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# Immune Response to Biologic Scaffold Materials

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## Abstract

Biologic scaffold materials composed of mammalian extracellular matrix are commonly used in regenerative medicine and in surgical procedures for the reconstruction of numerous tissue and organs. These biologic materials are typically allogeneic or xenogeneic in origin and are derived from tissues such as small intestine, urinary bladder, dermis, and pericardium. The innate and acquired host immune response to these biologic materials and the effect of the immune response upon downstream remodeling events has been largely unexplored. Variables that affect the host response include manufacturing processes, the rate of scaffold degradation, and the presence of cross species antigens. This manuscript provides an overview of studies that have evaluated the immune response to biologic scaffold materials and variables that affect this response.

## Introduction

Biologic scaffold materials composed of mammalian extracellular matrix (ECM) are commonly used for the surgical reconstruction of musculotendinous, dermal, cardiovascular, gastrointestinal, and lower urinary tract tissues, among others [1–14]. Examples of commercially available products include Mosaic™, Freestyle™, Prima™, Restore™, Oasis™, Surgisis™, CuffPatch™, GraftJacket™, Alloderm™, TissueMend™, and OrthAdapt™ (Table 1). These products are all composed of ECM, but differ in their tissue source (e.g., heart valve, small intestine, dermis, pericardium), species of origin, (e.g., porcine, bovine, equine, human), and methods by which they are processed.

Despite the extensive use of allogeneic and xenogeneic biologic scaffold materials, very little is understood, and even less is published, regarding the host immune response to these materials. The present manuscript provides a review of literature relevant to the host immune response to biologic scaffold materials, and the potential relationship between the host immune response and downstream remodeling events.

Biologic materials composed of extrcellular matrix are typically processed by methods that include decellularization and/or chemical crosslinking to remove or mask antigenic epitopes, DNA, and damage associated molecular pattern (DAMP) molecules [15–17]. The effect of various processing steps upon the host immune response has not been systematically examined. In a recent study that compared five ECM products, all of which were processed by different methods, the acute host response was uniformly characterized by an intense mononuclear cell infiltrate. The long term remodeling response, however, varied from chronic inflammation, fibrosis, scarring, and encapsulation to the formation of organized, site-appropriate tissue

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remodeling [18]. It seems clear that biologic scaffold manufacturing methods play an important role in determining the host response. It seems just as clear that traditional histologic methods of evaluating the host cellular response are not necessarily predictive of the long term remodeling outcome.

The present manuscript will first review the role of xenogeneic antigens, specifically the Gal epitope and DNA, on the host response to biologic scaffold materials. The influence of manufacturing methods, in particular the effect of chemical crosslinking upon the host response to ECM scaffold devices will also be discussed followed by a review of the phenotypic response of T lymphocytes and macrophages to various ECM scaffold materials. Finally, the importance of scaffold degradation upon the host response to biologic scaffold materials composed of ECM will be reviewed.

## The Gal epitope

Hyperacute rejection following organ xenotransplantation can be caused by the presence of cell membrane antigens, such as the oligosaccharide α-Gal (Galα1,3-Galβ1–4GlcNAc-R) (i.e., “Gal epitope”). This epitope is found in high density as a cell surface molecule in most species with notable exceptions of humans and Old World monkeys. The presence of the Gal epitope on the surface of the vascular endothelium is the primary cause of rejection of xenogeneic organ transplants [19–22]. The Gal epitope has also been found on cell associated glycoproteins and glycolipids [23,24], secreted glycoproteins including thyroglobulin, fibrinogen, and immunoglobulin G (IgG) [25,26], and basement membrane proteins such as laminin [27]. Humans and Old World monkeys do not normally express the Gal epitope due to two frameshift mutations in the the α1,3-galactosyl-transferase gene [28,29], and produce large amounts of anti-Gal antibodies (Ab), including IgG, IgM, and IgA [21,30–33], as a result of the constant exposure to intestinal bacteria that carry the Gal epitope. It has been estimated that up to 1% of circulating human IgG is anti-Gal [21,31].

