 (clone 104), PECy7-anti-Ly5.1 (clone A20), PE-anti-H2Kb (clone AF6-88.5), PercpCy5.5-anti-CD3 (clone 145-2C11), APC-H7-anti-CD4 (clone GK1.5), V450-anti-CD8 (clone 53-6.7), APC-anti-NK1.1 (PK136), and V450-anti-CD3 (clone 17A2) were all purchased from BD Pharmingen (San Diego, CA). Cells were analyzed on a LSR II flow cytometer (BD Biosciences, San Diego, CA, USA). APC-anti-CD19 (clone MB19-1) was purchased from eBiosciences (San Diego, CA, USA). Data were analyzed using FlowJo (Ashland, OR, USA). IL-2, TNF- α , and IFN- γ ELISA kits were purchased from eBioscience. APC-anti-BrdU (Clone BU20A) and anti-APC beads were purchased from Miltenyi Biotec (San Diego, CA, USA). Mouse T-activator CD3/CD28 beads were purchased from Life Technology (Grand Island, NY, USA). Stain buffer, cytofix/cytoperm buffer, and perm/wash buffer were purchased from BD Biosciences.

Materials for T Cell Encapsulation

Sodium alginate was purchased from Sigma and further purified as previously described.²⁰ Chitosan (pharmaceutical grade, 80 kDa) was purchased from Weikang Biological Products Co. Ltd. (Shanghai, China). FITC-alginate was produced by the following steps. First, alginate (1.8%, w/v) was dissolved in 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 4.7) and mixed with 9 mM 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride and 9 mM *N*-hydroxysulfosuccinimide to activate the carbonyl groups on alginate. After being stirred for 2 h at room temperature, 2 mM FITC was added to the solution and stirred for 18 h. Then, the solution was dialyzed against 1 M NaCl solution and distilled water each for 24 h, followed by freeze-drying.

Magnetic Separation of T Cells

T cells were isolated from spleens of donor C57BL/6 (Ly5.2) mice by negative selection using the MACS (Miltenyi Biotec, San Diego, CA, USA) magnetic T cell separation kit. Briefly, the single-cell suspension from the spleens of pooled mice (approximately 10^8 cells) in 500 μ L of MACS buffer was incubated with PE-anti-CD19 for 20 min, followed by incubation with anti-PE beads for an additional 20 min. The stained cell suspension was applied onto the LS column (Miltenyi Biotec). After being washed with degassed buffer three times, unlabeled T cells were collected.³⁷

Encapsulation of T Cells and Their Characterization

To encapsulate the isolated T cells, solutions of 0.15% (w/v) alginate or FITC-alginate and 0.4% (w/v) chitosan in isotonic saline were titrated to pH 7.4 and filtered through a 0.22 μ m filter before use. The T cells isolated from mouse spleens were aliquoted into different tubes with 5×10^6 cells per tube, washed with 5 mL of PBS in each tube by gentle pipetting and centrifuging (514g, 5 min), followed by resuspending in 1 mL of PBS in each tube to obtain the single T cell suspension. Encapsulation was then conducted with the suspension of single T cells in each tube to ensure they are individually encapsulated. Briefly, the cationic chitosan solution (1 mL) was added to the 1 mL single T cell suspension in each tube and mixed by gentle pipetting. After 15 min of deposition, cells were centrifuged (514g, 5 min) to remove excess chitosan and further washed in 5 mL of PBS to remove any loosely

attached chitosan. Afterward, the cells were resuspended in 1 mL of PBS, and 1 mL of alginate solution was similarly added to the cell suspension, followed by washing in the same way as aforementioned. These two coating steps were then repeated one more time to achieve conformal encapsulation of the T cells by electrostatic deposition.