In an effort to eliminate the Gal epitope as a barrier to xenotransplantation, transgenic herds of pigs have been produced in which this epitope has been knocked out [34,35]. However, xenotransplants of Gal knockout hearts [36,37] and kidneys [38] were rejected over periods of 6 months and 1 month, respectively, due to an immune response that included the formation of anti-non-Gal Ab specific to porcine antigens.

The presence of the Gal epitope in biologic scaffolds composed of xenogeneic ECM has been investigated for porcine bioprosthetic heart valves [39], porcine anterior cruciate ligament (ACL) and cartilage[40–42], and porcine SIS-ECM [43]. All of these materials were found to be Gal positive. Porcine heart valves showed presence of the Gal epitope even after treatment with glutaraldehyde and patients receiving such bioprosthetic valves showed a significant increase in anti-Gal IgM. It has been speculated that this host response to the bioprosthetic heart valve may contribute to degeneration and calcification that ultimately leads to failure of the graft, especially in younger patients [39].

Non-decellularized porcine grafts for repair of cartilage and the ACL have also been shown to contain the Gal epitope. Treatment of the xenogeneic tissue with α-galactosidase has been proposed to minimize potential adverse immune responses to these graft materials [40–42]. The enzymatic treatment can effectively remove the Gal epitope from both porcine cartilage and ACL tissue, and since the cells within the tissue graft are not viable, the Gal epitope is not replaced through natural turnover [44]. Galactosidase treated cartilage grafts [41] have been shown to reduce the proportion of T lymphocytes present at the site of remodeling from 70% of the total cell population to 10%, with the balance of cells being primarily macrophages. In both Rhesus monkeys and humans [40,42], galactosidase treated porcine ACL grafts have performed comparably with an allograft.

The Gal epitope was found to be present in SIS-ECM, a biomaterial consisting of porcine small intestinal submucosa [43]. It is not known whether the Gal epitope detected within SIS represents a secreted product of the cells originally present in the native material or cellular debris retained during preparation of the SIS. *In vitro* studies using immunoprecipitation showed that the most abundant anti-SIS Ab subtype that bound to SIS following exposure to human plasma was $IgG_2$, a finding which is consistent with the large percentage of IgG specific for the Gal epitope is $IgG_2$ [45,46]. However, complement activation was not observed either due to the low density of Gal epitopes, or the fact that $IgG_2$ is known to be a poor activator of complement [47–49].

To examine the potential role of the Gal epitope in the host immune response to SIS-ECM, samples of SIS-ECM were implanted subcutaneously in wild type (WT) mice and mice in which the α1,3 galactosyltransferase gene was knocked out (Gal $^{-/-}$ mice). The Gal $^{-/-}$ mice spontaneously produce anti-Gal Ab in a similar manner to that observed in humans [50]. The Gal $^{-/-}$ mice produced IgM anti-Gal antibodies in addition to $IgG_1$ SIS-specific antibodies, which did not bind to the Gal epitope. Histologically, the remodeling of the SIS-ECM material was complete by day 25 for the WT mice. In the Gal $^{-/-}$ mice, inflammatory cells were still present in the remodeling site after 25 days, but remodeling was complete by day 35. Immunization of the Gal $^{-/-}$ mice with sheep erythrocytes to enhance the anti-Gal Ab levels led to a more robust early inflammatory response following implantation, but did not alter the ultimate fate of the graft. Therefore, it appears that the presence of anti-Gal Ab delays, but does not prevent constructive remodeling of the ECM material.

## DNA

Remnant porcine DNA within biologic scaffold materials after decellularization has been implicated as the cause of “inflammatory reactions” following the implantation of porcine derived scaffolds for orthopaedic applications [51]. Considering the manner in which cells are naturally embedded within their surrounding ECM, especially in relatively dense tissues like the dermis, it is unlikely that complete removal of all cells and cell products is possible even with the most rigorous processing methods. Most commercially available biologic scaffold materials contain trace amounts of remnant DNA, including Restore™, GraftJacket™, and TissueMend™ [51–53]. The remnant DNA is typically present as small fragments, reducing the possibility that these remnants play any substantive role in an adverse tissue remodeling response. In most of the biologic scaffold materials that were investigated in a recent study, the remnant DNA consisted of fragments less than 300 bp in length [53]. DNA fragments of this length are not likely to be of concern. The only ECM device that appeared to contain full DNA strands was GraftJacket™, an ECM material manufactured from human dermis. In addition to the small amount and abbreviated length of the remnant DNA, the noncrosslinked forms of ECM are subject to rapid degradation after placement *in vivo* [18,54,55]. Any remnant DNA is logically subject to the same degradation fate via enzymatic breakdown. Toll-like receptors may play an important role in this regard as they bind soluble DNA so that they can be broken down into nucleotides for future use by the cells [56,57].

Despite the universal presence of DNA remnants in commercially available ECM devices, the clinical efficacy of these devices for their intended application has been largely positive [1–14]. It therefore appears unlikely that the remaining DNA fragments contribute to any adverse host response or are a cause for concern.

It is plausible and even likely that cytoplasmic proteins and cell membrane components are retained in ECM scaffold materials through the processing steps, just as small amounts of the Gal epitope remain in these biomaterials. Although it is known that non-self cell products are capable of eliciting a host inflammatory response and/or stimulating an immune reaction, it is

possible that a threshold amount of material is required to adversely affect the remodeling response. The existing processing/decellularization methods are effective for preventing adverse events in host tissue [17], however, more thorough methods of decellularization are desirable and quality assurance steps for assuring removal of cell remnants are indicated.

## Host Response to Biologic Scaffold Materials

The host response to biologic scaffold materials composed of ECM involves both the innate and acquired immune system and the response is affected by device specific variables including the intended clinical application, the source of the raw material/tissue from which the ECM is harvested, and the processing steps involved in manufacturing an approved medical device. A recent study examined the host response to five commercially available ECM devices [18], including GraftJacket™ (human dermis, proprietary cryogenic processing), Restore™ (porcine SIS, minimally processed), CuffPatch™ (porcine SIS, chemically crosslinked with carbodiimide), TissueMend™ (fetal bovine skin, proprietary processing), and Permacol™ (porcine dermis, chemically crosslinked with isocyanate). The results of the study showed profound differences in the acute and chronic host cellular response and in the downstream tissue remodeling outcomes. The intensity of the cell response and the temporal and spatial distribution of the cell response differed among the scaffold materials. GraftJacket™ and Restore™ elicited the most intense acute cell response, but this response was not necessarily predictive of an adverse remodeling outcome. Multinucleate giant cells, a cell type typically associated with a foreign-body response, was observed at the surgical site in which GraftJacket™, CuffPatch™, and Permacol™ was implanted. The cellular response to CuffPatch™ appeared to be predominantly a neutrophilic-type response throughout the entirety of the study, whereas the other devices showed a mainly mononuclear response. Conventional knowledge suggests that mononuclear cells follow neutrophils into a site of inflammation over time, phagocytose cellular debris and foreign material, and eventually exit from the site of inflammation [58,59]. The pattern of cell response and the remodeling outcome differed markedly for each of the ECM scaffold materials evaluated in this study. The GraftJacket™ device was replaced with fibrous connective tissue and a persistent low grade chronic inflammatory response. The host tissue response to Restore™ consisted of replacement of the biologic scaffold with a mixture of muscle cells and organized connective tissue, a finding consistent with an earlier report in which the SIS material was used as a body wall repair device in rat and dog models [60]. The CuffPatch™ device showed accumulation of dense collagenous tissue, a persistent foreign body response, and relatively slower remodeling compared to the Restore device™. The host response to TissueMend™ and Permacol™ was consistent with the expected response to a nonresorbable foreign material; that is, low grade chronic inflammation, minimal scaffold degradation, and fibrous encapsulation. This study, although limited in scope to the histomorphologic response, showed that biologic scaffolds composed of ECM differ markedly in the elicited host tissue remodeling response. There are both similarities and differences among ECM scaffold materials, but it is apparent that a more detailed investigation of the host immune response, the ECM constituents that affect the response, and the effect of these factors upon tissue/scaffold remodeling and outcomes is warranted for such materials.