To visualize the porous film conformally encapsulated over T cells, the FITC-labeled alginate was used for encapsulation with the same method. The thickness of the conformal film was examined using an Olympus FluoView FV1000 confocal microscope. We randomly chose three different locations (120° apart) for each cell to measure the thickness using NIH (Bethesda, MD, USA) ImageJ (version 1.48v), and a total of 30 encapsulated T cells were measured to obtain the mean and standard deviation of the film thickness. The encapsulated cells were further monitored using a Zeiss (Oberkochen, Germany) Axio Observer.Z1 fluorescence microscope to observe the degradation or disassembly of the film. The coating efficiency was quantified by identifying FITC-positive cell population using flow cytometry (BD LSR II). In addition, to evaluate the change of surface morphology of T cells after the encapsulation, T cells with and without encapsulation were observed using an FEI Nova NanoSEM 400 scanning electron microscope. The cells were fixed using 2.5% glutaraldehyde overnight and then dehydrated in ethanol and dried using hexamethyldisilazane. Before examination, the samples were sputter-coated using a Cressington 108 sputter coater at 17 mA for 120 s. The ζ -potential on the cell surface was measured with a Brookhaven (Holtsville, NY, USA) 90 Plus/BI-MAS dynamic light scattering instrument. After the reading was stable, at least five readings were recorded to calculate the ζ -potential during each step.

Cell Proliferation Measured by the BrdU Intake Assay

The proliferation of encapsulated T cells was measured by BrdU intake analysis. In brief, 10 μ L of BrdU solution (1 mM BrdU in 1 $\times$ DPBS) was added directly to encapsulated T cells cultured in the presence of IL-2 and incubated for 2 h. Non-encapsulated T cells were also studied as control. Then, the cells were surface-stained with anti-CD3 antibody for 15 min. Subsequently, the cells were washed and then fixed with BD cytofix/cytoperm buffer for 30 min, followed by incubation with BD cytoperm permeabilization buffer plus for 10 min. After being washed, the cells were treated with DNase at 37 °C for 1 h to expose incorporated BrdU. The cells were further stained with FITC-anti-BrdU for 20 min at room temperature. The stained CD3⁺BrdU⁺ cells were analyzed by flow cytometry.³⁸

Cytotoxicity Assays of Encapsulated T cells

T cell suspension (1×10^6 /mL) and 5×10^5 anti-CD3/CD28 beads (Dynabeads mouse T-activator CD3/CD28; Life Technologies) were added (1 mL/well) to a 24-well plate. Additionally, IL-2 (30 IU) was added to each well. The T cells were then incubated for 120 h and further encapsulated as aforementioned. A20 or P815 cells were incubated with encapsulated effector T cells at various ratios of effector to target cells, and ⁵¹Cr release was quantified by analyzing the supernatants using a gamma counter (Wallac, Turku, Finland). Non-encapsulated T cells were studied in the same way as control. The percentage of specific release was calculated with the following formula: $100 \times [(\text{experimental release} - \text{spontaneous release})/(\text{maximal release} - \text{spontaneous release})]$. Spontaneous and maximum

releases were determined from samples containing medium alone or completely lysed by buffer containing 1–2% detergent SDS, respectively.³⁹

Cytokine Analysis by ELISA

A total of 4×10^5 encapsulated T cells (2×10^6 cells/mL) were cultured with anti-CD3/CD28 beads in a 96-well plate for 48 or 96 h, and then the supernatant was collected. The levels of IL-2, TNF- α , and IFN- γ were detected by using ELISA kits following the manufacturer's instructions. Non-encapsulated T cell groups were studied in parallel as control.

Establishment of the GVHD Model

In order to observe changes of BM-derived donor cells and engrafted donor T cells in GVHD mice, Ly5.1 μ_B Cs from Ly5.1 C57Bl/6 mice and donor T cells from Ly5.2 C57Bl/6 mice were used. The GVHD model was established as previously described with slight modification.^{21,40,41} Briefly, BMCs from donor C57Bl/6 (Ly5.1) mice were flushed from femur and tibia bones using PBS. Afterward, the cells were washed once in PBS and resuspended in Dulbecco's modified Eagle's minimal essential medium. BALB/c recipients were irradiated at a single dose of 6 Gy. Four hours after irradiation, mice were simultaneously injected (i.v.) with Ly5.1 μ_B Cs (5×10^6) and encapsulated Ly5.2 T cells (1.0×10^6). Because the BMCs contain few CD3+ T cells,⁴² we isolated T cells from spleens of C57Bl/6 (Ly5.2) mice instead of bone marrow as previously reported.^{40,41} Non-encapsulated Ly5.2 T cells were also studied as control.