## Th1 vs. Th2 Lymphocyte Response

The role of T lymphcytes, especially the Th1 and Th2 lymphocyte phenotypes, in cell mediated immune responses to xenografts has been widely studied [61,62]. Th1 lymphocytes produce cytokines such as interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-β leading to macrophage activation, stimulation of complement fixing Ab isotypes ($IgG_{2a}$ and $IgG_{2b}$ in mice) and differentiation of $CD8^+$ cells to a cytotoxic phenotype [63,64]. Activation of this pathway is associated with both allogeneic and xenogeneic transplant rejection [61, 62,65]. Th2 lymphocytes produce IL-4, IL-5, IL-6, and IL-10, cytokines that do not activate

macrophages and that lead to production of non-complement fixing Ab isotypes ($IgG_1$ in mice). Activation of the Th2 pathway is associated with transplant acceptance [66–68].

The only ECM scaffold material for which the Th1/Th2 response has been characterized is SIS-ECM [69,70]. In one study, SIS-ECM was implanted subcutaneously into mice and the host response was compared to the response elicited by either xenogeneic or syngeneic muscle tissue. Histologically, the xenogeneic muscle tissue showed the presence of polymorphonuclear leukocytes (PMNs) within 1 day, with a transition to a mixed population of neutrophils, T lymphocytes, and multinucleate giant cells by day 10. After 28 days, the xenogeneic muscle showed evidence of necrosis, granuloma formation, and encapsulation; all of which are indicative of graft rejection. The syngeneic muscle tissue showed an acute inflammatory response, most of which disappeared by day 10. After 28 days, the residual mild chronic inflammatory response had resolved and the graft site showed organized tissue morphology, consistent with graft acceptance. The host response to the SIS-ECM was similar to that for the syngeneic muscle tissue. At Day 1, PMNs were present at the interface of the host tissue with the SIS-ECM device. After 10 days, the cellular infiltration was reduced and consisted primarily of mononuclear cells. By 28 days, the mononuclear cell infiltrate had diminished and the remodeling response was nearly complete. The mice did not develop an acquired adverse immune response to the SIS-ECM, and analysis of tissue cytokines showed that SIS-ECM strongly increased the expression of IL-4 (Th2), while the expression of IFN-γ (Th1) was 100 fold less than the response elicited by the xenogeneic muscle group. The SIS-ECM implanted mice developed an Ab response that was restricted to the $IgG_1$ isotype, which is most consistent with the Th2 pathway. Thus, although there was a vigorous immune response to the SIS-ECM after implantation, the response was dominated by the Th2 pathway mediators.

To confirm that the immune response to SIS-ECM was due to Th2 restriction rather than lack of sufficient antigen stimulation, Ab responses were measured in mice that received two sequential implants of SIS-ECM 28 days apart [69]. These animals showed a significant secondary antibody response, but the response was still exclusively of the $IgG_1$ isotype. There was no evidence of Th1 cytokines at the secondary graft site. No deposits of $IgG_1$ or $IgG_{2a}$ Ab were found in the SIS-ECM graft. This double implantation study was also conducted with ECM derived from a different tissue source, specifically the porcine urinary bladder submucosa (UBS). The results were very similar confirming that the source of ECM did not alter the restricted Th2 immune response.

The SIS-ECM has been implanted in T cell KO mice and B cell KO mice [69]. In the T cell KO mice, no IL-4 expression was found, showing that T cells are the source of the IL-4 mRNA observed in SIS implanted in WT mice. Anti-SIS Ab were absent in both T cell KO mice and B cell KO mice; however, in both cases, the SIS-ECM scaffold was completely remodeled within 28 days. These results confirm that T and B cells do indeed respond to SIS-ECM, but are not required for SIS-ECM acceptance and a constructive remodeling response.

Two mouse models were used to examine the effects of SIS-ECM implantation upon systemic immunity [70]. Mice implanted with SIS-ECM expressed levels of influenza specific Ab of the $IgG_1$ and $IgG_{2a}$ subtypes after vaccination with a T-dependent subunit vaccine. The vaccine response was comparable to that of mice not implanted with SIS-ECM. Furthermore, challenge of immunized and SIS-implanted mice showed the same survival rate as mice that did not receive the SIS implants. A second model of immune function examined the response to a deliberate bacterial infection following SIS implantation. The mice were immunized with a T-independent polysaccharide vaccine, produced Ab to *S. pneumoniae*, and survived a lethal dose of the bacteria with or without SIS-ECM implantation. In several other studies, ECM scaffolds with deliberate bacterial exposure have been shown to resist infection even without previous immunization [14,71–74]. The Th2 response elicited by SIS-ECM does not adversely affect

the host's ability to mount a protective systemic immune response to T-dependent or T-independent vaccines, and to overcome viral or bacterial infections.

Cell mediated immune responses were analyzed using delayed type hypersensitivity and cytotoxic T cell reactions [70]. In a mouse model of contact dermatitis, topical application of dinitrofluorobenzene led to similar levels of cellular infiltration in both SIS-ECM implanted mice and in mice not implanted with SIS-ECM. Similarly, SIS-ECM implantation did not increase or decrease the ability of mice to reject xenogeneic skin grafts. Thus, SIS-ECM implantation does not impair cell mediated immune responses to antigens.

Since similar studies have not been conducted for other forms of ECM scaffold materials, it is not possible to determine whether they would elicit the same type of host response. Considering the diversity of tissue sources and processing methods from which ECM scaffolds are produced, it seems likely that the host response to biologic scaffold prepared from different sources will vary to a large degree following implantation.

## M1 vs. M2 Macrophage Response

Phenotypic and functional polarization of the mononuclear macrophage population has recently been described [75–78]. A distinct phenotypic polarization profile is described for the macrophage polarization, similar to the Th1/Th2 polarization schemes for lymphocytes described above [61,62,79,80]. The pro-inflammatory, cytotoxic macrophage phenotype, signified as M1, is characterized by cells that promote pathogen killing and cells that are associated with classic signs of inflammation, especially chronic inflammation. The anti-inflammatory macrophage phenotype, signified as M2, promotes immunoregulation, tissue repair, and constructive tissue remodeling. Although morphologically indistinguishable by routine methods of histologic examination, mononuclear macrophages from these two pathways can be identified and distinguished by their cell surface markers and by their cytokine and gene expression profiles [78,81,82]. M1 macrophages are characterized by $CD68^+$ and $CD80^+$ cell surface markers in rats (species differences exist), the production of large amounts of nitric oxide and other reactive oxygen intermediates, and copious amounts of pro-inflammatory cytokines such as IL-12 and TNFα. Conversely, M2 macrophages produce high levels of IL-10 and TGF-β expression, produce large amounts of arginase, inhibit release of proinflammatory cytokines, scavenge debris, promote angiogenesis, and recruit cells involved in constructive tissue remodeling. M2 macrophages express CD163 surface markers in rats, but again, species differences do exist.

A recent study was conducted to evaluate the macrophage polarization profile in response to native SIS-ECM (Restore™), SIS-ECM crosslinked with carbodiimide (CuffPatch™), and autologous abdominal wall muscle in a rat model of abdominal wall muscle repair [83]. The native SIS-ECM showed an intense mononuclear cell response at 1, 2, and 4 weeks that was predominantly of an M2 phenotype (i.e., $CD163^+$) at all time points. Only remnants of the device were distinguishable by histomorphologic examination after 4 weeks. After 16 weeks of remodeling, the implant site was characterized by organized collagenous connective tissue, islands of skeletal muscle tissue, and occasional $CD163^+$ positive mononuclear cells.

The cellular response to SIS-ECM device that had been chemically crosslinked included an abundant mononuclear cell presence with PMN leukocytes surrounding the device at 1 and 2 weeks. The mononuclear macrophages were characterized by an equal number of $CD163^+$ and $CD80^+$ cells at weeks 1 and 2, but by 4 weeks the polarization profile shifted to a shifted to a predominantly $CD80^+$ cell presence, consistent with an M1 phenotype. After 16 weeks, mononuclear cells and multinucleate giant cells were present within and surrounding the graft site and showed the classic histologic picture of chronic inflammation and fibrosis.

The acute cellular response to the autologous abdominal wall muscle tissue graft was characterized by a dense infiltration of both neutrophils and $CD68^+$ mononuclear cells at 1 and 2 weeks after implantation. Morphologically, necrosis of muscle fiber bundles was observed. The mononuclear cell population showed a predominantly M2 phenotype at 1 week. By 2 weeks, approximately equal numbers of $CD163^+$ and $CD80^+$ cells were present. By 4 weeks and all time points thereafter, the muscle tissue graft was largely replaced by moderately well organized collagenous connective tissue and the few macrophages still present showed the M2 phenotype. After 16 weeks, the fibrous connective tissue was poorly organized and consisted of a mixture of scar tissue and adipose tissue.