Assessment of GVHD

Recipient mice were ear punched, and weights of individual mice were obtained and recorded on day 1 and every 3 days thereafter. Meanwhile, recipient mice were monitored every day for clinical signs of GVHD and survival. GVHD severity was scored according to a scoring system that incorporates six clinical parameters: weight loss, posture (hunching), activity, fur texture, diarrhea, and skin integrity. Weight loss greater than 10% was considered indicative of significant GVHD. Mice were evaluated and graded from 0 to 2 for each criterion. A clinical index score was subsequently generated by the summation of the six criteria scores (the maximum index score is 12).²¹ GVHD was also assessed by histopathology analysis of target tissues. Mice were sacrificed, and liver tissues were harvested, cryo-embedded, and subsequently sectioned. Tissue sections were fixed in 10% buffered formalin and stained with hematoxylin and eosin (H&E) for histological examination. All the samples were examined by two experienced pathologists in a blinded fashion. GVHD pathological scoring was performed according to tissue damage.⁴³ In the scoring system, 0 indicates normal, and 1, 2, and 3 represent mild, moderate, and severe tissue damage caused by donor T cells, respectively.^{44,45} Six parameters were evaluated in the liver: portal inflammation, percentage of portal tracts involved, bile duct damage, lobular inflammation, bile duct loss, and fibrosis in each section. The grading scheme consisted of ordinal categories ranging from "0" (no effect) to "4" (severe effect) as follows.^{44,45} (1) Portal inflammation and lobular inflammation: 0 = none, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe. (2) Fibrosis: 0 = none, 1 = portal, 2 = periportal, 3 = bridging fibrosis, and 4 = cirrhosis.

Analysis of BM-Derived Donor Cells and Donor T Cells by Flow Cytometry in GVHD and GVHD/GVL Models

Blood was collected from the tail vein of GVHD mice on days 19, 33, and 50. The erythrocytes were lysed in lysis buffer. The mononuclear cells were stained with PE-anti-H2Kb, FITC anti-Ly5.2, PEcy7 anti-Ly5.1, percpCy5.5 anti-CD3, V450 anti-CD8, APC-H7 anti-CD4, and APC anti-NK1.1 and incubated for 15 min at 4 °C in the dark. A total of 100 μ L of peripheral blood withdrawn from the tail vein of mice bearing A20 tumor was collected on day 45. The samples were lysed with red blood cell lysis buffer, and the cells were stained with the aforementioned antibodies. The numbers of BM-derived donor cells (Ly5.1), Ly5.2 CD3⁺ donor T cells, Ly5.2 CD4⁺ donor T cells, Ly5.2 donor CD8⁺ T cells, and NKT cells were determined by flow cytometry. Data were analyzed using FlowJo (TreeStar).

Establishment of the GVHD/GVL Model

To examine the GVL effect of the encapsulated T cells in a hematologic malignancy model, a murine A20 tumor model was used to study the antitumor effect of encapsulated T cells after transplantation. Luciferase-transfected A20 cells (A20-luc) were used to allow noninvasive visualization of tumor progression. BALB/c recipient mice were irradiated using a RS 2000 Irradiator⁴⁶ at 6 Gy on day 1 and injected intravenously with 0.5×10^6 A20 cells at 4 h after irradiation. On day 2, recipient mice were injected intravenously with Ly5.1 donor BMCs (5×10^6) and Ly5.2-encapsulated T cells (1.0×10^6). Mice with no T cell injection or injected with non-encapsulated Ly5.2 donor T cells were studied in parallel to serve as controls.²

The body weights of transplanted mice were obtained and recorded on day 1 and every 3 days thereafter. The degree of systemic GVHD was also assessed by the aforementioned scoring system.² In addition, the mice were monitored for survival, body weight, and clinical GVL and GVHD symptoms.