This study showed that macrophages respond differently to ECM scaffold materials depending upon the ECM source and processing methods. Chemical crosslinking of the SIS-ECM with carbodiimide resulted in a switch from an M2 dominant profile to an M1 dominant profile. An M2 phenotype profile was associated with constructive remodeling, while an M1 phenotype profile was associated with chronic inflammation. Interestingly, the autologous tissue graft showed an M2 response early followed by a duality of the M1 and M2 response, which may have been a consequence of pro-inflammatory cytokines produced as a product of cell death, or DAMP molecules released by dying cells within the autologous tissue graft. Additional work is needed to determine if macrophage phenotype can be predictive of downstream remodeling outcomes.

## Degradation of the ECM Scaffold Materials

The length of time that a host is exposed to foreign antigens certainly affects the type of immune response that will be elicited, but the effect of bioscaffold degradation rate upon the immune response has not been investigated. Naturally-occurring biologic scaffold material, when not chemically crosslinked, is rapidly degraded after implantation. Approximately 60% of the mass is degraded and resorbed within 4 weeks of implantation and complete degradation typically occurs by 3 months [54,55]. The resorbed degradation products are eliminated completely from the body primarily via urinary excretion [54,55].

The effect of ECM scaffold persistence upon the host immune response is not known. Chemical crosslinking of ECM scaffolds provides increased strength and inhibition of degradation [8, 84–87], but recent studies suggest that degradation of the ECM scaffold is an essential component of a rapid constructive remodeling response. Low molecular weight peptides formed during the degradation of ECM scaffolds have been shown to have chemoattractant potential for several cell types *in vitro*, including multipotential progenitor cells [88]. *In vivo* studies have shown that bone marrow-derived cells are recruited to the site of healing, and that they participate in the long-term remodeling of the ECM [89,90]. Stated differently, degradation of an ECM scaffold may be a requisite process with bioactive consequences that contribute to the overall remodeling events. It is possible that chemoattraction by degradation products contributes to the recruitment of host cells, and ultimately to site specific tissue remodeling. The role of the immune response in these important biologic processes is almost totally unexplored.

## Summary

In summary, allogeneic and xenogeneic biologic scaffolds composed of extracellular matrix are commonly used in numerous tissue engineering and regenerative medicine applications, and in many reconstructive surgical procedures. The effect of such scaffolds upon the host immune response has been largely unexplored. In addition, the association between the host immune response and tissue remodeling events is a factor that logically plays an important, if not determinative, role in the successful clinical application of these devices. There are many

variables in the manufacturing of matrix derived scaffolds and all of these variables can affect the host immune response. An improved understanding of the immune response to biologic scaffold materials can only lead to greater safety and efficiency of devices and applications that utilize such materials.

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**Table 1**

Source tissue and processing methods for commercially available devices produced from extracellular matrix scaffold material

| Test Article | Species/Tissue of Origin | Crosslinking |
|---|---|---|
| Oasis™, Surgisis™ (Cook Biotech, Inc.) | Porcine small intestinal submucosa | n/a |
| Restore™ (DePuy Orthopaedics) | Porcine small intestinal submucosa | n/a |
| CuffPatch™ (Organogenesis, Inc) | Porcine small intestinal submucosa | Carbodiimide |
| Acell Vet (Acell, Inc.) | Porcine urinary bladder basement membrane and mucosa | n/a |
| Alloderm™ (Lifecell, Corp.) | Human dermis | n/a |
| GraftJacket™ (Wright Medical Techology) | Human dermis | n/a |
| Zimmer Collagen Repair Patch™ (Zimmer, Inc.) | Porcine dermis | Isocyanate |
| TissueMend® (Stryker) | Bovine dermis | Proprietary |
| Mosaic®, Freestyle® (Medtronic, Inc.) | Porcine heart valve | Glutaraldehyde |
| Prima™ (Edwards Lifesciences) | Porcine heart valve | Glutaraldehyde |
| OrthAdapt™ (Pegasus, Inc.) | Equine Pericardium | Proprietary |