In Vivo Bioluminescence Imaging

The Xenogen IVIS whole-animal imaging system (Caliper Life Sciences, Waltham, MA, USA) was used for noninvasive live imaging of tumor cells. Mice were anesthetized using isoflurane (Webster Veterinary, St. Webster, SD, USA) and injected intraperitoneally with firefly luciferin substrate (5 mg/mL in PBS, Caliper Life Sciences) at 150 mg/kg body weight. The IVIS imaging was performed at 15 min after the substrate injection. Whole-body bioluminescent signal intensity was recorded weekly. The pseudocolor bioluminescent images were overlaid on conventional photographs and presented as photon counts per area. The images were analyzed using the Living Image Software (Xenogen, Alameda, CA, USA).²

Analysis of the Effects of Allogeneic Macrophages on Proliferation of Encapsulated T Cells by the Mixed Lymphocyte Reaction

Briefly, lymphocytes were seeded into 96-well plates at 10^5 cells/well, and purified allogeneic macrophages (1×10^4) were added to each well and incubated for 24, 48, 72, and 96 h at 37 °C in a humidified incubator in air and 5% CO₂. For the last 2 h, 10 μ L of 3-(4,5-

dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (G3580 CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, USA) was added to each well. Two hours later, the OD value (absorbance at 450 nm wavelength) was measured using an enzyme microplate reader. Cell viability was expressed by OD values. Each experiment was performed in triplicate.

Statistical Analysis

All data were presented as mean $\pm$ standard deviation and were analyzed by Student's two-tailed *t* tests using GraphPad Prism assuming equal variance. The Kaplan–Meier product limit estimator was used to construct survival curves, which were compared using the log-rank test. A *p* value of less than 0.05 was reported as significant for all comparisons.

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Figure 1.

Conformal nanoencapsulation of T cells by electrostatic deposition and its characterization.

(A) Schematic illustration of the procedure for conformal encapsulation. The cationic chitosan (orange) was deposited on the negatively charged cell membrane first. Subsequently, negatively charged alginate (green) was coated based on electrostatic deposition. The sequential coating of chitosan and alginate was repeated once. (B) Surface ζ -potential of T cells. The surface ζ -potential of T cells before coating was approximately -19.5 mV, while after first chitosan coating, it increased to -0.3 mV. The subsequent coating of alginate reversed the surface ζ -potential back to -24.6 mV. Then, the second chitosan and alginate coatings changed the ζ -potential to $+27.4$ and -26.9 mV, respectively. (C) Left, confocal fluorescence images of T cells before and after conformally encapsulated with the porous film of chitosan and alginate that was labeled with green fluorescent probe FITC. Right, scanning electron microscope (SEM) images of encapsulated and non-encapsulated T cells showing the morphology changes due to the conformal encapsulation. (D) Flow cytometry analysis of the encapsulation efficiency by labeling the alginate for coating with FITC, indicating that at least 85.7% of T cells were successfully encapsulated in the nanoscale porous film of chitosan and alginate with strong green fluorescence.

Figure 2.

Encapsulated and non-encapsulated T cells have similar proliferation, function, and capability of binding to antibody *in vitro*. (A) Fluorescence images of encapsulated T cells at different times after encapsulation. The images show the degradation of conformally coated porous film of FITC-alginate and chitosan over 3 days. (B) Flow cytometry data showing encapsulated T cells had similar capability of binding to CD3 antibody *in vitro*, compared to non-encapsulated T cells. (C) Proliferation of encapsulated *versus* non-encapsulated T cell. The number of T cells was counted using a hemocytometer at 48, 72, 96, and 120 h after culture. Encapsulated T cells had a proliferation rate similar to that of non-encapsulated T cells. (D) Proliferation of encapsulated *versus* non-encapsulated T cell analyzed by BrdU intake assay *in vitro*. Encapsulated T cells had proliferation similar to that of non-encapsulated T cells at 48 and 120 h after culture.

Figure 3.

Encapsulated and non-encapsulated T cells show similar levels of cytotoxicity against A20 and P815 tumor cells *in vitro*. (A,B) Percentage of lysed A20 and P815 cells after being incubated with encapsulated and non-encapsulated T cells for 6 h. No significant difference was observed. (C–E) Levels of IFN- γ , TNF- α , and IL-2 secreted by encapsulated and non-encapsulated T cells after being stimulated with CD3–CD28 beads; * $p < 0.05$.

Figure 4.

Encapsulated T cells reduce GVHD severity and improve animal survival. (A) Images of GVHD mice. The mice treated with allogeneic non-encapsulated T cells have more severe GVHD symptoms (hair loss, ruffled fur, hunched posture) than mice treated with allogeneic encapsulated T cells. (B) GVHD score. From day 21 to day 50, the clinical GVHD symptom score of mice in the encapsulated T cell group was significantly lower than that of mice in the non-encapsulated T cell group. (C) Survival analysis of BALB/c mice. Mice in the non-encapsulated T cell group started to die on day 27, and all of them died of GVHD within 60 days after transplantation. In contrast, transplantation with encapsulated T cells significantly prolonged the animal survival. (D–F) Pathohistology of the livers from mice with GVHD. Portal inflammation, bile duct damage, lobular inflammation, and fibrosis were detected in mice treated with non-encapsulated T cells, and 100% of portal tract inflammation was observed. The scores of portal inflammation, lobular inflammation, and fibrosis and the percentage of portal tract inflammation in recipient mice transplanted with the encapsulated T cells were significantly lower than those transplanted with non-encapsulated T cells; * $p < 0.05$, ** $p < 0.01$.

Figure 5.

BM-derived cells have greater quantity while engrafted donor CD8⁺ and total T cells are fewer in the peripheral blood of mice transplanted with encapsulated than non-encapsulated allogeneic T cells. (A) Number of BM-derived cells. The number of donor BM-derived cells in the encapsulated T cell group was higher than that of the non-encapsulated T cell group. (B–E) Number of T cells and T cell subsets in peripheral blood. The percentages of donor CD3⁺ T cells, CD8⁺ T cells, and CD4⁺ T cells in the encapsulated T cell group were significantly lower than those in the non-encapsulated T cell group, while no difference was observed for NKT cells. (F) Number of donor BMCs and T cell subsets in the spleen of recipient mice. No differences were observed for all the cell populations in spleens; * $p < 0.05$, ** $p < 0.01$.

Figure 6.

Conformal encapsulation of T cells does not compromise their GVL activity. (A) GVL effect assessed by bioluminescence imaging. Lymphoma was observed in mice treated with BMCs alone, while no evidence of lymphoma was observed in mice treated with BMCs together with either encapsulated or non-encapsulated T cells throughout the 70 day observation period. (B) Survival analysis of the mice in the GVHD/GVL model. Mice transplanted with BMCs and encapsulated T cells had significantly longer survival than mice treated with BMCs and non-encapsulated T cells or treated with BMCs alone. (C) Quantification of A20 tumor cells in the GVHD/GVL model by flow cytometry, showing that both the non-encapsulated and encapsulated T cells could effectively kill the A20 tumor cells *in vivo*; * $p < 0.05$.

Figure 7.

Encapsulation of donor T cells mitigates GVHD in GVHD/GVL model. (A) Clinic GVHD symptom scores of mice implanted with A20 tumor cells after transplanted with donor BMCs in combination with encapsulated or non-encapsulated T cells. (B) Body weights of the mice from (A), showing mice treated with non-encapsulated T cells having significantly greater weight loss after 40 days. (C) Numbers of donor BM-derived cells and engrafted T cells. Blood of mice was obtained from the tail vein on day 45 after transplantation, further processed into a single-cell suspension, and stained to analyze with flow cytometry. The numbers of donor BM-derived T cells, donor CD3+ T cells, donor CD4+ T cells, donor CD8+ T cells, and donor NKT cells from the encapsulated and non-encapsulated groups were compared. (D) Effect of allogeneic macrophages on the proliferation of encapsulated *versus* non-encapsulated T cells. The proliferation of encapsulated T cells incubated with the allogeneic macrophages was lower than that of non-encapsulated T cells; * $p < 0.05$, ** $p < 0.01$.